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In vitro anti-inflammatory activity of lignans isolated from *Magnolia fargesii*

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ABSTRACT

The overproduction of nitric oxide (NO) and prostaglandin E_2 (PGE_2) causes neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Four lignans, (+)-eudesmin (**1**), (+)-magnolin (**2**), (+)-yangambin (**3**) and a new structure named as epimagnolin B (**4**) were isolated from *Magnolia fargesii* (Magnoliaceae) as the inhibitors of NO production in LPS-activated microglia. The most potent compound **4** inhibited the production of NO and PGE_2 and the expression of respective enzyme iNOS and COX-2 through the suppression of I-κB-α degradation and nuclear translocation of p65 subunit of NF-κB.

Nitric oxide (NO) is produced via oxidation of L-arginine by nitric oxide synthase (NOS). There are three isoforms of NOS.[1] The constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS) release small amounts of NO required for the control of blood pressure and neurotransmission, respectively.[2] As a neuromodulator in central nervous system (CNS), NO participates in brain development, pain perception, memory, neuronal plasticity and behavior.[3] Unlike the constitutive NOS, inducible NOS (iNOS) is induced by several immunological responses[4] and produces large amounts of NO for extended periods of time.[5] Although iNOS plays a beneficial role in host defense against pathogens, uncontrolled production of NO is claimed to be responsible for chronic inflammation,[6] carcinogenesis,[7] sepsis,[8] and neurodegenerative disorders.[9] NO also activates the cyclooxygenase-2 resulting in markedly increased release of prostaglandins (PGs).[10]

PGs are derived from arachidonic acid through cyclooxygenase (COX) pathway. COX-1 is a constitutively expressed form in normal conditions, but COX-2 is expressed in response to pro-inflammatory effectors such as cytokines and bacterial endotoxin, lipopolysaccharide (LPS). COX-2 produces large amounts of PGE_2 that is a key inflammatory mediator. COX-2 is associated with inflammation, carcinogenesis, mitogenesis and several neuronal diseases.[11]

Microglial cells play an active role in CNS as an immune regulator and they can be activated in response to neurodegenerative and neuro-inflammatory conditions. Activated microglia produce pro-inflammatory and neurotoxic mediators including nitric oxide that causes the neuronal cell death.[12] COX-2 expression has been detected in activated microglia,[13] and the inhibition of COX-2 was protective against the neuronal cell death caused by COX-2.[14]

Flos magnoliae, the dried flower buds of *Magnolia* species (Magnoliaceae), well known as 'Shin-i', have been traditionally used for the treatment of allergic rhinitis, nasal empyema and sinusitis.[15] It has been reported that compounds isolated from flos magnoliae showed anti-inflammatory, anti-allergic, anti-rheumatoid arthritis, and anti-angiogenic activities.[16] Magnone A and B, fargesone A and B, denudatin B, magnolin, lirioresinol-B dimethylether, fargesin, and aschantin isolated from flos magnoliae showed Ca^{2+} antagonistic and platelet-activating factor (PAF) antagonistic activities.[17] The lignans from this plant were reported to inhibit the expression of cell adhesion molecules on the surface of human monocytic cells.[18]

As part of our searching for the anti-inflammatory compounds from medicinal plants, we isolated lignans as inhibitors of NO production in activated microglia. Herein we report the isolation and structure elucidation of a new lignan, epimagnolin B along with the identification of three known lignans from the flower buds of *Magnolia fargesii*. We also report their inhibitory activity of PGE_2 production and iNOS and COX-2 expression. The MeOH extract of the dried flower buds of *M. fargesii* was partitioned between EtOAc and water. Compounds **1**–**4** were purified from the EtOAc soluble fraction through silica gel column chromatography and reverse phase semi-preparative HPLC with activity-guided fractionation.[19] Compounds **1**, **2** and **3** were identified as (+)-eudesmin, (+)-magnolin and (+)-yangambin, respectively,[20] by comparison of their spectroscopic and physico-chemical data with those reported in the literature. The ^1H and ^{13}C NMR spectra of **1** and **3** showed *bis* symmetric structures. Compound **2** showed five methoxy peaks in ^1H NMR and four different environments of methoxy groups in

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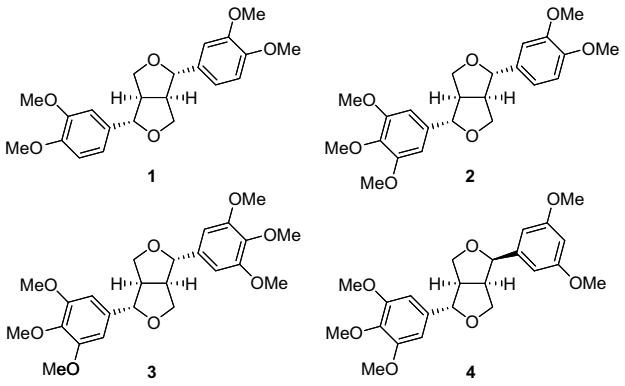

^{13}C NMR spectra. The protons of 3,4-disubstituted phenyl ring were found in ^{1}H NMR spectrum of compound **2**. Compounds **1**, **2** and **3** were previously reported from the flower buds of *M. fargesii*.[21] According to the literature, the *cis* arrangement of the fused tetrahydrofuran rings in natural lignans restricts the configurations of the bridge atoms of C-1 and C-5 to be either (*R*)/(*R*) or (*S*)/(*S*).[21] It was reported that all (+)-tetrahydrofuran type lignans have the absolute configuration (*R*) at the bridge carbons of C-1 and C-5.[22] In the present work, all compounds showed positive optical rotations implying the (1*R*)/(5*R*) absolute configuration.[23] Compound **4** was isolated as yellow oil. High resolution EI-MS of **4** supported the $C_{23}H_{28}O_7$ molecular formula $[M]^+$ *m/z* 416.1873 (calculated 416.1835). Compound **4** showed signals for five methoxy groups around δ 3.83–3.89 and three singlet peaks in the aromatic region at δ 6.59 (2H), 6.86 (2H) and 6.93 (1H) in ^{1}H NMR spectrum. The overall NMR pattern of proton and carbon peaks from ^{1}H-^{1}H COSY and HSQC spectra suggested that the structure of **4** might be furofuran type lignan of 2,6-diaryl-3,7-dioxabicyclo-[3.3.0]octane.[24] Two aryl rings have unsymmetrical stereochemistry. The downfield chemical shift of H-1 (δ 3.34) and H-2 (δ 4.88) suggested the β-orientation of aryl at C-2, while the upfield chemical shift of the respective H-5 (δ 2.92) and H-6 (δ 4.43) suggested the α-orientation of aryl at C-6. The coupling constants of H-2 (J = 5.2 Hz) and H-6 (J = 7.2 Hz) supported further this assignment.[25] In HMBC spectrum (Fig. 1), the singlet peak at δ 4.88 (H-2′/6′) correlated with C-4′ that was correlated with singlet peak (1H) at δ 6.93 in HSQC spectrum. This suggested the presence of a 3′,5′-disubstituted phenyl ring, which was assigned to C-2 from the correlation of H-2′/6′ with C-2 in HMBC spectrum. Peak of H-2′/6′ also correlated with quaternary carbons C-3′ (δ 148.7) and C-5′ (δ 147.9), which correlated with respective methoxy peaks at δ 3.91 and 3.88 in HMBC. These data suggested that 3′,5′-dimethoxyphenyl group is linked to C-2 of the tetrahydrofuran group of the dioxabicyclo-[3.3.0]octane unit. The presence of 3′,5′-disubstituted phenyl group has not been reported in furofuran type lignans. Another phenyl group was assigned to C-6 from the correlation of singlet aromatic peaks (2H, H-2″/6″) with C-6 in HMBC. As shown in Figure 1, the position of methoxy groups were identified as C-3″, 4″ and 5″ by the correlation with their respective aromatic carbons. Compound **4** was isolated as a new lignan structure having 3′,5′-disubstituted phenyl group and designated with the trivial name of epimagnolin B.[23]

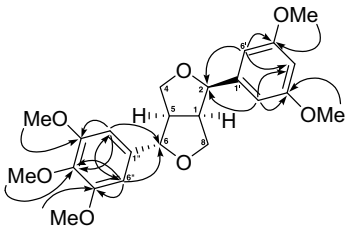

Figure 1. HMBC correlations of compound **4**.

For the screening of biological activity, BV-2 cells were stimulated with 0.1 μg/mL of LPS in the presence or absence of various concentrations of test compounds for 20 h, and the amounts of NO released into culture media were determined by the Griess method.[26] The concentrations required for 50% inhibition (IC_{50} value) of NO production by compounds **1**, **2**, **3**, and **4** were 30.0 ± 2.1, 20.5 ± 1.8, 28.6 ± 2.8, and 10.9 ± 1.6 μM, respectively. Compound **4** showed the most potent inhibition of LPS-induced NO production.

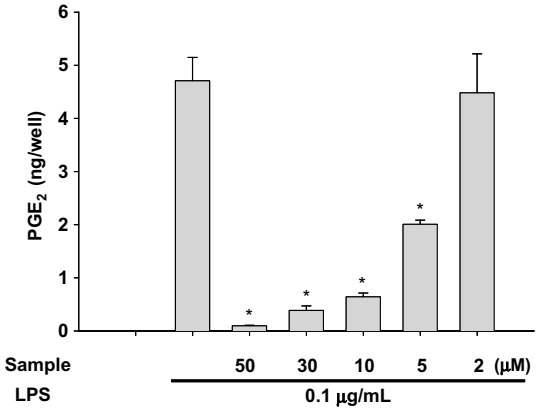

Figure 2. Effects of compound **4** on the production of PGE_2 by LPS-activated microglia. BV-2 cells were treated for 20 h with compound during LPS (0.1 μg/mL) activation. The levels of PGE_2 released into culture media were evaluated by enzyme immunoassay. Values represent means ± SD (n = 3). $^{*}p < 0.05$ indicate significant difference between LPS alone control and sample treatment.

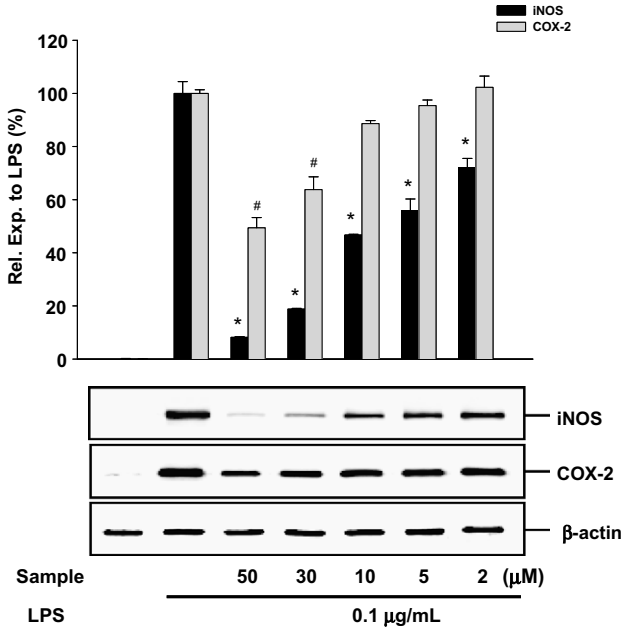

Figure 3. Effects of compound **4** on the expression of LPS-induced iNOS and COX-2 protein by activated microglia. BV-2 cells were treated for 20 h with compound during LPS (0.1 μg/mL) activation. Cell lysates were prepared and the iNOS, COX-2, and β-actin protein levels were determined by Western blotting. The blot is the representative results of three separate experiments. The relative intensity of iNOS/COX-2 to β -actin bands were measured by densitometric analysis. Values represent means ± SD (n = 3). $^{*,\#}p < 0.05$ indicate significant difference (*iNOS, $^{\#}$COX-2) between LPS alone control and sample treatment.

N^G-Monomethyl-L-arginine (L-NMMA, IC_{50} value of 19.2 ± 1.8 μM), a well known NOS inhibitor was used as a positive control for nitrite assay. Enzyme immunoassay was performed for the determination of PGE_2 produced by COX-2 in activated microglia as reported previously.[27] Figure 2 showed that compound **4** decreased PGE_2 synthesis in a dose dependent manner in LPS-activated BV-2 cells.

Western blot analysis was performed to clarify the underlying mechanism of **4** for the inhibition of NO and PGE_2 production.[28] The treatment of **4** decreased the expression of iNOS and COX-2 protein in activated microglia (Fig. 3). RT-PCR analysis also showed the inhibitory activity of **4** on the expression of iNOS and COX-2 mRNA in LPS-activated microglia in a dose dependent manner (Fig. 4). The amounts of protein and mRNA expressions were quantitated from the volume intensity of the bands corresponding to iNOS, COX-2 and β-actin. The gene expression of iNOS and COX-2 can be modulated by NF-κB. NF-κB can be activated by the degradation of inhibitor-κB (I-κB) and followed by translocation into nucleus. Compound **4** (30 μM) inhibited the I-κB-α degradation in LPS-activated microglia, and resulted in the decrease of nuclear translocation of NF-κB subunit, p65 (Fig. 5). These results suggest that compound **4** inhibited the expression of iNOS/COX-2 through the inhibition of I-κB-α degradation and NF-κB activation. All the treatment of samples did not show any specific toxicity against microglia compared to control group.

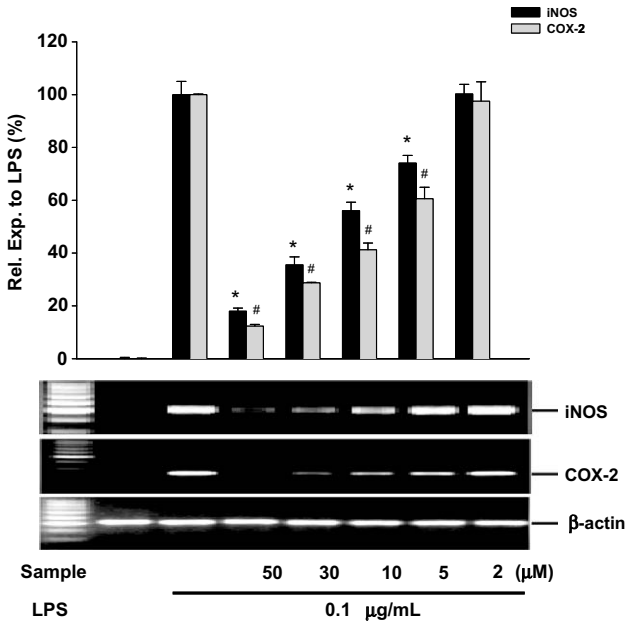

Figure 4. Effects of compound **4** on the expression of LPS-induced iNOS and COX-2 mRNA in activated microglia. BV-2 cells were treated for 4 h with compound during LPS (0.1 μg/mL) activation. The mRNA levels for iNOS, COX-2, and β-actin were determined by RT-PCR from total RNA extracts. The gel shown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

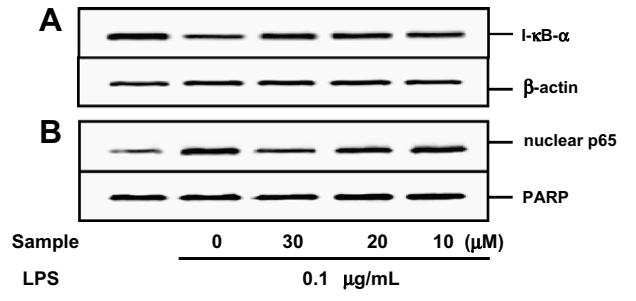

Figure 5. Effects of compound **4** on I-κB-α degradation and nuclear translocation of p65 in LPS-activated microglia. Cells were pretreated with **4** (30, 20, 10 μM) for 30 min and incubated further with LPS (0.1 μg/mL) for 30 min. The protein levels of I-κB-α in cell lysate (A) and the protein levels of p65 in nuclear extracts were determined by Western blotting (B).

Microglia serves as representative of the immune system in the brain. Microglia is thought to be similar in nature to macrophages in the blood system.[29] Activated microglia induces the expression of iNOS and COX-2, which produce pro-inflammatory mediators, such as NO and PGs. The over expression of iNOS causes neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[3,9,11] Therefore, inhibition of these mediators may have beneficial effects in the treatment of neuro-inflammatory reaction and oxidative neuronal diseases.

In summary, four lignans were isolated from the flower buds of *M. fargesii* and 3′,5′-dimethoxy substituted tetrahydrofuran lignan was purified for the first time from nature. Compounds **1**–**4** inhibited the production of NO in LPS-activated microglia. The most potent compound **4** inhibited the protein and mRNA expression of iNOS and COX-2 through the suppression of NF-κB activity. These results imply that the flower buds of *M. fargesii* might be beneficial in the treatment of nuero-inflammatory diseases through the inhibition of NO and PGs production.

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.2008.11.103.

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19. *Procedures of extraction and isolation*. The dried flower buds of *M. fargesii* (3 kg) were extracted for 3 h with MeOH under reflux and evaporated in vacuo. The residue (710 g) was suspended in water and extracted with EtOAc (382 g). A portion of the EtOAc fraction (10 g) was subjected to silica column chromatography eluting with *n*-hexane/ EtOAc gradient system (10:1 → 1:1) to yield 10 subfractions (fr. 1–10). A portion of fr. 9 (450 mg) was rechromatographed by semi-preparative HPLC (Inertsil ODS-2, 10 × 250 mm; 45% CH_3CN as eluent, 2.0 mL/min, UV 254 nm) to obtain (+)-eudesmin (**1**) (13.7 mg), (+)-magnolin (**2**) (37.6 mg), (+)-yangambin (**3**) (10.9 mg) and epimagnolin B (**4**) (13.5 mg). The purity of compounds **1**–**4** were confirmed by HPLC analysis (Inertsil ODS-2, 4.6 × 100 mm; 50% CH_3CN 1.0 mL/min, UV 254 nm) eluting as a single peak at 7.4, 8.0, 8.4 and 8.7 min, respectively. The structures of compounds were identified by the analysis of Mass, NMR spectroscopic data.
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23. *Representative characterization data for four compounds*. (+)-Eudesmin (**1**): yellow oil; $[\alpha]_D$ +37.2° (*c* 1.0, $CHCl_3$); EI-MS *m/z* 368 $[M]^+$. (+)-Magnolin (**2**): yellow oil; $[\alpha]_D$ +53.4° (*c* 3.1, $CHCl_3$); EI-MS *m/z* 416 $[M]^+$. (+)-Yangambin (**3**): yellow oil; $[\alpha]_D$ +12.0° (*c* 0.5, $CHCl_3$); EI-MS *m/z* 446 $[M]^+$. (+)-Epimagnolin B (**4**): yellow oil; $[\alpha]_D$ +92.9° (*c* 0.9, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 206.6 (4.84), 274.4 (3.85) nm; IR (KBr) ν_{max} 3020, 1585, 1510, 1465, 1338, 1250 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.93 (1H, s, H-4′), 6.86 (2H, s, H-2′, 6′), 6.59 (2H, s, H-2″, 6″), 4.88 (1H, d, *J* = 5.2 Hz, H-2), 4.43 (1H, d, *J* = 7.2 Hz, H-6), 4.02 (1H, dd, *J* = 0.4, 9.6 Hz, H-4a), 3.91 (3H, s, O*CH*$_3$-3′), 3.89 (2H, m, H-4b and H-8a), 3.88 (3H, s, O*CH*$_3$-5′), 3.87 (6H, s, O*CH*$_3$-3″, 5″), 3.83 (3H, s, O*CH*$_3$-4″), 3.34 (2H, m, H-1 and H-8b), δ 2.92 (1H, m, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.3 (C-3″ and 5″), 148.7 (C-3′), 147.9 (C-5′), 137.4 (C-4″), 136.7 (C-1″), 130.7 (C-1′), 117.6 (C-6′), 110.9 (C-2′), 108.8 (C-4′), 102.8 (C-2″ and 6″), 88.0 (C-6), 82.0 (C-2), 71.0 (C-4), 69.8 (C-8), 60.8 (O*CH*$_3$-4″), 56.2 (O*CH*$_3$-3″ and 5″), 55.93 (O*CH*$_3$-5′), 55.90 (O*CH*$_3$-3′), 54.6 (C-5), 50.1 (C-1); EI-MS *m/z* 416 $[M]^+$ (100), 249 (38), 195 (56), 165 (47); HREIMS *m/z* 416.1873 (calculated for $C_{23}H_{28}O_7$, 416.1835).
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