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# Nitric Oxide and Prostaglandin $E_2$ Synthesis Inhibitory Activities of Diarylheptanoids from the Barks of *Alnus japonica* Steudel

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Nine known diarylheptanoids (**1**-**9**) isolated from the barks of *Alnus japonica* were evaluated for their inhibitory activities on nitric oxide (NO) and prostagrandin $E_2$ (COX-2) production in interferon-$\gamma$ (INF-$\gamma$) and lipopolysaccharide (LPS)-activated RAW 264.7 cells *in vitro*. The NO and COX-2 levels were moderately reduced by the addition of compounds (**1**-**9**). Among these compounds, compounds **6** and **8** inhibited NO production in a dose dependent manner with an $IC_{50}$ of 16.7 and 27.2 μg/mL, respectively (positive control, L-NMMA; 22.8 μg/mL), and compounds **6**, **7**, **8**, and **9** reduced the COX-2 level in a dose dependent manner with an $IC_{50}$ of 20.7, 25.7, 25.0, and 27.3 μg/mL, respectively (positive control, indomethacin; 26.2 μg/mL). An analysis of the structural activity relationship among these diarylheptanoids suggests that the presence of a keto-enol group in the heptane moiety or a caffeoyl group in the aromatic ring were important for the efficacy on the inhibitory activities of NO and COX-2 production.

**Key words:** *Alnus japonica,* Betulaceae, Diarylheptanoid, Nitric oxide, Cyclooxygenase-2, Anti-inflammation, Cancer chemoprevention

## INTRODUCTION

Nitric oxide (NO) radical play important biological roles in physiological systems as vasodilation, neurotransmission and platelet aggregation as well as in pathophysiological systems such as acute and chronic inflammation. In addition, the over production of NO is also implicated in the pathogenesis of cancer (Moncada S *et al.*, 1991). Cyclooxygenase-2 (COX-2) is regarded as an inducible enzyme that is responsible for prostaglandin biosynthesis in the inflammatory process as well as in malignant or transformed cells (Subbaramiah *et al.*, 1996). Therefore, compounds that regulate the NO and COX-2 activities might provide a target for the development of new anti-inflammatory (Je *et al.*, 2004; Rie *et al.*, 1999) and cancer chemopreventive (Jang *et al.*, 2003) agents.

Previously, the inhibitory activities of some diaryheptanoids, oregonin and hirsutanonol, from *Alnus hirsuta* on inducible nitric oxide synthesis (Lee *et al.*, 2000) and COX-2 expression were reported (Lee *et al.*, 2000). A detailed study on the constituents of the bark from *Alnus japonica* led to the isolation of several diarylheptanoids (Kim *et al.*, 2004). This paper describes a biological evaluation of those of isolated compounds from the barks of *Alnus japonica* on the NO and COX-2 level for the purpose of developing new anti-inflammatory and cancer chemopreventive agents.

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## MATERIALS AND METHODS

### Materials

Nine diarylheptanoids, 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane-3-*O*-β-D-xylopyranoside (**1**), 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-*O*-β-D-apiofuranosyl(1→6)-β-D-glucopyranoside (**2**), 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-glucopyranoside (**3**), 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane (**4**), 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-glucopyranoside (**5**), oregonin (**6**), hirsutanonol (**7**), hirsutenone (**8**), and platyphylloside (**9**) were isolated from the 80% acetone extract of the fresh bark from *A. japonica* (Kim *et al.*, 2004)and were used in this experiment (Fig. 1).

### Biological assay

#### Cell culture

Raw 264.7 cells were purchased from the Korean Cell Line Bank. The cells were grown at 37 °C in a humidified atmosphere (5% $CO_2$) in a DMEM medium containing

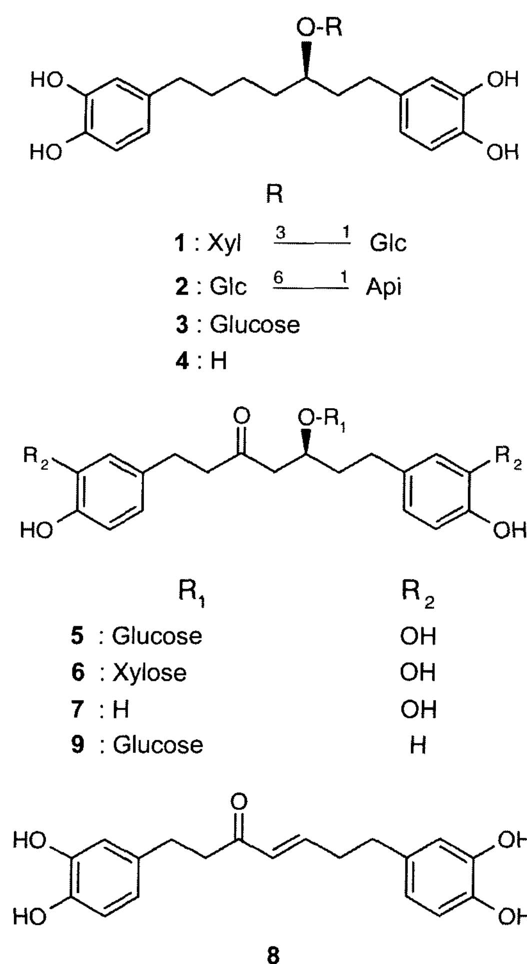


**Fig. 1.** Structures of compounds **1-9**

10% fetal bovine serum.

### MTT assay

The cytotoxicity was measured by the motochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to formazan (Mosmann, 1983). The cells were seeded at a density $1 \times 10^6$ cells/mL in 96 well-plates. After incubating for 2 h, the cells were treated with the samples. The cells were incubated for an additional 24 h, and the medium was replaced with fresh medium. The medium contained MTT (final concentration : 0.5 mg/mL), and the incubation continued for a further 1 h at 37 °C. The medium was then removed and the MTT-formazan produced was dissolved in 200 μL DMSO. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance at 570 nm using an ELISA reader.

### Nitrite assay

Raw 264.7 macrophage cells were cultured in a 24-well plate and preincubated for 2 h at 37 °C in a humidified atmosphere (5% $CO_2$). The cells were then incubated in a medium containing 10 μL of LPS, IFN-γ and the test samples. After incubating for an additional 24 h, the media were removed and analyzed for the level of nitrite accumulation, as an indicator of NO, using the supernatant by a Griess assay. The Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% $H_3PO_4$ solution, 100 μL) was added to 100 μL of each of the supernatants from the cells treated with the samples. The samples were then read at 540 nm against a standard sodium nitrite curve. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve.

### COX-2 enzyme assay

Raw 264.7 macrophages were plated in a 24-well plate and preincubated for 24 h at 37 °C in a humidified atmosphere (5% $CO_2$). The cells were preincubated for 2 h, and 10 μL LPS, IFN-γ was then added. The COX-2 enzyme was then induced for 16 h. After the cells had been washed by the medium, they were added to 170 μL of fresh medium, 20 μL of the test sample and incubated for 15 min. The cells were added to 10 μL arachidonic acid (600 μM) and incubated for 40 min. The inhibitory effects of the test samples upon COX-2 were then determined by a $PG_{E_2}$ assay.

## RESULTS AND DISCUSSION

The diarylheptanoids were isolated from the bark from *A. japonica* and identified by a direct comparison with either the authentic samples or with the reported spectral and physical data (co-TLC, IR, MS, NMR), previously. The inhibitory activities of the isolated diaryheptanoids **1-9** against NO and COX-2 were evaluated.

The MTT assay showed that all compounds **1-9** did not cause cell cytotoxicity in the treatment ranges of the compounds, 0 40 μg/mL.

The NO levels were moderately reduced as a result of the addition of the compounds **1-9** to the RAW 264.7 cell stimulated by LPS, IFN-γ. Compounds **6** and **8** were found to inhibit NO production in a dose dependent manner with an $IC_{50}$ value of 16.7 μg/mL and 27.2 μg/mL, respectively. As a positive control, L-NMMA (NO synthesis inhibitory agent) showed significant inhibition with an $IC_{50}$ value of 22.8 μg/mL (Table I). The viability of the RAW 264.7 cells were not altered and the NO production level was similar the presence or absence of compounds **6** and **8**, as determined by the MTT assay.

The COX-2 levels were also moderately reduced by the addition of the diarylheptanoids **1-9**. Among them, compounds **6**, **7**, **8**, and **9** were found to inhibit the COX-2 level in a dose dependent manner with an $IC_{50}$ value of

**Table I.** Effects of compounds **1-9** on the LPS, INF-γ-induced NO and COX-2 production in the Raw 264.7 marcrophages.

| Compounds | NO | COX-2 |
|---|---|---|
| | $IC_{50}$ (μg/mL) | $IC_{50}$ (μg/mL) |
| **1** | 48.6 | 45.2 |
| **2** | 49.4 | 34.2 |
| **3** | 32.5 | 32.2 |
| **4** | 28.4 | 45.3 |
| **5** | 29.4 | 33.7 |
| **6** | 16.7 | 20.7 |
| **7** | 44.3 | 25.7 |
| **8** | 27.2 | 25.0 |
| **9** | 53.1 | 27.3 |
| L-NMMA | 22.8 | - |
| Indomethacin | - | 26.2 |

20.7 μg/mL, 25.7 μg/mL, 25.0 μg/mL and 27.3 μg/mL, respectively. As a positive control, indomethacin significantly inhibited the COX-2 level with an $IC_{50}$ value of 26.2 μg/mL (Table I).

An analysis of the structural activity relationship among these diarylheptanoids showed that the presence of a keto-enol group in the heptane moiety or a caffeoyl group in the aromatic ring were important for the inhibitory activities on NO and COX-2 production.

These results suggest that the diarylheptanoids with a ketone group in the heptane moiety (**6**, **7**, **8**, and **9**) and a caffeoyl group in the aromatic rings (**6**, **7**, and **8**) are potential anti-inflammatory and cancer chemopreventive agents.

## ACKNOWLEDGEMENT

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