

# Inhibitory effect of *Panax notoginseng* and emodin on LPS-induced iNOS, COX-2 and prostaglandin E2

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Many traditional herbal remedies exhibit several beneficial effects including anti-inflammation. The exact mechanism of the anti-inflammatory action of *Panax notoginseng* Buck F.H. Chen, however, has not been determined. In the present study, we have isolated the acting compound, emodin, from *P. notoginseng* and examined the effects of *P. notoginseng* and emodin on lipopolysaccharide (LPS)-induced nitric oxide (NO) production, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in RAW264.7 macrophages. The results indicated that *P. notoginseng* concentration-dependently inhibited LPS-induced NO production. Furthermore, *P. notoginseng* inhibited the expression of LPS-induced iNOS and COX-2 proteins without an appreciable cytotoxic effect on RAW264.7 cells. Emodin also inhibited LPS-induced iNOS protein as potently as *P. notoginseng*. This was consistent with the findings that *P. notoginseng* but not emodin inhibited prostaglandin E2 synthesis induced by LPS.

Key words : iNOS, Cytokines, Cyclooxygenase-2(COX-2), lipopolysaccharide(LPS), Prostaglandin E2, emodin, *Panax notoginseng*

## Introduction

NO has been shown to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and host defense<sup>1,2</sup>. Molecular cloning and sequencing analysis have revealed the existence of at least three main types of NOS isoforms<sup>3,4,5</sup>. NOS present in the vascular endothelium (eNOS) and that in central and peripheral neurons (nNOS) are constitutive (cNOS), and their activation is Ca<sup>2+</sup>-dependent. Continuous release of NO by cNOS plays a role in keeping the vasculature in an active state of vasodilation. Various agonists such as bradykinin and acetylcholine have been shown to trigger cNOS-mediated NO production through increasing intracellular Ca<sup>2+</sup>. NOS in macrophages and hepatocytes, on the other hand, is inducible (iNOS), and its activation is Ca<sup>2+</sup>-independent<sup>6,7</sup>. After exposure to endotoxin and/or cytokines, iNOS can be induced in various cells such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes. The induced iNOS

catalyzes the formation and release of a large amount of NO, which plays a key role in the pathophysiology of a variety of diseases including septic shock<sup>8,9,10,11</sup>. NO production catalyzed by iNOS, therefore, may reflect the degree of inflammation and provides a measure by which effects of drugs on the inflammatory process can be assessed. Expression of cyclooxygenase-2 (COX-2) in various tissue preparations following lipopolysaccharide (LPS)-treatment also has been reported<sup>12,13,14</sup>. This enzyme is considered to play a major role in the inflammatory process by catalyzing the production of prostaglandins.

A number of traditional plant-derived medicines have been found to be rich in polyphenolic compounds<sup>15</sup>. These include flavonoids, tannins, and anthraquinones and have been shown to exhibit anti-inflammatory activities<sup>16,17,18</sup>. The exact mechanism of anti-inflammatory action of these compounds is not established. One of the herbal medicines, *Panax notoginseng*, is the most common drug to treat chronic liver disease in Korea. A Korean herbal medicine, *Panax notoginseng* Buck F.H. Chen, is highly prized in Korea for its therapeutic abilities to stop haemorrhages, to influence blood circulation and to act as a tonic agent. *P. notoginseng* is cultivated on a large scale in Korea. It is an oral medicine. The main root of this plant, named notoginseng, is used for treatment of trauma and bleeding due to internal and external injury. *P. notoginseng* has

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many reported actions such as limitation of liver injury, anti-tumor effect, and alteration of the functional balance of the immune system<sup>19</sup>. Recently, *P. notoginseng* is widely used by patients with chronic hepatitis in Korean. The preparation prevented liver fibrosis as well as the development of HCC in patients with cirrhosis<sup>20</sup>. In addition, *P. notoginseng* was found to inhibit the activation of stellate cells, the rodent equivalent of human stellate cells. This is believed to be the mechanism of prevention of liver fibrosis by *P. notoginseng*<sup>20</sup>. As the principal constituents of this medicinal herb, various dammarane-type triterpene saponins were isolated from the roots, leaves, and seeds<sup>21</sup>. Furthermore, its immunological adjuvant activities of the principal dammarane-type triterpene saponins from notoginseng and American ginseng were characterized<sup>22</sup>. Because of its major pharmaceutical effects, *P. notoginseng* is presumed to generally and gradually improve biological defense mechanisms, and it has been reported to have an anti-inflammatory action via an increase in blood corticosterone levels. However, its mode of action has not been fully elucidated. It was also found that the saponin fraction from the flower buds of *P. notoginseng* showed hepatoprotective effect on liver injury induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) in mice. The flower buds of *P. notoginseng* have been used for treatment of hypertension, vertigo, tinnitus, and laryngopharyngitis, and several known dammarane-type triterpene saponins were hitherto isolated from the flower buds<sup>23</sup>. New saponins from the flower buds of *P. notoginseng* as well as the hepatoprotective effects of the principal dammarane-type triterpene saponins have been characterized from the flower buds and roots<sup>24</sup>. The possibility that these compounds exhibit their biological effects by blocking iNOS and COX-2 expression, therefore, was examined in the present study. Specifically, we studied the effect of *P. notoginseng* on LPS-induced NO and PGE<sub>2</sub> production and expression of iNOS and COX-2 in RAW264.7 macrophages. The data demonstrated that *P. notoginseng* was the most potent among the compounds tested in blocking LPS-induced iNOS and COX-2 gene expression. These inhibitory effects of *P. notoginseng* were also demonstrated in Bcl-2-overexpressing RAW264.7 cells.

## Materials and Methods

### 1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml

of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco/BRL) and maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

### 2. Plant Material

The flower buds of *Panax notoginseng* was purchased in Kyungju, Kyungbuk, Korea. A voucher of the plant is on file in our laboratory (2002.09. PN-563). The dried flower buds of *P. notoginseng* (500 g) were finely cut and extracted three times by boiling water.

### 3. Water extraction of *P. notoginseng*

Voucher specimen is present at the Herbarium of Botany Department, Dongguk University, Kyungju, Korea. More specifically, stems of *P. notoginseng* were cut into small pieces and 200 g of the material was boiled in 4 ℓ of water until the liquid volume had been reduced by 50%. The fluid was then filtered through a 1 mm-pore-size filter, the water-insoluble component was discarded, and the *P. notoginseng* extract was lyophilized to a dry powder. Then, the *P. notoginseng* extract was filtered. The filtrate was evaporated to dryness in an oven at 40°C. The dried extract was weighed (yield, 1.5%).

A sample of the dry powder (2.5 g) was dissolved in 100 ml of milli-Q water at 37°C and stirred for 60 min. The sample was then centrifuged at 4000 × g for 15 min, and the supernatant was passed through a 0.22 mm-membrane bottle filter system (Corning, COstar, NY, USA). The water-insoluble component was discarded and the water-soluble ingredients were used in the experiments. The concentration of SB extract in the solution was calculated by lyophilizing the water-soluble component that passed through a 0.22 μm membrane bottle filter. The concentration of LPS in *P. notoginseng* (600 mg/ml) was 0.03 pg/ml.

### 4. Isolation and determination of emodin from *P. notoginseng* stems

Emodin (chemical structures shown in Fig. 1.) were isolated from Korean herbal plants. Emodin was isolated from *Panax notoginseng* Buck F.H. Chen.

HPLC and column chromatography were used to separate and purify emodin from the *P. notoginseng* stems, as described below. The HPLC system was from Shimadzu, equipped with a HPLC pump, a variable-wave-length monitor, a LC controller, column oven and a solvent conditioner. The system was equipped with UV monitor operating at 254 nm, recorder and a manual injection valve with a 10 ml loop. A two hundred grams of dry mass of *P. notoginseng* stems was extracted by 2 ℓ methanol, the mixture

was centrifuged and the supernatant was washed with light petroleum (b.p. 60-90°C). The remaining methanol phase was evaporated to form a syrup. The syrup was then dissolved and fractionated in 200 ml EtOAc. Emodin existed in the EtOAc phase and EtOAc solution was vacuum evaporated at 40°C, and 18.93 g residue of EtOAc was obtained. In separation process, the column was filled with the upper organic stationary phase, then the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were separately collected for analysis. A solvent system consisting of chloroform-methanol-water (4:3:2) was used and five fractions were obtained. Each fraction was analyzed by thin-layer-chromatography (TLC) with precoated silica G-25 UV254 plates. TLC plates were developed with hexane-EtOAc-formic acid (30:10:0.5), and detected at 254 nm. Fraction 5, a completely separated peak, was supposed to be emodin, 546.4 mg dried emodin was obtained from the peak.

#### 5. Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction<sup>25</sup>. One hundred microliters (100 µl) of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories).

#### 6. Western blots

Total cellular extract, cytosolic fractions (for IB), and nuclear fraction (for p65) were prepared according to Muller *et al.*<sup>26</sup>, separated on SDS-polyacrylamide minigels (8% for iNOS or COX-2, and 10% for IB or p65), and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was incubated overnight at 4°C with 1% bovine serum albumin and then incubated with anti-iNOS, anti-COX-2, or anti-tubulin monoclonal antibodies (Transduction Laboratories), or with anti-IB or anti-p65 polyclonal antibodies (Santa Cruz Biochemicals). iNOS, IB, p65, COX-2, and α-tubulin were detected by NBT and BCIP staining (Sigma Chemical Co.).

#### 7. Measurement of PGE2 production

*P. notoginseng* extract RAW264.7 cells were subcultured in six-well plates and were incubated with selected compounds (oroxylin A, emodin, and physcion) for 12 hr. One hundred microliters (100 µl) of supernatant of culture medium was collected for the determination of PGE2 concentrations by

ELISA (Cayman Enzyme Immunoassay kit).

## Result

### 1. Effects of *P. notoginseng* and emodin on LPS-induced NO production in RAW264.7 macrophages

The chemical structure of the anthraquinone, emodin, that were used in the present study are shown in Fig. 1. The effects of the compound and *P. notoginseng* on NO production in RAW264.7 macrophages were investigated. The accumulated nitrite, estimated by the Griess method, in the culture medium was used as an index for NO synthesis from these cells. *P. notoginseng*, at a concentration of 20 g/ml, did not interfere with the reaction between nitrite and Griess reagent (data not shown). Unstimulated macrophages, after 24 hr of incubation in culture, produced background levels of nitrite (Fig. 2). When the resting cells were incubated with each indicated compound alone, the concentration of nitrite in the medium was maintained at a background level similar to that in the unstimulated samples. After treatment with LPS (100 ng/ml) for 24 hr, nitrite concentration was increased markedly about 20-fold (35 M). When macrophages were incubated with various concentrations of *P. notoginseng* and emodin (5, 10, or 20 g/ml) together with 100 ng/ml of LPS for 24 hr, significant concentration-dependent inhibition of nitrite production was found in the presence of *P. notoginseng* and emodin. Examination of the effects of *P. notoginseng* and emodin at 20 g/ml on RAW264.7 cell viability as determined by the MTT assay indicated that only emodin at this high concentration caused slight cytotoxicity (30%), whereas *P. notoginseng* did not affect cell viability (data not shown).

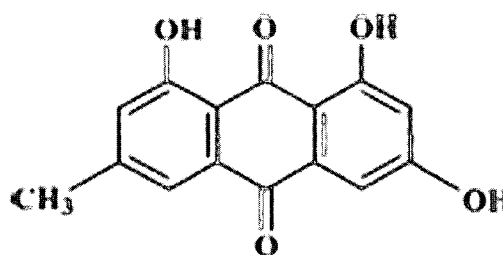


Fig. 1. Chemical structure of emodin

### 2. Effects of *P. notoginseng* and emodin on the expression of iNOS and COX-2 proteins in RAW264.7 macrophages

RAW264.7 macrophages did not express detectable iNOS protein when incubated in the medium alone for 24 or 7 hr, respectively. The basal level of iNOS in RAW264.7 cells was not affected when incubated with each of the eight compounds

alone (data not shown), whereas 100 ng/ml of LPS induced a dramatic increase in iNOS protein in these cells. *P. notoginseng* and emodin inhibited LPS-induced iNOS protein in a concentration-dependent manner. The amount of  $\alpha$ -tubulin protein as an internal control remained unchanged (Fig. 3).

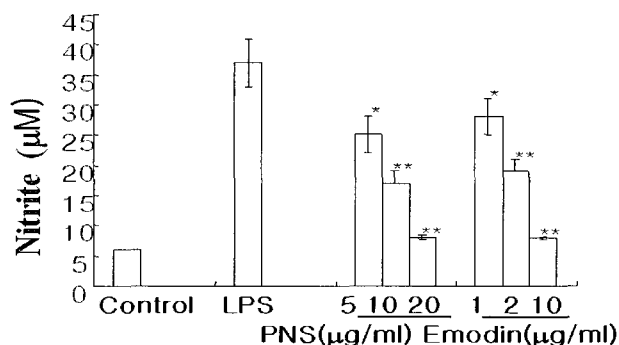


Fig. 2. Effects of *P. notoginseng* and emodin on LPS-induced nitrite production in RAW264.7 macrophages. The cells were treated with 100 ng/ml of LPS only or LPS plus 5  $\mu$ g/ml (1), 10  $\mu$ g/ml (2), or 20  $\mu$ g/ml (3) of *P. notoginseng* and emodin at 37°C for 24 hr. At the end of each incubation, 100  $\mu$ l of the medium was removed for measuring nitrite production. Control values were obtained in the absence of LPS or *P. notoginseng* and emodin. Data were obtained from three independent experiments and expressed as means  $\pm$  SD. Key: (\*)  $P < 0.05$  and (\*\*)  $P < 0.01$  indicate significant differences from the LPS-treated group.

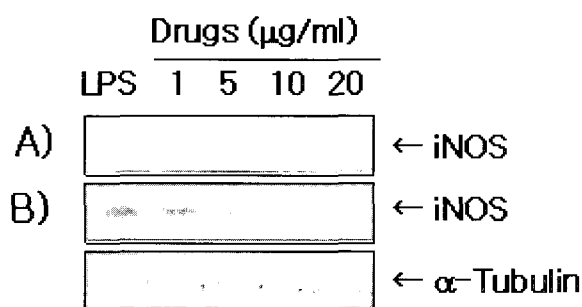


Fig. 3. Inhibition of LPS-induced iNOS protein in RAW264.7 macrophages by *P. notoginseng* and emodin. The cells were treated as described in Fig. 2. A) *P. notoginseng* B) emodin. Equal amounts of total proteins (50 g/lane) were subjected to 10% SDS-PAGE, and expression of iNOS and  $\alpha$ -tubulin protein was detected by western blotting using specific anti-iNOS and anti- $\alpha$ -tubulin antibodies.  $\alpha$ -Tubulin protein was used as an internal control. LPS = LPS-treated.

### 3. Effects of *P. notoginseng* and emodin on LPS-induced COX-2 protein expression

Since NO may directly activate expression of COX isoforms, and induction of COX gene expression has been shown to be involved in LPS-mediated response<sup>27,28,29</sup>, we investigated the effects of *P. notoginseng* and emodin on LPS-induced COX-2 protein expression. The results indicated that only *P. notoginseng* inhibited LPS-induced COX-2 expression at protein levels (Fig. 4. A,B). Furthermore, the marked increase in PGE<sub>2</sub>, a product of the COX-2 enzyme,

induced by LPS was inhibited significantly by *P. notoginseng* (1~20  $\mu$ g/ml). On the other hand, emodin at similar concentrations did not inhibit LPS-induced PGE<sub>2</sub> production (Fig. 4. C.).

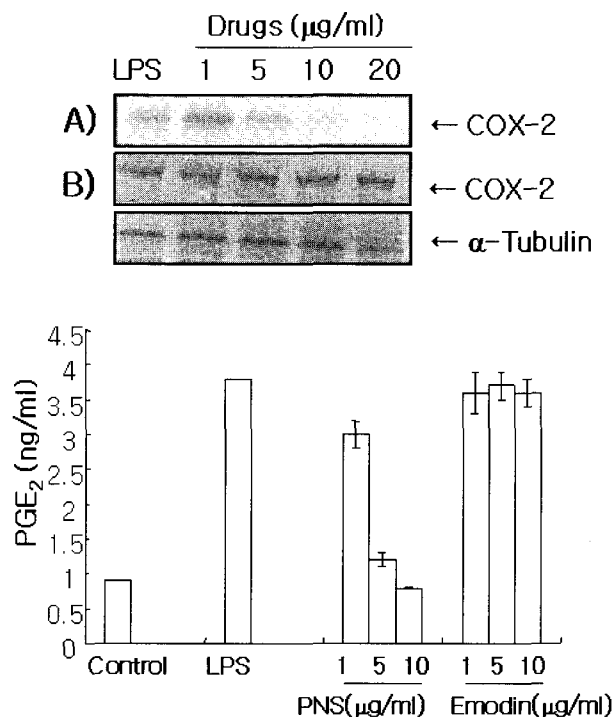


Fig. 4. Effects of *P. notoginseng* and emodin on production of COX-2 protein and PGE<sub>2</sub> in RAW264.7 macrophages. Cells were treated with LPS (100 ng/ml) only, or LPS plus *P. notoginseng* (1~20  $\mu$ g/ml) (A) or emodin (1~20  $\mu$ g/ml) (B) for 24 hr. Detection of COX-2 protein was performed by western blotting using a specific anti-COX-2 antibody.  $\alpha$ -Tubulin protein was used as an internal control among these eight compounds. (C) Effects of *P. notoginseng* and emodin on LPS-induced PGE<sub>2</sub> synthesis in cultured smooth muscle cells. PGE<sub>2</sub> concentrations in the culture medium were measured by ELISA. Key: (\*, \*\*) significantly different ( $P < 0.05$  and  $P < 0.01$ ) from LPS-treated samples.

## Discussion and Conclusion

The extract from the flower buds of *P. notoginseng* cultivated in Kyungbuk province of Korea was partitioned into a water to furnish a soluble fraction. From natural plants, several polyphenolic compounds showed their biological activities such as anti-inflammatory<sup>30</sup> and anti-carcinogenic activities<sup>31</sup>. Medicinal plants have been used as traditional remedies for hundreds of years. Among these herbs, *P. notoginseng*, which has been traditionally used for hepatitis, inflammation, osteomyelitis and gynecological diseases in Korea and China, was found to show potent anti-inflammatory effect. The water extract from the stems of *P. notoginseng* was found to inhibit inflammation. *P. notoginseng* has been used in Chinese and Korean medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases and

gynecological diseases. By bioassay-guided separation, emodin, emodin 8-O-glucopyranoside, resveratrol, baicalin, and berberine were isolated as active principles. The plant has been reported to contain a large number of flavonoids, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, and essential oils and amino acids, although the active principles in SB have not been determined fully.

*P. notoginseng* has also been used in Chinese medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases, hyperlipemia, and arteriosclerosis<sup>17</sup>. The active principle in *P. notoginseng* that exhibits these beneficial effects, however, has not been determined fully. Results of the present study indicated that *P. notoginseng* inhibited the LPS-induction of iNOS and COX-2 expression in macrophages without appreciable cytotoxic effects. These findings were consistent with a decrease caused by *P. notoginseng* in LPS-induced NO and PGE2 production. These results suggest that *P. notoginseng* may play an important role in inhibiting inflammatory processes.

Emodin, an anthraquinone derivative isolated from *P. notoginseng*, which also has been used in Chinese medicine to treat various diseases including inflammation<sup>32</sup>, is another potent inhibitor of LPS-induced NO production and iNOS protein synthesis. At 20  $\mu\text{g}/\text{ml}$ , it almost abolished LPS-induced iNOS protein synthesis and NO production. Emodin was the compound exhibiting cytotoxicity in about 30% of the cells at 24 hr post-treatment (not shown). This result suggests that the decrease in iNOS proteins and NO production 24 hr after treatment with emodin may be due, in part, to its cytotoxic effect on cell viability.

Results of the present study indicated that *P. notoginseng* and anthraquinones (emodin) differentially inhibited LPS-induced NO production and iNOS expression in RAW264.7 macrophages. *P. notoginseng* and emodin were potent inhibitors of LPS-induced NO production and iNOS expression. The mechanisms of inhibition of iNOS by *P. notoginseng* and emodin, however, were different. It was known that the emodin inhibits NF- $\kappa$ B activation. The finding is consistent with reports by others that the immunosuppressive effect of emodin in human mononuclear cells is not affected by indomethacin (a nonspecific COX-2 inhibitor)<sup>32</sup>.

It has been established that RAW264.7 macrophages are highly susceptible to endogenously generated or exogenously supplied NO<sup>33</sup>. Treatment of RAW264.7 macrophages with LPS and IFN has been shown to result in NOS induction and apoptosis. Both nitrite accumulation and apoptosis were blocked by the NOS inhibitor NG-monomethyl-arginine.

In summary, results of the present study indicated that *P. notoginseng* was an effective inhibitor of LPS-induced iNOS and COX-2 expression in RAW264.7 macrophages. Emodin, which also inhibited LPS-induced nitrite production and iNOS protein. *P. notoginseng* appears to be a potential therapeutic agent for treating LPS-induced sepsis syndrome.

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