

## ***Cedrela sinensis* Leaves Suppress Oxidative Stress and Expressions of iNOS and COX-2 via MAPK Signaling Pathways in RAW 264.7 Cells**

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### **Abstract**

Overproduction of reactive oxygen species (ROS), including nitric oxide (NO), could be associated with the pathogenesis of various diseases such as cancer and chronic inflammation. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are known to play key roles in the development of these diseases. *Cedrela sinensis* leaves have been used in Asian countries as a traditional remedy for enteritis, dysentery and itching. In the present study, we investigated the anti-inflammatory effects of *Cedrela sinensis* leaves in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Powder of *C. sinensis* leaves was extracted with 95% ethanol and fractionated with a series of organic solvents including *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and water. The dichloromethane (DCM) fraction strongly inhibited NO production possibly by down-regulating iNOS and COX-2 expression, as determined by Western blotting. Hydrogen peroxide-induced generation of reactive oxygen species (ROS) was also effectively inhibited by the DCM fraction from *C. sinensis* leaves. In addition, *C. sinensis* inhibited LPS-mediated p65 activation via the prevention of I $\kappa$ B- $\alpha$  phosphorylation. Furthermore, mitogen-activated protein kinases (MAPKs) such as ERK 1/2 and p38 were found to affect the expression of iNOS and COX-2 in the cells. Taken together, our data suggest that leaves of *C. sinensis* could be used as a potential source for anti-inflammatory agents.

**Key words:** *Cedrela sinensis* leaves, NO (nitric oxide), ROS (reactive oxygen species), iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), MAPK (mitogen-activated protein kinase), RAW 264.7 cells, anti-inflammation

### **INTRODUCTION**

*Cedrela sinensis* has been widely used as a traditional medicine in China and Korea. The leaves and stems have been used for the treatment of dysentery, enteritis, and itching in oriental medicine. In addition, the powdered roots have been used as a corrective, and the fruits have been used as an astringent and for the treatment of eye infection (1-3). Several flavonoids, limonoids, and phenolic compounds have been identified from *C. sinensis* (3-5). Crude extracts of *C. sinensis* have been shown to induce apoptosis of human ovarian cancer cells (6), enhance the glucose uptake in 3T3-L1 adipocytes (7), decrease the blood glucose levels of diabetic mice induced by alloxan (8), inhibit the steroidogenesis and activities of steroidogenic enzymes in normal mouse Leydig cell (9), and have cytotoxic activity on DU145 prostate cancer cells (10). It may be that the healing capacity of *C. sinensis* occurs through its ability to attenuate inflammation caused by factors such as reactive oxygen species (ROS), nitric oxide (NO), or cyclooxygenase-2 (COX-2).

Overproduction of ROS can cause oxidative damage to membrane lipids, DNA, proteins and lipoproteins, eventually leading to many chronic diseases such as cancer, chronic inflammation, and cardiovascular diseases. ROS also participate in the activation of intracellular signaling pathways, including those involved in modulating nuclear factor kappa B (NF- $\kappa$ B) activation (11).

Nitric oxide is another inflammatory mediator which is produced endogenously by a family of NO synthases (NOS) (12,13). NOS enzymes are classified into two groups; cNOS is constitutively present in several cell types while the inducible form, known as iNOS, is generally expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and astrocytes in response to proinflammatory cytokines, bacterial lipopolysaccharide (LPS), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), etc (14,15).

Another enzyme that plays a pivotal role in mediating inflammation is cyclooxygenase-2 (COX-2). Recent studies have suggested that COX-2 is barely detectable under normal physiological conditions, but is induced by pro-inflammatory mediators including LPS and mito-

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genic stimuli including cytokines in many different types of tumors and transformed cells (16-22). Furthermore, COX-2 is believed to be responsible for the production of pro-inflammatory prostaglandin (PGs) in various models of inflammation (23). Moreover, several studies indicated that mitogen-activated protein kinases (MAPKs) such as ERK, p38 and JNK have been implicated in the transcriptional regulation of the iNOS and COX-2 genes (24-27).

In the present study, we investigated the anti-inflammatory effects of *C. sinensis* leaves and studied the underlying molecular mechanisms through activity-guided fractionation. The results demonstrated that *C. sinensis* significantly inhibits LPS-induced NO and ROS formation as well as the expressions of iNOS and COX-2 via down-regulation of the ERK and p38 MAP kinase pathway and inhibition of p65 and I $\kappa$ B- $\alpha$  activation.

## MATERIALS AND METHODS

### Cell line and reagents

Murine macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Hyclone. 2',7'-dichlorodihydrofluorescein (DCF-DA) was purchased from Molecular Probe Inc. (OR, USA). Antibodies against iNOS, COX-2,  $\beta$ -actin monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phospho-JNK, phospho-ERK, phospho-p38, phospho-I $\kappa$ B $\alpha$ , and phospho-p65 monoclonal antibodies, p-p38 inhibitor (SB202190), p-JNK inhibitor (SP600125), and MEK1/2 inhibitor (U0126) were purchased from Cell Signaling Technology Inc. (OR, USA). Lipopolysaccharide (LPS, *Escherichia coli* O127:B8) was obtained from Sigma-Aldrich Co. (MO, USA). All other reagents used in this study were of the highest grade available.

### Plant material and extraction

The powder of *C. sinensis* leaves were kindly provided by Slow-life Chamjook Farm Corporation (Hamyang, Korea). The powder (7.3 kg) of *C. sinensis* leaves was extracted 3 times with 95% ethanol (EtOH) at room temperature for 24 hr ( $2 \times 10$  L). The EtOH extract was concentrated by rotary evaporation under 40°C. The concentrate was suspended in 10% EtOH and then fractionated with a series of organic solvent including n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), n-butanol (BuOH), water (H<sub>2</sub>O), in sequence. Each solvent fraction

was concentrated with rotary evaporator under 40°C and stored in a freezer (-20°C) until used for further experiments.

### Cell Culture

Murine macrophage RAW 264.7 cells were cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin. The cells were sub-cultured every 5~7 days at 1:5 split ratios. The medium was changed every 2 days. Cells were cultured for 24 hr (about 80 to 90% confluency). Then, the cells were treated with either vehicle (DMSO, 0.1%) or various concentrations of *Cedrele sinensis* and stimulated with LPS 1  $\mu$ g/mL.

### Cell viability assay

Cell viability was measured with a CellTiter 96 Non-radioactive Cell Proliferation Assay Kit (Promega Corporation, Madison, USA) according to manufacture's instruction. In brief, RAW 264.7 cells were seeded in 24-well plates and incubated at 37°C for 24 hr (about 80 to 90% confluency) and then treated with solvent soluble fractions from *C. sinensis* alone or in combination with LPS for 48 hr. After incubation time, medium was removed and culture media containing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was added and cultured for 1 hr at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, the absorbance was measured at 490 nm with ELISA reader (PowerWave XS, BioTek, USA). This assay was repeated three times with triplicate samples at each measurement.

### Nitrite determination

RAW 264.7 cells were plated at  $1 \times 10^5$  cells/well in 24-well plates and then incubated with or without LPS (1  $\mu$ g/mL) in the absence or presence of various concentrations of *C. sinensis* fractions for 12 hr. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels. Briefly, 100  $\mu$ L of cell culture medium was mixed with 100  $\mu$ L of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl], and incubated at room temperature for 10 min. Absorbance was then measured at 550 nm using a microplate reader (PowerWave XS, BioTek, USA). Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were read off of a standard sodium nitrite curve.

### ROS formation assay

The intracellular ROS production was measured using a cell permeable probe, 2',7'-dichlorofluorescein diacetate

(DCF-DA) (28). This method measures the formation of ROS generated by an oxidative metabolic burst. Viable cells can acetylate DCF-DA to 2',7'-dichlorofluorescein, which is not fluorescent. When this compound reacts quantitatively with oxygen species within the cell, the fluorescent 2',7'-dichlorofluorescein is produced, which remains trapped within the cells and can be measured to provide an index of intracellular oxidation. To study the effect of solvent soluble fraction from *C. sinensis* on ROS production, the cells were loaded with 20  $\mu\text{M}$  DCF-DA (dissolved in DMSO) for 1 hr at 37°C in darkness. After washing out the excess probe, the cells were treated for 1 or 24 hr and then media was removed and washed twice with 1X ice-cold PBS. The fluorescence was measured at 485/20 nm excitation and 528/20 nm emission in a fluorescence multi-detection reader (Synergy HT™ Multi-detection microplate reader, BIO-TEK Instruments, Inc, USA).

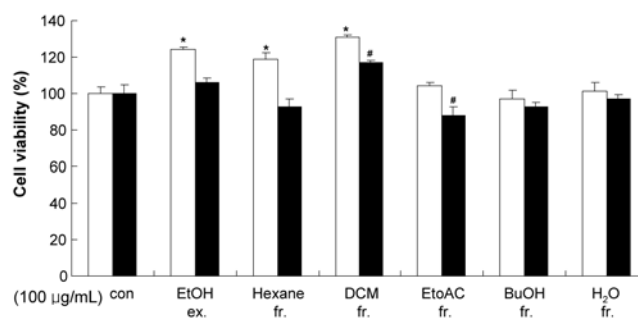
#### Western blot analysis

The RAW 264.7 cells were treated with various concentrations of *C. sinensis* fractions and stimulated with LPS, and then the treated cells were washed twice with ice cold PBS (pH 7.4) and harvested using a cell scraper. The cell pellets were resuspended in a lysis buffer on ice for 1 hr, and cell debris was removed by centrifugation at 13,000 rpm for 10 min. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, USA). Equal amounts of proteins were mixed with 4 $\times$ Laemmli loading buffer and pre-heated at 95° for 5 min. The samples were then loaded onto a 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane for 1 hr with a semidry transfer system (Bio-Rad). The membranes were blocked with 5% nonfat milk in PBST with 0.1% Tween 20 for 1 hr at room temperature, and then incubated overnight with primary antibodies. After hybridization with primary antibodies, the membrane was washed five times with PBST for 5 min, then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature and washed five times with PBST for 5 min. Final detection was performed with Immuno-Star horse-radish peroxidase Western blotting reagents (Pierce, Rockford, USA).

## RESULTS AND DISCUSSION

#### Cell viability

The cytotoxicity of organic solvent fractions from *C. sinensis* leaves on the RAW 264.7 cells were determined by MTS assay after 24 hr treatments. As shown in Fig. 1, ethanol extract and solvent fractions from *C. sinensis*

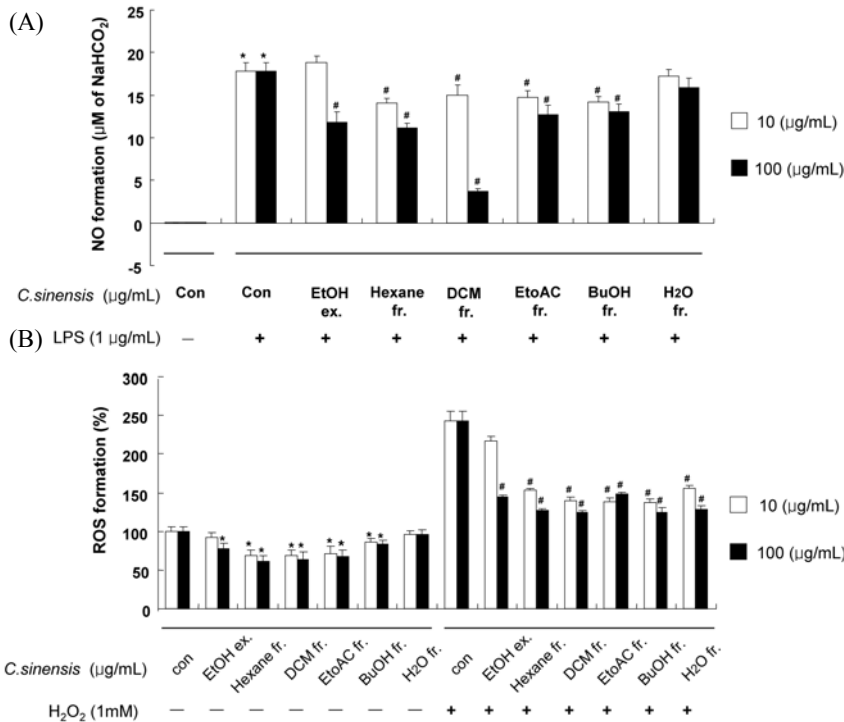


**Fig. 1.** Effect of *C. sinensis* leaves on cell viability in RAW 264.7 macrophages cells. RAW 264.7 cells were cultured with the indicated concentration of *C. sinensis* alone or in combination with LPS (1  $\mu\text{g}/\text{mL}$ ) at 37° in a 24-well plate for 24 hr. Cell viability was evaluated as described in Materials and Methods, and is expressed as a percentage of the control without the addition of *C. sinensis* leaves. Each bar (open bar, no treatment; closed bar, LPS treated) represents the mean  $\pm$  SD of three individual experiments. \*#Significantly different from the no treatment and LPS treated control, respectively ( $p < 0.05$ ).

(either in the presence or absence of 1  $\mu\text{g}/\text{mL}$  LPS) did not cause any cytotoxicity at 100  $\mu\text{g}/\text{mL}$ . Therefore, subsequent experiments were performed with concentrations at or below 100  $\mu\text{g}/\text{mL}$ .

#### NO and ROS production

To investigate the potential anti-inflammatory properties of solvent fractions from *C. sinensis* on the production of NO and ROS, we treated each solvent fraction or vehicle (0.1% DMSO) onto RAW 264.7 cells for 1 hr and then challenged with LPS (1  $\mu\text{g}/\text{mL}$ ) or H<sub>2</sub>O<sub>2</sub> (1 mM) for 24 hr, respectively. Increased production of NO plays a critical role in the process of macrophage activation and is associated with acute and chronic inflammations (29). In addition, an overproduction of ROS accompanied by oxidative damage may be deleterious to cells and tissues (30,31). Therefore, suppressing excessive NO and ROS by antioxidant molecules can be a potential protective tool against the development of inflammation and/or cancer. The amount of nitrite, a stable metabolite of NO, was used as an indicator of NO production in the cells. LPS (1  $\mu\text{g}/\text{mL}$ )-stimulated cells had significantly increased nitrite levels compared with control. In addition, treatments with DCM fraction at 100  $\mu\text{g}/\text{mL}$  dramatically inhibited the NO production stimulated by LPS (Fig. 2A). Treatment with H<sub>2</sub>O<sub>2</sub> (1 mM) resulted in 2.5-fold induction of ROS formation which was significantly suppressed by treatments with most of the solvent fractions (Fig. 2B). In addition, the fractions of hexane, DCM and EtOAc decreased the H<sub>2</sub>O<sub>2</sub>-stimulated ROS formation even at the basal levels.

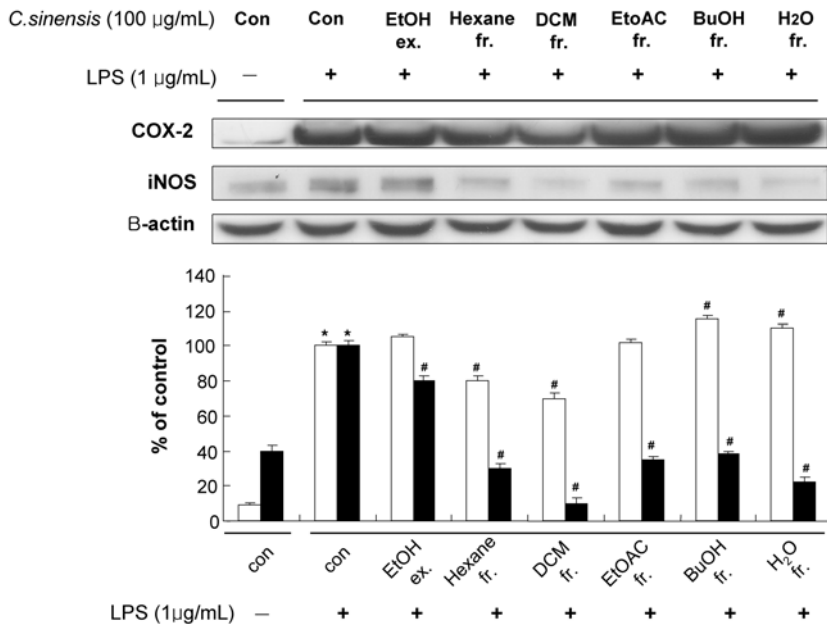


**Fig. 2.** Effect of *C. sinensis* on LPS/H<sub>2</sub>O<sub>2</sub>-induced NO formation and ROS formation in RAW 264.7 cells. (A) NO production. The cells were treated with LPS only or with different concentrations of *C. sinensis* for 24 hr. At the end of incubation time, the amount of nitrite oxide in the culture supernatants was measured by the Griess reaction assay, as described in Materials and Methods. The values are expressed as the means ± SD of three individual experiments. (B) ROS production. The level of intracellular ROS was measured with DCF-DA. RAW 264.7 macrophage cell was pre-treated with DCF-DA for 1 hr, and exposed to *C. sinensis* leaves for 1 hr more formation of ROS in the cells was evaluated by the arbitrary fluorescence unit and described as fold induction test via control vehicle (DMSO, 0.1%). Values are means of three independent experiments ± SD (n=6). \*#Significantly different from the no treatment and LPS treated control, respectively (p<0.05).

**iNOS and COX-2**

To investigate whether the inhibitory effects of solvent fractions from *C. sinensis* leaves on the production of NO and ROS are related to the corresponding gene expressions, the protein expression of iNOS and COX-2 were determined by Western blot analysis. As shown in Fig. 3, LPS treatment strongly stimulated the protein expressions of iNOS as well as COX-2 in RAW 264.7 cells. However, the DCM fraction significantly suppressed the LPS-stimulated expressions of iNOS and

COX-2. These results are consistent with the data we obtained from NO and ROS analyses in Fig. 2, suggesting that the inhibitory effects of DCM fraction on the formation of NO and ROS are at least in part due to its inhibition on iNOS and COX-2 expressions. There have been several studies showing that plant-derived extracts or isolated compounds can inhibit the expression of iNOS and COX-2. For example, extract of *Ginkgo biloba* has been shown to suppress the expressions of both iNOS and COX-2 (32). Also, curcumin inhibits ex-



**Fig. 3.** Effects of leaves from *C. sinensis* on LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages for 12 hrs. The total lysates of the proteins were subjected to Western blot analysis, as described in Materials and Methods. The ratio of immunointensity between the COX-2/iNOS and the β-actin calculated. Each bar (open bar, COX-2; closed bar, iNOS) represents the means ± SD from three independent experiments. \*#Significantly different from the no treatment and LPS treated control, respectively (p<0.05).

pression of COX-2 *in vivo* study, and capsaicin and deursin also inhibit of iNOS and COX-2 in macrophage cells (32-35). Taken together, these data suggest that iNOS and COX-2 serve as key mediators of inflammation; thus natural agents that suppress these proteins could be a potential anti-inflammatory agent.

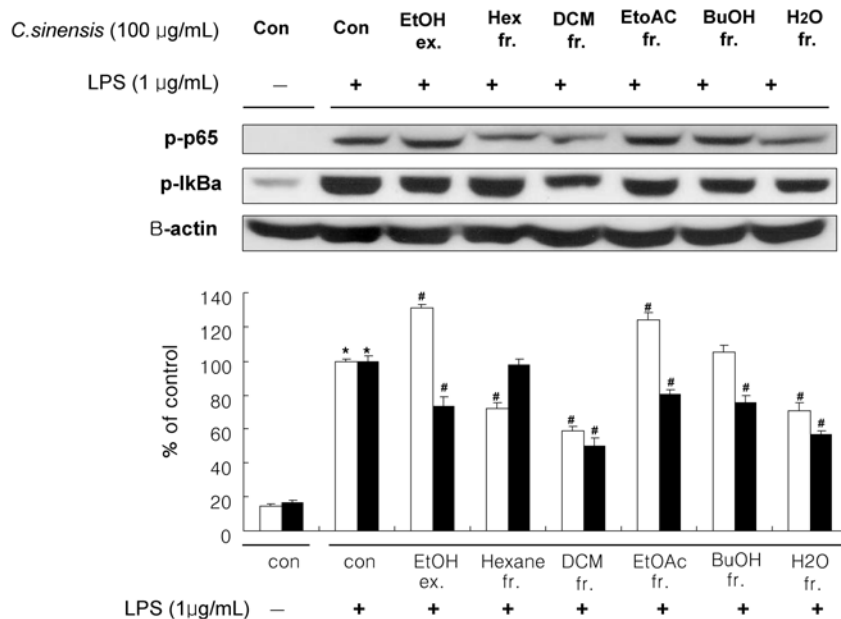
### Phosphorylation of p65 and I $\kappa$ B- $\alpha$

NF- $\kappa$ B plays an important role in the control the expression of cell survival and the expression of pro-inflammatory mediators such as iNOS, COX-2 and IL-6 (36). NF- $\kappa$ B is directly linked to I $\kappa$ B- $\alpha$  phosphorylation, since the signal-induced phosphorylation of I $\kappa$ B- $\alpha$  is considered as a key event that ultimately leads to the activation of NF- $\kappa$ B (37). Reactive oxygen species are one of the important activators of the I $\kappa$ B kinase complex (38). We investigated whether solvent fractions from *C. sinensis* leaves could prevent the phosphorylation of p65, which is a major component of NF- $\kappa$ B, and I $\kappa$ B- $\alpha$  by Western blot analysis. Our results indicate that the phosphorylations of p65 and I $\kappa$ B- $\alpha$  are strongly inhibited by DCM fraction (Fig. 4), which is again correspondent with the data obtained from analyses of iNOS and COX-2 (Fig. 3). These findings are consistent with other studies, which have shown that NF- $\kappa$ B response elements are present the promoters of the iNOS and COX-2 (39,40).

### MAPKs

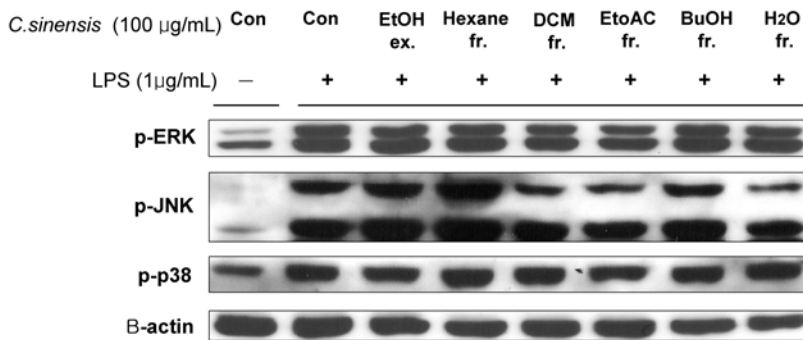
MAPKs including ERK, p38 and JNK are known to be involved in LPS-induced iNOS and COX-2 expression via activation of the related transcription factors (41-44). We examined the effects of solvent fractions

from *C. sinensis* leaves on the LPS-induced phosphorylation of MAPKs by using Western blot analysis in RAW 264.7 cells. As shown in Fig. 5(A), solvent fractions from *C. sinensis* leaves inhibited the phosphorylation of MAPKs. These results suggest that inhibition of iNOS and COX-2 by *C. sinensis* leaves might be due to the inhibition of MAPK phosphorylation. To further confirm the MAPK signaling pathway mostly involved in the inhibitory effect of DCM fraction on LPS-induced iNOS and COX-2 expression, each specific inhibitor of MAPKs (U0126, ERK inhibitor; SB202190, p38 inhibitor; SP600125, JNK inhibitor) was employed. As shown in Fig. 5(B), pretreatment of inhibitors profoundly inhibited LPS-induced iNOS and COX-2 expression. Among those inhibitors, the U0126 and SB202190 strongly recovered the DCM-inhibited expression of iNOS protein, suggesting involvement of ERK and p38 in the anti-inflammatory mechanism of *C. sinensis* leaves. On the basis of our findings, MAPKs are involved in the inhibition of pro-inflammatory mediator expression by *C. sinensis* leaves in RAW 264.7 cells. In conclusion, our findings indicate that *C. sinensis* leaves, especially the DCM fraction, inhibited LPS-induced NO production as well as H<sub>2</sub>O<sub>2</sub>-induced ROS production. In addition, the anti-inflammatory activity of *C. sinensis* results from the inhibition of iNOS and COX-2 expression in RAW 264.7 cells, and these effects are mediated by the inhibition of NF- $\kappa$ B/ I $\kappa$ B- $\alpha$  activity via down-regulation of the p38 and ERK of MAPK signal pathways. These results suggest that the extract from *C. sinensis* leaves, especially the DCM fraction, may be a potent natural anti-inflammatory agent.

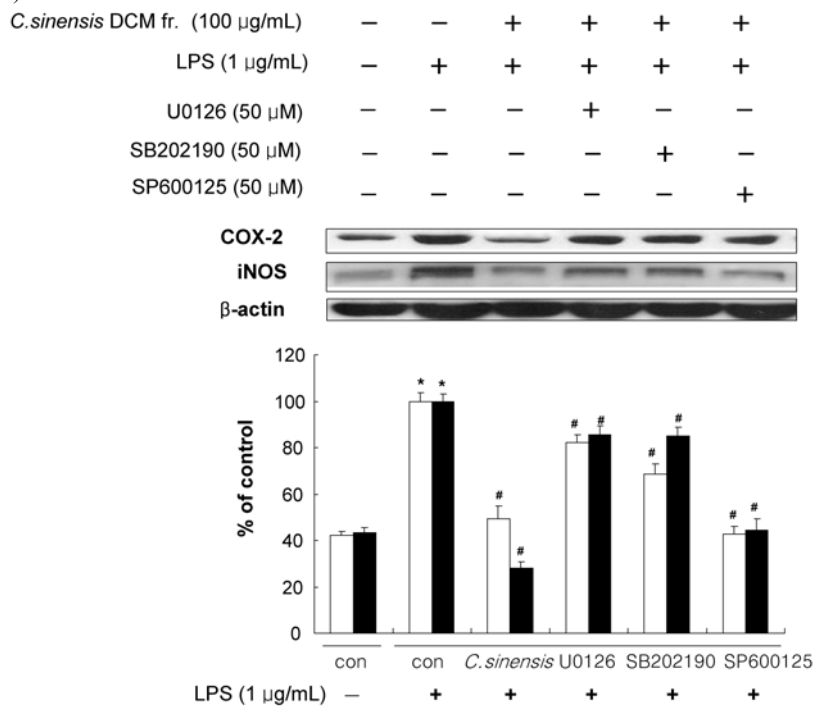


**Fig. 4.** Effect of *C. sinensis* on LPS-induced phosphorylation of NF- $\kappa$ B (p65) subunit and degradation of I $\kappa$ B in RAW 264.7 cells for 1 hr. The total lysates of the proteins were subjected to Western blot analysis. The ratio of immunointensity between the p-p65/ I $\kappa$ B and the  $\beta$ -actin calculated. Each bar (open bar, p-p65; closed bar, I $\kappa$ B) represents the means  $\pm$  SD from three independent experiments. \*#Significantly different from the no treatment and LPS treated control, respectively (p<0.05).

(A)



(B)



**Fig. 5.** Effect of *C. sinensis* on LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages. (A) The expression of phospho-ERK1/2 (p-ERK), phospho-JNK1/2 (p-JNK), and phospho-p38 (p-p38) were analyzed by Western blot. Cells were treated with LPS (1 µg/mL) for 1 hr. (B) Effects of MAPKs inhibitors on LPS-induced iNOS and COX-2 production. Cells treated with LPS (1 µg/mL) for 1 hr in the presence of JNK (SP600125), ERK (U0126), and p38 (SB202190) inhibitors. B-actin was used as a loading control. Each bar (open bar, COX-2; closed bar, iNOS) represents the means  $\pm$  SD from two independent experiments. \*#Significantly different from the no treatment and LPS treated control, respectively ( $p < 0.05$ ).

## ACKNOWLEDGEMENTS

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