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1. ABSTRACT

In this study, the chemical constituents of *Lindera erythrocarpa* Makino essential oil (LEO) were investigated useding GC–MS. The main constituents of LEO were Nerolidol (18.73%),Caryophyllene (14.41%), α-Caryophyllene(7.73%), Germacrene-D (4.82%) and α-pinene (4.47%). We also investigated the effects of LEO on the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), tumour necrosis factor (TNF)-α, and IL-6, in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Western blotting tests indicated that LEO has suppression effects on pro-inflammatory cytokines and mediators, dose-dependent manner. We investigated the action mechanism by which LEO suppressed NO and PGE_2 by examining the level of nuclear factor-κB (NF-κB) activation within the mitogen-activated protein kinase (MAPK) pathway, which is an inflammation suppressed signal pathway in RAW 264.7 cells. LEO suppressed LPS-induced ERK, JNK, and p38 phosphorylation. Furthermore, LEO inhibited the LPS-induced phosphorylation and degradation of Iκ-B-α, which is required for the nuclear translocations of the p50 and p65 NF-κB subunits in RAW 264.7 cells. Our results suggest that LEO might demonstrate an anti-inflammatory effect by suppressing the expression of pro-inflammatory cytokines. Such an effect is mediated by a blocking of NF-κB activation which accordigly suppress inflammatory mediators in RAW 264.7 cells. In conclusion, LEO is suggested as a potential candidate treating inflammatory diseases.

Keywords : *Lindera erythrocarpa* Makino essential oil (LEO), lipopolysaccharide (LPS), pro-inflammatory cytokines, nuclear factor-κB (NF-κB), mitogen-activated proteinkinase (MAPK)

2. Introduction

Inflammation is involved in a variety of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and inflammatory brain diseases(Chung, *et al*., 2007). Lipopolysaccharide (LPS) activates macrophages to produce pro-inflammatory mediators , such as nitric oxide(NO), prostaglandin E_2 (PGE_2), as well as proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2(COX-2)(Korhonen *et al*.,2005). NO is overproduced endogenously by iNOS which is induced in response to proinflammatory cytokines and LPS(D. Salvemini and Marino, 1998). COX-2 is also induced by several stimuli and is responsible for the production of large amounts of proinflammatory prostaglandins at the inflammatory sites(Tsatsanis *et al*.,2006). Therefore, the inhibition of these inflammation mediators is an important target pathway in the treatment of disease with anti-inflammatory components. (Surh *et al*.,2001, A. Murakami and Ohigashi,2007)

Many studies have shown that the expressions of several cytokines genes, including tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), are associated with Nuclear transcription factor kappa-B (NF- κB)(Kim *et al.*,2009).

Nuclear transcription factor kappa-B (NF- κB) regulates different genes associated with immune and acute phase inflammatory responses. NF-κB activation, in reaction to pro-inflammatory stimuli, associates the phosphorylation of IκBs by the IKK signalosome complex. NF-κB activeted by this process translocates to the nucleus, where it binds to κB-binding receptor. It then induces the transcription of pro-inflammatory mediators such as iNOS, COX-2, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 (Ghosh and Hayden, 2008, Edwards *et al*., 2009, Wong and Tergaonkar, 2009). Recently, many studies have demonstrated the role of phytochemicals in anti-inflammatory activity through

downregulation of NFκB pathway(Hsieh *et al.*,2011, Kim *et al.*,2012).

The mitogen-activated protein kinases (MAPKs) are one of the major kinase families associated with cellular processes such as differentiation, stress responses, apoptosis, and immune defense(Liu, *et al.*,2007). MAPKs are consist of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. They play a crucial role in inducing cytokine production and the expression of iNOS and COX-2(Rao, 2001, Rajapakse, *et al*.,2008). So, NF-κB and MAPK are main factors in the inflammatory process.

Lindera erythrocarpa Makino belongs to the large family Lauraceae, which consists mostly of trees or shrubs from the warmer regions of the earth,It is widely distributed in Republic of Korea, Japan, China(Sun BY, Chung YH 1988). *Lindera* species, containing *L. lucida, L. strychnifolia,L. aggregate* and *L. chunii* are significant medicinal plants. The fruit of *L. erythrocarpa* is used as a traditional medicine for analgesic, digestive, diuretic, antidote, and antibacterial purposes; also, its leaves have been used as a folk medicine for stomach-ache, thirst, and neuralgia(Hong, *et al.*,2009, Liu, Ogihara, 1975, Liu, *et al.*,1976, Oh, M.,2005). Recently *L.erythrocarpa* has been reported suppresses adipogenesis, attenuates obesity, suppresses melanin, antioxidant, anti-inflammatory, antifungal(Hwang,2007, Hsieh, H., Wang, 2000, Kumar, *et al.*, 2010, Wang, Lan *et al.*, 2008). However, the action mechanisms by which the essential oil from *L. erythrocarpa* essential oil(LEO) exerts its anti-inflammatory effect is unknown.

Therefore, we examine the anti-inflammatory effects of *Lindera erythrocarpa* essential oil(LEO) in LPS-stimulated RAW 264.7 cells. The results show that LEO suppressed LPS-induced NO, PGE_2, IL-6 and TNF-α by inhibiting activation of NF-κB pathway, as well as the MAPKs signaling pathway in LPS-stimulated RAW 264.7 cells.

3. Materials and methods

3.1. Reagents

Lipopolysaccharide (LPS, *E. coli* 0111:B4) was bought from Sigma-Aldrich Chemical Co. (St. Louis, MO,USA). dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) were bought from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from Invitrogen-Gibco(GrandIsland, NY, USA). ELISA kits for TNF-α, IL-6 and PGE_2 were bought from R&D Systems, Inc. (St. Louis, MO, USA) and BD Biosciences (San Diego, CA, USA). Antiphosphorylated IκB-α (anti-p-IκB-α), anti-NF-κB, anti-JNK, anti-phosphorylated JNK (anti-p-JNK), anti-ERK1/2, anti-phosphorylated ERK1/2 (anti-p-ERK1/2), anti-p38, and anti-phosphorylated p38 (anti-p-p38) mouse or rabbit antibodies were bought from CellSignaling Technology (Beverly, MA, USA). All other reagents were bought from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

3.2. Essential oil extraction of leaves of *L.eryherocarpa*

Leaves of *L.erythrocarpa* were collected from Namwon(a region in Jeju Island, Korea) in May 2014 . The LEO was extracted by hydrodistillation. Briefly, approximately 300g of fresh *Lindera erythrocarpa* leaves was immersed in 3ℓ of distilled water in a 5-l three-neck flask. Subsequently The acquired essential oil was dried over sodium sulfate, filtered, and stored in a sealed vial at 4℃ until tested. The essential oil yield was approximately 0.067% (v/w).

3.3. GC–MS analysis

An analysis of the principal components in the most active essential oil was carried out using an Agilent 6890 gas chromatograph (GC) connected to an Agilent 5975 mass spectrometer (MS,Agilent Technologies Inc., Santa Clara, Calif., USA). The GC was equipped with a DB1-HT column with a 30 m × 0.32 mm × 0.1 μm film thickness. The oven temperature was programmed to increase from 40 to 100℃ at a rate of 2℃ per min, and then from 100 to 230℃ at a rate of 5℃ per min;temperature was then held at 230℃ for 5 min (71 min analysis time). The injector and detector temperatures were 240 and 280℃, respectively. The flow rate of the carrier gas (He) was 1.5 ㎖ per min and the split ratio was 1:10. For the injection (split less), 10 ㎕ of essential oil was diluted in 500 ㎕ of CH_2Cl_2; 1 ㎕ of this diluted solution was injected. Identification of the compounds was achieved by comparison of their mass spectra with those of the Wiley libraries. The GC–MS retention indices were also calculated using a n-alkanes C6-C31.

3.4. Cell culture

The murine macrophage cell line RAW 264.7 was bought the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium(DMEM; GIBCO Inc., NY, USA) added with 100 units/㎖ of penicillin, 100 ㎍/㎖ of streptomycin and 10% fetal bovine serum (FBS; GIBCO). The cells were incubated in an atmosphere of 5% CO_2 at 37℃ and were subcultured every 3 days.

3.5. Lactate dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1.8×10^5 cells/㎖) plated on 96-well plates were treated with aliquots of LEO at 37℃ for 24 h. The medium was cautiously removed from each well, and the medium was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). In brief, 100 ㎕ of reaction mixture were added to each well, after the reaction was incubated for 30 min at room temperature in darkness. The absorbance was measured a UV spectrophotometer (490nm).

3.6. Cell viability assay

The cytotoxicity of LEO against the RAW 264.7 cells was assessed via a colorimetric MTT assay. RAW 264.7 cells (1.8×10^5 cells/㎖) plated on 96-well plates were treated with aliquots of LEO at 37℃ for 24 h. MTT stock solution (50 ㎕; 2 ㎎/㎖ in PBS) was added to each well to accomplish a total volume of 250 ㎕. After 4 h of incubation, the plates were centrifuged for 10 min at 2000 rpm, and the supernatants were cautiously aspirated. The formazan crystals were dissolved by DMSO. The purple formazan absorbane of each well was measured a UV spectrophotometer (540 nm).

3.7. Determination Nitric oxide(NO) production

After pre-incubating RAW 264.7 cells (1.8×10^5 cells/㎖) with LPS (1 ㎍/㎖) for 24 h, the quantity of nitrite in the culture medium was measured and used as an indicator of NO production. Briefly, 100 ㎕ of cell culture medium was mixed with 100 ㎕ of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated at room temperature for 10 min; absorbane of each well was measured a UV spectrophotometer (540 nm). New culture medium was used as a blank.

The quantity of nitrite was determined on the basis of a sodium nitrite standard curve. All experiments were performed in triplicate.

3.8. Determination of PGE_2 production

LEO was diluted with DMEM prior to treatment. Cells were treated for 24 h with LPS (1 ㎍/㎖) to stimulate PGE2 production. The PGE2 concentration in the medium was quantified using a enzyme immunoassay kit (R&D Systems Inc., MN, USA) in accordance with the use manual. The production of PGE2 was determined relative to that observed after control treatment.

3.9. Determination of TNF-α, IL-6 prodction

ELISA was used to determine the suppress effects of 0.01%, 0.02%, and 0.04% of oil on the production of cytokines TNF-α, IL-6 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated for 24 h before the supernatant was cautiously harvested and tested according to the use manual for the ELISA kit (R&D Systems Inc., MN, USA).

3.10. Western blot analysis

RAW 264.7 cells were treated to LPS with absence or presence of the elapsed concentration of LEO. After the elapsed times of incubation, the cells were washed twice with cold phosphate buffered saline (PBS) and were lysed in a protein lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 ㎍/㎖ a protinin, and 25 ㎍/㎖ leupeptin], and then the cells were collected. Then The cell lysates were isolated using centrifugation at 15,000 rpm (4℃ for 15 min). The protein concentration in

the supernatants was measured by the Bradford assay (Bio-rad, Herculers, CA, USA) and all proteins were adjusted to equal protein content. Aliquots of the lysates (20~30 μg protein/lane) were separated on a NuPAGE 4-12% bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and the separated proteins were transferred from the electrophoresis gel to the surface of polyvinylide nedifluoride (PVDF) membranes using an iBlot gel transfer device (Invitrogen, Carlsbad, CA,USA). Then membranes were treated with 5% non-fat skim milk as a blocking solution, and incubated with primary antibodies (1:1,000) at 4℃ overnight. After incubating, the membranes were washed by Tween 20-Tris-buffered saline (TBST) several times and incubated with secondary HRP-linked anti-rabbit or anti-mouse IgG (1:5,000, Cell signaling) for 90 min at room temperature. After washing, immunoactive proteins were detected by the WEST-ZOL (plus) Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Korea).

3.11. Statistical analysis

All data are expressed as means ± S.D. Statistical analyses of data were done by Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

4. Results

4.1. Chemical composition of LEO

The general chemical profile, percentage content, and retention indices of the constituents of LEO are summarized in Table 1 and Figure. 1. A total of 15 volatile constituents were identified on the basis of their mass spectra, which were compared to those in the computer library. Only the components with matches exceeding 95% were characterized; these represented 63.73% of the LEO. The main constituents of LEO were Nerolidol (18.73%), Caryophyllene (14.41%), α-Caryophyllene (7.73%), Germacrene-D (4.82%) and α-pinene (4.47%).

Table 1. Chemical composition of the essential oil from *Lindera erythrocarpa*

RT (min)	Constituent	Peak area (%)
6.881	3-Hexen-1-ol	0.39
8.998	α-pinene	4.47
9.570	Camphene	3.03
10.726	β-pinene	1.76
11.447	β-Myrcene	0.92
13.026	Dl-Lionene	0.61
14.004	β-ocimene	2.23
16.791	β-Linalool	0.67
24.052	Geranlol	0.73
25.265	Bornyl acetate	2.56
30.570	**Caryophyllene**	**14.41**
31.966	α-Caryophyllene	7.34
33.087	Germacrene-D	4.82
34.787	δ-Cadinene	1.05
36.704	**Nerolidol**	**18.73**
Total identified		63.73

RT, retention time.

Components were identified by comparison of their mass spectra in the GC-MS library (wiley 138)

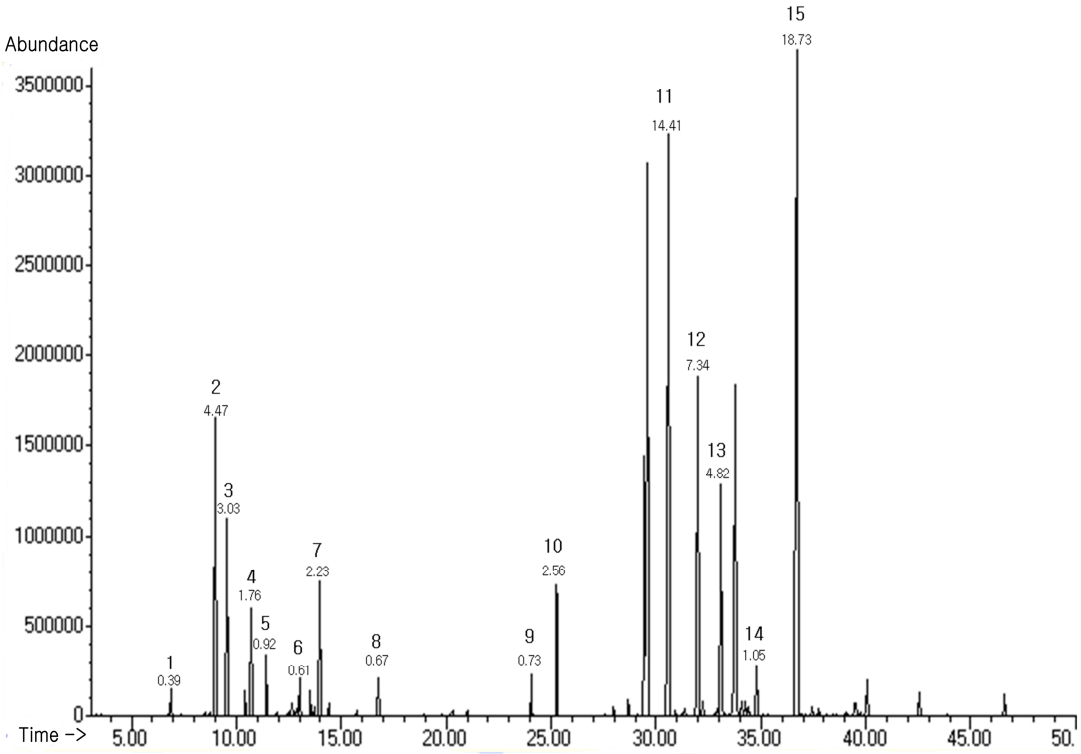

Figure. 1. GC–MS total ion chromatogram of the of LEO. The compound labels in the chromatogram correspond to the Arabic numerals given in Table 1.

4.2. Cytotoxicity and cell viability on LEO in RAW264.7 cells.

We fist measured the cytotoxicity of LEO in RAW264.7 cells by using the LDH and MTT assay. LEO did not influence the cytotoxicity of RAW 264.7 cells at the employed concentrations (0.01, 0.02, and 0.04%).(Figure. 2). Therefore, in all subsequent experiments, we used concentrations of LEO ranging from 0.01% to 0.04%.

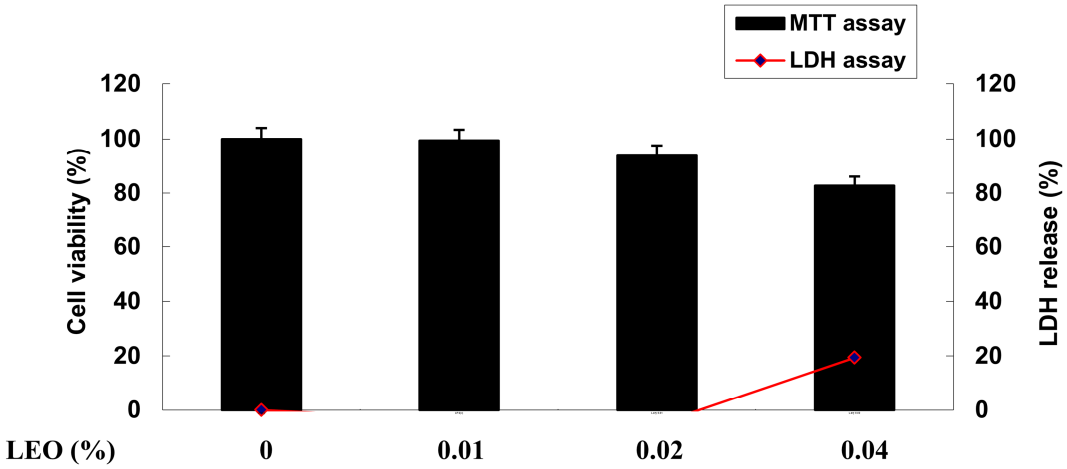

Figure. 2. Cytotoxicity of LEO on the viability of RAW 264.7 cells. RAW 264.7 cells (1.8 × 10^5 cells/㎖) plated on 96-well plates were treated with aliquots of LEO at 37℃ for 24 h. Cytotoxicity of LEO was assessed by MTT and LDH assays. Values are expressed as means ± S.D. of triplicate experiments. Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*

4.3. Effect of NO production on LEO in LPS-stimulated RAW 264.7 cells

LPS-induced production of NO from macrophages occurs in the inflammatory response. Nitrite levels, as measured with Greiss reagent, are used as a measure of NO. The LEO(0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced on increases in NO.(Figure. 3).

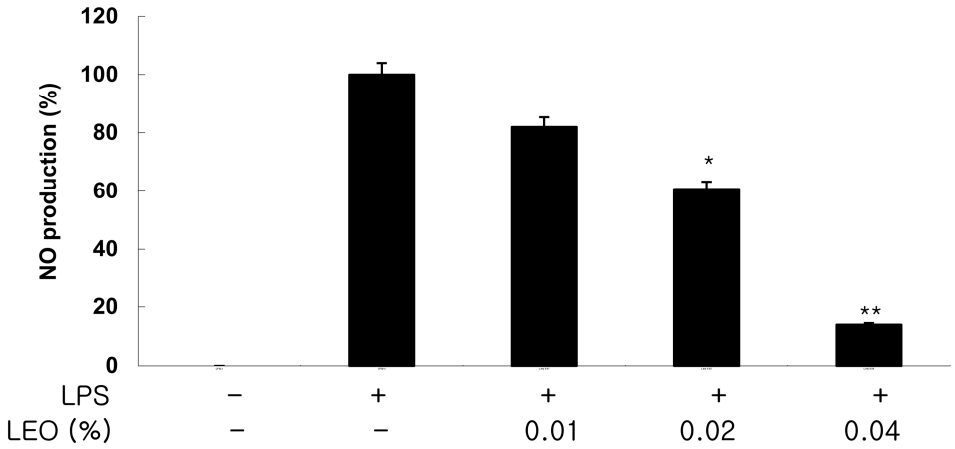

Figure 3. Inhibitory effect of nitric oxide production on LEO in RAW 264.7 cells. The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 ㎍/㎖) for 24h in the presence of LEO (0.01, 0.02, and 0.04 %). Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*

4.4. Effect of PGE_2 production on LEO in LPS-stimulated RAW 264.7 cells.

LPS was used to stimulate the release of PGE_2 from macrophage cells. PGE_2 is an inflammatory mdediator produced from the conversion of arachidonic acid by cyclooxygenase. In varity of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount of PGE_2 at inflammatory sites. LPS can stimulate PGE_2 release from RAW 264.7 macrophages. LPS (1㎍/㎖) treatment for 24h increased PGE_2 levels in the culture medium. The LEO(0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced on increases in PGE_2 production. (Figure. 4).

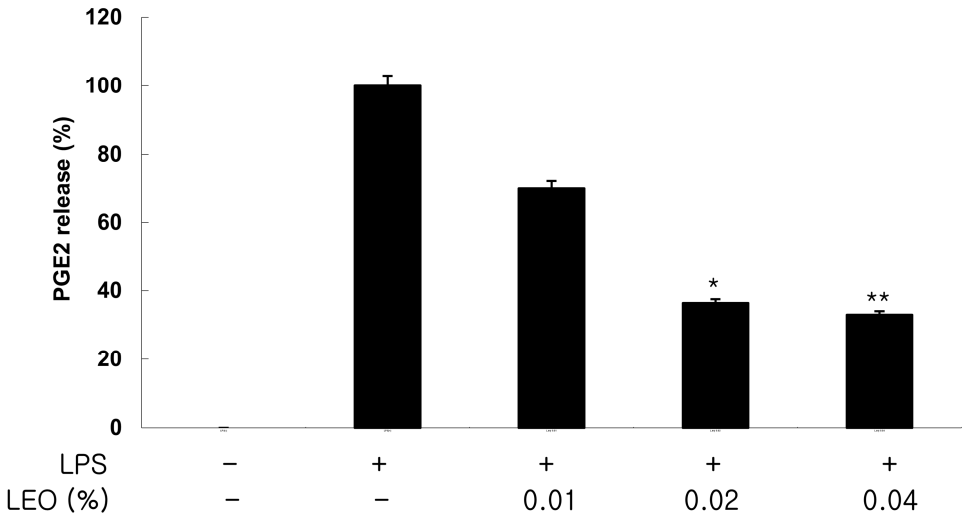

Figure 4. Inhibitory effect of PGE_2 production on LEO in RAW 264.7 cells. Cells (1.8×10^5 cells/㎖) were stimulated by LPS (1㎍/㎖) for 24h in the presence of LEO(0.01, 0.02, and 0.04 %) Supernatants were collected, and the PGE_2 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*

4.5. Effects of IL-6 and TNF-α production on LEO in LPS-stimulated RAW 264.7 cells

LEO was found to potently inhibit the pro-inflammatory mediators NO and PGE2, after we investigated its effects on LPS-induced in IL-6 and TNF-α release; this was done by enzyme immunoassay(EIA). The LEO (0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced increases in IL-6 and TNF-α production. (Figure. 5-1,2).

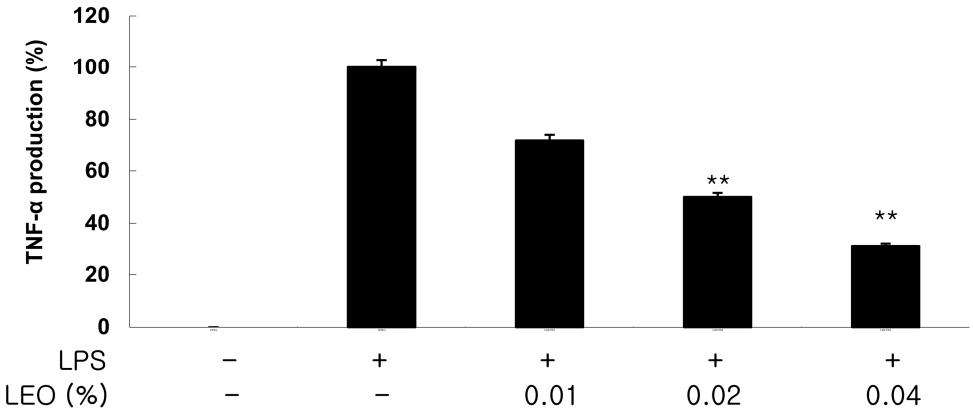

Figure 5-1 . Inhibitory effect of TNF-α production on LEO in RAW 264.7 cells. Cells (1.8×10^5 cells/ml) were stimulated by LPS (1 ㎍/㎖) for 24 h in the presence of LEO(0.01, 0.02, and 0.04 %). Supernatants were collected, after the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*

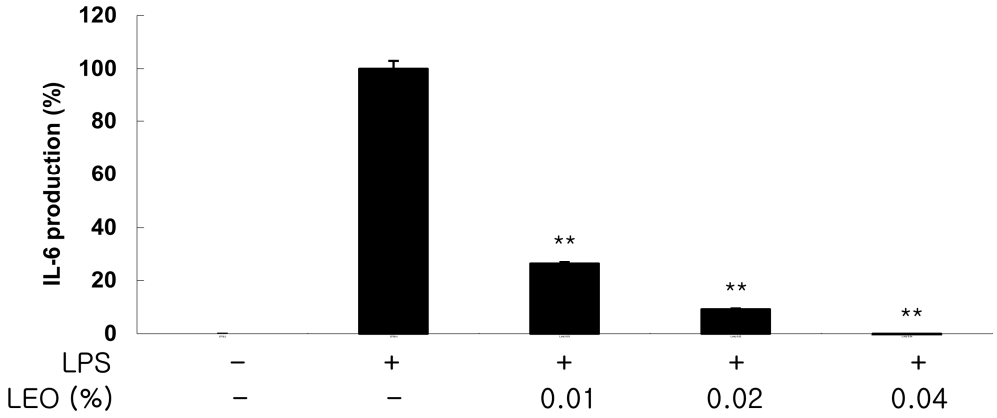

Figure 5-2. Inhibitory effect of IL-6 production on LEO in RAW 264.7 cells. Cells (1.8×10^5 cells/㎖) were stimulated by LPS (1 ㎍/㎖) for 24 h in the presence of LEO(0.01, 0.02, and 0.04 %). Supernatants were collected, after the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*

4.6.Effects of iNOS and COX-2 protein level on LEO in LPS-stimulated RAW 264.7 cells.

Decreased NO and PGE_2 production may result form lower iNOS and COX-2 enzymatic activity or decreased expression. We therefore tested the effects of LEO on iNOS and COX-2 levels after LPS induction. The LEO (0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced increases in iNOS and COX-2 levels. (Figure. 6).

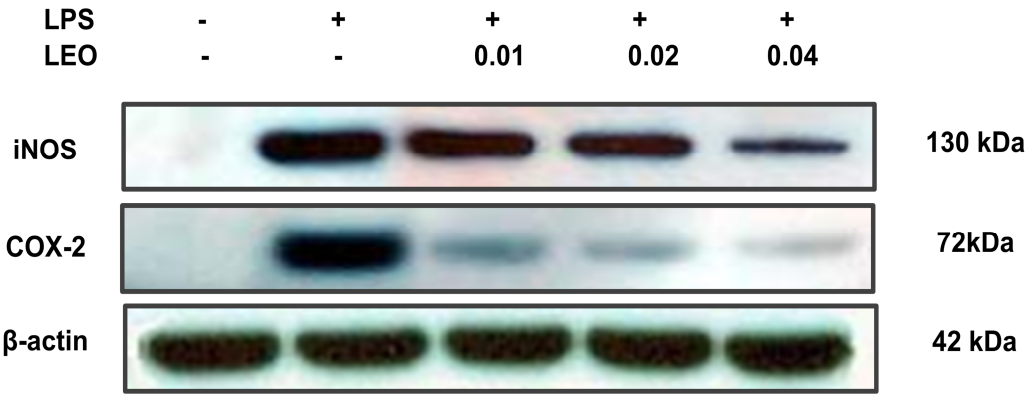

Figure 6. Inhibitory effects of iNOS and COX-2 protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.2×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were stimulated with LPS (1㎍/㎕) in the presence of LEO (0.01, 0.02, and 0.04 %) for 24 hr. iNOS and COX-2 protein levels were determined using immunoblotting method.

4.7.Effcet of MAPKs phosphorylation on LEO in LPS-stimulated Raw 264.7 cells.

MAPKs play critical roles in the regulation of cell growth and differentiation as well as in the control of cellular responses to cytokines and stressors. Moreover, they are also known to be important for the activation of NF-κB. To investigate whether LEO inhibits NF-κB activation via the MAPK pathway, we used Western blotting to examine the effects of LEO on the LPS-stimulated phosphorylation of ERK, JNK, and p38 MAPKs in RAW 264.7 cells. The LEO (0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced activation of ERK, JNK, and p38 MAPKs levels. (Figure. 7-1,2,3).

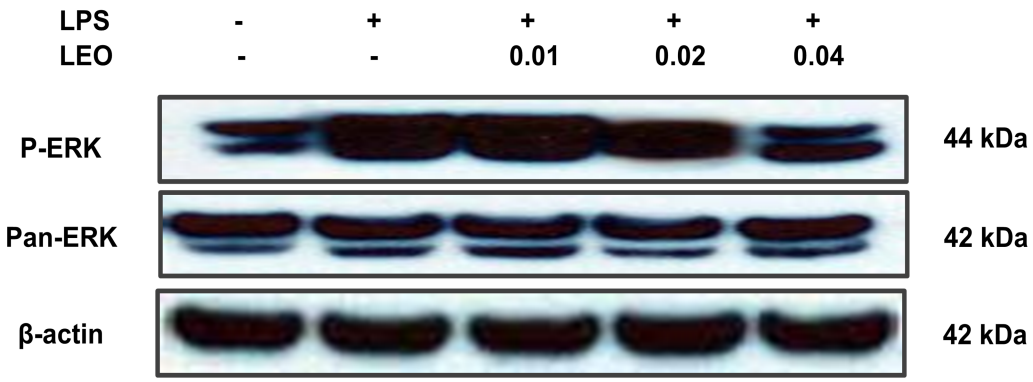

Figure 7-1. Inhibitory effect of ERK protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO (0.01, 0.02, and 0.04 %) at indicated concentrations and then stimulated for 30 min with LPS (1㎍/㎖). The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.

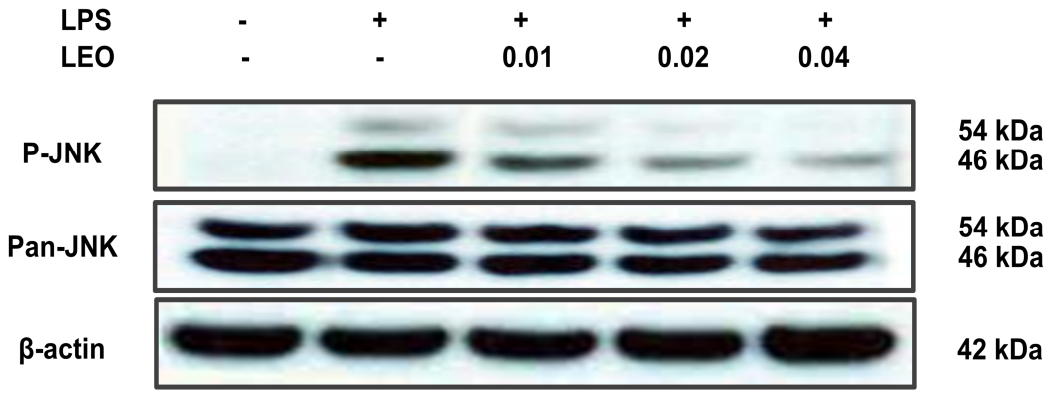

Figure 7-2. Inhibitory effect of JNK protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO (0.01, 0.02, and 0.04 %) at indicated concentrations and then stimulated for 20 min with LPS (1㎍/㎖). The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.

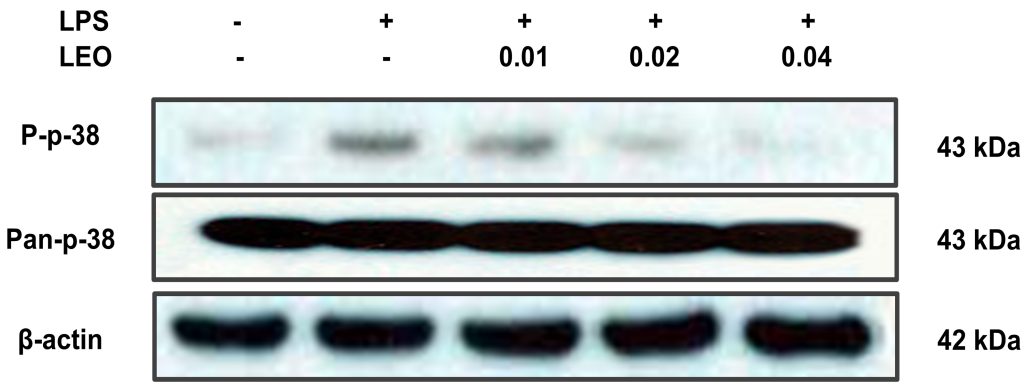

Figure 7-3. Inhibitory effect of P-38 protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO (0.01, 0.02, and 0.04 %) at indicated concentrations and then stimulated for 10 min with LPS (1㎍/㎖). The levels of p-p38 (phosphorylated-p-38) and pan-p-38 were determined using immunoblotting method.

4.8.Effcet of NF-κB activation on LEO in LPS-stimulated Raw 264.7 cells.

LPS also activated NF- κB in RAW 264.7 Cells via phosphorylation of p65 and p50. LEO (0.01, 0.02, and 0.04 %) dose-dependently inhibited this translocation, and also inhibited phosphorylation and degradation of IκB-α. This of this translocation and IκB-α degradation may mediate the effects on LPS-induced NO and PGE_2 production as well as iNOS and COX-2 expression.(Figure. 8-1~8-3).

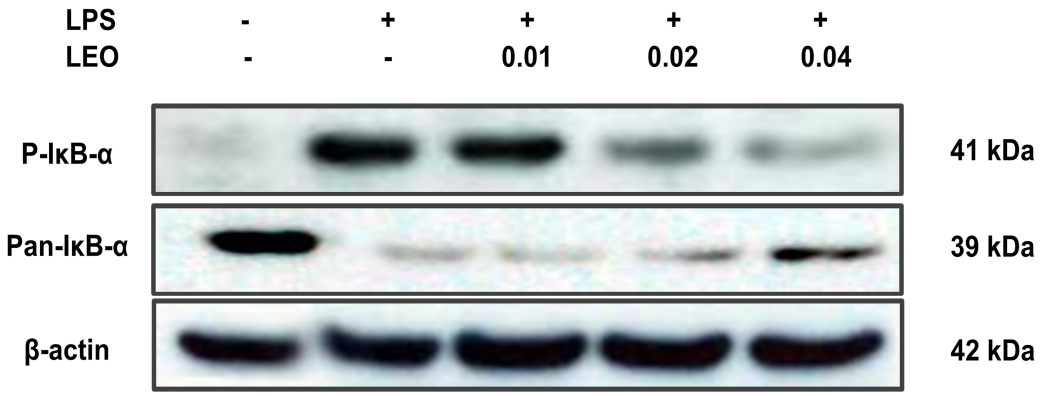

Figure 8-1. Inhibitory effect of IκB-α protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO (0.01, 0.02, and 0.04 %) at indicated concentrations and then stimulated for 30 min with LPS (1㎍/㎖). The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblotting method.

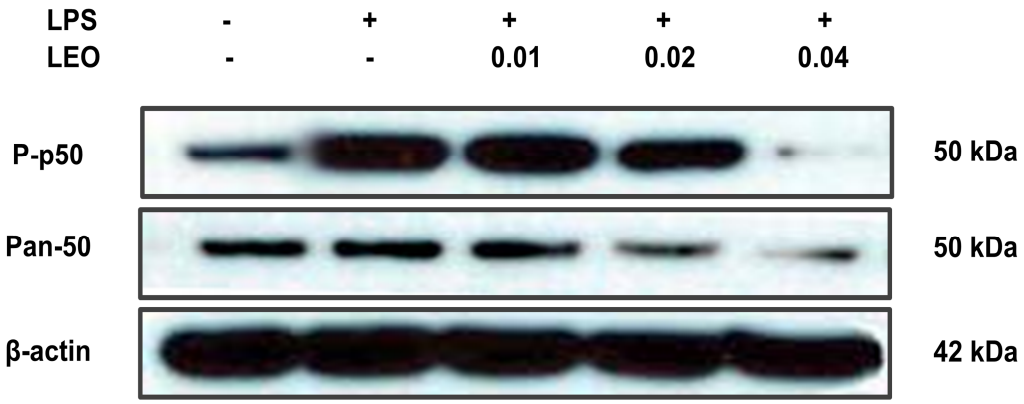

Figure 8-2. Inhibitory effect of P-50 protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO (0.01, 0.02, and 0.04 %) at indicated concentrations and then stimulated for 20 min with LPS (1㎍/㎖). The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblotting method.

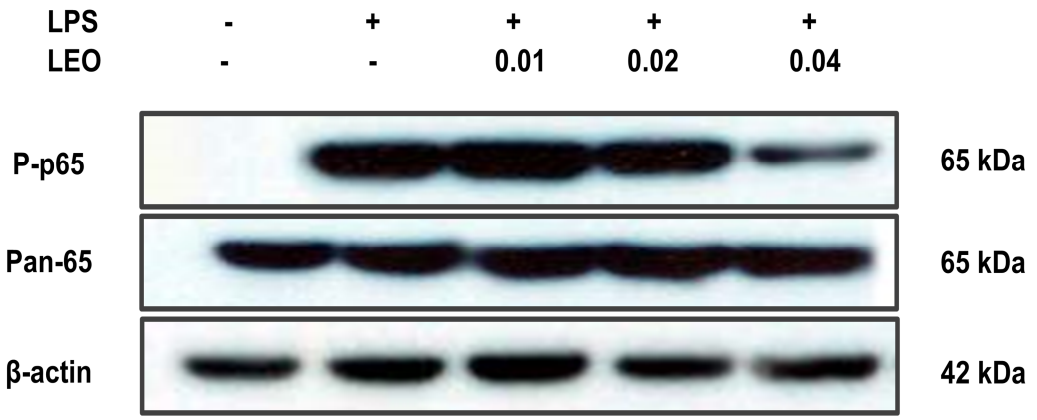

Figure 8-3. Inhibitory effect of P-65 protein level on LEO in RAW264.7 cells. RAW 264.7 cells (1.8×10^6 cells/㎖) were pre-incubated for 18 hr, and the cells were pre-incubated for 2 hr with LEO(0.01, 0.0,2 and 0.04 %) at indicated concentrations and then stimulated for 30 min with LPS (1㎍/㎖). The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblotting method.

5. DISCUTION

The present study demonstrates for the first time that *Lindera erythro carpa* essential oil(LEO), LEO possesses anti-inflammatory properties that are due to the down-regulation of the MAPKs signal pathways and inhibition of NF-κB activation in LPS-stimulated RAW 264.7 cells. The excessive amounts of NO produced by activation of iNOS in response to LPS play an important role in inflammation(McCann *et al.*, 2005).In addition, Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 are known to inflammatory diseases, including rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and inflammatory brain diseases(Chung, *et al*., 2007). They are considered to be important initiators of the inflammatory response and mediators of a variety of many inflammatory diseases (Glauser, 1996). Lipopolysaccharide (LPS) activates macrophages to produce pro-inflammatory mediators , such as nitric oxide(NO), prostaglandin E_2 (PGE_2), as well as proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2(COX-2)(R. Korhonen *et al*.,2005). NO is overproduced endogenously by iNOS which is induced in response to proinflammatory ctokines and LPS(Salvemini and Marino, 1998). Therefore, we investigated effects of LEO on NO and PGE_2 production in LPS-stimulated RAW 264.7 cells. The LEO(0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced on increases in NO and PGE_2 production (Figs. 3,4). Also, Effect of LEO on the protein level of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The LEO(0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced on increases in iNOS and COX-2 levels(Fig. 6). In addition pro-inflammatory cytokines, TNF-α and IL-6 are LEO(0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced increases in IL-6 and TNF-α production(Fig. 5-1, 5-2). The mitogen-activated protein kinases (MAPKs) are one of the major kinase families associated with cellular processes such as differentiation, stress responses, apoptosis, and immune defense (Liu,Y et al.,2007)

There are three major subgroups of MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. They play a crucial role in inducing cytokine production and the expression of iNOS and COX-2(Rao, 2001, Rajapakse, *et al.*,2008). So we used Western blotting to examine the effects of LEO on the LPS-stimulated phosphorylation of ERK, JNK, and p38 MAPKs in RAW 264.7 cells. The LEO (0.01, 0.02, and 0.04%) dose-dependently inhibited the LPS-induced activation of ERK, JNK, and p38 MAPKs levels(Fig. 7-1~7-3). Nuclear transcription factor kappa-B (NF-κB) regulates various genes involved in immune and acute phase inflammatory responses. NF-κB activation, in reaction to pro-inflammatory stimuli, associates the rapid phosphorylation of IκBs by the IKK signalosome complex. NF-κB produced by activated translocates to the nucleus, where it binds to κB-binding sites receptor. It then induces the transcription of pro-inflammatory mediators such as iNOS, COX-2, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 (Ghosh and Hayden, 2008, Edwards *et al*., 2009, Wong and Tergaonkar, 2009).

So we used Western blotting to examine the effects of LEO on degradation of IκB-α and Phosphorylation of p50, and p65 in LPS-stimulated RAW 264.7 cells. The LEO(0.01, 0.02, and 0.04%) dose-dependently suppress the LPS-induced phosphorylation and degradation of IκB-α,and the nuclear this translocation of p65 and p50(Fig. 8-1~8-3).

In conclusion, the present study shows that *Lindera erythro carpa* essential oil (LEO) has anti-inflammatory activity,which depends on its ability to regulate the production of NO, PGE_2 and other cytokines (TNF-α, IL-6) by suppressing the activation of NF-κB and the phosphorylation of MAPKs in LPS stimulated RAW 264.7 cells (**Fig.9**). LEO is suggested as a potential candidate treating inflammatory diseases.

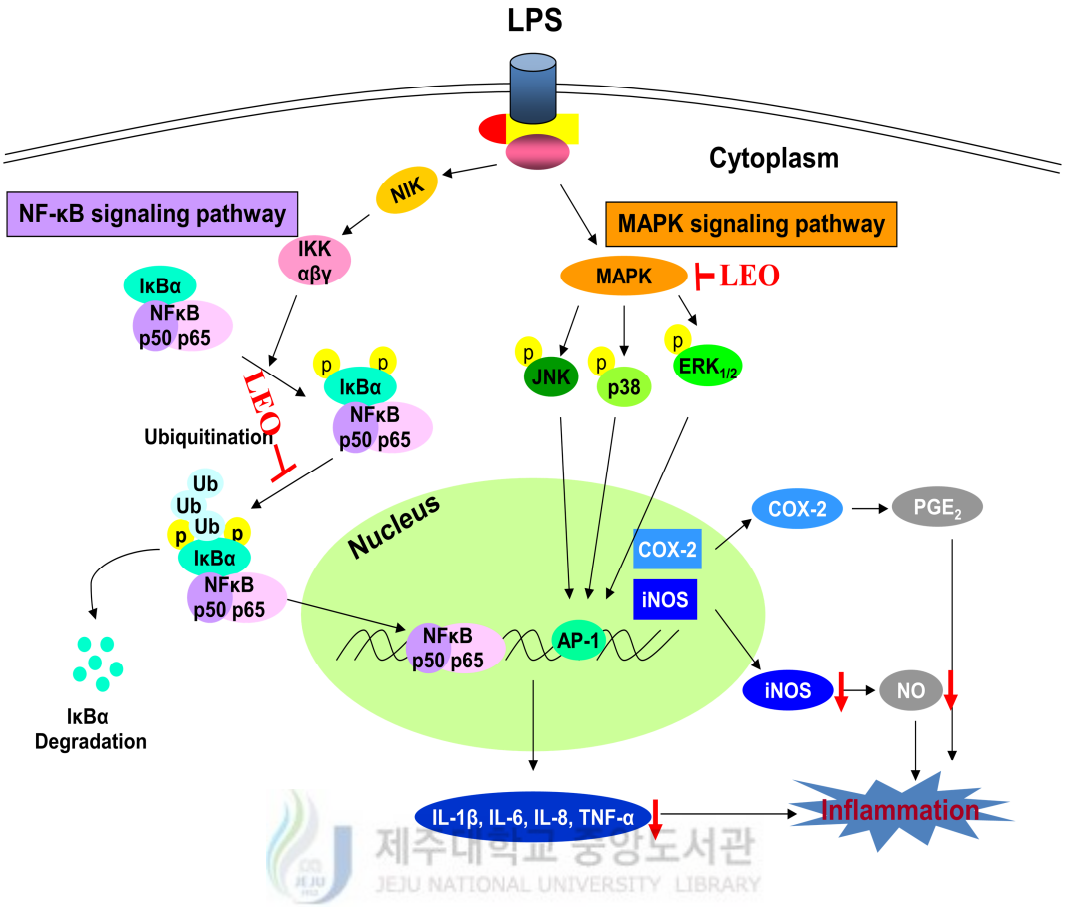

Figure. 9. Inhibitory mechanism of LEO on LPS-induced inflammation.

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감사의 글

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저를 알고 있는 모든 분들 항상 건강하고 행복하게 사세요. 사랑합니다.