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# Anti-inflammatory Effect of Yongseollan on the LPS-activated RAW 264.7 Cells

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## ABSTRACT

This study was conducted to evaluate the inhibitory effects of Yongseollan(YSL) on the production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated RAW264.7 cells. YSL is tropical plant originating from Mexico. The biological activity of this plant is not yet evaluated systematically. The aim of the present work is to investigate a potential anti-inflammatory activity of YSL. The RAW264.7 cells were cultured in DMEM/F12 medium for 24 hrs. After serum starvation, cells were treated with YSL for 1 hr, followed by stimulating NO production with a LPS. We found that YSL has an inhibitory effect on the production of NO, iNOS expression and phospho-IκB expression. YSL also inhibited tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1 β. Moreover, YSL inhibited cyclooxygenase (COX)-2 expression and prostanglandin E2 (PGE2). These findings showed that YSL could have some anti-inflammatory effects which might play a role in therapy in Gram-negative bacterial infections.

*Key word :* Yongseollan(YSL), LPS, iNOS, COX-2

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# Ⅰ. INTRODUCTION

Yongseollan (YSL; 龍舌蘭) is used as an important source for not only textile fiber, but also steroidal sapogenins such as hecogenin and tigogenin in china. The plant is also used as folk medicine for its anti-inflammatory and anti-fungus effects on the treatment of uterus bleeding, scabies, etc[1].

Nitric oxide (NO) is a gaseous molecule synthesized from L-arginine in the presence of nitric oxide synthase (NOS) enzyme and is involved in inflammation, immune function, bone metabolism, and apoptosis[2]. Three NOS isoforms, i.e., neural NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) are known[3].

LPS-activated marcrophages have usually been used for evaluating the anti-inflammatory effects about various materials. LPS is a principle component of the outer membrane of Gram-negative bacteria, is an endotoxin that induces septic shock syndrome and stimulates the production of pro-inflammatory mediators such as NO, tumor necrosis factor-α (TNF-α), interleukins, prostanoids and leukotrienes[4-6]. Therefore, LPS plays an important role in not only eliciting an inflammatory response but also causing septic shock during a Gram-negative bacterial infection. Inflammatory responses are advantageous for eradicating bacteria, as long as the responsesare under control. However, when out of control, deregulated inflammatory responses lead to the massive production of pro-inflammatory cytokines such as TNF-α, interleukin-1 (IL-1) and IL-6 by macrophage[7,8], which can cause tissue injury and multiple organ failure[9]. TNF-α is recognized to be an pivotal mediator in the development of endotoxicity or septic shock. TNF-α is considered to be a major mediator in the systemic inflammatory response syndrome observed during gram-negative sepsis[10].

Moreover, cyclooxygenase-2 (COX-2) is also involved in pathological mechanism of chronic inflammation. COX-2, an inducible isoform of cyclooxygenase, is a key enzyme that synthesizes PGE2 in response to inflammatory stimuli such as LPS[11].

Therefore, this study evaluated the effect of methanol extract of YSL on the regulatory effects of NO, cytokines and PGE2 in the LPS-activated RAW264.7 cells.

# Ⅱ. MATERIAL AND METHODS

## 1. Reagents

LPS (Escherichia coli 026:B6) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleu m (MTT) were obtained from Sigma (St. Louis, MO, USA). The fetal bovine serum (FBS) and antibiotics were purchased from Gibco/BRL (Eggenstein, Germany). The antibodies were obtained from BD Bioscience (USA), Cayman (USA) and Zymed (USA), and the NC paper used was Schleicher & Schuell (USA). The TNF-α, IL-6 and IL-1β ELISA Kits were purchased from Pierce endogen (Rockford, IL, USA).

## 2. Preparation of Methanol Extract of YSL

YSL (300g), obtained from Jeju island in

Korea, was extracted with 1000 ml of methanol at room temperature for 24 h. The extract was filtered through a 0.2 μm filter (Nalgene, New York, NY, USA), lyophilized and stored at -20℃ until needed. The yield amount of YSL MeOH extract was 17.85%.

## 3. Cell Culture

Raw264.7 cell, which is a murine macrophage cell line (KCLRF, Korean Cell Line Research Foundation, Seoul, Korea), was cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco, Germany) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. For all experiments, the cells were grown to 80~90% confluence, and were subjected to no more than 20 cell passages. The cells were maintained at 37℃ in a humidified atmosphere containing 5% $CO_2$. The RAW264.7 cells were plated at a density of $2\sim3\times10^6$/ml and pre-incubated at 37℃ for 24h. After serum starvation for 12 h, the cells were exposed to either LPS (1 μg/ml) or LPS+YSL for the indicated time (6-24 h). YSL was dissolved in DMSO + medium (EMEM, Cambrex Bio Science, MD, USA) and added to the incubation medium 1 h prior to adding the LPS.

## 4. Assay of NO Production

The level of NO production was monitored by measuring the nitrite concentration in the cultured medium. Briefly, the samples were mixed with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid) and incubated for 10 min at room temperature in dark. The absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL).

## 5. Cell Viability (MTT assay)

The Raw264.7 cells were plated at a density of $1\times10^5$cells/well in a 24 well plate to determine the cytotoxic concentrations of YSL. The cells were exposed to YSL at concentrations of 0.1 and 0.3 mg/ml at 37℃under 5% $CO_2$. After incubating the cells in the presence of YSL, the viable cells were stained with MTT (0.5 mg/ml) for 4 h. The media were then removed and the formazan crystals produced in the wells were dissolved in 200 μl of dimethylsulfoxide (DMSO). The absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL). The cell viability was defined as the % of untreated control cells [i.e. viability (% control) = 100 × {(absorbance of YSL - treated sample)/(absorbance of control)}].

## 6. Immunoblot Analysis

The cells were lysed in the buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 mg/ml leupeptin. The total cell lysate was prepared by centrifuging the cells at 10,000×g for 10 min and collecting the supernatant. The expression of

iNOS, COX-2 and p-IκB was immunochemically monitored with the total lysate fraction using anti-mouse iNOS, COX-2 and p-IκB antibodies, respectively. The bands for the iNOS, COX-2 and p-IκB proteins were visualized using ECL western blotting detection reagents (Amersham Biosciences, New Jersey, USA) according to the manufacturer's instructions.

## 7. Measurement of Cytokines Production

For the cytokine immunoassays, the cells ($1\times10^6$/ml) were pre-incubated with YSL for 1 h and further cultured for 24 h with 1 μg/ml of LPS in 6-well plates. The supernatants were removed at the allotted times and the level of TNF-α, IL-6 and IL-1β production was measured using an ELISA Kit (Pierce endogen, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, 50 μl of biotinylated antibody reagent and the samples were added to the anti-mouse TNF-α, IL-6 and IL-1β pre-coated 96-well strip plates. The plates were covered and kept at room temperature for 2 h and washed three times in a prepared washing buffer. This was followed by the addition of 100 μl of Streptavidin-HRP Concentrate. After 30 min incubation at room temperature, the wells were washed three times, and 100 μl of TMB Substrate Solution was then added and developed in the dark at room temperature for 30 min. The reaction was quenched by adding 100 μl of TMB Stop Solution, and the absorbance of the plates was measured at 450 nm to 550 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6 and IL-1β in serial dilutions. The level of TNF-α, IL-6 and IL-1β was quantified from standard curve.

## 8. Measurement of PGE2 Production

YSL was treated into culture medium 1 h before the addition of 1 μg/ml LPS. LPS-treated cells were further cultured with vehicle or YSL for 24 h. The cultured medium was collected and assayed with ELISA kit (RnD Systems, Minneapolis, MN, USA). Cultured medium was incubated in goat anti-mouse IgG coated plate with acetylcholinesterase linked to $PGE_2$ and $PGE_2$ monoclonal antibody for 18 h at 4°C. The plate was empted and rinsed five times with wash buffer contained in the kit. And then, 200 μl of substrate reagent was added to each well and incubated for 1 h at 37°C. The developed plate was read at 405 nm and the $PGE_2$ concentration of each sample was determined according to the standard curve.

## 9. Statistical Analysis

The data were expressed as a mean ± S.D. of the results obtained from a number of experiments. One-way analysis of variance (ANOVA) was used to assess the significant differences between the treatment groups. For each significant effect of treatment, the Tukey test was used to compare the multiple group means. A $P$ value $<0.01$ was considered significant.

## III. RESULTS

### 1. YSL Inhibits LPS-Activated NO, Cytokines and PGE2 Release

The inhibition of NO production by YSL was investigated by measuring the level of NO production in RAW264.7 cells treated with 0.01, 0.03, 0.1, 0.3 and 1.0 mg/ml of YSL. As shown in Fig. 1A, in the LPS plus YSL groups, the level of NO production decreased in a concentration and time-dependent manner compared with the LPS group. In the 0.01, 0.03, 0.1, 0.3 and 1.0 mg/ml of YSL group, the level of NO was significantly inhibited at 24 h (Fig. 1A).

Cell viability was measured at different YSL concentrations for 24 h using an MTT assay in order to determine if the concentrations of YSL used in this study induce cytotoxicity. The results showed that YSL concentration dose had no cytotoxicity for 24 h incubation (Fig. 1B).

We treated RAW264.7 cells with LPS, collected media for 24 h, detected cytokines from the collected media. TNF-α, IL-1β and IL-6 were highly induced by LPS at 24 h. As show in Fig. 2, 1.0 mg/ml of YSL significantly reduced the level of TNF-α (Fig. 2A), IL-1β (Fig. 2B) and IL-6 (Fig. 2C) at 24 h incubation. LPS-activated PGE2 also decreased by YSL as compared with control (Fig. 2D).

### 2. Inhibitory Activities of YSL on LPS-Activated iNOS and COX-2 Expression

The expression level of iNOS protein in the cytosol fraction was examined using immunoblotting analysis. The iNOS protein expression was reduced by YSL in a concentration-dependent manner. As shown in Fig 3, 1.0 mg/ml of YSL strongly suppressed the induction of iNOS by LPS.

COX-2 plays a key role in the development of inflammation12,13). YSL was further investigated to determine if it can reduce COX-2 expression levels. As shown in this experiment, the COX-2 protein was strongly induced by LPS. 0.1, 0.3 mg/ml of YSL slightly suppressed the induction of COX-2, and 1.0 mg/ml of YSL with LPS strongly suppressed the induction of COX-2 (Fig. 3).

### 3. Inhibitory Activities of YSL on IκB Phosphorylation

In order to determine if YSL can directly affect p-IκB expression in macrophage cells, the level of p-IκB protein expression was assessed immunochemically in RAW264.7 cells incubated with or without YSL. LPS increased the p-IκB level. However, 0.3 mg/ml of YSL reduced the LPS-inducible p-IκB expression level, and 1.0 mg/ml of YSL markedly reduced the protein levels of p-IκB expression in a dose-dependent manner (Fig. 3).

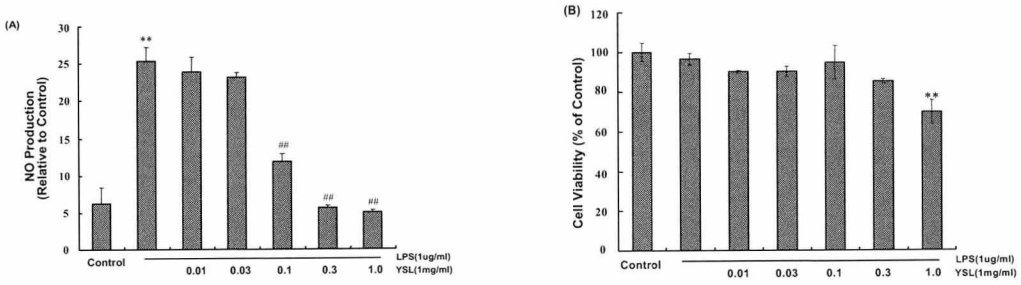


Fig. 1. NO release and the cytotoxicity by YSL in LPS-activated RAW264.7 cells. The NO concentration (A) in the culture medium and cytotoxicity (B) were measured for 24 h. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test (*: significant compared with the control, **P<0.01, #: significant compared with the LPS alone, ##P<0.01).

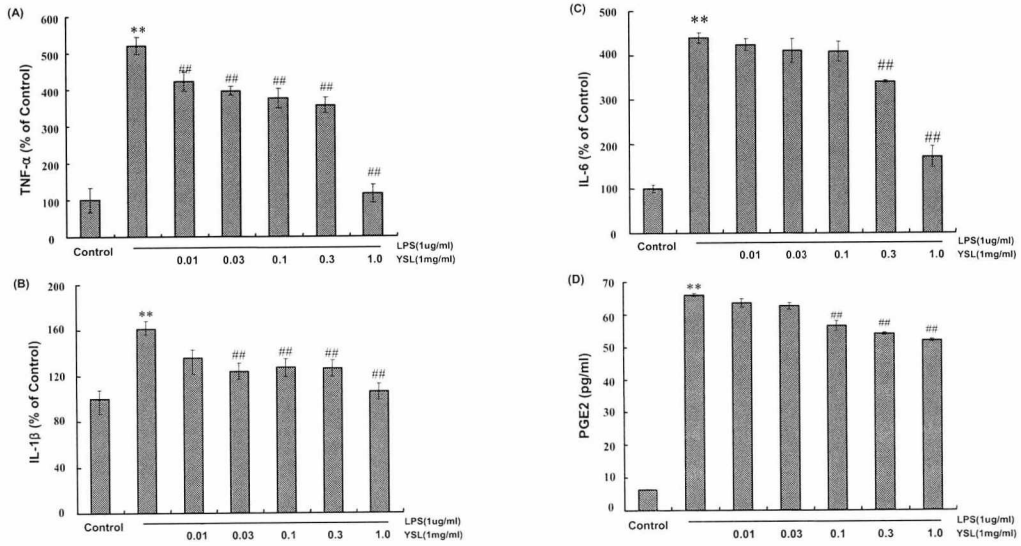


Fig. 2. Inhibition of LPS-activated cytokines and $PGE_2$ production by YSL. RAW264.7 cells were cultured with LPS(1 ug/ml) in the presence or absence of YSL for 24 h to determine the level of TNF-α (A), IL-1β(B), IL-6(C) and $PGE^2$(D). The cells were incubated for 24 h under same conditions. The cultured medium was collected and directly assayed for cytokines and PGE2. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test (*: significant compared with the control, **P<0.01, #: significant compared with the LPS alone, ##P<0.01).

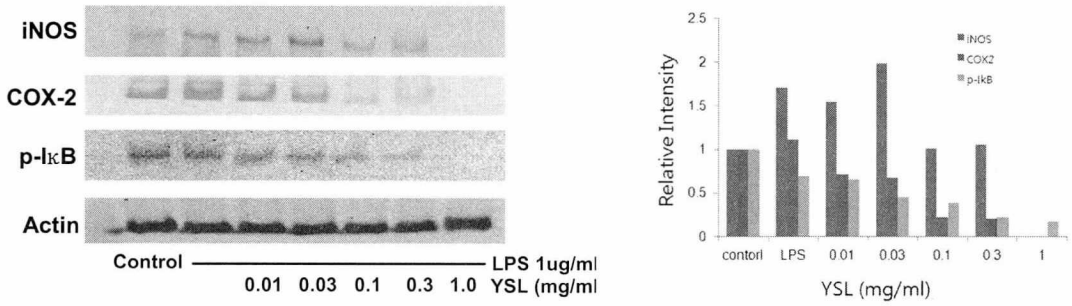


Fig. 3. Reduction of LPS-activated iNOS, COX-2 and p-IκB expression by YSL. A) The levels of iNOS, COX-2, p-IκB and actin were monitored. Actin is used as a loading control. B) Values indicate the relative densitometric intensities (relative level in untreated control/1).

## Ⅳ. DISCUSSION

Inflammation is the first response of the immune system to infection or irritation. It is caused by cytokines such as tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6)[7], and by eicosanoid such as prostaglandin E2 (PGE2)[14]. Thus, inhibitors of these cytokines have been considered as a candidate of anti-inflammatory drugs.

In this study, we showed that Yongseollan (YSL) could modulate the regulatory mechanism of nitric oxide (NO), cytokines and PGE2 in the lipopolysaccharide (LPS)-activated RAW264.7 cells. YSL inhibited the levels of NO, PGE2, TNF-α, IL-6 and IL-1β, and the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) activated by LPS. These inhibitory effects were mediated though the inhibition of inhibitory kappa B (IκB) phosphorylation.

NO is a free radical produced from L-arginine by nitric oxide synthases (NOSs), and an important cellular second messenger[15]. The modulation of iNOS-mediated NO release is one of the major contributing factors during the inflammatory process[16]. At adequate concentrations, NO can generate and modify intracellular signals, thereby affecting the function of immune cells, as well as tumor cells and resident cells of different tissues and organs. However, its uncontrolled release can cause inflammatory destruction of target tissue during an infection[17-19].

Nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) have been well defined, to be associated with iNOS and COX-2 expression. Among these, the NF-κB is a functionally transcriptional factor[20]. NF-κB plays an important role in the regulation of immune and inflammation response, such as major histocompatibility complex-Ⅰ (MHC-Ⅰ), major histocompatibility complex-Ⅱ (MHC-Ⅱ), interferon regulatory factor-1 (IRF-1), and diverse cytokines (TNF-α, IL-1, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor

(G-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cell expressed and secreted (RANTES)). Wide stimuli including toxic materials like virus and bacteria activate NF-κB. Activated NF-κB translocates into nucleus[21], and modulates the expression of iNOS or TNF-α in nucleus[22]. In resting cells, NF-κB is sequestered in the cytoplasm as an inactive form through association with one of several inhibitory molecules like IκB α, IκBβ, IκBε, p105, p100[23]. Activation of the signaling cascade of NF-κB results in a complete degradation of IκB or partial degradation of the carboxyl termini of p105 and p100 precursors. So the translocation of NF-κB to the nucleus induces transcription of COX-2, iNOS, Bcl-XL and cellular inhibitor of apoptosis proteins (cIAPs) in nucleus. The kinds of IκB protein have been known to IκBα, IκBβ and IκBε. Among them IκBα is the most abundant inhibitory protein for NF-κB[24]. YSL suppressed the phosphorylation of IκB. Thus, we supposed that YSL could inhibit NF-κB activation by blocking IκB phosphorylation.

In conclusion, we determined that the YSL can have anti-inflammatory activity by suppressing the phosphorylation of IκB, and by inhibiting the expression of iNOS and COX-2 in LPS-activated RAW264.7 cells.

## V. ACKNOWLEDGMENTS

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