Thesis for the Degree of Master of Science

The Anti-inflammatory Effects of *Persicaria thunbergii* Extracts on Lipopolysaccharide-stimulated RAW264.7 Cell Line

by
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The Anti-inflammatory Effects of *Persicaria thunbergii* Extracts on Lipopolysaccharide-stimulated RAW264.7 Cell Line
(LPS로 처리된 RAW264.7 세포에서의 고마리 추출물의 항염증 효과)

Advisor: Prof. Gun Do Kim

by

Sang Bo Kim

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ABSTRACT

In the present study, we investigated the anti-inflammation effect of *Persicaria thunbergii* (*P. thunbergii*) on RAW 264.7 murine macrophage cells. The anti-inflammatory activity of *P. thunbergii* was investigated by measuring expression of the LPS-induced inflammatory proteins, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nuclear factor-κB (NF-κB), and the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2). Methanol extract of *P. thunbergii* decreased the expression of iNOS, COX-2 and NF-κB, and increased the expression of HO-1 (Heme oxygenase-1) in LPS-stimulated RAW264.7 cells. Methanol extract was fractioned by *n*-butanol, hexane and ethyl acetate (EtOAc) and each fraction was tested for inhibitory effects on inflammation. Among the sequential solvent fractions, the EtOAc soluble fraction was investigated by the expression of prostaglandin E_2 (PGE_2), and showed decreasing form in the dose-dependent manner. EtOAc extract showed the most effective inhibitory activity of the expression of iNOS, COX-2 and NF-κB, and the production of NO. Our study showed manifest result that *P. thunbergii* has anti-inflammatory activity through the decrease of NO and inhibition of iNOS, COX-2, PGE_2 and NF-κB expression, and by the increase of HO-1 enzyme. This study needs for more investigation to find out the most effective single compound in anti-inflammatory activity.

INTRODUCTION

Inflammation is a major defense mechanism against pathogens and is stimulated by a range of microbial products. In macrophages and dendritic cells, toll-like receptors (TLRs) expressed at high levels for the attachment of various microbial products (Akira et al., 2003). This binding triggers a wide spectrum of responses from phagocytosis to various cytokine production, which enhances the inflammatory and adaptive immune responses (Kaisho and Akira, 2006, Takeda and Akira, 2004). TLR4 is One of the TLRs isoform of macrophage that binds to the lipopolysaccharide (LPS) (D'Aiuto et al., 2004). LPS is a product and a major constituent of Gram negative bacteria. It initiates a number of major cellular responses that play critical roles in the pathogenesis of inflammatory responses and is also employed to induce activation of RAW264.7 cell (Woo et al., 2004) This eventually triggers NF-κB and iNOS and COX-2 enzymes. However, excessive inflammatory cytokines and protein production need to be regulated, since it can lead to harmful inflammatory responses such as rheumatoid arthritis, septic shock and other chronic inflammatory diseases (D'Aiuto et al., 2004).

During the inflammatory processes, large amount of toxic factors are released. Nitric oxide (NO) and Plostaglandin E_2 (PGE_2) are produced by iNOS and COX-2 enzymes, respectively (Vane et al., 1994).

NO is a short-lived free radical and is a signaling molecule that mediates many physiological and pathophysiological processes, including neurotransmission and inflammation

(Nathan and Xie, 1994, Garthwaite, 1995). NO, as generated in activated macrophages by iNOS, is an important event in host defenses. NO modulates the synthesis of prostaglandins, tormboxans and other inflammatory molecules (Moncada et al., 1991). Despite the beneficial roles of NO in host defense mechanism against tumor cells, viral replication and other factors, and over expression of NO can be harmful to the host, leading to rheumatoid arthritis (St. Clair et al., 1996), experimental allergic encephalomyelitis (Cross et al., 1994), and allograft rejection (Worrall et al., 1995). Thus, selective inhibition of iNOS can be beneficial.

COX is an enzyme the catalyzes the conversion of arachidonic acid to prostaglandin H2, the precursor of a variety of biologically active mediators such as PGE_2, prostacyclin and thromboxane A2 (Hawkey,1999). Two COX isoforms are described namely COX-1 and COX-2. COX-1 is ubiquitously expressed and it produces prostanoids that are involved in normal cellular functions. COX-2 expression , on the other hand can be induced in several cell types by cytokines, mitogens, bacterial endotoxins and other growth factors. Also, it plays a critical role in the damage produced by inflammation (Arakietal.,2001).

Prostaglandins and glucocorticoids are potent mediators of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) exert certain effects by the inhibition of prostaglandin production. The pharmacological target of NSAIDs is cyclooxygenase, which catalyze the first committed step in arachodonic-acid metabolism. NSAIDs act on the cyclooxygenase active site and inhibits expression of both COX-1 and COX-2 with little specificity. This eventually leads to serious side effects

such as gastric lesions and renal toxicity. COX-2 selective inhibitors are also identified to show potent anti-inflammatory activity, *in vivo,* with minimal gastric side effects (Kurumbail et al., 1996). It was then investigated that whether *P. thunbergii* extracts can suppress the expression of COX-2.

Inactive NF-κB is constitutively present as a homo- or a heterodimer, and binds to inhibitory IκB proteins. Pro-inflammatory cytokines or bacterial infections can induce phosphorylation, ubiquitination and proteasome mediated degradation of the IκB proteins. this is followed by the translocation of NF-κB to the nucleus, binding to the relevant DNA sites on the promoter region of the genes and induction of gene transcription (Tak and Firestein, 2001). LPS is known to stimulate the degradation of one of the isoforms of IκB, IκBα and to promote the activation of NF-κB DNA binding activity (Watters et al., 2002). The effects of *P. thunbergii* extracts on the nuclear translocation of NF-κB in LPS-stimulated RAW264.7 cells were examined through immunofluorescence.

P. thunbergii is as an annual plant and is widely distributed in Korea. It has been used as a folk medicine to treat rheumatism, hemorrhage and measles in both Korea and China (Oh et al., 2005). Thus, the effects of *P. thunbergii* on the pro-inflammatory response in LPS-stimulated RAW264.7 cells were investigated.

MATERIALS AND METHODS

Plant materials

Dried *P. thunbergii* powder was extracted three times with 80% methanol and was filtered. The filtrate was evaporated at 40℃ to obtain the methanol extract. It was dissolved in water, then, partitioned with ethyl acetate, hexane and *n*-butanol.

Cell culture and treatments

RAW264.7 cells from the mouse macrophage cell line were obtained from the American Tissue Culture Collection (Manassas, VA, USA). RAW264.7 cells were growth in Dulbecco's Modified Eagle's Medium (DMEM) -High Glucose (HyClone Laboratories, Logan, UT, USA), supplemented with 10% heat inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) containing, penicillin and streptomycin (PAA Laboratories GmbH, PA, Austria) were incubated in a humidified atmosphere of 5% CO_2 at 37℃. *P. thunbergii* extract was then added to the culture media with the final concentration as specified on the text.

Cell viability assay

For the cell viability study, RAW264.7 cells, approximately $1x10^4$ in number were resuspended in 100 ㎕ medium and were plated in a 96-well plate. The cells were treated with 50~150 ㎍/㎖ of *P. thunbergii* extract with LPS for 24 h. After the treatment, 10 ㎕ of WST-1 ® (Daeil Lab service, Jong-No, Korea) solution was added into each well and the cells were incubated at 37℃ for 3 h. the absorbance was read at 460 nm.

Nitric oxide (NO) assay

The cells ($5X10^5$ cells/㎖) were pre incubated with *P. thunbergii* extract for 2 h, and were incubated with indicated concentrations of LPS for 24 h. The nitrite accumulation in the supernatant was assessed by the Griess reaction (Sigma-Aldrich, st. Louis, MO, USA). Each 100 ㎕ of the culture supernatant was mixed with an equal volume of Griess reagent and was incubated at room temperature for 10 min. The absorbance was then measured at 540 nm in a microplate absorbance reader and a series of known concentrations of sodium nitrite was used as a standard.

Western blot analysis

RAW264.7 cells were cultured as described above, were pre incubated with *P. thunbergii* extract (100 ㎍/㎖) for 2 h, and then, were incubated with LPS (1 ㎍/㎖). The RAW264.7 cells were harvested and lysed after 24 h with ice-cold Cell lysis

buffer (Intron Biotechnology Inc., Gyeonggi, Korea). After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min. The protein content of the cell lysates determined by using a Protein Quantification Kit (CBB solution) (Biosesang Inc., Gyeonggi, Korea) with bovine serum albumin (BSA) (Thermo Scientific, Rockford, Il, USA) was used as standard. An aliquot from each sample (50 μg of protein) was boiled with the sample buffer for 5 min, and then, was resolved in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was electrotransferred to a nitro cellulose membrane (Pall, Pensacola, Fl, USA) and was blocked overnight in PBST buffer (135 mM Sodium chloride, 2.7 mM Potassium chloride, 4.3 mM sodium phosphate, 1.4 mM Potassium dihydrogen phosphate and 0.5% Tween-20) containing 5% Skim Milk powder at 4℃. The blots were then probed overnight with primary antibodies (anti-HO-1, anti-NF-κB p65, anti-i-NOS, anti-COX-2 and anti-β-actin) (Cell Signaling Technology Inc., Beverly, MA, USA) were washed three times with PBST and were incubated for 1 hr with anti-rabbit IgG or anti-mouse IgG conjugated with HRP. The blots were washed in PBST and then visualized using an enhanced chemiluminescent (ECL) detection solution (Pierce, IL, USA).

Immunofluorescence

Cells were cultured (37℃, 5% CO_2) in coverglass-Bottom dishes (SPL Lifesciences, Gyeonggi, Korea) for 24 h. For the experiment, cells were fixed with 4% formaldehyde (Junsei Chemical Ltd., Japan) for 15 min at room temperature and were blocked for 1 h in 5% normal serum of the host against primary antibodies and 0.3% Triton X-100. The fixed and blocked cells were then incubated with 0.1 ㎍/㎖ of primary antibodies (anti-NF-κB p65) for 3 h and thereafter, with 0.1 ㎍/㎖ of anti-rabbit IgG Fab2 fragment Alexa Fluor 488 conjugate (Cell Signaling Technology Inc. Beverly, MA, USA) for 1 h. Stained cells on the slides were mounted with Prolong Gold Antifade Reagent (Invitrogen, Eugene, OR, USA) and were observed under Nikon FCLIPS 50i microscope, equipped with a charged-coupled device (CDD) camera. Images were captured and were processed with High-Content Analysis Software (Cambridge Healthtech Institute, Needham, MA, USA).

PGE2 measurement

For the quantitative determination of Prostaglandin E2 (PGE2) in cell culture supernatants, the cells were pre incubated with *P. thunbergii* extract for 2 h, and were incubated with indicated concentrations of LPS for 24 h. Prostaglandin E2 Parameter Assay Kit (R&D systems, Minneapolis, MN, USA) was employed to measure PGE2 concentrations in cell supernatants based on manufacturer's protocol. The absorbance was then measured at 450 nm within 30 min in a microplate absorbance reader.

RESULTS

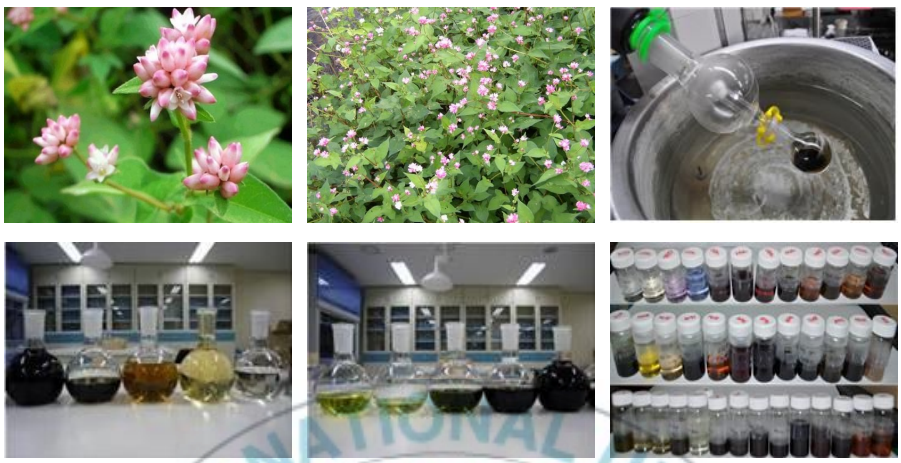

Fig. 1. Extraction and fractionation of *P. thunbergii*

P. thunbergii is as an annual plant and is widely distributed in Korea. Dried *P. thunbergii* powder was extracted three times with 80% methanol and was filtered. The filtrate was evaporated at 40℃ to obtain the methanol extract. It was dissolved in water, then partitioned with ethyl acetate, hexane and *n*-butanol. Among them, ethyl acetate extrat was more separated and numbers are listed in bottle.

Cellular viability

For the cell viability study, lipopolysaccharide (LPS) and methanol extract were treated on RAW264.7 cells. LPS is employed to induce RAW264.7 cell activation. After 24 h, LPS did not influence cell survival and methanol extract of *P. thunbergii* have a little effect on RAW264.7 cells viability. As shown Fig. 2, LPS and methanol extract were not cytotoxic to RAW264.7 cells. therefore, 100 ㎍/㎖ concentration was selected for anti-inflammatory experiment.

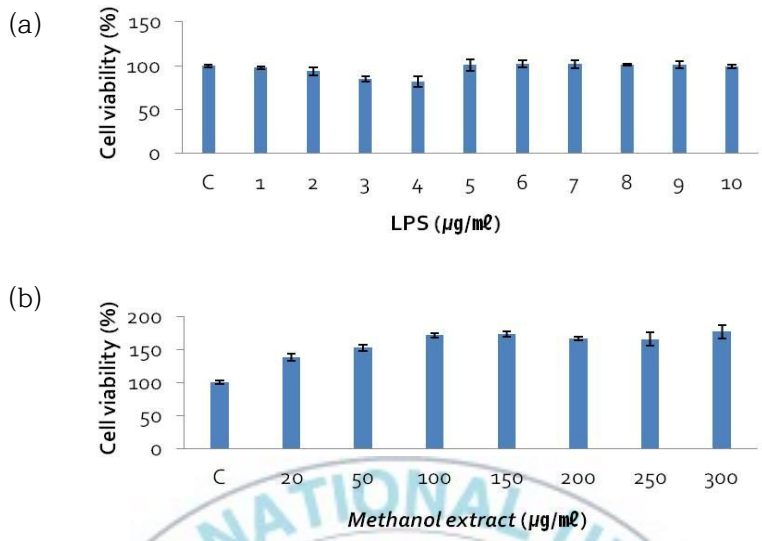

Fig. 2. The effects of LPS and methanol extract of *P. thunbergii* on cell proliferation

The cell viability of LPS and methanol extracted *P. thunbergii* was measured by WST-1 assay. cells were treated with various concentrations for 24 h. LPS (1 μg/mℓ) is employed to induce RAW264.7 cell activation and produce nitric oxide without cell cytotoxicity (Fig. 2a). The filtered and evaporated methanol extract didn't have cytotoxicity in a dose dependent manner (Fig. 2b).

Effects of methanol extract of *P. thunbergii* on NO Production in LPS-stimulated RAW264.7 cells.

In order to observe the effects of methanol extract of *P. thunbergii* on generation of NO, the RAW264.7 cells were pre incubated with methanol extract for 2 h and were stimulated with LPS, subsequently, the levels of nitrite were measured. The results of NO generation showed that methanol extracted *P. thunbergii* inhibit NO generation of LPS-stimulated RAW264.7 cells after 24 h in a dose-dependent manner (Fig. 3).

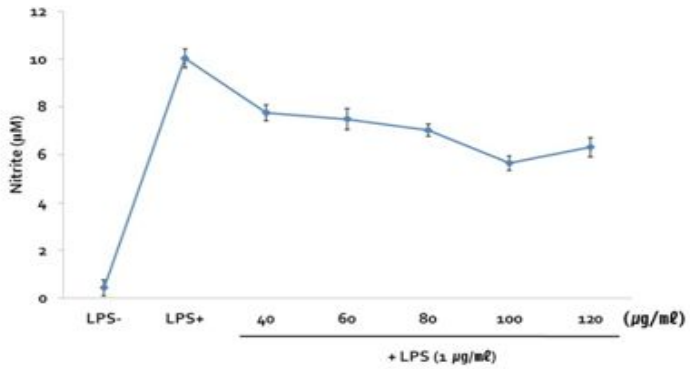

Fig. 3. The effects of methanol extracted *P. thunbergii* on the generation of NO in LPS stimulated RAW264.7 cells.

The cells were treated with indicated concentrations of methanol extract for 24 h. Methanol extracted *P. thunbergii* inhibited NO generation on RAW264.7 cells in a dose-dependent manner. NO production was inhibited at concentration of 100 ㎍/㎖ especially.

Expression levels of pro-inflammatory proteins.

To investigate the anti-inflammatory activity of *P. thunbergii*, RAW264.7 cells were stimulated for 24 h with LPS (1 μg/mℓ) or LPS with methanol extracted *P. thunbergii*. It down-regulates the expression of pro-inflammatory proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Beneficial protective effects of HO-1 in inflammation (Paine et al., 2010) are increased in cells treated with LPS and methanol fraction of *P. thunbergii*. Nuclear factor kappa b (NF-κB) is known for regulatory protein by inducing iNOS and COX-2 expression levels. It was suppresed by methanol extract in a dose dependent manner (Fig. 4).

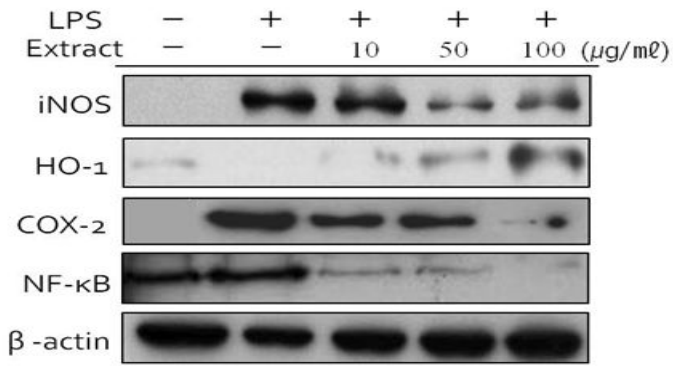

Fig. 4. Down-regulation of pro-inflammatory protein expression levels.

The expression levels of pro-inflammatory proteins were examined by western blot analysis. methanol extracted *P. thunbergii* (10, 50, 100 μg/mℓ) were treated with LPS (1 μg/mℓ) in RAW264.7 cells. After 24 h, iNOS, COX-2 and NF-κB was decreased. However, beneficial protective HO-1 protein was increased by methanol extracted *P. thunbergii*.

Nulear translocation of NF-κB.

NF-κB is one of the principal factors for the expression of COX-2 and iNOS as mediated by LPS or pro-inflammatory cytokines (Jung et al., 2009). The present study identified that NF-κB is responsible for the maintenance of iNOS expression (Gookin et al., 2006). To identify NF-κB translocation from the cytosol to the nucleus, immunofluorescence was performed. The specific DNA binding of NF-κB showed that treating RAW264.7 cells with LPS (1 ㎍/㎖) enhanced NF-κB activation. However, when, the methanol extracted *P. thunbergii* (100 ㎍/㎖) was added to the RAW264.7 cells, NF-κB activity was decreased markedly (Fig. 5).

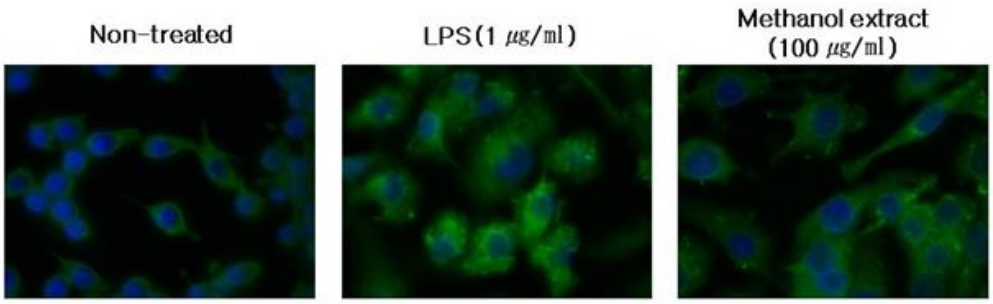

Fig. 5. Detection of NF-κB activity.

RAW264.7 cells were treated with LPS (1 ㎍/㎖) or LPS with methanol extracted *P. thunbergii* (100 ㎍/㎖). LPS led to NF-κB activation. However, treated with methanol extract showed that NF-κB activation was decreased when compare to non-treated RAW264.7 cells.

Cellular viability

The cytotoxicities of *n*-butanol, hexane and ethyl acetate fractions on RAW264.7 cells were examined. After 24 h of incubation, the viabilities of the cells were determined by WST-1 assay. As shown in Fig. 6, the various concentrations of *P. thunbergii* fractions did not influence cell survival.

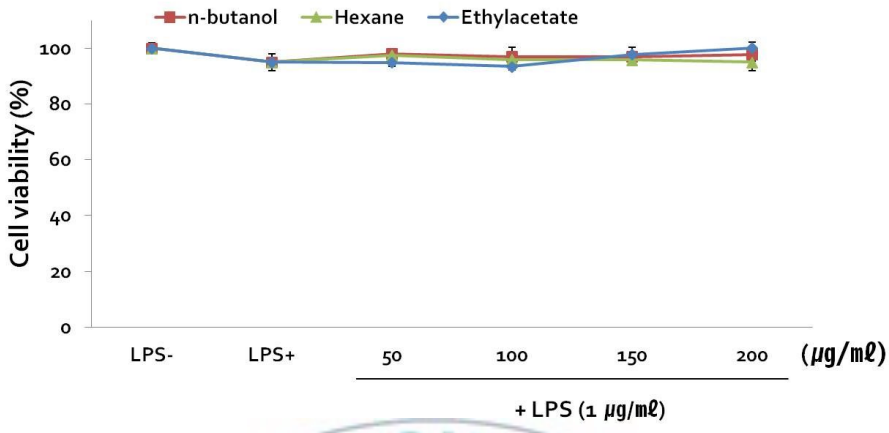

Fig. 6. The effects of *n*-butanol, hexane and ethyl acetate fractions on cell proliferation

Cells were treated with various concentrations of *P. thunbergii* extracts (*n*-butanol, hexane and ethyl acetate) for 24 h. The rates of cell viability were measured by WST-1 assay.

Effects of *n*-butanol, hexane and ethyl acetate extracted *P. thunbergii* on NO Production in LPS-stimulated RAW264.7 cells.

In order to observe the effects of *n*-butanol, hexane and ethyl acetate extracted *P. thunbergii* on generation of NO, the RAW264.7 cells were pre incubated with indicated concentration of sequential fractions for 2 h and were stimulated with LPS (1 ㎍/㎖). Thereafter, the levels of nitrite were measured. The results of NO generation showed that sequential fractions are inhibit NO generation of LPS-stimulated RAW264.7 cells. The most potent influence of all, ethyl acetate fraction was selected for the anti-inflammatory study.

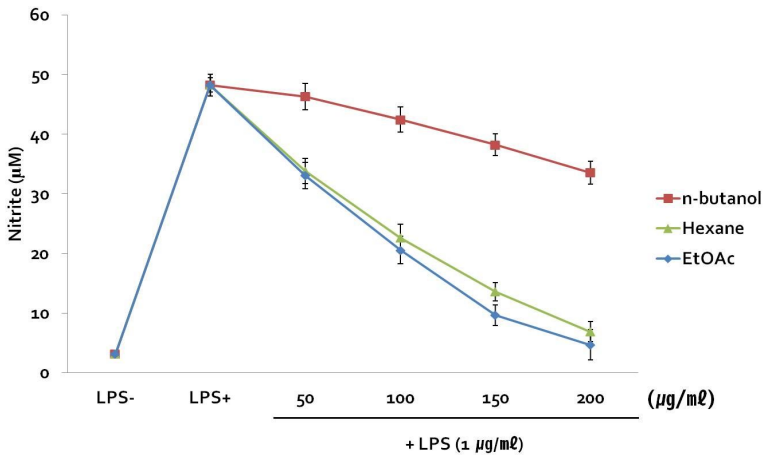

Fig. 7. The effects of *n*-butanol, hexane and ethyl acetate fractions on the generation of NO in LPS stimulated RAW264.7 cells.

Cells were treated with indicated concentrations of *n*-butanol, hexane, ethyl acetate fraction. sequential solutions are decrease nitric oxide production than treated with LPS only. Among them, ethyl acetate extract of *P. thunbergii* decreased NO production markedly.

Expression levels of iNOS, COX-2 and PGE_2 in LPS stimulated RAW264.7 cells.

To investigate the anti-inflammatory activity of ethyl acetate extract, RAW264.7 cells were stimulated with LPS (1 ㎍/㎖). It leads to the down-regulated expression of pro-inflammatory proteins such as iNOS and COX-2. PGE_2 (Prostagalndin E_2) represents the most important inflammatory product of COX-2 activity and, thus, it was quantified in the supernatant. To assess whether each solutions could inhibit production of LPS-induced PGE_2 in RAW264.7 cells, the cells were pre-treated with solution for 2 h and then stimulated with LPS (1 ㎍/㎖). After incubation for 24 h, the cell culture medium was harvested and the production of PGE_2 was measured using ELISA.

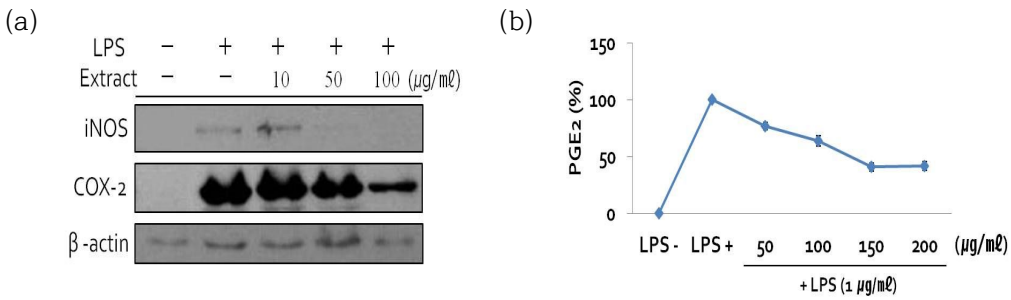

Fig. 8. Effects of ethyl acetate extract on NO and PGE_2 production in LPS stimulated RAW264.7 cells.

The expression levels of iNOS and COX-2 proteins were examined by western blot analysis. Indicated concentration of ethyl acetate extract was treated with LPS (1 μg/mℓ) in RAW264.7 cells. Following 24 h, iNOS was markedly decreased at 50 μg/mℓ and COX-2 was suppressed in a dose dependent manner (Fig. 8a). Pre-treatment of the cells with ethyl acetate extract (50, 100, 150 or 200 μg/mℓ) and LPS resulted in a significant dose-dependent reduction in PGE_2 production (Fig. 8b).

Nuclear translocation of NF-κB by ethyl acetate extract.

To identify NF-κB translocation from the cytosol to the nucleus, immunofluorescence was performed. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. The specific DNA binding of NF-κB showed that treating RAW264.7 cells with LPS (1 μg/mℓ) enhanced NF-κB activation. However, when the ethyl acetate extract (100 μg/mℓ) was added to the RAW264.7 cells, NF-κB activity was decreased significantly (Fig. 9).

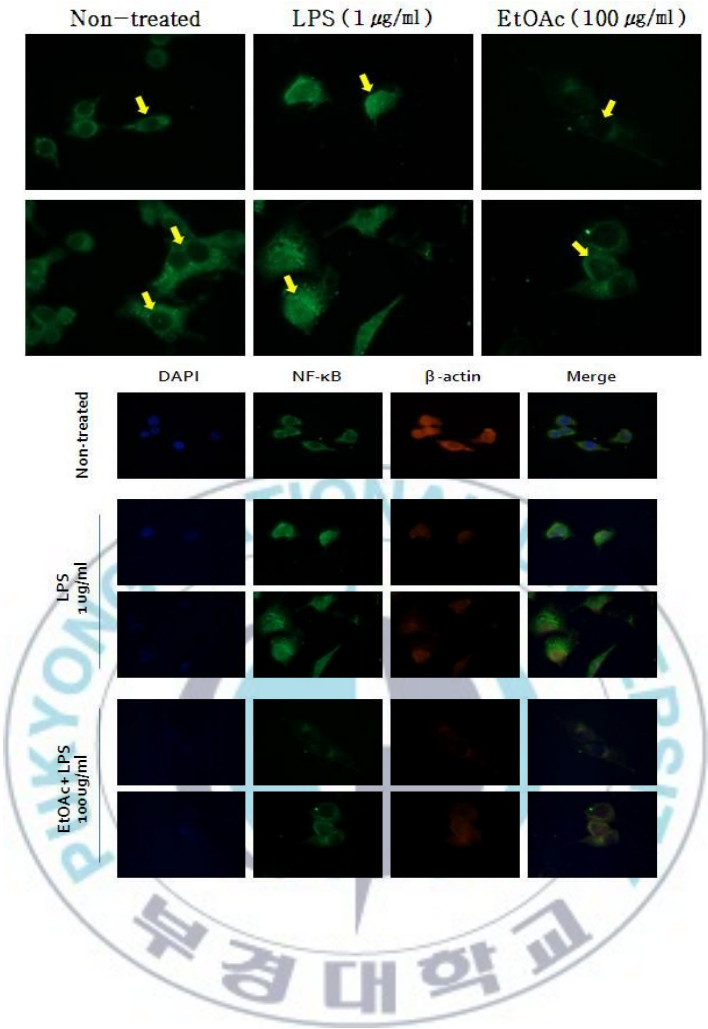

Fig. 9. Detection of NF-κB activity by ethyl acetate extract.

To identify of translocation activity, the RAW264.7 cells were pretreated with ethyl acetate extract (100 ㎍/㎖) for 2 h and then stimulated by LPS (1 ㎍/㎖). The NF-κB activity was increased by treated with LPS only. However, ethyl acetate extract was decreases traslocation of NF-κB activity.

Cellular viability and NO analysis for selection of ethyl acetate fractions.

Ethyl acetate extract was more fractioned for investigation of anti-inflammatory effects. Fractions was arranged involved in serial numbers. The 5, 7 fractions of ethyl acetate extract was employed for the anti-inflammatory study. The 5 fraction have cytotoxicity at 150 ㎍/㎖ concentration but suppressed NO production completely at 100 ㎍/㎖ concentration without cytotoxicity (Fig 10b, c). The 7 fraction strongly inhibit of NO production without any cytotoxicity (Fig 10b, d).

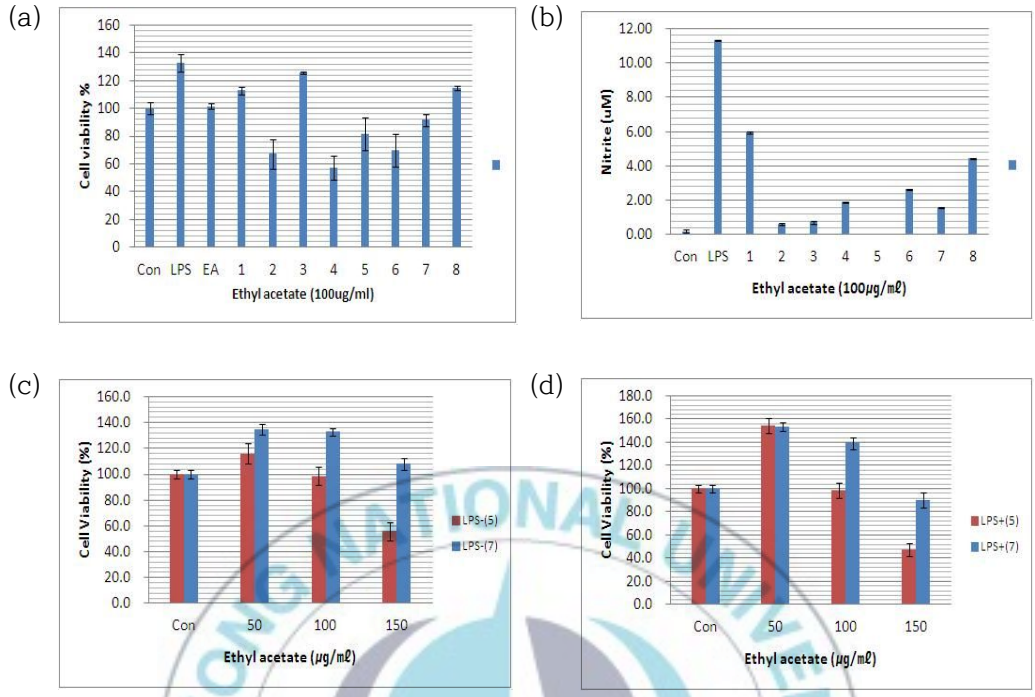

Fig. 10. The effects of fractioned ethyl acetate extract on cell proliferation and NO production.

Ethyl acetate extract was fractionated to study of anti-infalmmatory effects. fractionated ethyl acetate extracts were labeled and then measured the cell viability. The number 2,4 and 6 suppressed cell viability about 40%, number 1 and 3 increased cell viability about 20% (Fig. 10a). Down-regulation of NO production was further investigated. The number 5 and 7 strongly decreased NO production (Fig. 10b) without cell cytotoxicity in 100 μg/mℓ concentration (Fig 10c,d).

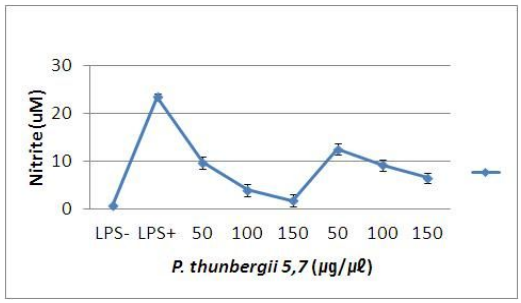

Fig. 11. Effects of 5 and 7 fraction on NO generation in LPS stimulated RAW264.7 cells.

The LPS activated RAW264.7 cells treated with 5 and 7 fractions showed reduced NO generation in a dose dependent manner. The concentration of 50 μg/mℓ decreased NO production about 50% and it is strongly decreased at concentration of 100 μg/mℓ.

Expression levels of pro-inflammatory proteins and protective proteins in LPS stimulated RAW264.7 cells.

To investigate the anti-inflammatory activity of 5 and 7 fraction, western blot analysis was conducted. RAW264.7 cells were stimulated with LPS (1 ㎍/㎖). The result showed that the fraction 5 and 7 down-regulates the expression of pro-inflammatory proteins such as iNOS and COX-2. Beneficial protective effects of HO-1 in inflammation are increased. These results suggest that 5 and 7 fractions possess a good anti-inflammtory activity.

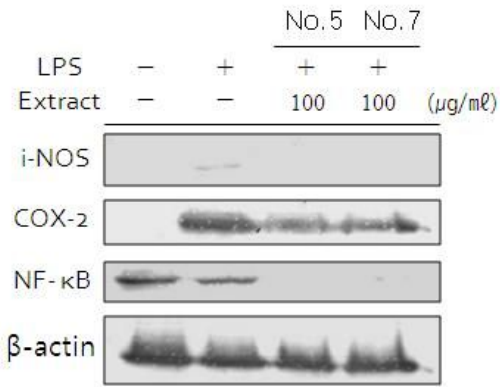

Fig. 12. Down-regulation of pro-inflammatory protein expression levels.

The expression levels of pro-inflammatory proteins were examined by western blot analysis. The ethyl acetate fraction (5 and 7) 100 μg/mℓ was treated with LPS (1 μg/mℓ) in RAW264.7 cells. After 24 h, iNOS, COX-2 and NF-κB was decreased. especially, 5 and 7 fractions suppressed to express of iNOS- and NF-κB protein.

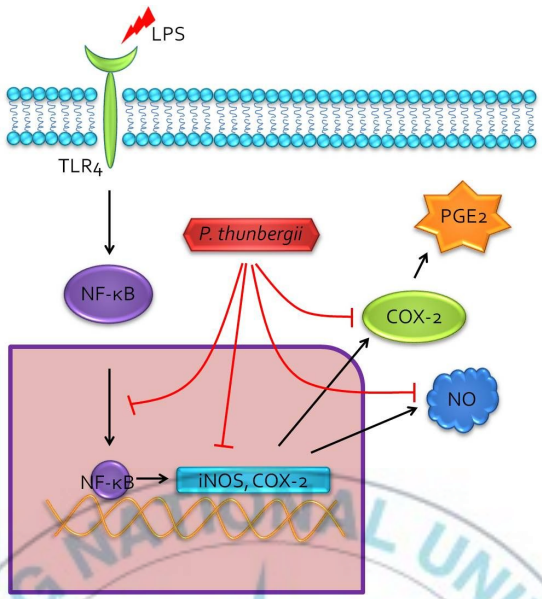

Fig. 13. Effects of *P. thunbergii* on inflammatory signaling.

In the inflammatory signaling, LPS was employed to induce the activation of RAW264.7 macrophage cells. NF-κB protein is one of the transcription factors for the expression of COX-2 and iNOS as mediated by LPS or pro-inflammatory cytokines. Following NF-κB activation, the iNOS and COX-2 expression levels were increased. The activation of sequential upstream signaling result in PGE_2 and NO productions are also increased. According to the results, the *P. thunbergii* extract inhibited proteins related to inflammatory signaling.

DISCUSSION AND CONCLUSION

Macrophages are produced by the differentiation of monocytes in tissues. Their role is to phagocytose (engulf and digest) cellular debris and pathogens. They also stimulate lymphocytes and other immnue cells to respond to pathogens (Khazen et al., 2005). In theory, lipopolysaccharide (LPS) induces the activation of macrophages. Over activated macrophages are generate pro-inflammatory responses that release nitric oxide, prostaglandin E_2 (PGE_2), inflammatory cytokines and ROS, which can all lead to harmful inflammatory responses.

Nitric oxide (NO) has beneficial roles in host defense system against tumor cells, viral replication and other factors. However, over production of NO cause various inflammatory diseases.

Relative to inflammatory response, iNOS and COX-2 are most important target proteins that produces NO and PGE_2. The iNOS produces NO in the cytoplasm. COX-2 activates synthesis of prostaglandins, prostacyclin and thromboxanes. Generally, COX-2 is barely detectable under normal physiological conditions, but the lipopolysaccharide induces over expression of COX-2 protein (Lee et al., 2010). It plays a vital role on the induction of inflammatory responses.

In this study, the effects of *Persicaria thunbergii* on pro-inflammatory responses were investigated. Pro-inflammatory responses was induced by LPS (1 ㎍/㎖) in RAW264.7 cells. For this research, the dried *P. thunbergii* powder was extracted by methanol and was partitioned with *n*-butanol, hexane and ethyl acetate (Fig. 1).

For the selection of non-cytotoxic concentrations, cell viabilities

were measured by WST-1 assay. Various concentrations of *P. thunbergii* methanol extracts did not influence the survival of RAW264.7 cells (Fig. 2). Under same conditions, the effects of NO production in LPS stimulated RAW264.7 cells were determined. As shown in Fig. 3, NO production was decreased by *P. thunbergii* methanol extracts in a dose dependent manner.

Following the NO analysis, western blot analysis was employed to analyze pro-inflammatory protein expression levels. Expression of iNOS, COX-2 and NF-κB was decreased but beneficial protective HO-1 protein is increased (Fig 4).

For the observation of translocated NF-κB in the nucleus, immunofluorescence was implemented. The NF-κB family of proteins comprises several transcription factors that regulate inducible gene expression in various immunological and antioxidant protective responses, including the up-regulation of major pro-inflammatory cytokines, adhesion molecules and antioxidant stress proteins (Perkins et al., 2006), (Gilmore. 2006). Under basal conditions, NF-κB is located in the cytoplasm in conjugation with inhibitory NF-κB (I-κB). In response to a wide range of signals, the regulatory NF-κB subunits, p50 and p65, dissociates NF-κB from I-κB and subsequently translocate to the nucleus (Ghosh and Hayden. 2008, Chen and Greene. 2004). In LPS-treated RAW264.7 cells, the NF-κB was translocated to the nucleus. However, methanol extract of *P. thunbergii* suppressed the translocation into the nucleus (Fig. 5).

For further study, methanol extract of *P. thunbergii* was partitioned by sequential solvent (*n*-butanol, hexane and ethyl acetate). Among them, ethyl acetate extract had the highest activation against NO production and was decreased by half on

the 100 ㎍/㎖ concentration without cell cytotoxicity (Fig. 6, 7)

Following the NO analysis, the expression levels of iNOS and COX-2 as pro-inflammatory marker proteins were determined by western blotting on the LPS stimulated RAW264.7 cells. As shown in Fig. 8a, cells treated with ethyl acetate extract were more efficient than cells treated with methanol extract. iNOS was completely decreased at 50~100 ㎍/㎖ concentration and COX-2 expression was suppressed in a dose-dependent manner.

Considering the results of NO analysis and western blotting, the decreased iNOS expression levels influenced NO production on the translation level. The decreased COX-2 expression levels are down regulate of PGE_2 up to 40% at 150 ㎍/㎖ concentration (Fig. 8b). To identify the translocation activity of ethyl acetate extract, immunofluorescence was performed. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. when the cells were treated with LPS (1 ㎍/㎖), it is activate the translocation of NF-κB in nucleus. However, when the ethyl acetate extract (100 ㎍/㎖) was added to the LPS stimulated RAW264.7 cells, NF-κB translocation was dramatically suppressed. (Fig. 9).

To find the more details, ethyl acetate was partitioned and arranged orderly. The number 5 and 7 fraction decreases the NO production without cytotoxicity at 100 ㎍/㎖ concentration (Fig. 10, 11). The effect of 5 and 7 fraction was further examined by western blotting, the result showed that the down-regulated expression of pro-inflammatory proteins (Fig. 12). In the inflammatory signaling, LPS activates the upstream signal proteins such as NF-κB, iNOS and COX-2, it leads to the over expression of NO and PGE_2. *P. thunbergii* suppressed each

upstream signaling steps and finally inhibitis NO and PGE_2 production (Fig. 13).

In this study, *P. thunbergii* showed that it could be a good anti-inflammatory agent by suppressing NO production, decreasing the expression levels of pro-inflammatory proteins and inhibiting NF-κB translocation on LPS stimulated RAW264.7 cells. However, the more fractioning of *P. thunbergii* extracts and additional research about its anti-inflammatory effects are needed.

LPS로 처리된 RAW264.7 세포에서의 고마리 추출물의 항염증 효과

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요 약

본 연구는 고마리의 추출물이 가지는 항염증 활성을 알아보기 위하여 쥐의 대식세포(RAW264.7 cell)에 Lipopolysaccharide (LPS)를 처리하여 염증반응을 유도하고 발생되는 Nitric oxide (NO)의 생성 억제를 확인하였다. 또한 염증에서 중요하게 알려져 있는 Inducible nitric oxide synthase (iNOS), Cyclooxygenase-2 (COX-2), Nuclear factor-kappa B (NF-κB)단백질의 발현을 비교하였고, 추가적으로 NF-κB 단백질의 핵 내로의 이전 및 활성을 확인하였다. 메탄올 추출물은 NO 생성 및 iNOS, COX-2, NF-κB 단백질을 억제 하고, 세포 보호 효과를 가지는 Heme oxygenase-1 (HO-1) 단백질을 증가시켰다. 위 결과를 바탕으로 하여 *n*-butanol, hexane, ethyl acetate 용매를 이용한 추가적인 분획을 실시하였고, 그 중에서도 고마리의 ethyl acetate 추출물은 Prostaglandin E_2 (PGE_2), NO생성을 억제 하였으며, iNOS, COX-2 발현 감소, NF-κB의 핵 내로의 이동억제에 높은 효과를 가지는 확인하였다. 이러한 연구 결과는 고마리 식물이 좋은 항염증 활성을 가지고 있음을 나타내며, 지속적인 분획으로 고마리 식물이 가지는 항염증 활성 단일 물질의 발견에 귀추가 주목된다.

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대학원에 입학하여 2년이라는 긴 시간동안 함께 실험실에서 생활하며, 저에게 많은 것들을 느끼게 해준 우리 세포신호전달실험실의 브레인 진수, 넘치는 끼를 가져 늘 피곤한 현일이, 항상 부지런한 순진이, 세포방 마스코트 조미, 큰 키와 똑똑한 머리를 가진 우석이, 바른 자세와 어깨를 잘 들썩이는 덕만이, 먹고 자고 몰카의 1인자 난희, 귀여운 필리핀 처자 초봉이, 푸근하고 편안해 보이는 은수, 듬직한 민석이, 키퍼 창원이, 회장님 지웅이, 있을 수 있을 까 동화, 파릇파릇한 예비군 민재강, 귀엽고 싹싹한 막둥이 보은이, 뭔가 있어 보이는 카신형, 항상 에너지 넘쳐 보이는 코이 모두 정말 고맙습니다.

그리고 제대하고 나서부터 학교에서 오랜 기간 우정을 쌓아온 현수, 편입해서도 기죽지 않고 학교생활 열심히해준 아름이 둘 다 대학원 동기라서 너무 좋았고, 지금은 다른 곳에서 활동 하고 있지만 두개의 심장 해진이, 재간둥이 민재리, 소탈하게 잘 웃던 석천이, 물리과 1등 의리도 1등 원석이, 여유 있어 보이는 승훈이, 바이러스 실험실에서 동고동락 했던 연지, 연정이, 나리, 은솔이, 한솔이, 조란이, 옥진이, 경용이형, 상은이 누나 모두 고맙습니다.

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