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THESIS FOR MASTER DEGREE

Isolation and Purification of Anti-inflammatory Compounds from Needles of *Pinus densiflora*

Graduate School of Chosun University

Department of Life Science

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ABBREVIATION

EtOAC	Ethyl Acetate
***n*-BuOH**	*n*-Butyl Alcohole
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric Oxide
TLC	Thin-Layer Chromatography
iNOS	Inducible nitric oxide synthase
COX-2	Cyclooxygenase-2
IL-6	Interleukin-6
IL-1 β	Interleukin-1 beta
TNF- α	Tumor Necrosis Factor - alpha
Real-time PCR	Real-time Polymerase Chain Reaction

ABSTRACT

Isolation and Purification of Anti-inflammatory Compounds from Needles of *Pinus densiflora*

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Pinus densiflora sieb. et zucc. contains several phenolic compounds that have various biological activities such as antimicrobial, antioxidant and antihypertension. However, the health beneficial effects of these compounds have rarely been reported.

Inflammatory response protects the host against tissue injury, microbial invasion but uncontrolled inflammation cause disease such as cardiovascular disease, autism, cancer and autouimmune disease. RAW264.7 murine macrophage cell line play a major role in inflammatory response.

In this study, we seperated n-Hexane, EtOAc n-BuOH and H_2O layer from

methanolic extract of *pinus densiflora* and tested anti-inflammation activity on LPS-induced inflammation in RAW264.7 murine macrophage cell line. EtOAc layer was not cytotoxic in RAW 264.7 and nitric oxide (NO) production decreased in LPS-stimuated RAW264.7 more than other layers. We fractionated EtOAc layer by silica gal (63-200um) open column chromatography and eluted with gradient mixtures of methylen chloride-MeOH (70:1 to 0:1) to yield nine fractions. We tested cytotoxicity, anti-inflammation activity. Most of activity fraction 5 (F.5) treatment reduced mRNA level of cytokines (IL-6) and pro-inflammatory genes such as iNOS and COX-2 in LPS-stimulated macrophage activation in a dose-dependent manner.

INTRODUCTION

Natural products, secondary metabolites, isolated from plants are important sources for bioactive molecules that in many cases have been developed into treatments for diseases (Francisco A. Villa, and Lena Gerwick et al. 2010). Clinical, pharmacological, and chemical studies of these natural products, which were derived predominantly from plants, were the basis of most early medicines such as aspirin (Newman, D.J. et al. 2000), digitoxin (Buss, A.D. et al. 2003), morphine (Grabley, S. et al. 2000), quinine (Sneader, W. et al. 1996), and pilocarpine (Mann, J. Muder et al. 1996).

Pine trees (*Pinus densiflora sieb. et zucc.*) belong to the family *pinaceae* and are widely distributed around the world. In East-Asian countries such as Korea, Japan and China, various parts of pine trees, including the needles, cones, cortices, and pollen are widely consumed as foods or dietary supplements to promote health (Deagao Wang et al. 2005). In Korea, Pinus densiflora has been used as a folk remedy for rheumatitis, hemorrhage, gastroenteric trouble, hypertension and asthma (K. H. Bae et al., 2000). Especially, pine needles habe been widely used as food materials, food additives and folk medicines (B. G. Lee et al. 2014). Pine needles have shown antibacterial (Su Feng et al. 2010), antioxidant (Y. S. Park et al. 2011), antihypertension (K. Y. Kim et al. 2000) activity. Moreover, pine needles have been shown to inhibit leukemia cell growth (Betty Hsu et al. 2006) and protect against oxidative DNA damage and apoptosis induced by hydroxyl radicals (J. B. Jeong et al. 2009)

Inflammatory response protects the host against tissue injury and microbial invasion. As such, this response should be short-lived, and failing can result in pathogenesis of many immunity-related diseases (Yu-Tang Tung ea al. 2008). Also,

inflammation is a variety of biological response to harmful stimui and is associated with many pathophysiological conditions. Macrophages play an essential role in local host defense and inflammatory response (M. E. Kim et al. 2014). Activation of macrophage plays in the initiation and propagation of inflammatory responses by the production of cytokines, interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), cycloxygenase-2 (COX-2) and other inflammatory mediators (H. G. Kim et al. 2006)

Lipopolysaccharide (LPS), a bacterial membrane component, has been shown to be a potent activator of microglia and inducer of brain inflammation-associated proteins and proinflammatory cytokines in many in vivo and in vitro experimental models (G. Li et al. 2004 and J. R. weinstein et al. 2008). The LPS-induced increases in levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) result in the productions of NO and prostaglandins, which contribute to cell damage(V. E. Laubach et al. 1995)

Production of NO is regulated by nitric oxide synthase (NOS) in the conversion of L-arginine to L-citrulline, and there are at least three types of NOS including inducible nitric oxide synthase (iNOS), eNOS and nNOS (H. Y. Lin et al., 2005). Activation of iNOS occurs in several human diseases such as inflammation, diabetes, stroke, and positive bacteria, and induce NO production by activation of iNOS gene expression (Kengatharan et al. 1996; Jungi et al. 1999)

In inflammatory process, several pro-inflammatory cytokines such as, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-4, IL-6, and IL-8, recruit activated immune and inflammatory cells to the site of lesions, thereby amplifying and perpetuating the inflammatory condition (H. H. Park et al. 2007). Tumor necrosis factor (TNF) is the term applied to a pair of cytokines originally called cachectin (TNF-α) and lymphotoxin (TNF-β). The TNF-α is released early in copious amounts in response to a wide variety of invasive stimuli, and induces directly and

potently many immune and inflammatory activation through induction of adhesion molecules and neutrophil chemotactic factors. Therefore, it can be hypothesized that an inhibitor of TNF-α release could be a possible anti-inflammatory agent (B. H. Kim et al. 2004)

In this study, anti-inflammatory compounds were isolated from methanol extract of *Pinus densiflora*. Cytotoxicity and anti-inflammatory activity confirmed on the regulation of NO production in macrophages stimulated by LPS. Futhermore, iNOS, COX-2, IL-6, IL-1β and TNF-α expression levels identified in LPS activated macrophages.

MATERIALS AND METHODS

1. Plant materials

Fresh needles of red pine (*Pinus densiflora Sieb. et Zucc*) had been picked up from red pine in Gokseong province, Jeollanam-Do, South Korea. Harvested needles of red pine were cleaned with tap water (cleaned with 5% charcoal) and dehydrated with the spin-drier. The dehydrated pine needles (100 kg) was dried in the sun.

2. Isolation and purification of anti-inflammation compounds from *pinus densiflora*

The dried pine needle (49.97kg) was extracted with 80% MeOH (500L) at 69 ℃ for 3h. The combined MeOH extracts were concentrated to yield (20 L). This crude extract was partitioned successively with *n*-Hexane (3 × 10L), EtOAc (3 × 10L) and *n*-BuOH (3 × 10L). The EtOAc fraction (590.18g), which exhibited strong anti-inflammatory activity, was chromatographed over a silica gel column (10 × 60 cm; 63-200㎛ particle size) and eluted with gradient mixtures of methylen chloride-MeOH (70:1, 60:1, ..., 0:1) to yield nine fractions.

3. Cell lines and culture conditions

Mouse lymphoid microvascular epithelium immortalized cell line, Raw 264.7 was obtained from the ATCC (TIB-71). Raw264.7 was grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 ㎍/mL penicillin-streptomycin at 5% CO_2 and 37 ℃ humidified atmosphere.

4. Cytotoxicity

MTT assay was performed by the method of Mosmann in the modification of Hansen, Nielsen, and Berg as Kuzma, Wysokinska, Rozalski, Krajewska, and Kisiel described previously. Briefly, Raw 264.7 cell in suspension were seeded at 2×10^4 cells per well in 96-well plate and these cells were grown in a humidified atmosphere of 5% CO_2 in air at 37℃. Then the cells were exposed to varying concentrations of the Pine needle extract (200 ㎍/mL to 25 ㎍/mL) for 24h. After that, culture medium was added 100 uL fresh one. MTT solution (5 mg/mL PBS) was then added and the plate was located in optimal atmosphere at 37 °C. The metabolically active cells reduced MTT to blue formazan crystals. After 4 h, MTT-formazan crystals were dissolved in 50% ethanol and 50% DMSO and absorbance was measured at 570 nm on a multifunctional plate reader (Eon, Bio-tek, USA) and compared with untreated cells.

5. Inhibition of NO production in LPS-induced RAW 264.7

Raw 264.7 cells (5×10^4 cells/well) were seeded in 96-well culture plate in DMEM. Cultured cells were pretreated with various concentrations of Pine needle extracts for 2h and then cells were incubated for 22h in absence or presence of LPS. After incubation, the cultured medium was mixed with an equivalent volume of 1 × Greiess Reagent (Sigma, USA) and incubated for 15min at room temperature. After incubation, absorbance was measured at 540 nm on a multifunctional plate reader (Eon, Bio-tek, USA).

6. Real-time PCR

Raw 264.7 cells (1 × 10^6 cells/well) were seeded in 6-well culture plate in DMEM. Cultured cells were pretreated with various concentrations of Pine needle extracts for 2h and then cells were incubated for 6h in absence or presence of LPS. After incubation, the cells were collected by centrifugation and total RNA was isolated from Pine needle extracts-treated cells using RiboEX (GeneAll, Seoul, Korea) according to protocol. To synthesize cDNA, 0.5 ㎍ of total RNA was primed with oligo dT and reacted with mixture of Hyperscript (GeneAll, Seoul, Korea). To measure the mRNA level of inflammatory cytokines including IL-6, IL-1β, iNOS and COX-2, we designed the primers for target genes. And cDNA was amplified using Rotor-Gene (Corbett, Hilden, Germany), SYBR Green (Takara, Shiga, Japan) and the primers.

COX-2	F	5' - TGG GTG TGA AGG GAA ATA AGG - 3'
	R	5' - CAT CAT ATT TGA GCC TTG GGG - 3'
TNF-α	F	5' - GGC CTC TCT ACC TTG TTG CC - 3'
	R	5' - TAG GCG ATT ACA GTC ACG GC - 3'
IL-1β	F	5' - GTG TCT TTC CCG TGG ACC TT - 3'
	R	5' - TCG TTG CTT GGT TCT CCT TG - 3'
IL-6	F	5' - CCT TCC TAC CCC AAT TTC CA - 3'
	R	5' - CGC ACT AGG TTT GCC GAG TA - 3'
iNOS	F	5' - CGG GTT GAA GTG GTA TGC AC - 3'
	R	5' - GCT GTG TGG TGG TCC ATG AT - 3'

Table 1. Sequence of pro-inflammatory gene primer designs

RESULTS

Partition of anti-inflammation compounds from *Pinus densiflora*

The dried pine needles was extracted with 80% MeOH at 69℃ for 3hr. The combined MeOH extracts were concentrated to yield (20L). The extract was suspended in H_2O and partitioned with *n*-BuOH, EtOAc and *n*-Hexane.

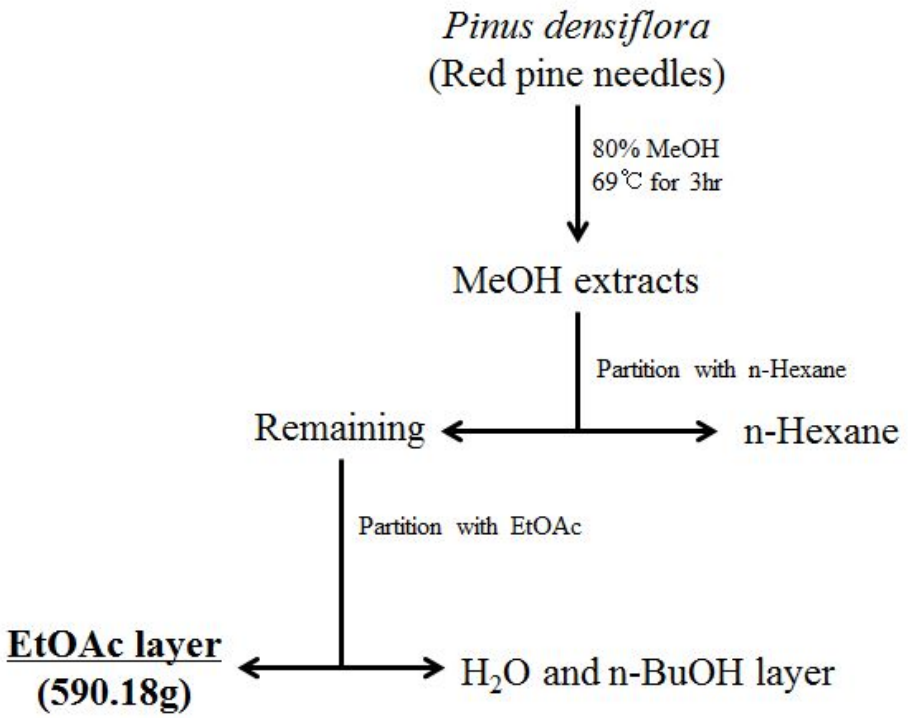

Fig 1. The dried pine needle (500kg) was extracted with 80% MeOH at 69℃ for 3h. The combined MeOH extracts were concentrated to yield (20L). This crude extract was partitioned successively with *n*-Hexane (3 × 10L), EtOAc (3 × 10L) and *n*-BuOH (3 × 10L). The EtOAc layer was 590.18g.

Cytotoxicity of anti-inflammation compounds from *Pinus densiflora*

The cytotoxic effects of 4 fractions at molecular and cellular levels were tested on RAW264.7 cultured cell lines. The results indicate that *n*-Hexane layer displayed cytotoxic and EtOAc, *n*-BuOH, H_2O no cytotoxic effects at concentrations of 25 μg/mL to 200 μg/mL on three tested cultured cell lines, as shown in figure 2, 3, 4 and 5.

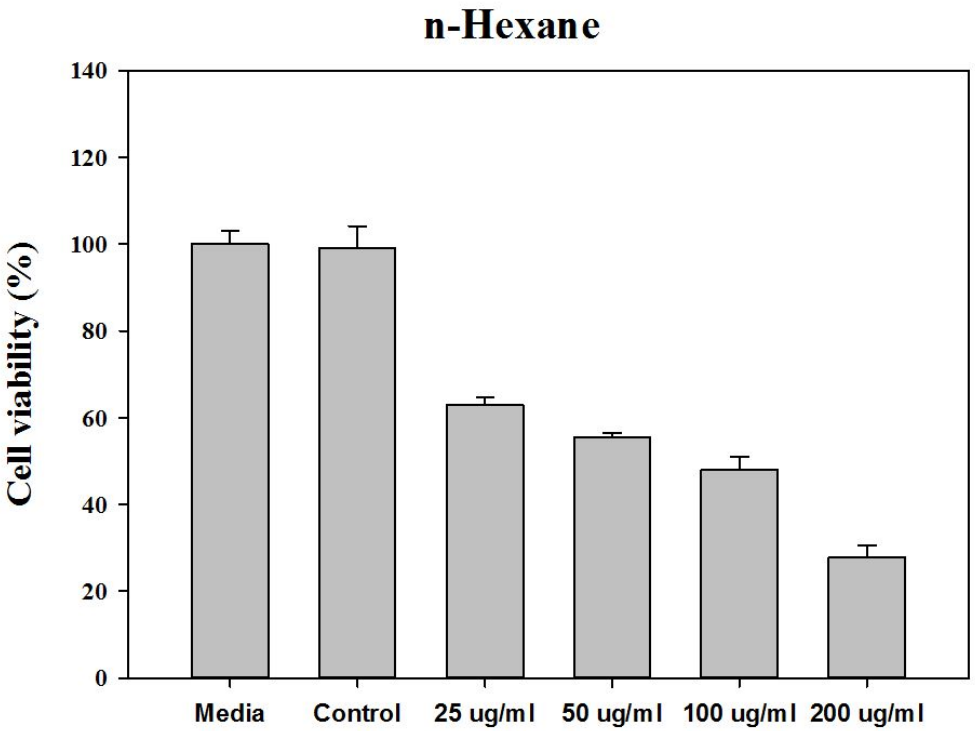

Fig 2. Cell viability of n-Hexane layer on RAW 264.7 cells. n-Hexane layer were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. n-Hexane layer had cytotoxicity.

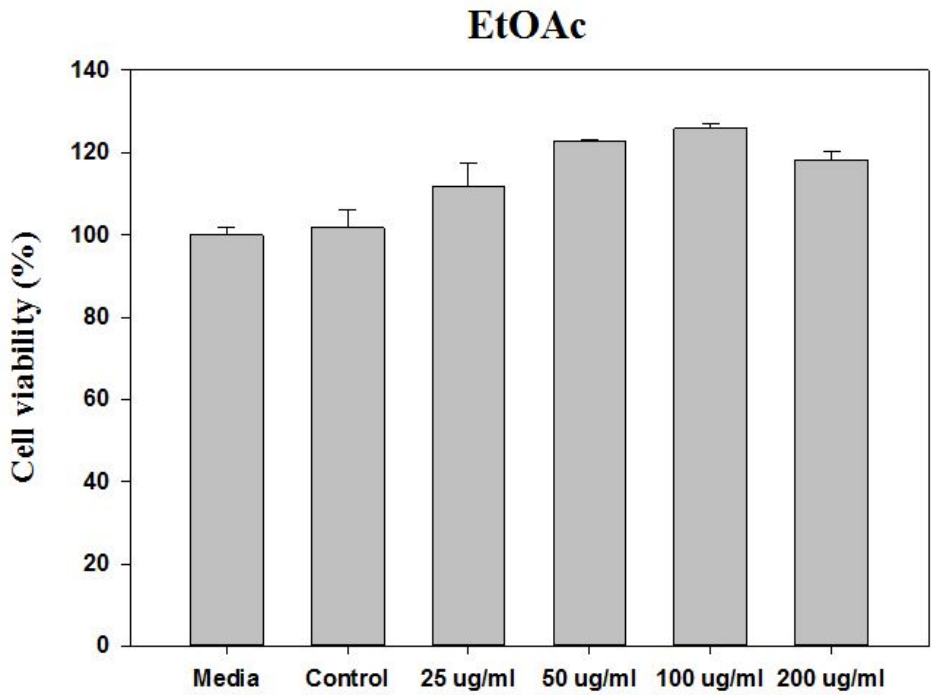

Fig 3. Cell viability of EtOAc layer on RAW 264.7 cells. EtOAc layer were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. EtOAc layer had no effect on cell viability.

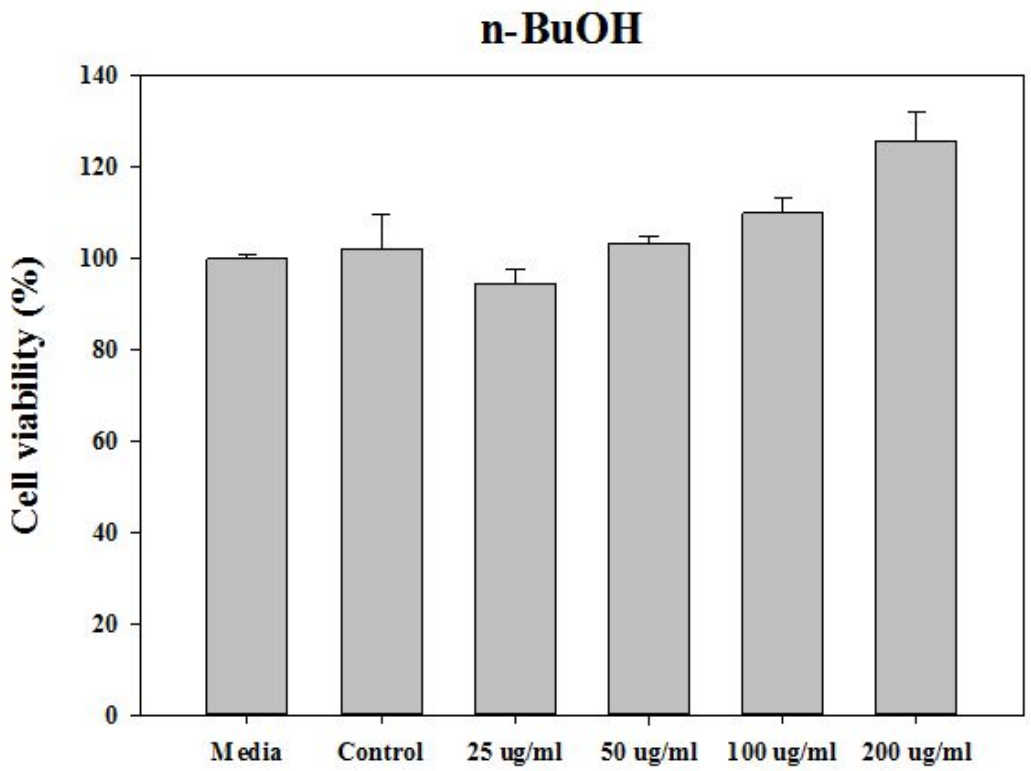

Fig 4. Cell viability of *n*-BuOH layer on RAW 264.7 cells. *n*-BuOH layer were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. *n*-BuOH layer had no effect on cell viability.

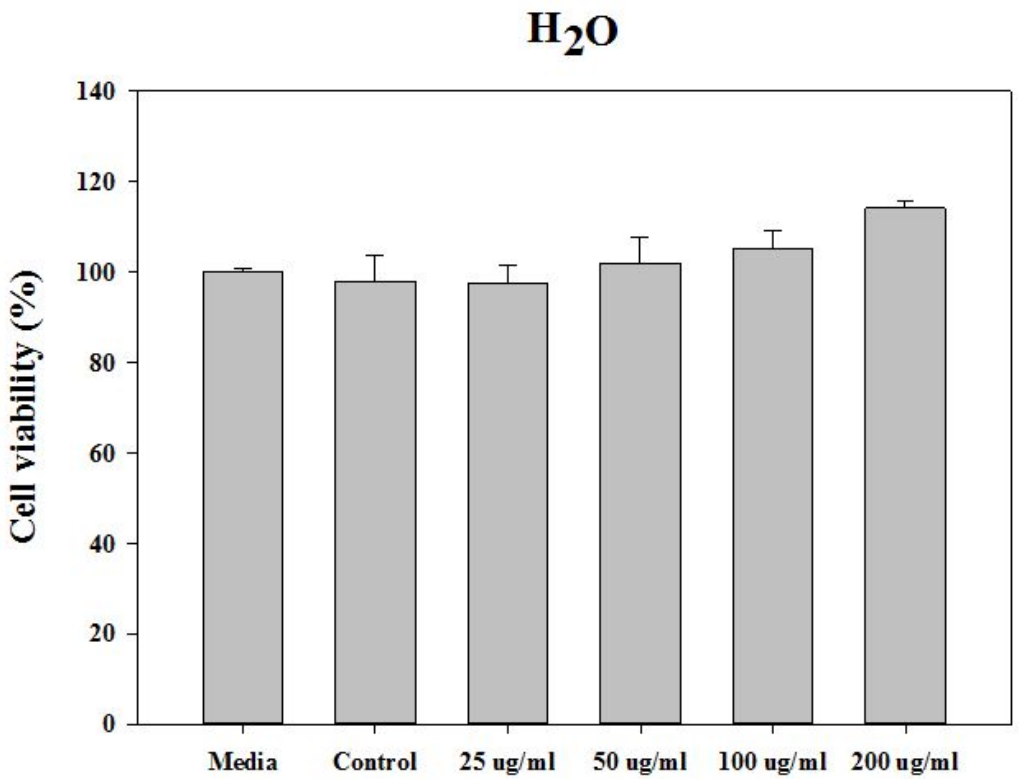

Fig 5. Cell viability of H_2O layer on RAW 264.7 cells. H_2O layer were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. H_2O layer had no effect on cell viability.

Inhibition of NO production in LPS-induced RAW 264.7

EtOAc, n-BuOH and H_2O layer were tested regulate NO production, and measured NO levels in LPS-stimulated macrophages after three layer treatment. Macrophages were pre-treated with ESH for 2h and then stimulated by LPS (200 ng/mL). LPS treatment significantly induced NO production compared with control (Figure 6, 7, 8). EtOAc layer decreased LSP-induced NO production but *n*-BuOH and H_2O had no effect of NO production in RAW 264.7 murine macrophages.

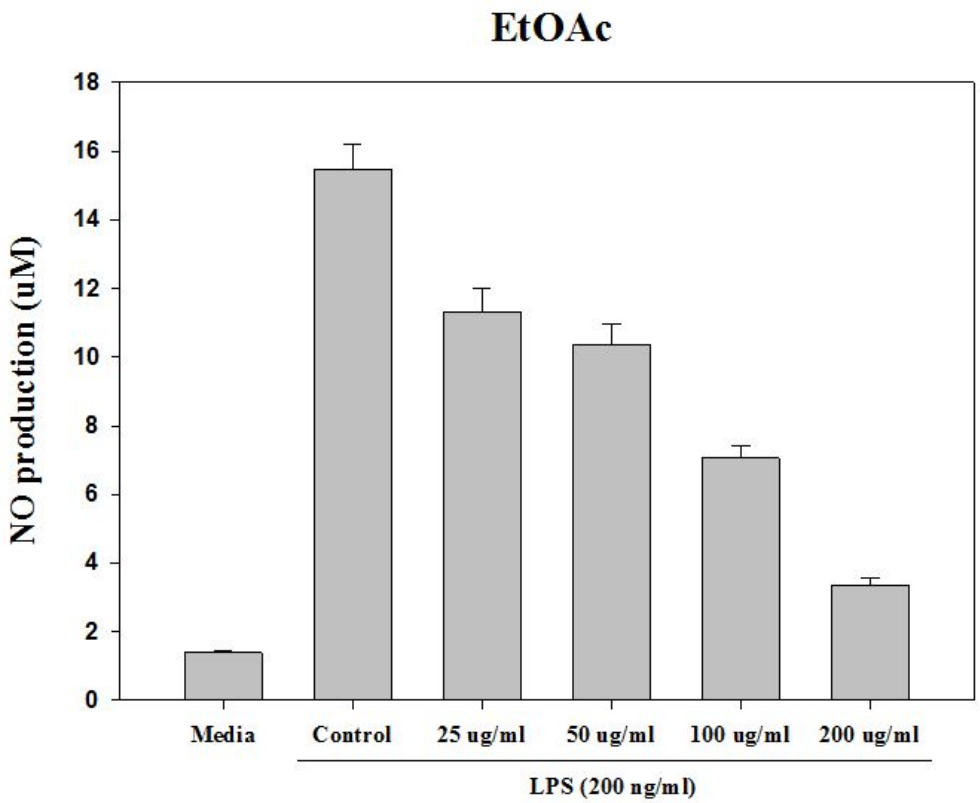

Fig 6. EtOAc layer decreased LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EtOAc layer (0-200 ug/mL) were pretreated on cells for 2h, and then the cells ere incubated for 22h in presence or absence of LPS (200ng/mL). Suprernatants were mixed with griess reagent and absorbance was measure by ELISA microplate reader.

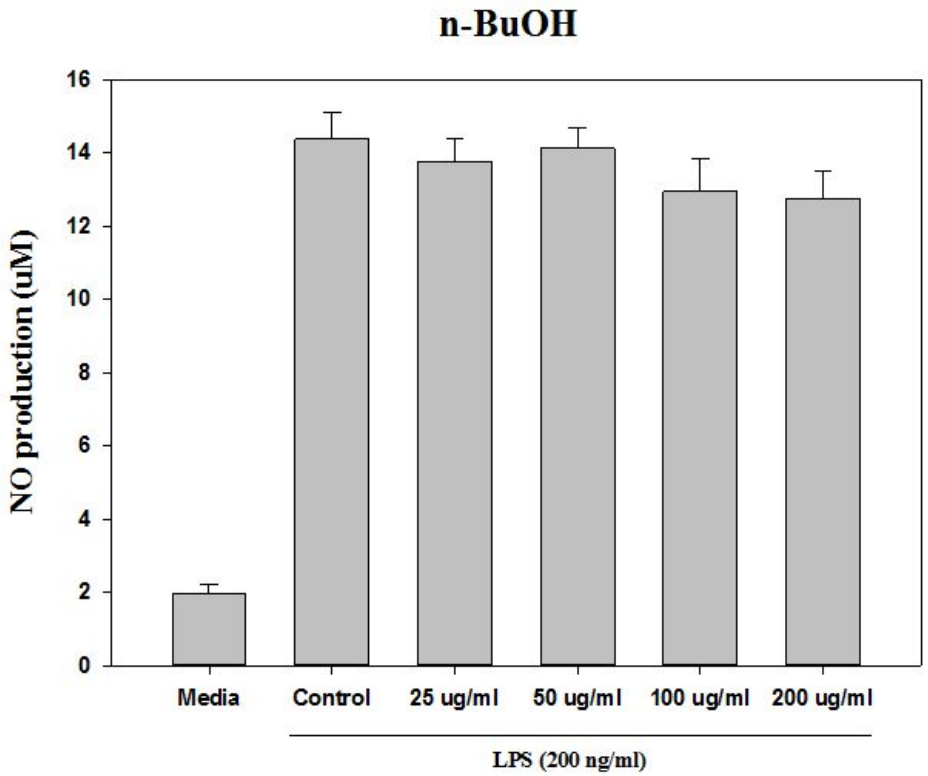

Fig 7. *n*-BuOH layer had no effect LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EtOAc layer (0-200 ug/mL) were pretreated on cells for 2h, and then the cells ere incubated for 22h in presence or absence of LPS (200 ng/mL). Suprernatants were mixed with griess reagent and absorbance was measure by ELISA microplate reader.

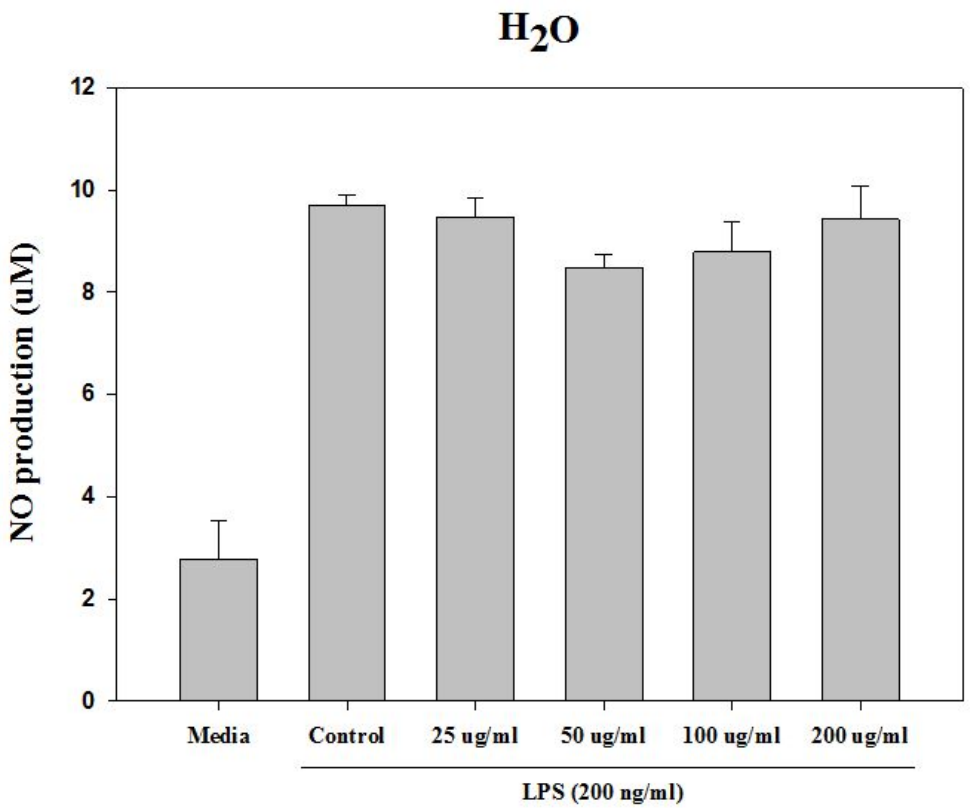

Fig 8. H_2O layer had no effect LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EtOAc layer (0-200 ug/mL) were pretreated on cells for 2h, and then the cells ere incubated for 22h in presence or absence of LPS (200ng/mL). Suprernatants were mixed with griess reagent and absorbance was measure by ELISA microplate reader.

Fractionation of EtOAc layer with column chromatography

The EtOAc layer (100g) was found to be most active among the solvent fraction. EtOAc layer fractionated by silica gel column chromatography (10 × 60 cm) using a gradient of CH_2Cl_2 : MeOH (from 70:1 to 0:1), to yield nine combined fractions according to their TLC profiles.

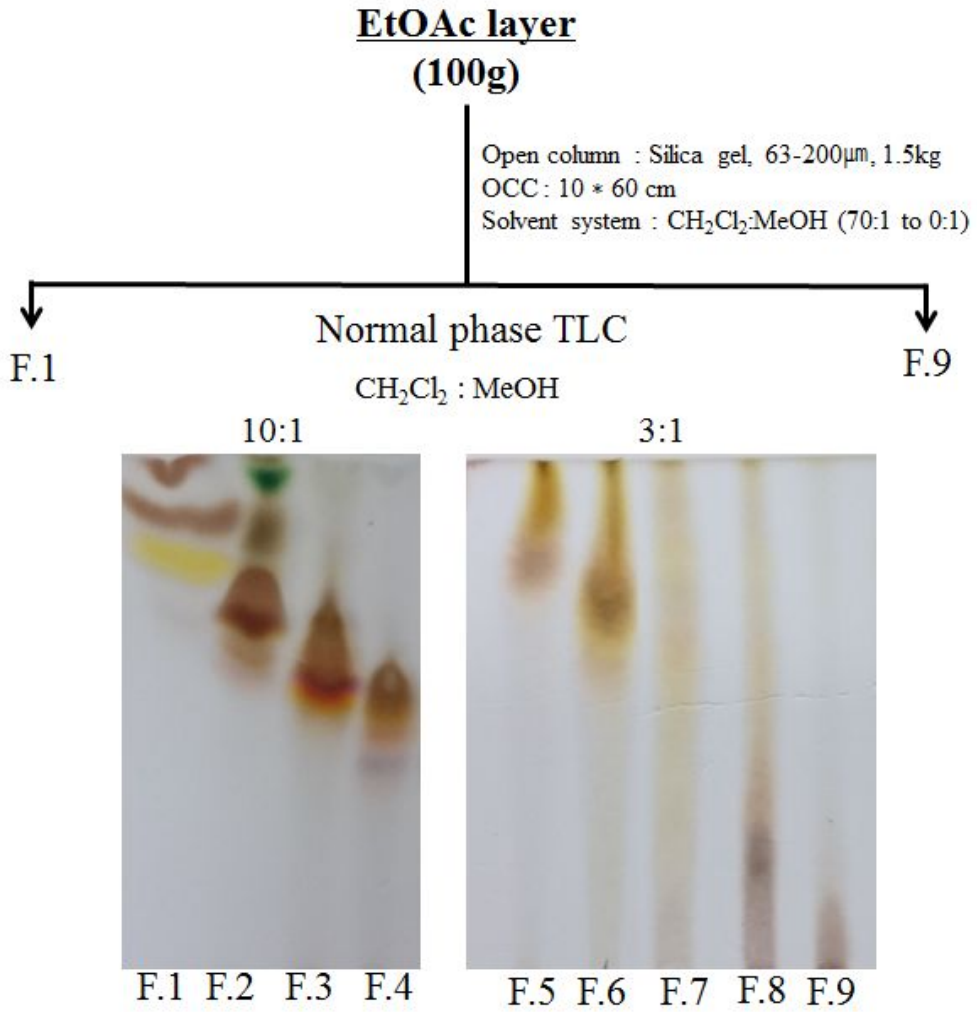

Fig 9. Isolation of nine fraction from EtOAc layer.

Cytotoxicity of nine fractions from EtOAc layer

The cytotoxic effects of nine fractions at molecular and cellular levels were tested on RAW264.7 cultured cell lines. The results indicate that F.1, F.2, F.3 and F.4 displayed cytotoxic and F.5, F.6, F.7, F.8, F.9 no cytotoxic effects at concentrations of 25 µg/mL to 100 µg/mL on three tested cultured cell lines, as shown in figure 10 and 11.

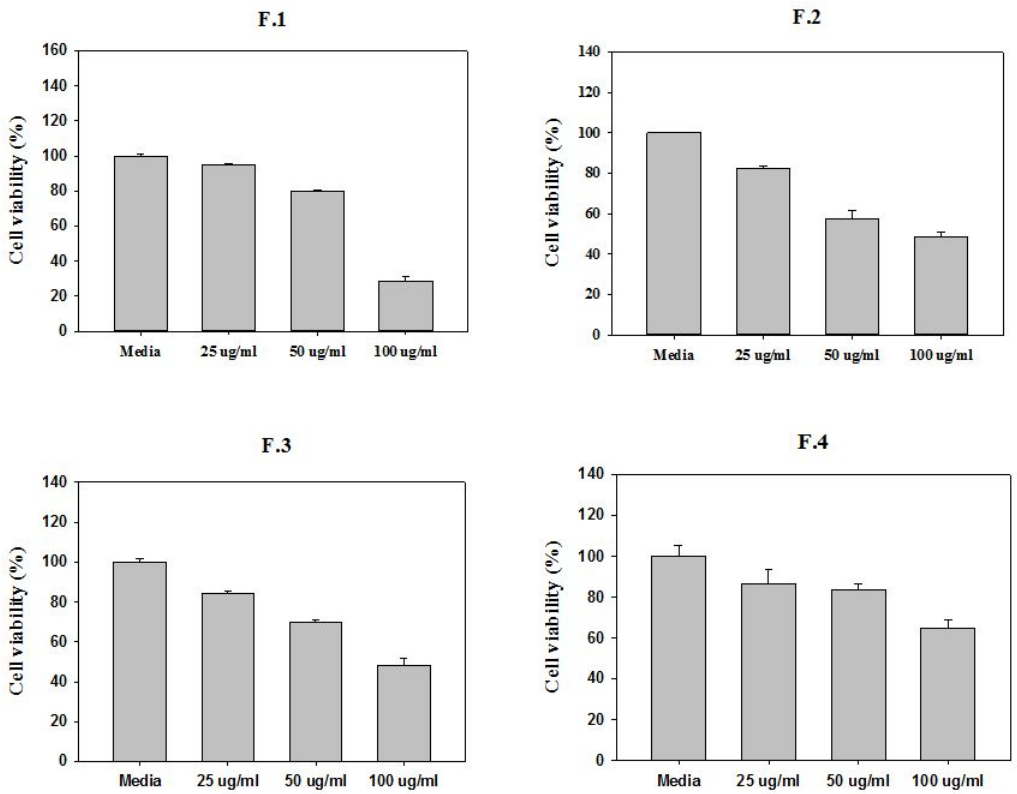

Fig 10. Cell viability of F.1, F.2, F.3 and F.4 on RAW 264.7 cells. 4 fractions were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. 4 fractions had effect on cell viability.

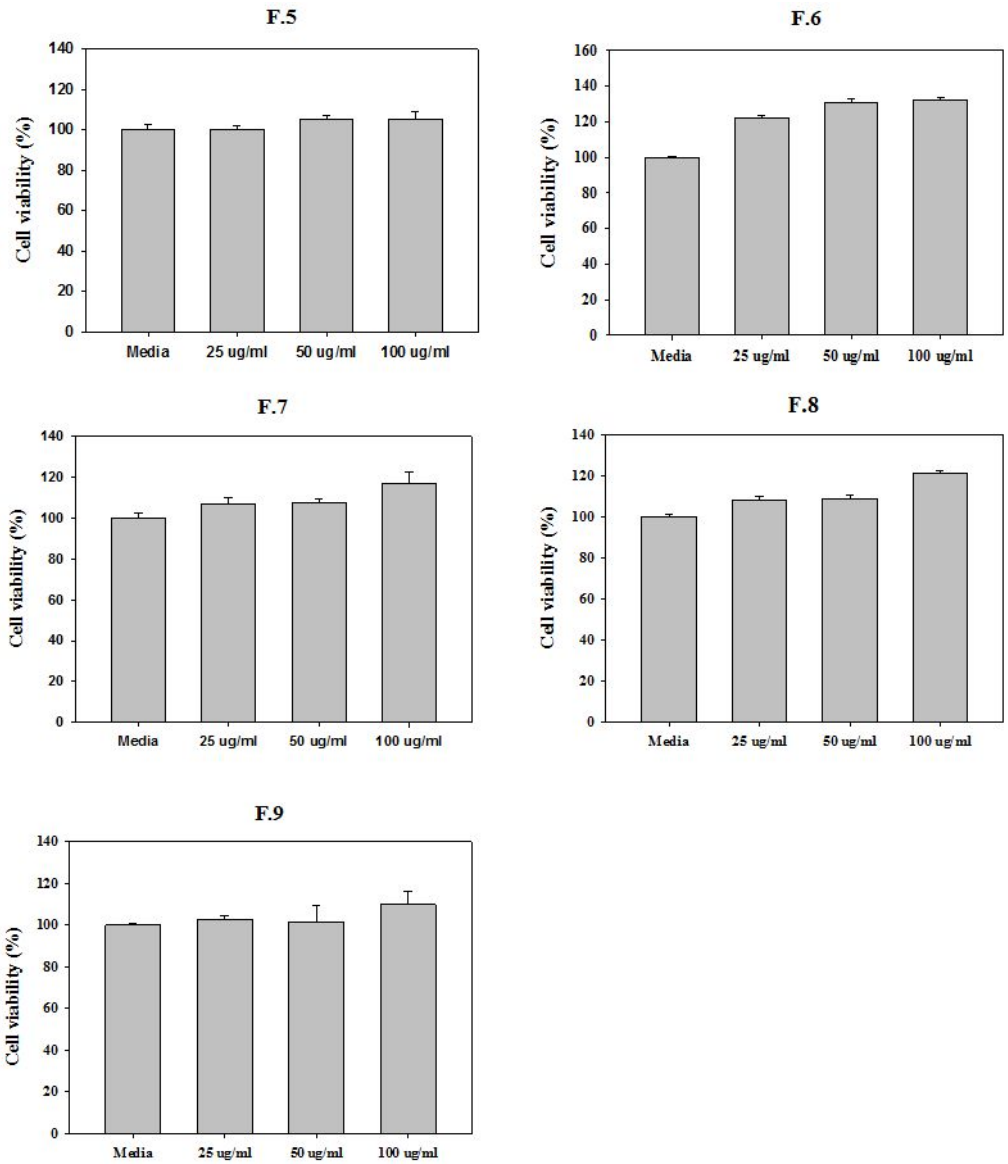

Fig 11. Cell viability of F.5, F.6, F.7, F.8 and F.9 on RAW 264.7 cells. 5 fractions were applied to the cells for 24h and cell viability was assessed by MTT assay as described in the text. 5 fractions had no effect on cell viability.

Inhibition of NO production of five fractions in LPS-induced RAW 264.7

Five fractions tested regulate NO production and measured NO levels in LPS-stimulated macrophages. Macrophages were pre-treated with fractions for 2h and then stimulated by LPS (200 ng/mL). LPS treatment significantly induced NO production compared with control (Figure 12). F.5 decreased LSP-induced NO production more than other fractions.

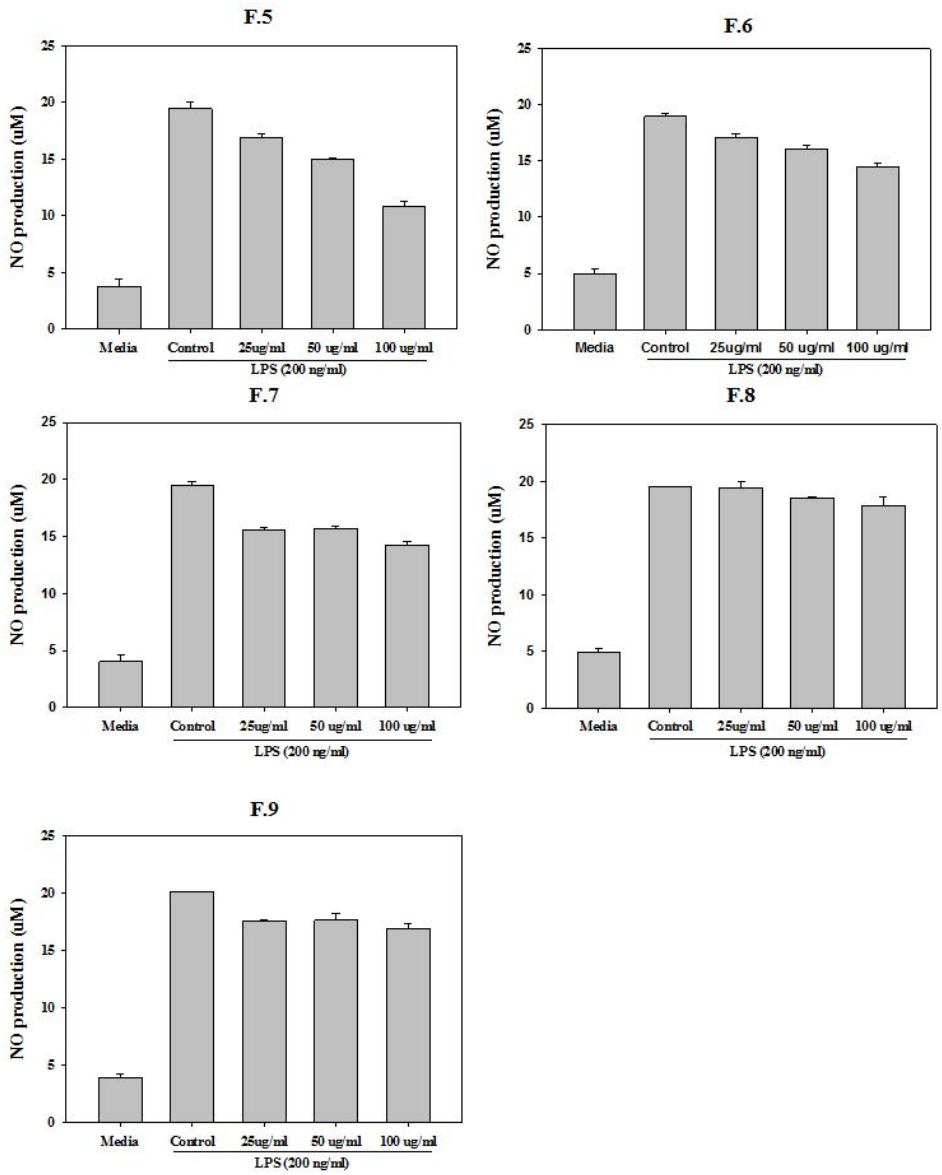

Fig 12. F.5 decreased LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EtOAc layer (0-200 ug/mL) were pretreated on cells for 2h, and then the cells ere incubated for 22h in presence or absence of LPS (200 ng/mL). Suprernatants were mixed with griess reagent and absorbance was measure by ELISA microplate reader.

Real-time PCR

IL-1β is pro-inflammatory cytokines that is released from macrophages upon stimulation with LPS or other inflammatory conditions. Real-time PCR performed to confirm the effect of F.5 on IL-1β mRNA expression level. For real-time PCR, macrophages were pretreated F.5 fo 2hr and then stimulated with LPS for 6h. F.5 reduced mRNA expression level of IL-1β. And F.5 confirmed inhibitory effect of iNOS, IL-6, COX-2 and TNF-α. mRNA expression levels determined of iNOS, IL-6, COX-2 and TNF-α in macrophages stimulated with LPS in the presence or absence of F.5. After treatment of LPS, the mRNA expression of iNOS, IL-6, COX-2 and TNF-α was significantly increased in macrophages. However pre-treatment of various concentrations of F.5 inhibited iNOS, IL-6, COX-2 mRNA expression levels.

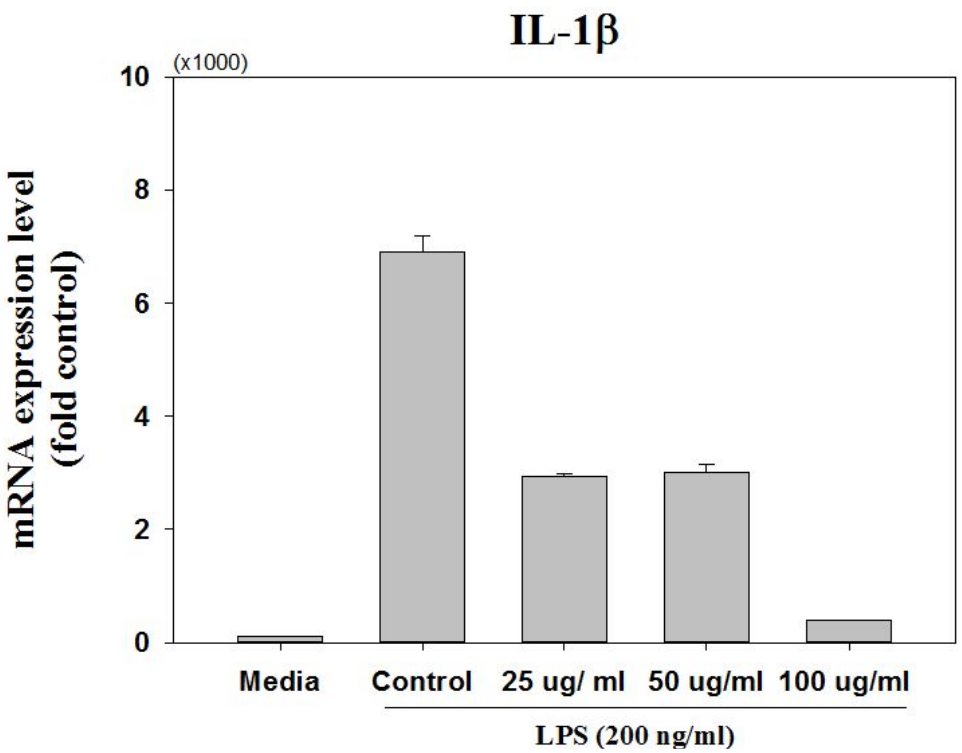

Fig 15. F.5 inhibited LPS-induced pro-inflammatory genes on RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 25, 50, 100 ug/mL of F.5 for 2h, and stimulated by LPS for 6h. mRNA expression level of inflammatory genes were determined by real-time PCR. F.5 treatment reduced mRNA expression level of IL-1 β .

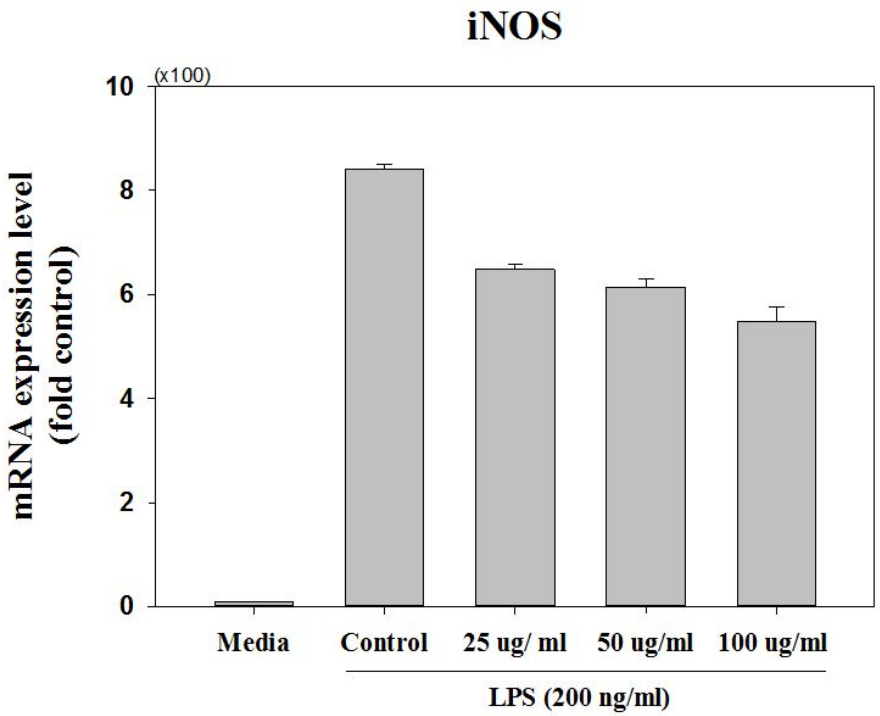

Fig 13. F.5 inhibited LPS-induced pro-inflammatory genes on RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 25, 50, 100 ug/mL of F.5 for 2h, and stimulated by LPS for 6h. mRNA expression level of inflammatory genes were determined by real-time PCR. F.5 treatment reduced mRNA expression level of iNOS.

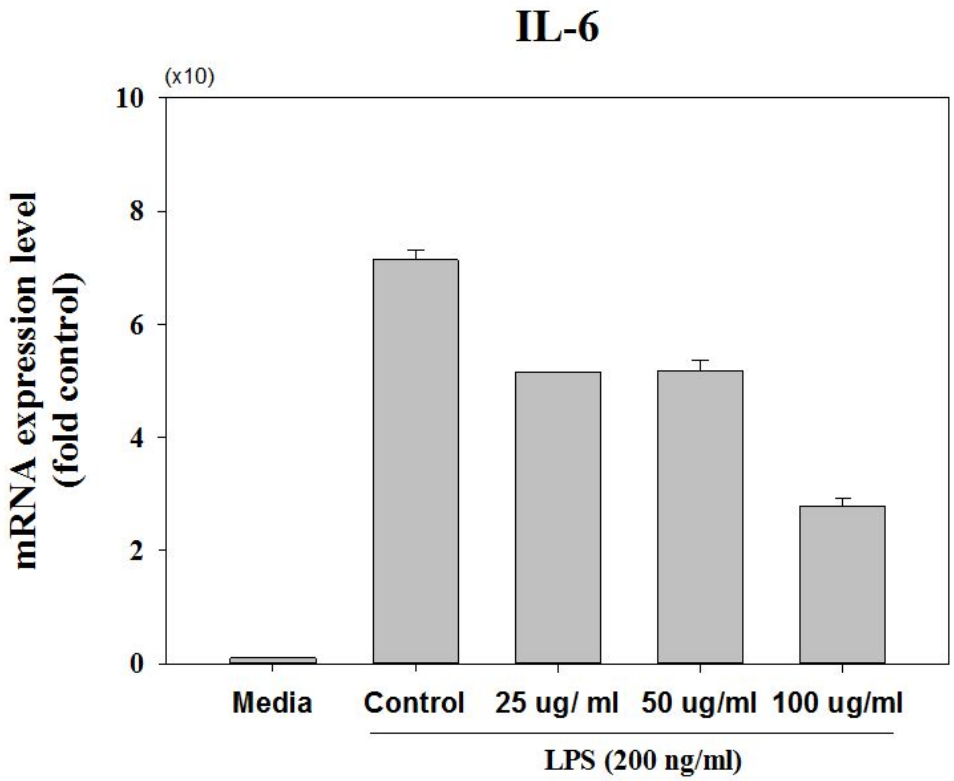

Fig 14. F.5 inhibited LPS-induced pro-inflammatory genes on RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 25, 50, 100 ug/mL of F.5 for 2h, and stimulated by LPS for 6h. mRNA expression level of inflammatory genes were determined by real-time PCR. F.5 treatment reduced mRNA expression level of IL-6.

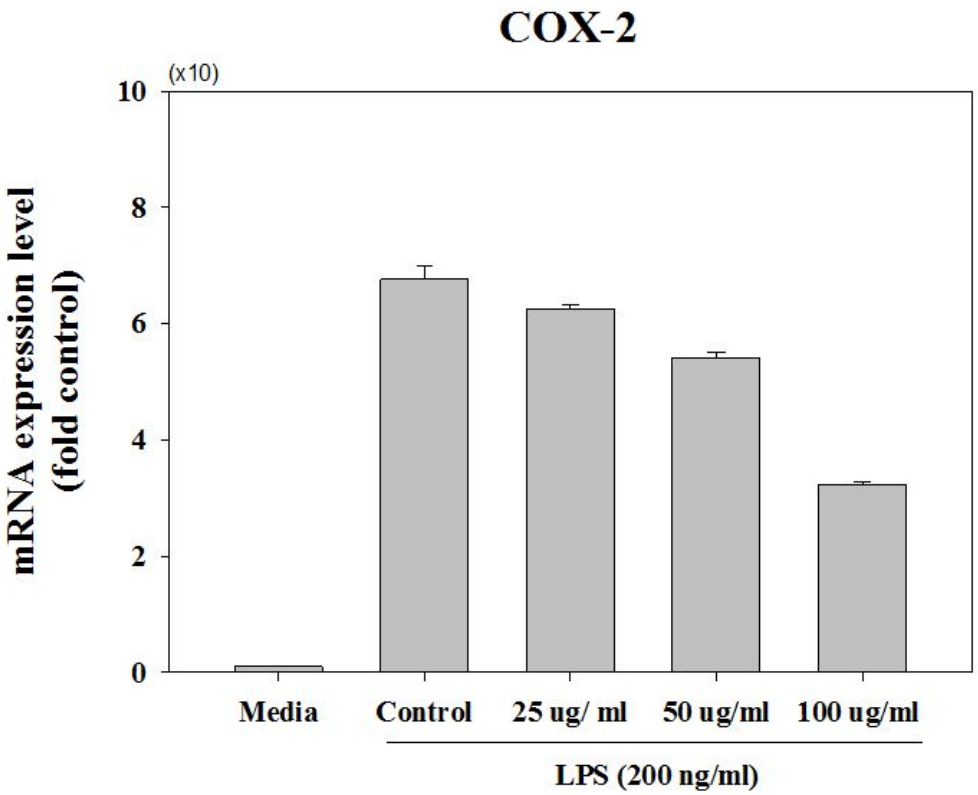

Fig 16. F.5 inhibited LPS-induced pro-inflammatory genes on RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 25, 50, 100 ug/mL of F.5 for 2h, and stimulated by LPS for 6h. mRNA expression level of inflammatory genes were determined by real-time PCR. F.5 treatment reduced mRNA expression level of COX-2.

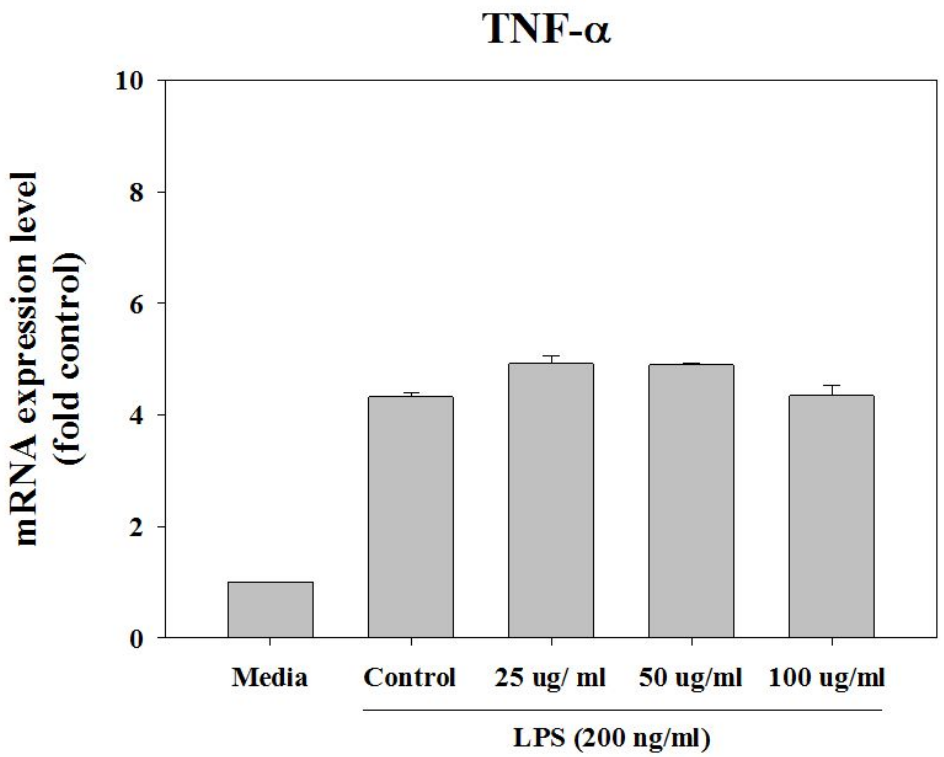

Fig 17. F.5 inhibited LPS-induced pro-inflammatory genes on RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 25, 50, 100 ug/mL of F.5 for 2h, and stimulated by LPS for 6h. mRNA expression level of inflammatory genes were determined by real-time PCR. F.5 treatment not decreased mRNA expression level of TNF-α.

DISCUSSION

The examination of traditionally used natural products has culminated in the development of a variety of drugs that are medically proven for their therapeutic effectiveness against a wide range of diseases. Current interest leads to the search for new natural products with anti-inflammatory activity (Gaofeng Yuan et al. 2006). Macrophage is one of the major cell types in inflammatory response and influence many chronic inflammatory disease.

In this study, Anti-inflammatory compounds were extracted from needles of *pinus densiflroa.* Needles of *pinus densiflora* extracted by MeOH and separated four layers, *n*-Hexane, EtOAc, *n*-BuOH and H_2O. Four layers tested NO assay, NO levels from LPS-stimulated RAW 264.7 cells decreased with increasing concentration of EtOAc layer in the range of 0-200ug/mL, indicating that EtOAc layer showed marked inhibitory action toward NO production in the LPS-stimulated RAW 264.7 cells. And, EtOAc layer were separated by column chromatography and tested anti-inflammation activity. EtOAc layer was chromatographed over a silica gel column (10 × 60 cm; 63-200㎛ particle size) and eluted with gradient mixtures of methylen chloride-MeOH (70:1 to 0:1) to yield nine fractions. And nine fractions tested NO assay, The F.5 was identified as the best fraction of anti-inflammatory in NO assay. Moreover, mRNA expression level of inflammatory factors were investigated in LPS-induced RAW264.7 murine macrophages. F.5 was confirmed suppresses the expression of IL-1 β, iNOS, IL-6, COX-2 and TNF-α.

Decrease NO production in the LPS-stimulated RAW 264.7 cells are beneficial in maintaining proper physiological homeostasis; however, excess NO act as a toxic radical and cause many diseases such as mutagenesis, cancer, atherosclerosis, cell apoptosis and necrosis (Tamir and Tannenbaum, 1996). According to the NO production decrease data, EtOAc and F.5 clearly showed scavenging effect on NO

production in macrophages, considering that EtOAc layer may be of therapeutic use in inflammation and pathogenical conditions arisen from NO.

The expression of iNOS, COX-2, IL-6, IL-1β and TNF-α is an important part of immune response to many inflammatory stimuli. However, excessive expression of these mediators is seen in many acute and chronic human diseases, including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis and atherosclerosis. Thus, the suppression of these mediators may be an effective therapeutic strategy for preventing inflammatory reaction and diseases.

NO and PGE2 are important in the process of macrophage activation and related with both acute and chronic inflammation (Evans, 1995; Robinson et al. 1999). Therefore decrease NO and PGE2 production by inhibition of iNOS and COX-2 expression level is a promising therapeutic strategy for the development of anti-inflammatory agents (M. E. Kim et al. 2014). iNOS and COX-2 mRNA expression levels from LPS-stimulated RAW 264.7 cells decreased with increasing concentration of F.5 in the range of 0-100 ug/mL, indicating that F.5 have anti-inflammatory activity.

IL-1β, IL-6 and TNF-α are multifunctional cytokines with widely overlapping activities. These inflammatory cytokines have a central rol in the pathology of chroni inflammatory diseases(Niina Pennanen et al. 1995). In this study clearly demonstrates the inhibitory F.5 effect of IL-1β, IL-6 expression levels on LPS-induced RAW264.7 murine macrophages. But F.5 had no inhibition effect of TNF-α mRNA expression levels.

In summary, NO assay and mRNA expression levels results demonstrated that F.5 isolated from methanolic extract of *Pinus densiflora* EtOAc layer suppresses NO synthesis through the down-regulation of iNOS expression in LPS-stimulated macrophages. Moreover F.5 suppresses COX-2, IL-6, IL-1β expression levels involved inflammation mechanism. Such a result, methanolic extract of *pinus densiflroa* have anti-inflammatory effect and *pinus densiflroa* has the potential to be used as inflammatory medicine.

적 요

솔잎은 여러 페놀화합물을 다량으로 함유 하고 있어 다양한 생리활성(항균활성, 항산화 활성, 항고혈압 활성 등.)을 지니고 있다. 그러나 다양한 생리활성에 대한 화합물은 많이 발견되지 않은 상태이다. 염증반응은 조직의 손상이나 미생물의 침입에 대해 방어하는 기작을 의미한다. 그러나 이러한 염증반응이 적절히 조절되지 않으면 심혈관계질환, 암, 자가면역 증후군, 류마티스 관절염 등 여러 질병을 일으키게 된다. 쥐의 대식세포인 RAW 264.7은 면역반응에서 중요한 역할을 하고 있다. 이 연구는 RAW264.7세포를 이용하여 솔잎의 항염증 활성을 확인 하였다. 솔잎을 메탄올로 추출하여 극성에 따라 헥산, 에틸아세테이트, 부탄올, 물층 으로 나누었다. 이 4층을 이용하여 먼저 RAW 264.7에 처리하여 세포독성을 확인 한 결과 헥산층에서 농도별로 높은 독성이 나타났으며 나머지 층에서는 독성이 나타나지 않음을 확인 하였다. 이후 RAW264.7세포에 LPS로 염증반응을 일으켜 일산화질소의 생성 시킨 후 RAW264.7세포에 에틸아세테이트, 부탄올, 에탄올 층을 처리하여 일산화질소의 감소량을 확인하는 NO Assay를 실시 하였다. 이 실험을 통해서 에틸아세테이트 층에서 항염증 활성을 확인 하였고 이후 오픈컬럼크로마토그래피를 통해서 에틸아세테이트 층을 세분화 하였다. 컬럼에 사용된 고정상은 실리카-겔을 이용 하였으며 이동상은 메틸렌클로라이드와 메탄올의 혼합물을 이용하였다. 메틸렌클로라이드와 메탄올의 비율을 70:1에서 0:1로 조절하여 분획을 나누었으며, 총 9개의 분획을 나누었다. 나누어진 9개의 분획을 이용하여 RAW264.7 세포에서 독성을 확인 한 결과 분획1~4번까지는 독성이 나타났으며 나머지 5~9번은 독성이 나타나지 않아 NO Assay를 실시하였다. NO Assay를 통해 가장 항염증 효과가 뛰어난 분획 5번을 선정하였으며, 분획 5번을 이용하여 염증에 관련된 유전자들의 발현량을 Real-time PCR을 통해서 확인 하였다. 이때 사용된 유전자는 iNOS, COX-2, IL-1β, IL-6, TNF-α로 각각의 발현량을 확인 하였다. 확인 결과 분획 5번은 일산화질소 생성과 관련이 높은 iNOS 유전자의 발현을 낮추는 것으로 확인 되었다. 또한 염증을 악화시키고 발열에 관련된

프로스타글란딘을 생성하는 COX-2유전자의 발현량 역시 낮추는 것으로 확인 되었다. 이 밖에도 염증과 깊은 연관이 있는 IL-1β, IL-6 발현량 역시 현저히 낮추는 것으로 확인 되었다. 그러나 분획 5번은 종양세포의 자살 촉진 및 염증반응을 돕는 TNF-α의 발현에는 영향이 없는 것으로 나타났다. 모든 실험결과들을 종합해 보았을 때, 솔잎은 높은 항염증 활성을 가지고 있는 것으로 확인 되었으며 항염증과 관련된 건강식품, 건강보조제, 의약품등 폭 넓은 범위로 사용 될 것으로 생각된다.

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