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# *Desmarestia tabacoides* Ameliorates Lipopolysaccharide-induced Inflammatory Responses via Attenuated TLR4/MAPKs/NF-κB Signaling Cascade in RAW 264.7 Cells

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*Desmarestia tabacoides* Okamura is a brown macroalgae that is found worldwide. Although several genera of *Desmarestia* have been reported as having anti-tumorigenic, anti-melanogenic, and photo-protective properties, the anti-inflammatory activity of *D. tabacoides* Okamura has not yet been evaluated. In this study, we analyzed the anti-inflammatory mechanisms of *D. tabacoides* Okamura ethanol extract (DTEE) via the inhibition of nitric oxide (NO) and prostaglandin (PG) $E_2$ production and the expression of their corresponding enzymes, inducible NO synthase (iNOS), and cyclooxygenase (COX)-2. In addition, their upstream signaling molecules were evaluated by Western blot analysis, such as nuclear factor (NF)-κB, mitogen-activated protein kinase (MAPK), and phosphoinositide-3-kinase (PI3K)/Akt, in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The DTEE treatment significantly inhibited LPS-induced NO and $PGE_2$ production as well as the expression of their corresponding enzymes, iNOS, and COX-2 without cytotoxicity. The stimulated transcription factor NF-κB and upstream signaling molecules extracellular signal-regulated kinase (ERK), c-Jun $NH_2$-terminal kinase (JNK), and p38 were attenuated by the DTEE treatment, which was statistically significant, while Akt did not provide any inhibitory effect. Moreover, the DTEE treatment significantly mitigated the LPS-activated adaptor molecules, toll-like receptor 4 (TLR4), and myeloid differentiation primary response 88 (MyD88) in the RAW 264.7 cells. These results suggest that DTEE attenuates TLR4-mediated inflammatory responses by inhibiting NF-κB activation and suppressing MAPK phosphorylation in LPS-stimulated RAW 264.7 cells.

**Key words :** *Desmarestia tabacoides* Okamura, nuclear factor-κB, mitogen activated protein kinase, toll-like receptor 4

## Introduction

Inflammatory responses triggered by lipopolysaccharide (LPS) exposure make result in excessive secretion of various proinflammatory mediators including nitric oxide (NO) and prostaglandin (PG) $E_2$ in a variety of cell types including macrophages, microglial cells, and hepatocytes [3, 14]. Excessively generated NO, synthesized from L-arginine by inducible NO synthase (iNOS), contributes to the promotion of liver fibrosis and cancer through promoted oxidative stress and inflammatory process [8, 25]. Another critical proinflammatory mediator, $PGE_2$, is generated by cyclooxygenase (COX)-2 from arachidonic acid and is usually overexpressed in inflammatory lesions and even in malignant tissues [16]. The pathway for COX-2-mediated $PGE_2$ synthesis has been considered as one of the hallmarks of chronic inflammation, which means inhibitors of this pathway might be promising candidates for anti-inflammatory agents. Both inflammatory mediators synthesized by their corresponding enzymes, iNOS, and COX-2, are modulated by the transcription factor, nuclear factor (NF)-κB. This inflammatory transcription factor becomes active form when the immune cells are exposed to an inflammatory stimulus, such as LPS. Then, the activated transcription factor is able to translocate into the nucleus and

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bind the promotor regions for inflammation-related genes including iNOS and COX-2 [20]. Many studies have exhibited that LPS exposure that stimulates NF-κB induces the mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)/Akt activation [4, 9, 15]. MAPK and PI3K/Akt signaling pathways are the most extensively investigated intracellular cascades related to inflammatory responses that lead to the induction of iNOS, COX-2, and NF-κB. In addition, LPS can stimulate Toll-like receptors (TLRs), especially TLR4, which contribute to the activation of NF-κB, MAPKs, and PI3K/Akt signaling pathways. Then, exaggerated inflammatory responses could be driven to subsequently triggered abnormal inflammatory lesions [18]. Therefore, any dietary phytochemicals that are possible to regulate proinflammatory mediators' expression through the modulation of a transcription factor and its upstream signaling molecules might play a role in potential anti-inflammatory agents.

Over the years, seaweeds have been considered as rich sources of beneficial functional compounds that exhibit anti-inflammatory, antioxidative, and antiviral activities [2]. Among them, Desmarestia spp. is a genus of brown macroalgae distributed worldwide. One of Desmarestia genera, *D. viridis*, reported its anti-inflammatory activity through the inhibited proinflammatory mediators, including NO and $PGE_2$ production in LPS-stimulated RAW 264.7 cells [11]. Fucoxanthin from *D. anceps* exhibited photoprotective activity against ultraviolet (UV) through the inhibited reactive oxygen species (ROS) formation in HaCaT cells and roled for sunscreen on reconstructed human skin (RHS) [23]. A marine plastoquinone, 9′-hydroxysargaquinone (9′-HSQ), from *D. menziesii* showed potent cytotoxic activity and induced apoptosis in Jurkat and Daudi cells. In addition, these results were confirmed in *ex vivo* assays with lymphoid neoplasm cell lines [21]. Nanoparticles from aqueous extract of *D. Antarctica* present higher concentrations of total phenolic compounds and lower DPPH scavenging activity than those of *Iridaea cordata* [5]. *D. tabacoides* has reported its antioxidative, anti-melanogenic activities in B16F10 cells, and antimicrobial activity against *Cutibacterium acnes* [13, 19]. By the way, there is no previous study about the anti-inflammatory mechanism of *D. tabacoides* against LPS-stimulated inflammatory responses in RAW 264.7 cells. Therefore, the present study tried to analyze the inhibitory effect of DTEE in LPS-stimulated inflammatory responses that focused on the regulatory mechanisms of pro-inflammatory mediators' generation, in LPS-stimulated murine macrophage cells.

## Materials and Methods

### Reagents

Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine were obtained from Cytiva (Marlborough, MA, USA). The whole plant of *D. tabacoides* Okamura was extracted by 80% of ethanol (DTEE) and obtained from Jeju Biodiversity Research Institute (Jeju, Korea, Specimen No. JBR10003), which was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Antibodies against iNOS, COX-2, phospho-p65, p65, phospho-Akt, Akt, phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho-c-jun $NH_2$-terminal kinase (p-JNK), JNK, phospho-p38, p38, and actin, as well as horseradish peroxidase (HRP) conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for TLR4 and myeloid differentiation primary response (MyD) 88 were obtained from Abcam (Cambridge, UK). All antibodies were diluted in a ratio of 1:1,000. Polyvinylidene fluoride (PVDF) membrane was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

### Cell culture and treatment

RAW 264.7 cell line was obtained from Korean Cell Line Bank (KCLB No. 40071, Seoul, Korea) and was cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine (Cytiva). To analyze the inflammatory mediators, cells were preincubated with indicated concentrations of DTEE for 2 hr and treated with LPS (1 μg/ml) for 18 hr. In addition, cells were treated with various concentrations of DTEE with 1 μg/ml of LPS for 4 hr to evaluate the expression levels of MAPK and NF-κB [26].

### Nitrite and cell viability assays

The amount of nitrite produced in the culture medium was analyzed by the Griess reaction, and cell viability was determined by the cell proliferation assay which was purchased from Promega Corporation (Madison, WI, USA). Cells were incubated with various concentrations of DTEE and 1 μg/ml of LPS for 24 hr. Then, cells were treated with cell proliferation assay solution for 1 hr at 37℃, which was measured at 490 nm (xMark Microplate Spectrophotometer, Bio-Rad Laboratories).

### $PGE_2$ concentration

The $PGE_2$ concentration was measured by an enzyme-

linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions. Cells were preincubated with indicated concentrations of DTEE for 2 hr and then incubated with 1 μg/ml of LPS for 18 hr. Then, mixed equal volumes of culture medium and $PGE_2$ tracer were incubated in the $PGE_2$ ELISA plate for 18 hr at 4℃. The absorbance was measured at 405 nm after the addition of Ellman's reagent to develop.

### Western blot analysis

Cells were washed with PBS and harvested by protein extraction reagent (M-PER, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature. The cell lysate was centrifuged at 13,000 × g for 10 min and the protein concentration was determined by Bradford assay. The protein sample (50 μg) was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad Laboratories). Blotted membrane was blocked with 5% skim milk for 2 hr at room temperature and incubated with primary antibody overnight at 4℃. The membrane was incubated with HRP-conjugated anti-rabbit IgG for 2 hr at room temperature and developed by ECL substrate solution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The result was obtained and quantified from the ChemiDoc imaging system (Bio-Rad Laboratories)

### Immunofluorescence staining

Cells were seeded onto a cell culture slide (SPL Life Sciences, Pocheon, Korea) and treated with DTEE (500 μg/ml) and LPS (1 μg/ml) together for 4 hr. Cells were washed with 1× PBS and fixed in freshly prepared 4% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin (BSA) for 30 min. Primary antibodies (anti-COX-2 and anti-p-p65) were incubated overnight at 4℃ and then the secondary antibody conjugated to rabbit Alexa Fluor 594 (Cell Signaling Technology) was incubated at room temperature for 2 hr. The slide was then mounted with Prolong Gold Antifade Reagent with DAPI (Cell Signaling Technology) and the images were obtained using a fluorescence microscope (Leica, Wetzlar, Germany).

### Statistical analysis

All data were expressed as means ± S.D. and SPSS version 25.0 (IBM Corp., Armonk, NY, USA) was used to analyze the statistical significance. One-way ANOVA with Duncan's multiple range test was applied to evaluate the difference between each group. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

### Attenuated expression of inflammatory mediators by DTEE treatment

The anti-inflammatory activity of DTEE and its underlying molecular mechanism was investigated in LPS-stimulated RAW 264.7 cells. As shown in Fig. 1A, LPS-induced NO production, which plays a critical role in the promotion of inflammation, was significantly attenuated by DTEE treatment in a dose-dependent manner, without causing cytotoxicity (data not shown). An additional inflammatory mediator, $PGE_2$ which is regarded as an important marker for in-

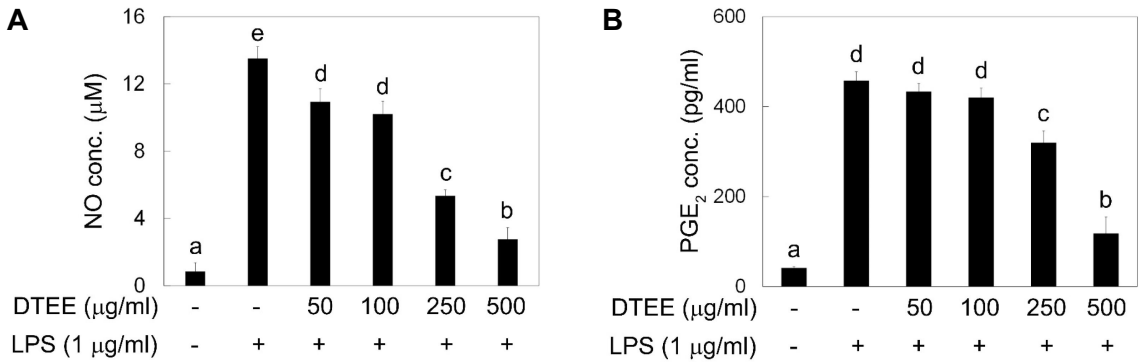


Fig. 1. DTEE inhibited NO and $PGE_2$ production in LPS-stimulated RAW 264.7 cells. Cells were pre-incubated with or without the indicated concentrations of DTEE for 2 hr, then incubated with LPS (1 μg/ml) for 18 hr at 37℃ in a humidified atmosphere containing 5% $CO_2$. (A) NO production was analyzed by the Griess reaction. (B) $PGE_2$ concentration was determined by ELISA assay. Data are represented as the mean ± SD of triplicate experiments. DTEE, *D. tabacoides* Okamura ethanol extract; NO, nitric oxide; $PGE_2$, prostaglandin $E_2$; LPS, lipopolysaccharide.

flammation, was also mitigated by 250 and 500 μg/ml of DTEE treatment with statistical significance. The protein expression levels of each corresponding enzyme for NO and $PGE_2$ formation, iNOS and COX-2, were also significantly attenuated by DTEE treatment (Fig. 2A). Immunofluorescence analysis of COX-2 also reflected the anti-inflammatory activity of DTEE in LPS-stimulated RAW 264.7 cells (Fig. 2B).

### Inhibited activation of NF-κB by DTEE treatment

Western blot analysis was applied to analyze the phosphorylated status of p65, one subunit of NF-κB that plays a critical role in inflammatory responses. As shown in Fig. 3A, phosphorylated p65 was weakly detected in the untreated group, but LPS stimulation potently increased p65 activation. Elevated p65 phosphorylation was significantly inhibited by 250 and 500 μg/ml DTEE treatment while 50 and 100 μg/ml DTEE treatment slightly attenuated p65 activation in RAW 264.7 cells. The result of immunoblot was in accordance with the immunofluorescence analysis in 500 μg/ml DTEE treatment (Fig. 3B). These results imply that DTEE treatment contributes to LPS-induced NF-κB inactivation and consequently attenuates the expression of inflammatory mediators in LPS stimulated RAW 264.7 cells.

### Regulated expression of MAPKs *via* TLR4/MyD88 inactivation by DTEE treatment

Signaling molecules including MAPKs and PI3K/Akt play an important role in the regulation of LPS-stimulated inflammation and transcriptional regulation [22]. In order to investigate whether DTEE treatment might affect MAPKs and Akt activation, the phosphorylation levels of ERK, JNK, p38, and Akt were measured by western blot analysis to identify the upstream signaling molecules that can regulate NF-κB activation in RAW 264.7 cells. As shown in Fig. 4, DTEE treatment significantly inhibited ERK, JNK, and p38 phosphorylations, but had no visible effect on the phosphorylation of Akt (Fig. 4).

LPS is initially recognized by TLR4 and its signal is transmitted to MyD88 which is able to activate NF-kB and MAPKs signaling pathways [24]. Therefore, the effect of DTEE on TLR4 and MyD88 activation was also analyzed by western blot analysis to identify their roles as adaptor molecules in the development of NF-κB and MAPKs in RAW 264.7 cells [6]. As shown in Fig. 5, DTEE treatment significantly attenuated LPS-induced TLR4 and MyD88 activations in a dose-dependent manner, following the inhibited NF-κB, ERK, JNK, and p38 MAPK expression levels in RAW 264.7 cells. Consequently, these results imply that inhibited phosphorylation levels of ERK, JNK, and p38 through the regulation

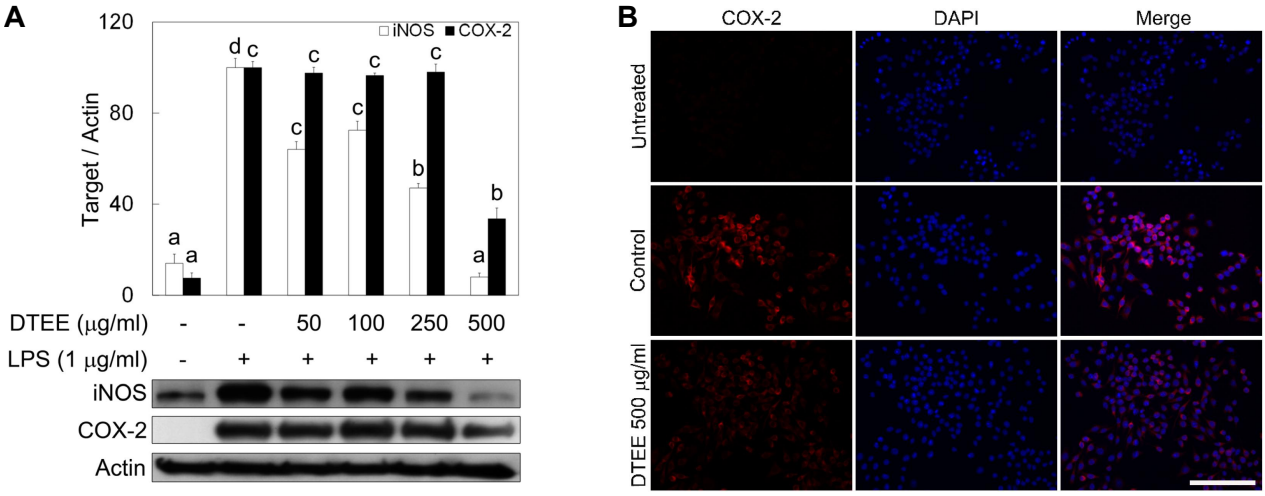


Fig. 2. DTEE inhibited iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Cells were pre-incubated with or without the indicated concentrations of DTEE for 2 hr, then incubated with LPS (1 μg/ml) for 18 hr at 37℃ in a humidified atmosphere containing 5% $CO_2$. (A) Protein expression levels of iNOS and COX-2 were assessed following DTEE treatment. The relative induction of iNOS and COX-2 expression was quantified by densitometry and actin was used as an internal control. Data are represented as the mean ± SD of triplicate experiments. (B) COX-2 expression was visualized by immunofluorescent analysis with anti-COX-2 antibody-bound Alexa Fluor 594 (red)-conjugated secondary antibody. The nuclei were stained by DAPI (blue). The scale bar is 100 μm. DTEE, *D. tabacoides* Okamura ethanol extract; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

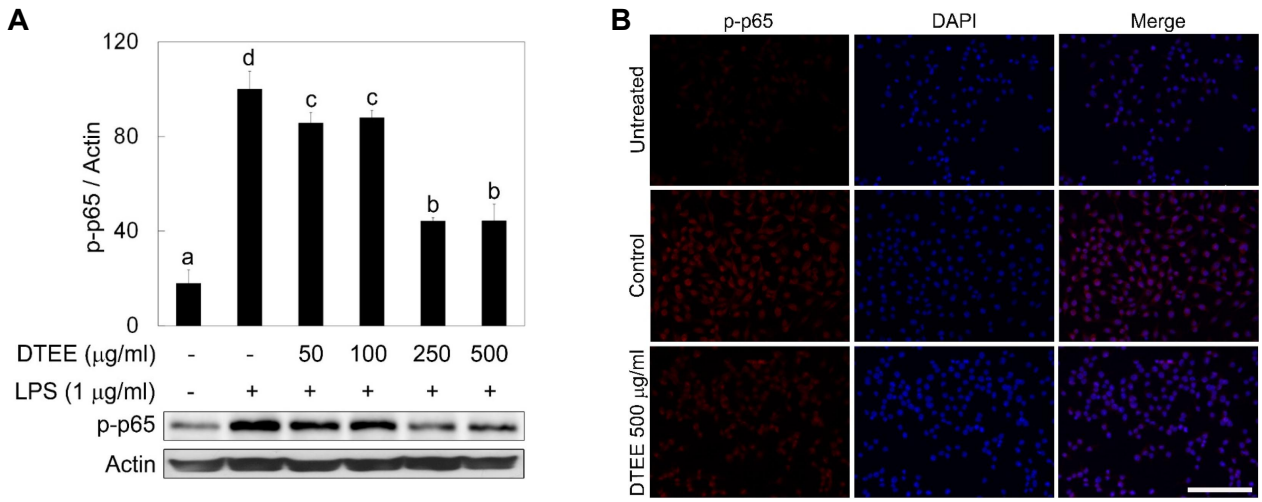


Fig. 3. DTEE inhibited the phosphorylation of p65 in LPS-stimulated RAW 264.7 cells. Cells were pre-incubated with or without the indicated concentrations of DTEE for 4 hr with LPS (1 μg/ml) at 37℃ in a humidified atmosphere containing 5% $CO_2$. (A) The phosphorylated status of p65 was assessed following DTEE treatment. The relative induction of p-p65 expression was quantified by densitometry and actin was used as an internal control. Data are presented as the mean ± SD of triplicate experiments. (B) The phosphorylated status of p65 was visualized by immunofluorescent analysis with anti-p-p65 antibody-bound Alexa Fluor 594 (red)-conjugated secondary antibody. The nuclei were stained by DAPI (blue). The scale bar is 100 μm. DTEE, *D. tabacoides* Okamura ethanol extract; LPS, lipopolysaccharide.

of TLR4/MyD88 activation by DTEE treatment may contribute to reducing NF-κB activation, resulting in reduced iNOS and COX-2 expressions, as well as NO and $PGE_2$ productions in LPS stimulated RAW 264.7 cells.

## Discussion

Among several kinds of research about Desmarestia genera, anti-inflammatory activity was exhibited in *D. viridis* through the inhibited production of proinflammatory mediators in LPS-stimulated RAW 264.7 cells [11]. *D. tabacoides* showed antioxidative, antimelanogenic, and antimicrobial activities in a cell line and bacterium [13, 19]. Therefore, the present study tried to analyze the inhibitory mechanism of DTEE through the regulation of TLR4-mediated signaling molecules and proinflammatory mediators in LPS-stimulated RAW 264.7 cells.

Inflammatory response, occurred in the immune system, is one of the defensive mechanisms that can be activated by infection, tissue injuries, or xenobiotic toxins [1]. Among them, LPS, which existed in the outer membrane of Gram-negative bacteria, can activate immune responses through the interaction with TLR4, which can induce MAPK, PI3K/Akt activations and subsequently induces the phosphorylation of transcription factor, NF-κB [12]. Then, proinflammatory mediators, including NO and $PGE_2$, are excessively generated in inflammatory lesions by iNOS and COX-2 which are usually considered common markers for inflammation [17]. In this study, DTEE treatment significantly inhibited NO and $PGE_2$ productions as well as their corresponding enzymes, iNOS, and COX-2 expression in LPS-stimulated RAW 264.7 cells (Fig. 1, Fig. 2). NF-kB is a transcription factor that can regulate the gene expression related to inflammation, immunity, cell proliferation, and differentiation. This transcription factor ubiquitously exists in the cytoplasm as a heterodimer with p65 and p50, which retain an inactive state bound to an inhibitor of NF-κB proteins (IκBs). Upon inflammatory stimulus, the dimer is freed from phosphorylated IκBs and then translocates into the nucleus, which induces gene expression of proinflammatory mediators, NO, and $PGE_2$ in RAW 264.7 cells. As shown in Fig. 3, DTEE treatment inhibited phosphorylation of p65, one component of NF-κB, which means DTEE attenuated iNOS and COX-2 expressions through the inhibition of NF-kB activation in LPS-stimulated RAW 264.7 cells. In addition, LPS-stimulated NF-κB activation is regulated by signaling molecules including MAPK and PI3K/Akt, which were one of the most abundantly investigated intracellular signaling molecules related to inflammatory responses [6]. Three groups of well-characterized MAPK subfamily members including ERK, JNK, and p38 MAPK are serine-threonine protein kinases that regulate proliferation, differentiation, and inflammation [10]. PI3K also regu-

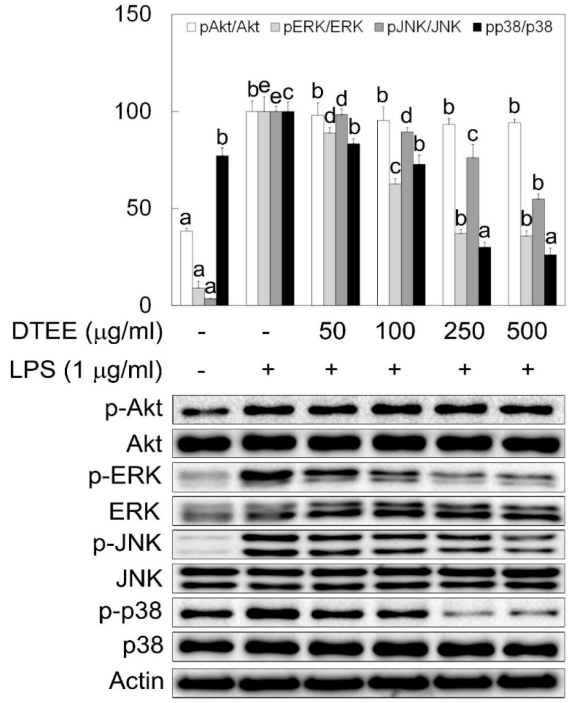


Fig. 4. DTEE inhibited the phosphorylation of ERK, JNK, and p38 in LPS-stimulated RAW 264.7 cells. Cells were incubated with or without LPS (1 μg/ml) and with the indicated concentrations of DTEE for 4 hr at 37℃ in a humidified atmosphere containing 5% $CO_2$. Protein expression levels of p-Akt, p-ERK, p-JNK, and p-p38 were assessed following DTEE treatment. Unphosphorylated forms of signaling molecules and actin were used as internal controls. Akt, ERK, JNK, and p38 phosphorylation were quantified by densitometry, and unphosphorylated forms of each signaling molecule were used as an internal control. Data are represented as the mean ± SD of triplicate experiments. DTEE, *D. tabacoides* Okamura ethanol extract; LPS, lipopolysaccharide; p, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun $NH_2$-terminal kinase.

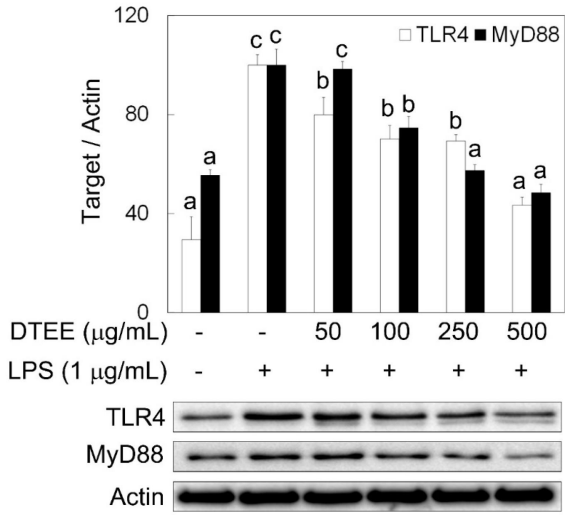


Fig. 5. DTEE inhibited the activation of TLR4 and MyD88 in LPS-stimulated RAW 264.7 cells. Cells were incubated with or without LPS (1 μg/ml) and with the indicated concentrations of DTEE for 4 hr at 37℃ in a humidified atmosphere containing 5% $CO_2$. TLR4 and MyD88 protein expressions were quantified *via* densitometry and actin was used as an internal control. Data are represented as the mean ± SD of triplicate experiments. TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response 88; LPS, lipopolysaccharide.

lates several critical cellular events in the inflammatory response [7]. All of these signaling molecules are activated through phosphorylation in response to a wide array of extracellular stimuli and phosphorylated molecules then activate other kinases or transcription factors, thereby altering the expression of the target genes. TLR4 is one of the pattern recognition receptors (PRRs) which can activate various biological activities through the interaction with LPS exposure [10]. LPS selectively binds TLR4 on the surface of macrophages and then triggers the activation of MyD88, which subsequently induces downstream inflammation-mediated signaling events, MAPK and PI3K/Akt activation as well as NF-κB induction. TLR4-initiated signaling cascades eventually contribute to the overexpression of transcripts for proinflammatory mediators, NO and $PGE_2$, in macrophages [6].

In this study, DTEE treatment dose-dependently inhibited TLR4 and MyD88 activation (Fig. 5), followed by inhibited ERK, JNK, and p38 phosphorylations while DTEE did not affect Akt activation (Fig. 4). Attenuated MAPK by DTEE treatment might inhibit NF-κB activation (Fig. 3) and proinflammatory mediators, NO and $PGE_2$, were significantly ameliorated by DTEE treatment (Fig. 1, Fig. 2) in LPS-stimulated RAW 264.7 cells. In conclusion, these findings suggest that DTEE significantly inhibits NO and $PGE_2$ production through the regulation of TLR4 and MyD88-mediated MAPK phosphorylation as well as NF-κB activation in LPS-stimulated RAW 264.7 cells.

Though several biological activities of Desmarestia spp. have been analyzed so far, the identification of functional compounds has not been sufficiently performed yet [5, 21, 23]. Therefore, it is considered that comprehensive research on the functional ingredients and especially anti-inflammatory components contained in DTEE should be needed in future studies.

## The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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## 초록 : RAW 264.7 세포에서 담배잎산말의 TLR4/MAPKs/NF-κB 신호전달체계 조절을 통한 항염증 효과

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*Desmarestia tabacoides* Okamura는 전 세계적으로 널리 분포하는 갈조류 중 하나이다. 몇몇 산말류의 항종양, 멜라닌 생성 억제 및 광보호 활성에 대한 연구는 있었으나 *D. tabacoides* Okamura의 항염증 기전에 대해서는 보고되지 않아 본 연구에서는 LPS (lipopolysaccharide)로 자극된 RAW 264.7 세포에서 *D. tabacoides* Okamura 에탄올 추출물(DTEE)의 항염증 기전을 inducible nitric oxide synthase (iNOS)와 cyclooxygenase (COX)-2의 발현 및 이들의 상위신호전달물질인 nuclear factor (NF)-κB, mitogen-activated protein kinase (MAPK) 그리고 phosphoinositide-3-kinase (PI3K)/Akt의 인산화 조절 정도를 통해 분석하였다. DTEE의 처리는 세포 독성 없이 LPS로 유도된 NO와 prostaglandin (PG) $E_2$의 생성과 이들의 생성 효소인 iNOS 및 COX-2의 발현을 유의하게 억제하였다. 그리고 LPS에 의해 활성화된 NF-κB 및 상위 신호 전달 물질인 extracellular signal-regulated kinase (ERK), c-Jun $NH_2$-terminal kinase (JNK) 및 p38은 DTEE 처리에 의해 유의적으로 억제되었다. DTEE의 처리는 RAW 264.7 세포에서 LPS에 의해 활성화되는 adaptor molecule인 Toll-like receptor (TLR) 4 및 myeloid differentiation primary response (MyD) 88 또한 유의적으로 억제하였다. 이 결과를 통해 DTEE는 LPS에 의해 유도된 TLR4와 NF-κB 및 MAPK의 활성을 억제함으로써 염증 매개인자의 발현을 조절하였고, 이는 DTEE가 염증을 완화할 수 있는 기능성 식품의 소재로써 유용하게 사용될 수 있음을 시사한다.