**Thesis for the Degree of Master of Fisheries Science**

# Anti-inflammatory Effect of an Ethanolic Extract of *Myagropsis yendoi* in Lipopolysaccharide-Stimulated BV-2 Microglia Cells

By

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KOICA-PKNU International Graduate Program of Fisheries Science

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# LPS 로 자극된 BV-2 microglia 세포에서 애기외톨개모자반 에탄올 추출물의 항염증효과

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A dissertation

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## Abstract

*Myagropsis yendoi* is a brown alga that was shown to have anti-inflammatory. In this study, the molecular mechanism of the anti-inflammatory action of *Myagropsis yendoi* ethanolic extracts (MYE) was investigated using lipopolysaccharide (LPS)-stimulated microglia BV-2 cells. MYE inhibited LPS-induced nitric oxide (NO) production in a dose-dependent manner and suppressed the expression of inducible nitric oxide synthase (iNOS) in BV-2 cells. MYE also reduced the production of pro-inflammatory cytokines in LPS-stimulated BV-2 cells. LPS-induced nuclear factor-κB (NF-κB) transcriptional activity and NF-κB translocation into the nucleus were significantly inhibited by MYE treatment through the prevention of the degradation of inhibitor κB-α (IκB-α). Moreover, MYE inhibited the phosphorylation of Akt, ERK, JNK and p38 MAPK in LPS-stimulated BV-2 cells. The results indicate that the MYE can be considered as a potential source of therapeutic agents for neuro-inflammatory diseases.

# 1. Introduction

Inflammation is a complex process of the host towards the exterior challenge or cellular injury that leads to the release of a complex array or inflammatory mediator, finalizing the restoration of tissue structure and function. However, prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases such as cancer, diabetes, arthritis, atherosclerosis and Alzheimer.

Microglia are the primary immune cells in the brain, and play an important role in maintaining the integrity of central nerve system (Kim et al., 2012; Kim et al., 2011a). They support synaptic integrity, and their main function is modulation of innate immune response. The developing brain is damaged by a variety of factors and mechanisms including inflammation, excitotoxicity, and oxidative stress. In these conditions, activated microglia secrete excessive levels of inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (Bozinovski et al., 2002; Joung et al., 2012a; Lee et al., 2012a; Silva et al., 2008). These mediator and cytokines are known to exacerbate a neuronal tissue injury and subsequent

neurodegeneration. Therefore, modulation of activated microglia is an effective strategy for therapeutics in the neuroinflammation.

NO is primarily produced by enzymatic action of inducible nitric oxide synthase (iNOS). Expressions of the enzyme as well as pro-inflammatory cytokines are regulated by the activation of nuclear factor-kappaB (NF-κB) (Bozinovski et al., 2002). iNOS gene promoter contains a NF-κB consensus sequence, which is responsible for binding of activated NF-κB. In most cell types, NF-κB is located in the cytosol as an inactive complex by bound to inhibitory kappaB (IκB) family (Kim et al., 2011a). NF-κB is activated by diverse external stimuli including inflammatory cytokines, bacterial components, and viral infection (Pahl, 1999). Lipopolysaccharide (LPS) stimulates the phosphorylation, ubiquitination and proteasomal degradation of IκB by the activation of IκB kinase complex, resulting in the translocation of NF-κB into the nucleus by dissociation of NF-κB-IκB-α complex (Janssen-Heininger et al., 2000). The activation of NF-κB is also controlled by cellular signalling kinases such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/AKT (Cantley, 2002; Jang et al., 2005). MAPK family including

extracellular signal regulated kinase (ERK), p38 MAPK, and c-Jun $NH_2$-terminal kinase (JNK) is associated with the transcriptional regulation of inflammatory genes via NF-κB activation (Herlaar and Brown, 1999; Jang et al., 2005). The elucidation of detailed molecular mechanisms involved in these regulations is important for counteracting the harmful effects of pro-inflammatory mediators (Jana et al., 2007).

Marine macroalgae contain an abundance of polysaccharide, minerals, pigments and polyunsaturated fatty acids, thus they are known to be beneficial for human health. Recently, diverse studies have revealed that the compounds, such as phlorotannins (Gupta and Abu-Ghannam, 2012) presented in the extracts from *Sargassum horneri*, *Sargassum fulvellum*, *Undaria pinnatifida*, and *Laminatia japonica*, have various biological activities including anti-oxidation (Airanthi et al., 2011) and anti-inflammation (Kang et al., 2008; Khan et al., 2008; Lee et al., 2012c). *Myagropsis yendoi* which grows in subtidal zone of the coast of East Asia belongs to the family Sargassaceae in phaeophyta. It was shown to have a strong antimicrobial activity and it showed growth inhibition against *Candida tropicalis* and Gram-positive bacteria such as *Bacillus subtilis,*

*Listeria inoccua, Listeria monocytogenes and Staphylococcus aureus* (PARK et al., 2009) .

We found strong anti-inflammatory activity in *M. yendoi* ethanolic extract (MYE) among various brown macroalgae. To the best of our knowledge, there have not been any reports about the anti-inflammatory activity of MYE. Hence, we investigated molecular mechanisms of anti-inflammatory activity of MYE using LPS-stimulated BV-2 microglia cells. The findings support that MYE may be used as a source of functional food for the prevention of neuro-inflammatory diseases.

# 2. Materials and Methods

## 2.1. Plant material and preparation of MYE

*M. yendoi* was collected along the coast of Tongyoung, South Korea in March 2012. The samples were rinsed using tap water to remove salt. They were air-dried under shade for 2 days and ground with hammer grinder. The resulting dried powder was stored at -20°C until used.

Dried powder (100 g) of *M. yendoi* was extracted three times with 95% (v/v) ethanol for 3 h at 70°C. The combined extracts were concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 40°C and lyophilized to give a powder (9 g), and then stored at -20°C until used. The extract was dissolved in DMSO and further diluted with culture media before use. The final concentration of DMSO in cell culture medium was less than 0.1%.

## 2.2. Reagents

LPS (*Escherichia coli* O55:B5), 2, 2-diphenyl-l-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). CellTiter$^{96}$ AQ$_{ueous}$ One Solution Cell

Proliferation assay kit, dual luciferase assay kit, murine NF-kB promoter/ luciferase DNA, pRL-TK DNA, and Moloney murine leukemia virus reverse transcriptase were obtained from Promega (Madison, WI, USA). Primary and secondary antibodies were purchased from Cell Signaling Biotechnology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. LipofectaminePlus Reagent and TRIzol reagent were purchased from Invitrogen (Carlbad, CA, USA).

### 2.3. Cell cultures and sample treatment

The murine BV-2 microglial cell line (ATCC, Rockvile, MD, USA) was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulphate (100 μg/ml) in a humidified atmosphere of 5% $CO_2$. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of MYE.

### 2.4. Cytotoxicity assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

using CellTiter$^{96}$ AQ$_{ueous}$ One Solution Cell Proliferation assay kit according to the manufacturer's manual. Cells were inoculated at a density of $3\times10^5$ cells into 96-well plates and cultured at 37°C for 24 h. Cells were then treated with LPS (1 μg/ml) in the presence or absence of MYE in different concentration for 24 h. The culture medium was removed and replaced by 95 μl of fresh culture medium and 5 μl of MTS solution. After 1 h, the absorbance at 490 nm was measured using a microplate reader (Glomax Multi Detection System, Promega).

### 2.5. Measurement of nitric oxide and pro-inflammatory cytokines

Cells ($5\times10^4$ cells/well) were pre-treated with MYE (0-100 μg/ml) for 2 h prior to LPS treatment for 24 h. After treatment of LPS, culture media of BV-2 cells were collected and stored at -75°C until tested. For the measurement of NO, 100 μl of culture supernatant mixed with the same volume of Griess reagent (0.1% naphthylethylenediaminedihydrochloride and 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance of the mixture was measured with a microplate reader at 540 nm. Levels of TNF-α and IL-6 in cultured media

from each group were quantitatively determined by ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.6. Western blot Analysis

Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were washed with Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl, PH 7.5) supplemented with 0.05% Tween 20 (TBST) followed by blocking with TBST containing 5% non-fat milk. The membranes were incubated overnight with primary antibodies. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at room temperature. The membranes were washed three times with TBST at room temperature. Immuno-reactivities were detected by ELC reagents. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II (ATTO & Rise Co., Tokyo, Japan) and CS analyzer ver. 3.00 software (ATTO).

## 2.7. Preparation of cytosolic and nuclear extracts

BV-2 cells plated in a 6-well cell culture plates at a density of $3\times10^6$ cells per well were pretreated with or without MYE for 2 h and then treated with LPS for 0.5 h. Cells were washed two times with ice-cold PBS, scraped in PBS and centrifuged at 12,000 g for 5 min at 4°C. Pellets were suspended in 180 μl of hypotonic buffer A [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.02% $NaN_3$, 0.5 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF)] on ice, and afterward, 20 μl of 5% Nonidet P-40 was added for 5 min. The mixture was centrifuged at 1,800 g for 5 min. Supernatant was collected as cytosolic extract. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 25% glycetol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM Methylenediaminetetraacetic acid, 0.02% $NaN_3$, 0.5 mM DTT and 1 mM PMSF] for 1 h on ice and centrifuged at 14,000 g for 10 min. The supernatant containing nuclear proteins was collected and stored at a -70°C after determination of the protein concentration.

## 2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

BV-2 cells plated in a 6-well cell culture plate at a density of $3.0 \times 10^5$ cells/well were pretreated without or with MYE for 1 h and then treated with LPS for 6 h. Total RNA from each group was isolated with the TRIzol reagent. Five microgram of total RNA was used for reverse transcription using oligo-dT and M-MLV reverse transcriptase. PCR was carried out using the resulting cDNA as a template, with the following condition: 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products were visualized by agarose gel electrophoresis. Verification of PCR product of specific genes was established by their predicted sizes under ultraviolet light illuminator. The primer sequences were presented in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to evaluate relative expression of iNOS. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software.

**Table 1. RT-PCR primers used this study.**

| Gene | Direction | Sequence |
|---|---|---|
| iNOS | Sense | 5`-ACCACTCGTACTTGGGATGC-3` |
| | antisense | 5`-CACCTTGGAGTTCACCCAGT-3` |
| GAPDH | Sense | 5`-GACCCCTTCATTGACCTCAA-3` |
| | antisense | 5`-CTTCTCCATGGTGGTGAAGA-3` |

## 2.9. Immunocytochemical analysis

To analyze nuclear localization of NF-κB in BV-2 cells, cells were cultured on glass coverslips (SPL Lifesciences Co., Gyeonggi-do Korea) in 24-well plates for 24 h. After preincubation with MYE for 2 h, cells were stimulated with or without LPS (1 μg/ml). Cells were fixed in 4.0% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Permeabilized cells were washed with PBS and blocked with 3% BSA in PBS for 30 min. Thereafter, cells were incubated in an anti-NF-κB polyclonal antibody diluted in 3% BSA/PBS for 2 h, rinsed three times for 5 min with PBS, and incubated in Alexa Fluor® 488-conjugated secondary antibody diluted in 3% BSA/PBS for 1 h. Cells were stained with 2 μg/ml DAPI and images were captured using an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

## 2.10. Statistical Analysis

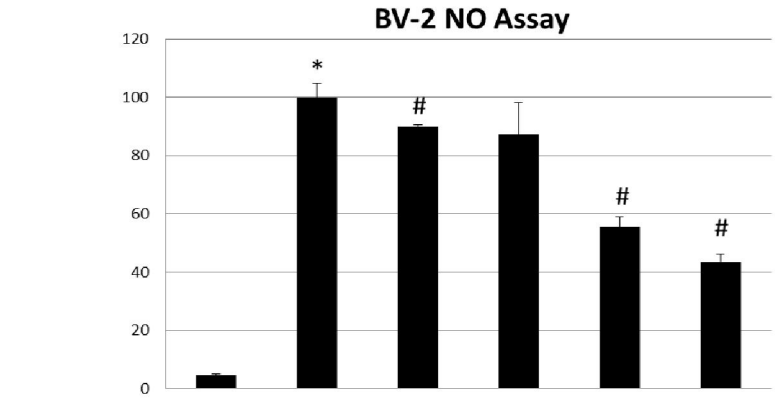
Data were expressed as means ± standard deviations (SDs) of at least three independent experiments unless otherwise indicated. Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair of Student's *t*-test for multiple comparisons. Differences with a value of $P < 0.05$ were considered statistically significant. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL, USA).

# 3. Results

## 3.1. Effect of MYE on NO production in LPS- stimulated BV-2 cells.

Anti-inflammatory effect of MYE was determined by the inhibition of NO using LPS-stimulated BV-2 cells. Cells pre-treated with MYE were stimulated with or without LPS (1 μg/ml) for 24 h. As shown in (Figure 1A), MYE significantly suppressed LPS-induced NO production in a dose dependent manner. MYE at concentrations higher than 25 μg/ml significantly suppressed the production of NO in LPS-stimulated BV-2 cells ($P < 0.05$). MTS assay showed no cytotoxicity at the concentrations below 100 μg/ml of MYE in BV-2 cells (Figure 1B). These data indicate that MYE inhibited LPS-induced NO secretion in BV-2 cells with no cytotoxicity.

(A)




(B)

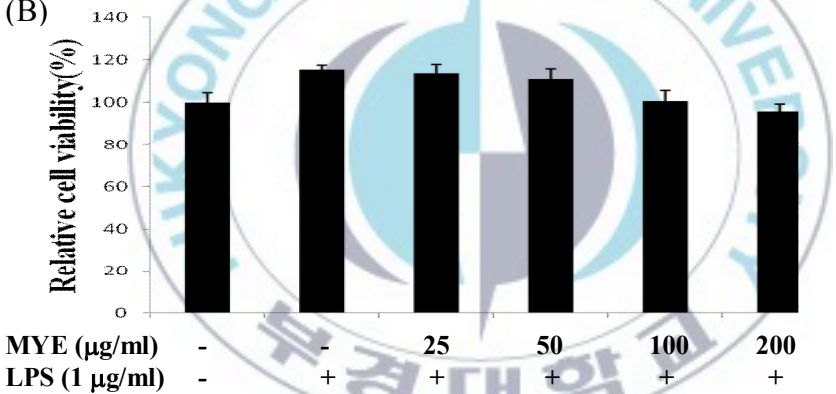



**Figure 1. Effect of ethanol fraction from *Myagropsis yendoi* (MYE) on cell viability and on the inhibition of NO production in LPS-stimulated BV-2 cells.**

Cells pretreated with various concentrations of MYE for 2 h were stimulated with LPS (1 μg/ml) for 24 h. The culture media of the treated cells were used to measure the amount of nitrite to evaluate NO level (A). Cell viability was analyzed by MTS assay (B). All data are presented as means ± SDs of three independent experiments. $^{\#}p < 0.05$ indicates significant differences from the control group. $^{*}p < 0.05$ indicates significant differences from the LPS-only group.

### 3.2. Effect of MYE on the expression of iNOS in BV-2 cells

To investigate whether the suppressions of NO production by MYE treatment is related with the regulation of iNOS protein, the level of iNOS protein was analyzed by Western blot. As shown in Figure 2A, treatment of BV-2 cells with LPS (1 μg/ml) for 16 h induced the expressions of iNOS protein. However, pretreatment with MYE led to a dose-dependent inhibition of the LPS-induced iNOS protein expression in BV-2 cells. MYE at 100 μg/ml almost completely inhibited the LPS-induced iNOS expression. To further examine the effect of MYE on the transcriptional regulation of iNOS expression, RT-PCR analysis was performed to examine the expression of iNOS mRNA. Figure 2B showed that MYE suppressed the expressions of iNOS mRNA in a concentration-dependent manner in LPS-stimulated BV-2 cells. Similar to the result of protein production, 100 μg/ml of MYE strongly inhibited iNOS mRNA expression. These results indicate that MYE-mediated down-regulation of iNOS protein production is mainly due to its transcriptional regulation of MYE in LPS-stimulated BV-2 cells.

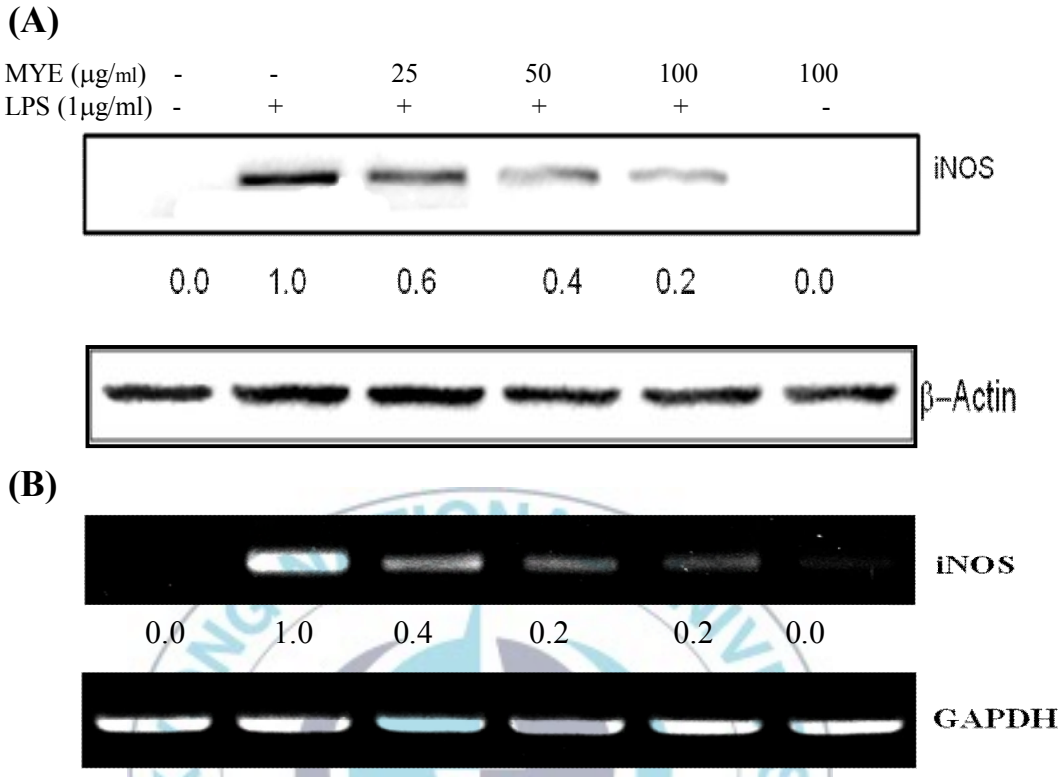



**Figure 2. Effect of MYE on LPS-induced iNOS expression in BV-2 microglia cells.**

Cells pretreated with various concentrations of MYE for 2 h and were stimulated with or without LPS (1mg/ml) for 16 h. Whole proteins were separated with SDS-PAGE and analyzed with Western blot (A) . Cell were incubated with various concentration of MYE for 2 h, and then stimulated with LPS (1 mg/ml) for 6 h. mRNA levels of iNOS and GAPDH were determined by RT-PCR analysis using corresponding gene-specific primers (B). The results presented are representatives of three independent experiments. Quantitative data represent means ± SDs of three independent experiments.

### 3.3. Effect of MYE on the production of TNF-α and IL-6 in LPS-treated BV-2 cells

Pro-inflammatory cytokines such as TNF-α and IL-6 are secreted in the early stage of inflammatory responses, so they are the key markers of the inflammation. We determined the levels of these cytokines in the media of LPS-stimulated BV-2 cells. BV-2 cells were incubated with various concentrations of MYE for 2 h and then stimulated with LPS for 24 h. the levels of the cytokines in the culture media were measured by ELISA. The stimulation of BV-2 cells with LPS induced significant increase in the levels of TNF-α (Figure 3A) and IL-6 Figure 3B), however, pre-treatment with MYE inhibited their increased productions in a dose-dependent manner. .

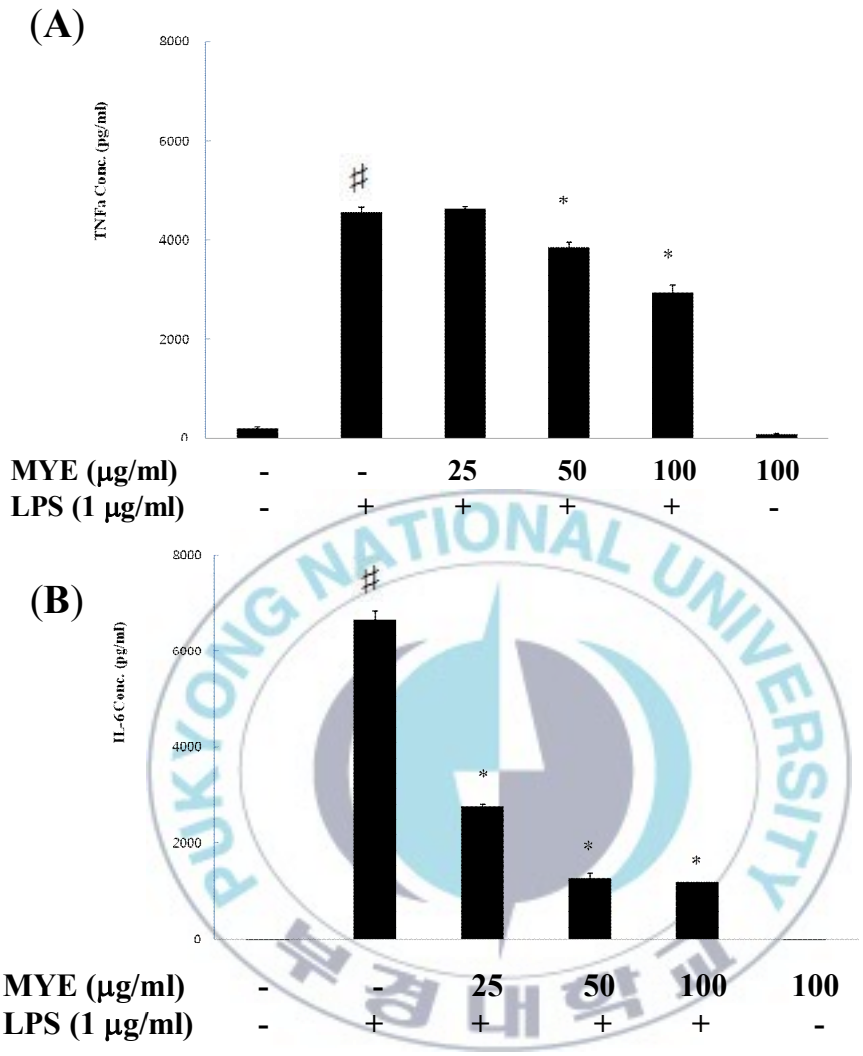



**Figure 3. Effect of MYE on the secretion of pro-inflammatory cytokines in LPS-stimulated BV-2 cells.**

Cells pretreated with various concentrations of MYE were stimulated with or without LPS (1 μg/ml) for 24 h. TNF-α (A) and IL-6 (B) in the culture media were measured by ELISA. Data are means ± SDs of three independent experiments. $^{\#}p < 0.05$ indicates significant differences from the control group. $*p < 0.05$ indicates significant differences from the LPS-only group.

### 3.4. Effect of MYE on the nuclear translocation of NF-κB in LPS-stimulated BV-2 cells

To analyze the effect of MYE on the regulation of transcriptional factor during the inflammatory response, the effect of MYE on the intercellular localization of NF-κB/p65 was assessed by Western bolt and confocal microscopy in LPS-stimulated BV-2 cells. Immunohistochemical analysis (Figure 4A) showed that NF-κB protein was mainly localized in the cytoplasm of unstimulated cells. After stimulation with LPS, NF-κB was translocated into the nucleus; however, pretreatment with MYE remarkably suppressed its translocation regardless of LPS treatment in BV-2 cells. Considering the inhibitory effects of MYE on the LPS-induced NF-κB translocation, the subcellular distribution of NF-κB was analyzed by Western blot. As shown in the Figure 4B, LPS treatment induced translocation of NF-κB into the nucleus in BV-2 cells. However, MYE pretreatment strongly suppressed the nuclear translocation of NF-κB in a dose-dependent manner, which is consistent with the result of the immunohistochemical analysis (Figure 4A). These results suggest that MYE

attenuated the expressions of iNOS and pro-inflammatory cytokines at least in part by the inactivation of NF-κB pathway.

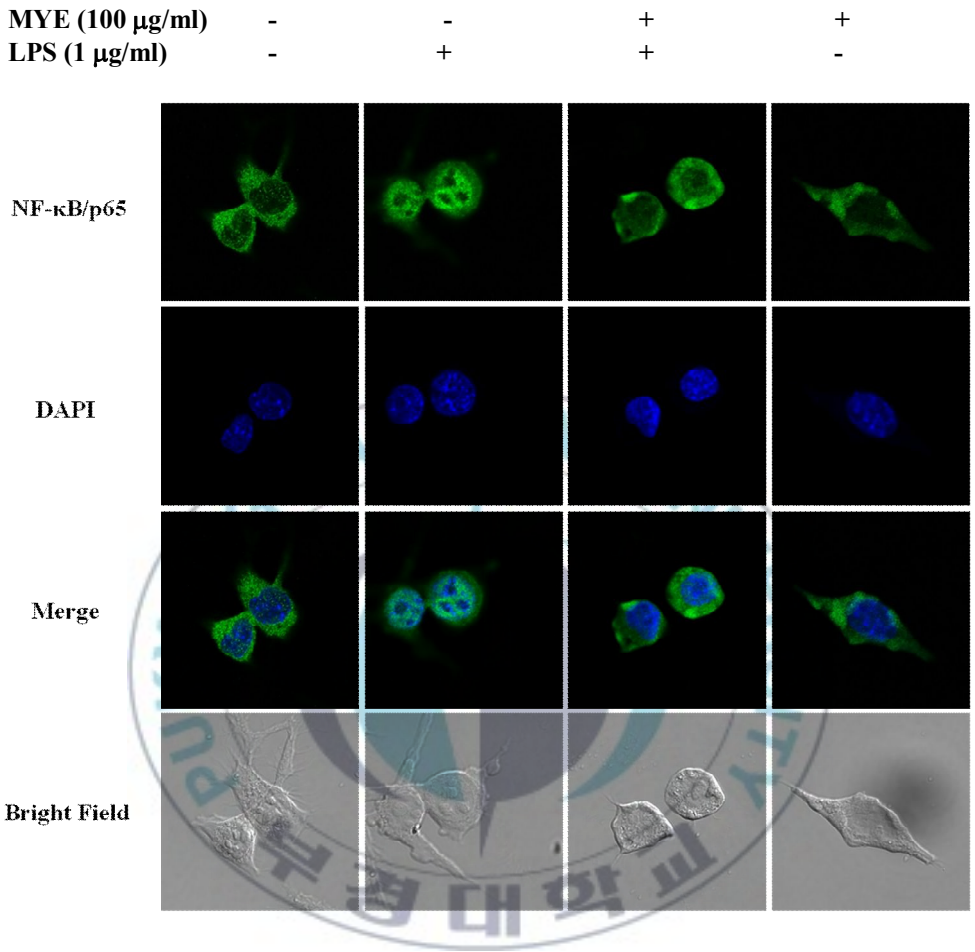



**Figure 4A. Effect of MYE on the nuclear translocation of NF-kB in LPS-stimulated BV-2 cells.**

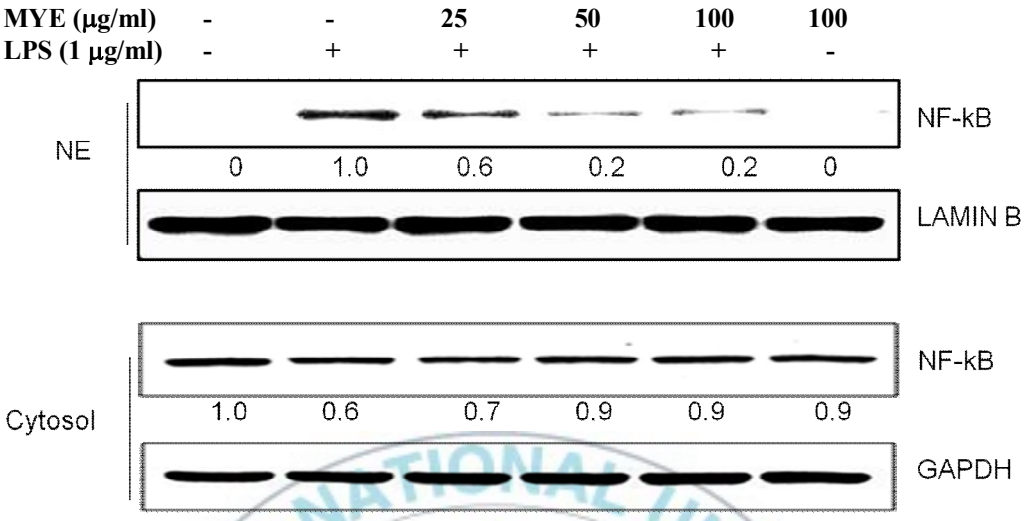



**Figure 4B. Effect of MYE on the nuclear translocation of NF-kB in LPS-stimulated BV-2 cells.**

Cells were pretreated with MYE (100 μg/ml) for 2 h, followed by LPS stimulation for 30 min. DAPI (blue) was used for staining nuclei (Figure 4A). Cells preteated with MYE for 2 h were stimulated with LPS for 30 min and levels of NF-κB in nucleus were analyzed with Western blot (Figure 4B). The results presented are representative of three independent experiments. Quantitative data represent means ± SDs of three independent experiments.

### 3.5. Effect of MYE on the activation of JNK, ERK, AKT and p38 in LPS-stimulated BV-2 cells

To evaluate the effect of MYE on the signalling pathway associated with NF-κB activation, the changes in the phosphorylation of intracellular signalling proteins in BV-2 cells was analyzed by Western blot. As shown in Figure 5 MYE inhibited the phosphorylations of JNK, ERK, AKT and p38 MAPK in dose-dependent manner in LPS-stimulated BV-2 cells, these data suggest that the reductions of inflammatory protein and pro-inflammatory cytokines by MYE are mediated by blocking the activations of ERK, JNK, AKT and p38 MAPK via NF-κB pathway.

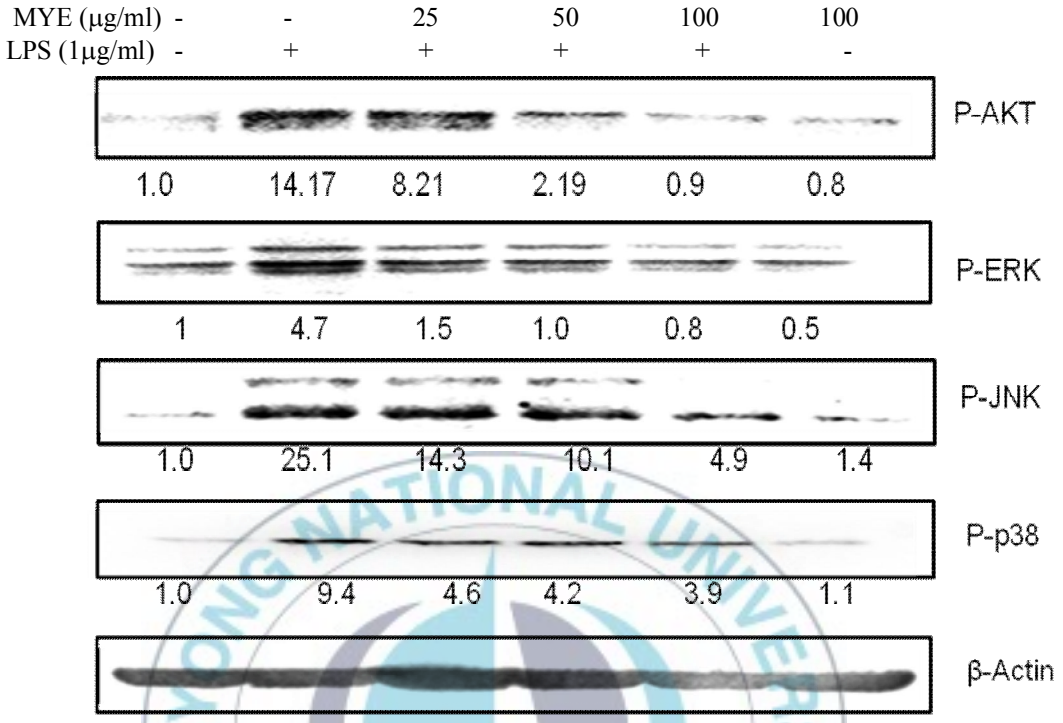



**Figure 5. Effect of MYE on the phosphorylations of MAPKs and AKT in BV-2 cells**

Cells pretreated with indicated concentrations of MYE for 2 h were stimulated with LPS (1 μg/ml) for 30 min. whole cell lysates (40 μg) were analyzed by Western blotting using corresponding antibodies.

# 4. Discussion

Various studies have concentrated on the biological activities of natural compounds or extracts to develop natural drugs or functional food since they are advantageous to human health due to their easy availability and low side effects or toxicity. Many phytochemicals from terrestrial plants have shown an anti-inflammatory effect, moreover, various studies have been demonstrated the molecular mechanisms of anti-inflammatory action of extracts from marine brown algae, such as *Myagropsis myagroides, Laminaria japonica, Eckolnia stolonifera* and *Sargassum fulvellum* (Gwon et al., 2013; Joung et al., 2012b; Kim et al., 2013; Lee et al., 2012a; Lee et al., 2012d). The present study was designed to investigate anti-inflammatory effect of MYE on LPS-stimulated murine BV-2 microglia cells. To further understand the molecular mechanisms of MYE, the effects of MYE on the secretion of NO, TNF-α and IL-6, the expression of iNOS and the activation of NF-κB were investigated. Results indicated that MYE effectively inhibited the secretion of NO, TNF-α and IL-6 via a blockade of the NF-κB pathway through the inhibition of ERK, JNK, p38 and Akt

phosphorylation in LPS-stimulated BV-2 microglia cells. The inhibitory effect of MYE on the expression of inflammatory mediators suggested one of the mechanisms responsible for its anti-inflammatory action and its potential for use as a therapeutic agent for treating neuro-inflammatory diseases.

NO is key inflammatory and neurotoxic mediators in neuro-inflammation. It is responsible for the detrimental effect under injury or disease state of central nervous system, including ischemia, Alzheimer's disease, and neuronal damage (Meda et al., 1995). Many studies revealed that abnormally high level of NO is found in various types of brain injuries and neurodegenerative diseases, that caused by the excessive expressions of iNOS (Brown and Neher; Cunningham, 2012; Teismann et al., 2003). The microglial cells that over-express iNOS protein was found in rodent brains treated with LPS (Minghetti et al., 1999). The treatment with inhibitors of iNOS resulted in neuroprotection against LPS-induced neurotoxicity, suggesting that NO is important mediator in neurotoxicity (Oh et al., 2009; Teismann et al., 2003). This study demonstrated that MYE has a potential to inhibit the production of NO in LPS-stimulated BV-2 cells. In addition, the result provided the

evidence that the inhibition of NO production by MYE results from the suppression of both mRNA and protein expressions of iNOS in LPS-stimulated BV-2 cells. Moreover, the inhibition of iNOS gene expression by MYE was detected at mRNA level, indicating that inhibition of NO by MYE is associated with down regulation of iNOS at transcriptional level in the LPS-stimulated cells. These data suggest the possibility that MYE has a protective effect on the neuro-inflammatory response in neurodegenerative diseases.

Activated microglia produce high levels of pro-inflammatory cytokines including IL-6 and TNF-α, which are involved in the pathogenesis of several neurodegenerative disorders (Lee et al., 2012c; Lee et al., 2011; Wong et al., 2000). Also, excessive production of pro-inflammatory cytokines such as TNF-α and IL-6 play a critical role in acute inflammatory responses as well as chronic inflammatory diseases (Joung et al., 2012b). MYE suppressed secretion of these pro-inflammatory cytokines in LPS-stimulated BV-2 cells; these results additionally support the potential of MYE as a neuroprotective agent against neuro-inflammation.

Transcriptions of iNOS and pro-inflammatory cytokine genes are mainly controlled by NF-κB transcription factor, which binds to respective *cis*-acting elements in iNOS promoter region (Kim et al., 2011b; Marks-Konczalik et al., 1998; Xie et al., 1993; Yoon et al., 2011). A variety of external stimuli induce the phosphorylation of IκB in the NF-κB-IκB-α complex, resulting in proteasomal degradation of IκB-α and subsequent translocation of NF-κB from the cytosol into the nucleus (Jang et al., 2005; Janssen-Heininger et al., 2000; Lee et al., 2012b). In this study, MYE inhibited the phosphorylation and subsequent degradation of IκB-α, resulting in the suppression of activation and nuclear translocation of NF-κB p65 subunit. The results in this study suggest that transcriptional repressions of iNOS and pro-inflammatory cytokines genes by MYE are primarily due to the inactivation of NF-κB in LPS-stimulated microglia cells.

Intercellular signalling proteins such as MAPKs and AKT are activated by LPS and involved in transcription of iNOS and pro-inflammatory cytokine genes in microglia cells (Herlaar and Brown, 1999; Jang et al., 2005; Jung et al., 2010; SKAPER, 2007). The

phosphorylations of ERK, JNK, p38, and AKT are decreased by MYE treatment in a concentration-dependent manner in LPS-stimulated BV-2 cells. Given the roles of ERK, JNK, p38 and AKT in inflammatory processes, these kinases can be another target of MYE's anti-inflammatory activity in LPS-stimulated BV-2 cells. These data indicate that MYE showed anti-inflammatory effects at least partly by down-regulating NF-κB pathway via suppression of the phosphorylation of MAPKs and AKT.

## 5. Conclusions

In the present study, MYE inhibit the secretion of inflammatory mediators, such as NO and pro-inflammatory cytokines, including TNF-α IL-6 in LPS-stimulated BV-2 microglia cells. Moreover, the inhibitory effect of MYE was associated with down-regulation of the NF-κB pathway via blocking the phosphorylation of MAPKs and Akt. However, the culture of LPS-stimulated BV-2 may not be the same condition as in *vivo* neuro-inflammatory response. Further studies are required to investigate anti-inflammatory and neuroprotective effects of MYE in the animal models of neurodegenerative diseases. Taken together, this study suggests that MYE has a therapeutic candidate for the treatment or prevention of neuro-inflammation in neurodegenerative diseases.

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