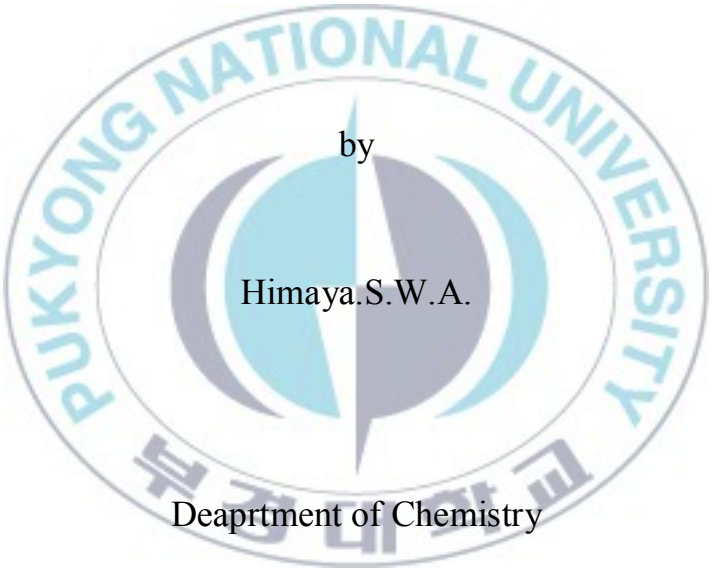
Thesis for the Degree of Master of Science

Attenuation of Neuroinflammatory Effects by *Hippocampus Kuda*Bleeler Derived Compounds, SE-1 and SE-2 via MAPK and NF-κB Signaling Pathways



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A thesis submitted in partial fulfillment of the requirements
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Abstract

Inflammation has recently been implicated as a critical mechanism responsible for neurodegenerative diseases. Microglial cells and macrophages are responsible for mediating inflammatory responses related to neurodegeneration. The two compounds isolated from sea horse *Hippocampus Kuda*Bleeler; 1-(5-bromo-2-hydroxy-4-methoxyphenyl) ethanone [SE-1] and 1-(2-hydroxy-4-methoxyphenyl) ethanone [SE-2] were studied as agents to suppress lipopolysaccharide (LPS) mediated inflammatory responses in cultured BV-2 microglial cells and RAW264.7 macrophage cells. SE-1 and SE-2 significantly attenuated LPS induced release of inflammatory products such as nitric oxide, prostaglandin E2, reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). The compounds also down regulated the protein and gene expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β and IL-6 in both cell lines. Molecular signaling pathway studies showed that SE-1 and SE-2 inhibited the nuclear translocation of nuclear factor-κB (NF-κB) p65 and p50 subunits by attenuating the IKKα/β (IκB kinase α/β) and IκBα. Also SE-1 and SE-2 suppressed the phosphorylation of MAPK molecules; JNK and p38 in both cell lines. These results suggest the potential *in vitro* anti-inflammatory activity of SE-1 and SE-2, which inhibited the LPS, stimulated inflammatory responses in BV-2and RAW264.7cells via suppressing MAPK and NF-κB signaling pathways.

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List of Abbreviations

AP-1	activatorprotien-1
COX-1	constitutive cyclooxygenase
COX-2	inducible cyclooxygenase
CNS	central nervous system
DCF	2,7-dichlorofluorescein
DCFH	2,7-dichlorodihydroxydrofluorescein
DCFH-DA	2,7-dichlorofluorescin diacetate
DMSO	dimethyl sulfoxide
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
IKK	iκB kinase
IL-1β	interleukin-1beta
IL-6	interleukin-6
iNOS	inducible nitricoxide synthase
JNK	c-Jun N-terminal kinase
LPS	bacterial lipopolysaccharide
MAPKs	mitogen activated protein kinases

MTT	3-(4,5_dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa b
NIK	Nf-κB inducing kinase
PBS	phosphate buffered saline
PGE_2	prostaglandin e_2
ROS	Reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinase
SDS	sodium dodysilsulphate
TNF-α	tumor necrosis factor-alpha

1. Introduction

1.1. Inflammation; beneficial or detrimental?

Inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function. However, prolonged inflammation can cease to be a beneficial event and it contributes to the pathogenesis of many disease states (Lawrence, 2002). Interactions of cells in the innate immune system, adaptive immune system, and inflammatory mediators orchestrate aspects of the acute and chronic inflammation that underlie diseases of many organs. Inflammation provides a unifying pathophysiological mechanism underlying many chronic diseases, including diabetes, cardiovascular disease, certain cancers and bowel diseases, arthritis, and osteoporosis (Lawrence, 2002; Libby, 2007) and neurodegenerative diseases. Common pathophysiologic scenarios apply to many of these diseases. New research on inflammation has created a shift in medical thinking. For two millennia it has been viewed mainly as a necessary, even beneficial, response to illness or injury. But now both observational studies and laboratory research are indicating that inflammation can be more of a bane than boon, the common, causative factor in many diseases.

The process of inflammation is divided into acute and chronic patterns. Acute inflammation is of relatively short duration, lasting for minutes, several hours, or a few days, and its main features are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is of longer duration and is associated histologically with the presence of lymphocytes and macrophages, the proliferation of blood vessels, fibrosis, and tissue necrosis. Many factors participate in the course and histologic features of both acute and chronic inflammation. As continual activation of the adaptive immune system is the driving force behind chronic inflammation, it is crucial to identify the stop signals that are present in self-limiting, self- resolving inflammatory lesions. These signals might be used therapeutically to control the activation of the adaptive immune response and the transition from acute to chronic inflammation, when these signals might be absent or become deregulated.

1.2. Inflammatory responses in neurodegeneration

Researchers once thought the central nervous system (CNS) were outside the reach of the immune system as the blood/brain barrier, formed by tightened capillaries, acts like a bouncer, screening out inflammatory cells and molecules so they can't enter the brain. Yet observational studies have found links between non-steroidal anti-inflammatory drugs (aspirin, ibuprofen, and naproxen), inducible cyclooxygenase (COX-2) inhibitors, and other anti-inflammatory medications and a lower risk of Alzheimer's disease and Parkinson's disease (Perry et al., 2007). In addition, the brain may have its own branch of the immune system. Cells inside the brain called microglia, the counterparts to macrophages, swarm and engulf foreign substances and release inflammatory molecules. Therefore macrophages and microglia are central to inflammation in chronic neurodegenaation.

In neurodegenerative brain diseases, inflammation is triggered by several factors including aggregated/modified proteins, stimulatory signals derived from injured/dying cells, or by pro-inflammatory and anti-inflammatory mediators from infiltrating immune cells and/or endogenous immune cells, such as microglia and astrocytes.

1.3. Role of Macrophage cells in inflammation

In inflammation, macrophages have three major function; antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors (Fujiwara and Kobayashi, 2005; Kasahara and Matsushima, 2001). In addition, macrophages produce biological active molecules that serve many important roles in innate and adaptive immune responses. No human has been identified as having congenital absence of this cell line, probably because macrophages are required to remove primitive tissues during fetal development as new tissues develop to replace them.

Macrophages express a set of pattern recognition receptors including various scavenger receptors and Toll-like receptors, (Krieger, 1997) whose ligands include pathogen-associated molecular patterns such as lipopolysaccharides, surface phosphatidylserine, and inflammation related

diseases specific receptors. Ligation of scavenger receptors can lead to endocytosis and lysosomal degradation of the bound ligands, while engagement of Toll-like receptors results in activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Faure et al., 2000; Libby, 2007). Ligation of Toll-like receptors can also heighten phagocytosis, production of reactive oxygen species, and release of cytokines, autacoids, and lipid mediators that coordinate and amplify the local inflammatory response (Libby, 2002; Takeda and Akira, 2005).

1.4. Microglial cells; a friend or foe?

Microglia are the major resident immune cells in the CNS and play a functional role in CNS defense which comprise approximately 12% of cells in the brain. Microglia density varies by brain region in the adult human (0.5–16.6%) (Blocketal.,2007). Similar to peripheral macrophages, microglia may derive from hematopoietic lineage and enter the CNS during development (Cuadros and Navascues, 1998;Tzeng et al., 2005).

In the mature brain, microglia typically exists in a resting state characterized by ramified morphology, and monitors the brain environment. The turnover rate of microglia in the healthy adult brain is probably low. In response to certain cues such as brain injury or immunological stimuli, however, microglia are readily activated (Davaloset al., 2005; Nimmerjahn et al., 2005) and both intrinsic proliferation of parenchymal microglia and recruitment of monocytes could be substantial (Ajami et al., 2007). Activated microglias undergo a dramatic transformation from their resting ramified state into an amoeboid morphology and present an up-regulated catalogue of surface molecules (Cho et al., 2006; Kreutzberg, 1996; Perry, 1994). Activated microglia are involved in regulating brain development by enforcing the programmed elimination of neural cells , and seem to enhance neuronal survival through the release of trophic and anti-inflammatory factors (Ekdahl et al., 2009; Marin-Teva et al., 2004). In addition, in the mature brain, microglia facilitate repair through the guided migration of stem cells to the site of inflammation and injury (Morgan et al., 2004), and might be involved in neurogenesis.

However, under some circumstances, microglia become over activated and can induce significant and highly detrimental neurotoxic effects by the excess production of a large array of

cytotoxic factors such as superoxide, nitric oxide (NO) and pro-inflammatory cytokines (Moss and Bates, 2001). The stimuli that cause microglial over activation and deregulation can be diverse, ranging from environmental toxins, such as the pesticide rotenone, to neuronal death or damage. In neurodegenerative diseases, activated microglia have been shown to be present in large numbers, a condition termed microgliosis, strongly implicating these cells in disease pathology (Block et al., 2007). Currently, the conditions defining whether microglial activation is detrimental or beneficial to neuronal survival are poorly understood. It can be suggested that microglia activation, as an indicator of inflammation, is not pro- or anti-neurogenic per se but the net outcome is dependent on the balance between secreted molecules with pro- and anti-inflammatory action.

However, it is becoming more widely accepted that although microglial activation is necessary and crucial for host defense and neuron survival, the over activation of microglia results in deleterious and neurotoxic consequences (Polazzi and Contestabile, 2002). Moreover, activated microglia are a major source of pro-inflammatory and neurotoxic molecules that augment brain inflammation and contribute to brain damage (Figure **1**). Thus, the activation of microglia is considered to be associated with the pathogenesis of various neurodegenerative diseases (Liu and Hong, 2003; Nakajima and Kohsaka, 2004).

As discussed above, microglia-mediated neurotoxicity tends to be progressive which could contribute to the progressive nature of several neurodegenerative diseases. This has been most effectively demonstrated in models using lipopolysaccharide (LPS), the polysaccharide component of the cell walls of gram-negative bacteria which results in acute excessive activation of microglia that contributes to the death of newly formed neurons (Hanisch and Kettenmann, 2007) Although LPS models cannot not precisely mimic the conditions under which microglia are activated in neurodegenerative disease, studies indicate that stimulation of microglia with LPS can initiate neuronal damage (Gibbons and Dragunow, 2006; Ling et al., 2002). For example, LPS is reported to induce microglial activation in vivo and in vitro and cause the progressive and cumulative loss of DA neurons over time. Furthermore, embryonic exposure to LPS has an impact on microglial activation and neuron survival into adulthood (Ling et al., 2006).

When the phenotype of the microglia is switched by the inflammatory challenge, increased production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), major inflammatory mediators

such as NO/iNOS (Cunningham at al., 2007) and PEG_2/COX-2 (Minghetti and Levi, 1998) were observed. A full understanding of the mechanisms underlying microglial deregulation and over-activation is of major interest because of the valuable insight it will provide into the aetiology, pathogenesis and treatment of neurodegenerative diseases.

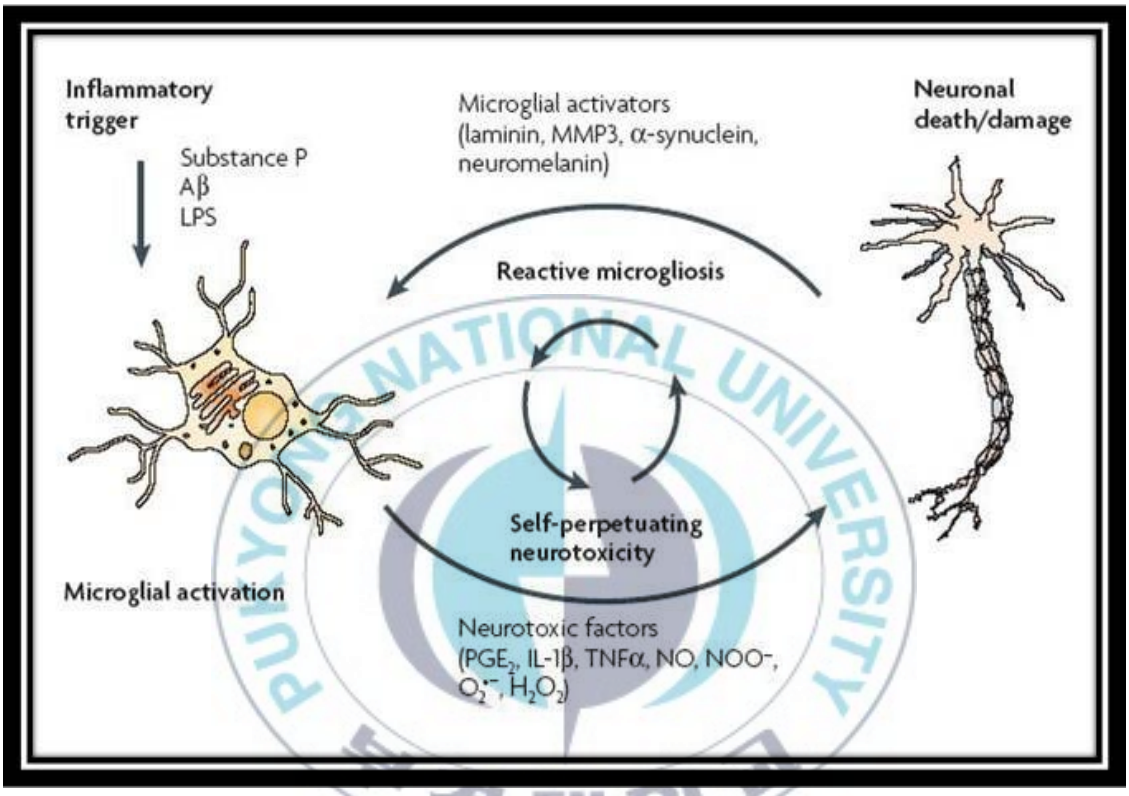

Figure 1: Microglia mediated neurotoxicity.Microglia can become over-activated and cause neurotoxicity through two mechanisms. First, microglia can initiate neuron damage by recognizing pro-inflammatory stimuli, LPS, becoming activated and producing neurotoxic pro-inflammatory factors. Second, microglia can become over activated in response to neuronal damage (reactive microgliosis), which is then toxic to neighboring neurons, resulting in a perpetuating cycle of neuron death.

1.5. NF-κB signaling pathway mediated inflammatory responses

A wealth of data suggests that the NF-kB signaling pathway plays a crucial role in the initiation, amplification and resolution of inflammation as a response to pro-inflammatory stimuli (Lawrence et al., 2001; Tak and Firestein, 2001; Xiao and Ghosh, 2005). Nuclear factor- B (NF- B) is a dimeric transcription factor formed by the hetero or homo-dimerization of proteins in the Rel family (Hanada and Yoshimura, 2002). In mammalian cells, there are five members of the NF-kB family: RelA (p65), RelB, C-Rel, p105 (NF-kB1; a precursor of p50) and p100 (NF-kB2; a precursor of p52). However the most frequently activated form of NF-kB is a heterodimer composed of RelA and p50 (Hayden et al., 2006). NF-kBproteins form homo- or hetero-dimers and regulate expression of distinct but overlapping genes involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, stress responses and cancer progression (Gilmore, 2006; Karin and Greten, 2005). Under un-stimulated conditions, NF-kB is sequestered in the cytoplasm as an inactive form by interaction with a family of inhibitor proteins known as IkB proteins which can mask the nuclear localization signal of NF-kB in the cytoplasm through direct interactions, to prevent nuclear translocation of NF-kB. Inflammatory stimulation triggers the rapid phosphorylation of specific serine residues of IkB proteins by a multi protein complex termed the IKK complex, which consists of two catalytic components, IKKα and IKKβ, and a regulatory component, NEMO (NF-kB essential modifier, also known as IKK□) (Kawai and Akira, 2007). Phosphorylated IkB proteins are subsequently poly-ubiquitinated and degraded by the 26S proteasome, allowing NF-kB to move into the nucleus.

NF-κB is thought to have a pivotal role in immune and inflammatory responses through the regulation of genes that encode pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes, such as COX2 and iNOS (Lawrence et al., 2002). However, uncontrolled accumulation of these defense molecules leads to a more severe inflammatory response. NO and PEG2 the end products of inflammatory enzymes, iNOS and COX-2. iNOS and COX-2 genes have putative binding sites for NF-kB at their promoter sites in the gene. Therefore activation of NF-kB pathway have a remarkable link with the production of NO and PEG2 (Kim et al., 2003). And also during inflammation leucocytes are continuously produce ROS. It has been revealed that ROS are closely associated with the initiation or aggravation of diverse pathological events via NF-kB pathway (Yunbiao and Wahl,

2005). NF-kB also triggers the production of excess ROS by breaking its physiological balance. The effects of ROS in inflammation are believed to be mediated through its ability in altering the biomolecules such as DNA, lipids and proteins (Sanlioglu et al., 2001). Taken together it is clear that suppression of NF-κB pathway is considered as an effective target for the treatment of inflammatory diseases.

1.6. MAPK signaling pathway in inflammation

Mitogen-activated protein kinase (MAPK) signaling pathways are also involved in activation of inflammation and it has been extensively studied in microglia in vitro. MAPK includes three subtypes which are upstream signaling molecules in inflammatory reactions: p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) (Pan et al., 2009). The activation of MAPKs requires phosphorylation of conserved threonine and tyrosine residues. Phosphorylation of MAPKs results in their direct translocation to the nucleus where they activate transcription factors, NF-κB and AP-1 by phosphorylation and leading to expression of inflammation related genes (Figure 2) (Adachi et al., 2000). MAPKs are largely cytoplasmic in unstimulated cells and translocate to the nucleus when cells are stimulated.

In neuro-inflammatory responses it has been proposed that an aberrant expression of MAPKs or activation of NF-κB induced microglia is directly correlated with pathogenic events in neurodegenerative diseases (Pan et al., 2009). Therefore, strategies to suppress MAPKs and/or NF-κB activation may attenuate neuro-inflammation and neuronal damage, which will be of benefit in neurodegenerative disease treatment.

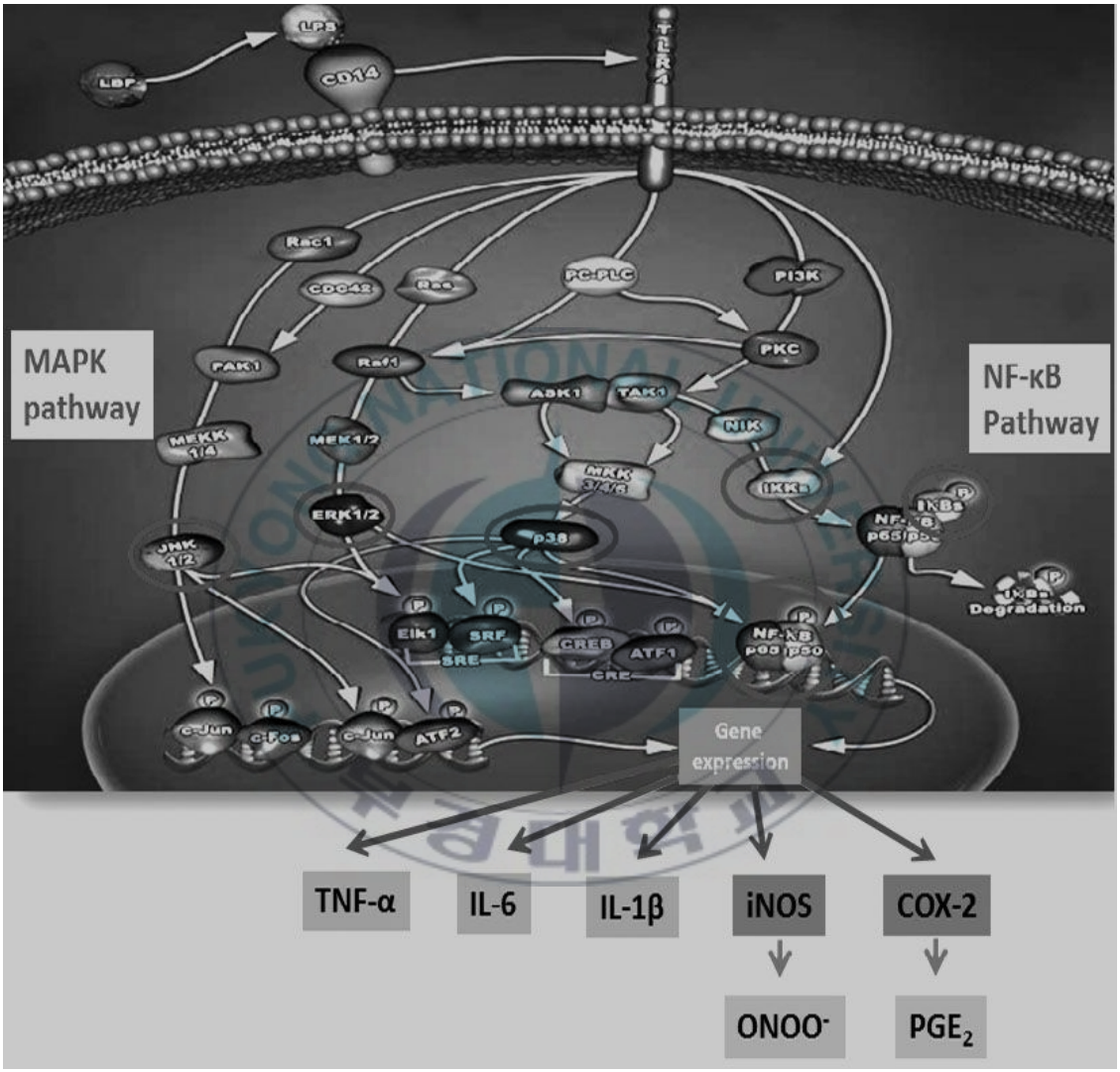

Figure 2: NF-κB and MAPK signaling pathways mediated transcription of inflammatory genes in response to LPS challenge.

1.7. Hydroxyacetophenone compounds SE1and SE2 as inhibitors of neuro-inflammatory responses

Seahorse, a marine teleost fish, is well known not only for its special medicinal composition but also for its unusual features including male pregnancy. Seahorse belongs to syngnathidae family of vertebrate phylum. Chinese seahorse (*Hippocampus kuda*Bleeler) is used as one of the most famous and expensive materials of traditional Chinese medicine. has been studied for many years for its various biological activities, including appetite enhancement, antioxidant, anti-tumor, antiaging, anti-fatigue and Ca2+ channel blocking (Li et al., 2008; Ryu et al., 2010; Zhang et al., 2003).

Two hydroxyacetophenone compounds; 1-(5-bromo-2-hydroxy-4-methoxyphenyl)ethanone (SE1) and 1-(2-hydroxy-4-methoxyphenyl)ethanone (SE2) (Figure. 3) were isolated from sea horse *Hippocampus kuda*Bleeler. Scientific evidences confirms that the compounds bearing methoxy (-OCH_3) groups are of high anti-inflammatory activity (Sirinivasan et al., 2009). And also it has been found that SE2 is a compositional compound in Moutan Cortex, a widely used traditional Chinese medicine for the treatment of various diseases. Furthermore it has been characterized for its inhibitory activity on TNF-α and IL-6 (Wu and Gu, 2007). Therefore with this background the two compounds have been tested for their anti-inflammatory activity in this study using an in vitro model of LPS stimulated BV-2 and RAW264.7 cells.

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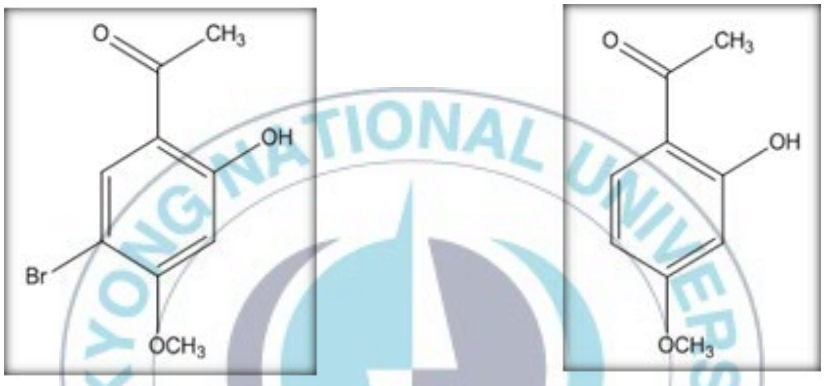


Figure 3: Chemical structures of 1-(5-bromo-2-hydroxy-4-methoxyphenyl)ethanone (SE1) and 1-(2-hydroxy-4-methoxyphenyl)ethanone (SE2).

Research objectives

The objective of the present study is to evaluate the effects of seahorse; *Hippocampus kuda*Bleeler derived compounds SE1 and SE2 on neuro-inflammatory responses. More specifically this study focus on their ability in blocking; the production of inflammatory mediators including NO, PGE_2, and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and the expression of their respective upstream enzymes and genes iNOS, COX-2 and cytokines. Furthermore, efforts have taken to identify the underling molecular mechanisms MAPK and NF-κB signalling pathways.

2. Materials and methods

2.1. Materials

Mouse microglial cells were a kind gift by professor Il-Whan Choi from Inje University, Korea. Mouse macrophages, RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture media [Dulbecco's modified Eagle's minimal essential medium (DMEM)], penicillin/streptomycin, fetal bovine serum (FBS) and the other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY). The MTT reagent [(3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide)], the Griess reagent and LPS of *Escherichia coli* 026:B6 were purchased from Sigma Chemical Co. Primary and secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Amersham Pharmacia Biosciences (Piscataway, NJ). Other chemicals and reagents used were of analytical grade commercially available.

2.2. Cell culture and cell viability assay

BV-2 and RAW264.7 cellswere maintained in a 95% air, 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin. RAW264.7 cells and BV-2 cells were maintained via 2 times passage per week and cells were utilized for experimentation at 70–80% confluency.

Cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay as described by Hansen et al. (1989). MTT is used as an indicator of cell viability through its mitochondrial reduction to formazan (Rajapakse et al., 2008). In brief, cells were pre-incubated overnight in 96-well plates at a density of 1×10^4 cells per well, and were then washed with phosphate-buffered saline (PBS). Cells were treated with various concentrations (5, 10 and 50 μM) of SE1 and SE2 for 24 h, 100 μl MTT was added and incubated at 37^oC for 4 h. After the culture supernatants were removed, the resulting dark blue crystals were dissolved with DMSO. Absorbance values were read at 540 nm on an enzyme linked immunosorbant assay

(ELISA) microplate reader (Tecan Austria GmbH). Relative cell viability was calculated compared with the absorbance of the untreated control group.

2.3. Nitric oxide production assay

Nitric oxide (NO) levels in the culture supernatants were measured by the Griess reaction as described earlier by Coker and Laurent (1998). In brief, BV-2 and RAW264.7 cells were pre-incubated overnight in 96-well plates using DMEM without phenol red at a density of 1×10^4 cells per well, followed by the of treatment different concentrations (5, 10 and 50 μM) of SE1 and SE2 for 1 h. After, the NO production was stimulated by adding LPS (1μg/ml final concentration) and incubated for 48 h. Then 50 μl of culture supernatants from each sample was mixed with the same volume of the Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediaminedihydrochloride/2.5% phosphoric acid] following incubation for 15 min. Absorbance values were read at 540 nm on an ELISA microplate reader (Tecan Austria GmbH). The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in DMEM, and the concentration of nitrite in conditioned media of sample treated cells were calculated.

2.4. Enzyme immuno assay of PGE_2

Assessment of PGE_2 synthesis was performed by enzyme immunoassay without prior extraction or purification using commercially available PGE_2 enzyme immunometric assay kit (Amersham Pharmacia Biosciences, NJ, USA). BV-2 and RAW264.7 cells were treated with different concentrations of SE1 and SE2 (5, 10 and 50 μM) for 1 h and stimulated with LPS (1 μg/ml) or 18 h. the conditioned media was collected to perform PGE_2 enzyme immune-metric assay (PGE_2-EIA) according to the instructions of the manufacturer. The concentration of PGE_2 was calculated according to the equation obtained from the standard curve plot using PGE_2 standard solution in the EIA kit.

2.5. Enzyme immuno assay of TNF-α, IL-1β and IL-6

The inhibitory effects of SE1 and SE2 on the production of pro-inflammatory cytokines; TNF-α, IL-1β and IL-6 were determined by an enzyme-linked immunosorbent assay (ELISA). Cells were treated with different concentrations of SE1 and SE2 (5, 10 and 50 μM) for 1 h following LPS (1 μg/ml) treatment for 18 h. Conditioned medium were analyzed per the manufacturer's recommendations with mouse cytokine-specific Biotral™ ELISA kits (Amersham Pharmacia Biosciences, NJ, USA). The concentrations of TNF-α, IL-1β and IL-6 were calculated according to the standard curve using each of the recombinant cytokines in the ELISA kits.

2.6. Determination reactive oxygen species (ROS) production by FACS

Intracellular oxidative stress was assayed by measuring intracellular oxidation of dichlorofluorescein (DCFH). The substrate was 2',7'-dichlorofluorescein diacetate (DCFH-DA) which easily diffuses into the cell and was next deacetylated by cellular esterases to the more hydrophilic, nonfluorescent DCFH. H_2O_2 produced in the cell oxidized DCFH to the fluorescent 2',7'-dichlorofluorescein (DCF). BV-2 and RAW264.7 cells were pretreated with DCFHDA (5 μM, Molecular Probes, Eugene, OR, USA) for 1 h following SE1 and SE2 treatment for another 30 min. Next, the cells (except for controls) were exposed to 1 μg/ml LPS at 37 °C for 2 h. Cells were detached and fluorescence was measured with a FACSCalibur flow cytometer 9488 nm excitation, 530 nm emission) using the Cell Quest analysis software (Becton Dikinson, Mountain View, CA, USA).

2.7. Reverse transcription (RT)-PCR analysis

RT-PCR was performed for the detection of the mRNA expressions of iNOS, COX-2, and cytokines (TNF-α ,IL-1β and IL-6). Total RNA was extracted from RAW264.7 cells after LPS (1 μg/ml) treatment followed by SE1 and SE2 treatment at different concentrations (5, 10 and 50 μM) for 18 h. The cells were lysed with Trizol® and centrifuged at 12,000 rpmfor 15 min at 25

°C following the addition of chloroform. Supernent was separated and isopropanol was added to it at a 1:1 ratio. By centrifugation for 10 min at 10000 rpm, RNA pellet was obtained. After washing with ethanol, extracted RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using the GENios® microplate reader (Tecan Austria GmbH). Resulted RNA (1 μg) were reverse transcribed into cDNA in a mastermix containing 1×reverse transcriptase (RT) buffer, 1 mMdNTPs, 500 ng of oligo (dT)15 primers, 140U of murine Moloneyleukaemia virus (MMLV) reverse transcriptase and 40U of RNase inhibitor, for 45 min at 42 °C. Polymerase chain reaction (PCR) was carried out in an automatic Whatmanthermocycler (Biometra, Kent, UK) to amplify iNOS, COX-2, TNF-α , IL-1β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Each transcript was identified using specific forward and reverse primers as manufacturer's instructions (Promega, Madison, WI, USA). GAPDH expression was included as an internal, housekeeping gene control. Primer sequences used to amplify the desired cDNA were as follows: iNOS forward and reverse primers: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′; COX-2 forward and reverse primers: 5′-GGGGTACCTTCCAGCTGTCAAAATCTC-3′ and 5′-GAAGATCTCGCCAGGTACTCACCTG-3′; TNF- α forward and reverse primers: 5′-ATGAGCACAGAAAGCATGATC-3′and 5′-TACAGGCTTGTCACTCGAATT-3′; IL-1βforward and reverse primers:5′-ATGGCAACTGTTCCTGAACTCAACT-3′ and 5′-TTTCCTTTCTTAGATATGGACAGGAC-3′; IL-6forward and reverse primers:5′-AGTTGCCTTCTTGGGACTGA-3′and 5′-CAGAATTGCCATTGCACAAC-3′; and G3PDH forward and reverse primers: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CA TGTAGGCCATGAGGTCCACCAC-3′. The conditions for the amplification cycles were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 35 cycles. After polymerase chain reaction (PCR) products electrophoresed on 1.5% agarose gels in 1× TAE buffer for 30 min at 100 V were visualized by ethidium bromide staining using AlphaEase® gel image-analysis software (Alpha Innotech, San Leandro, CA, USA).

2.8. Western blot analysis

Standard procedures were used for the Western blotting, where BV-2 and RAW264.7 cells treated with different concentrations of SE1and SE2 (5, 10 and 50 μM) followed by LPS treatment (1 μg/ml) for 18 h, were lysed in lysis buffer containing 50 mMTris-HCl (pH 7.5), 0.4% Nonidet P-40, 120 mMNaCl, 1.5 mM $MgCl_2$, 2 mMphenylmethylsulfonyl fluoride, 80 μg/ml of leupeptin, 3 mMNaF and 1 mM DTT at 4 ^{o}C for 30 min. Cell debris were removed by centrifugation followed by quick freezing of the supernatants. To obtain the nuclear extracts of BV-2 and RAW264.7 cells, CelLytic™NuCLEAR™ extraction kit (Sigma-Aldrich Co., Mo, USA) was used according to the manufacturer's instructions. The protein concentration was determined using Lowry method. Cell lysates (≈ 20 μg of total protein) were separated by SDS-PAGE gel electrophoresis and electro-blotted onto a nitrocellulose membrane. The membranes were blocked with 10% skim milk and then incubated with different antibodies (Santa Cruz Biotechnology Inc.), which were used to detect the respective proteins using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences), according to the manufacturer's instructions. Western blots were visualized using an LAS3000® Luminescent image analyzer and protein expression was quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

2.9. Statistical analysis

One-way analysis of varience was used for all statistical analysis using independent experiments and data are presented as means ±S.D. The mean values were calculated based on the data taken from at least three independent experiments conducted on separate days using freshly prepared reagents.

3. Results and Discussion

3.1. Cyto-compatibility of the compounds SE1 and SE2

Cyto-compatibility of SE1 and SE2 were tested at three concentrations (5, 10 and 50 μg/ml) using the MTT cell viability assay in cultured BV-2 and RAW264.7 cells. The experiments were carried out based on the fact that, the live cells converts MTT into visible formazan crystals while incubation and the absorbance values taken from solubilized formazan crystals in dimethylsulphoxide is proportional to the cell viability (Hansen et al., 1989). As depicted in Figure 4 neither SE1 nor SE2 showed a significant ($p<0.05$) cytotoxicity on both cell lines at all tested concentrations after 24 h of treatment. Non-toxic concentrations ranging from 5 –50 μg/ml were selected from both compounds for further analysis.

(a)

(b)

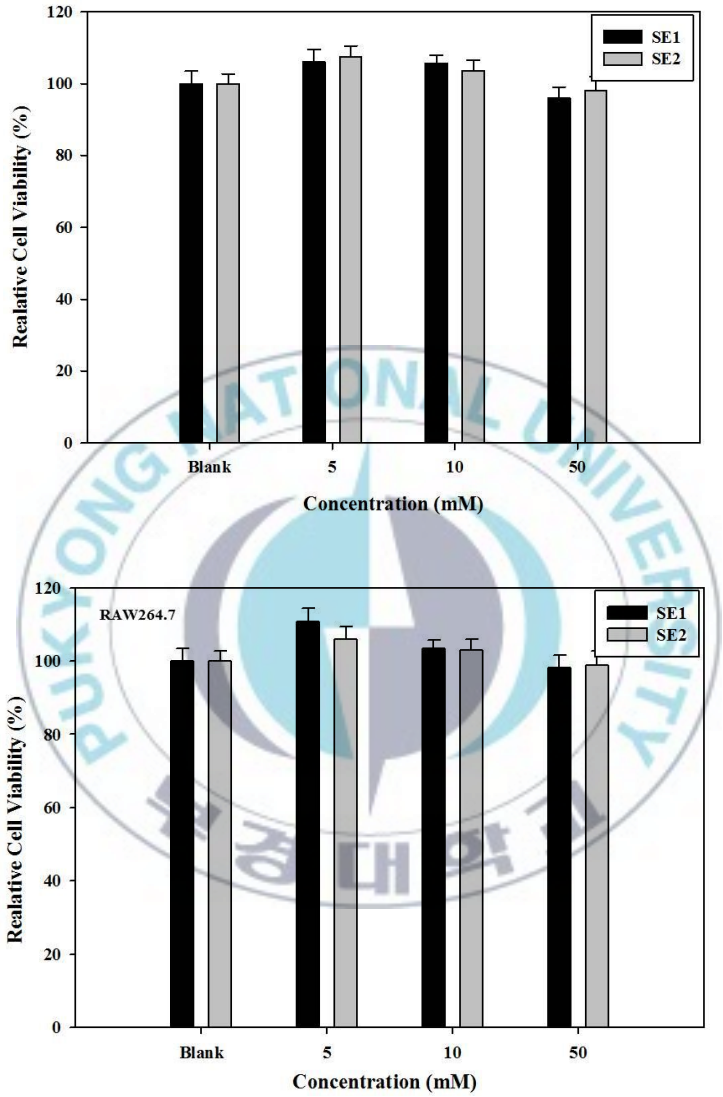


Figure 4: Effects of SE1 and SE2 on the viability of BV-2 (a) and RAW264.7 (b) cells. Cells grown in serum-free medium were treated with different concentrations (5, 10 and 50 μM) of SE1 and SE2 for 24 h and relative cell viability was assessed by the MTT assay, as described. Results of three independent experiments were averaged and are shown as percentage cell viability compared with the viability of untreated control cells.

3.2. SE1 and SE2 suppressed the LPS induced NO production in BV-2 and RAW264.7 cells

Stimulation of cells with LPS showed a significant increase in cellular nitrite levels compared to non-stimulated blank group in both cell types, which was approximately 6 fold increase(Figures 5 and 6). SE1 and SE2 were tested for their ability to inhibit this LPS stimulated NO production. Nitrite is the converted stable product of released NO by LPS stimulated cells. As shown in figures 5 and 6, both SE1 and SE2 showed a potential to inhibit NO production in LPS stimulated BV-2 and RAW264.7 cells and the inhibitions were dose dependent. The ability to inhibit the LPS stimulated NO production was used as the screening test for the anti-inflammatory activity of the metabolites (Himaya et al., 2010). During inflammation some pro-inflammatory cytokines and endotoxins induce the expression of iNOS and leading to generation of NO. Therefore, the ability to inhibit this NO production could be effectively used in screening of anti-inflammatory candidates. Therefore in this study, due to shown effectiveness in inhibiting the LPS challenged NO production in BV-2 and RAW264.7 cells, SE1 and SE2 were selected for further investigation of their anti-inflammatory activity.

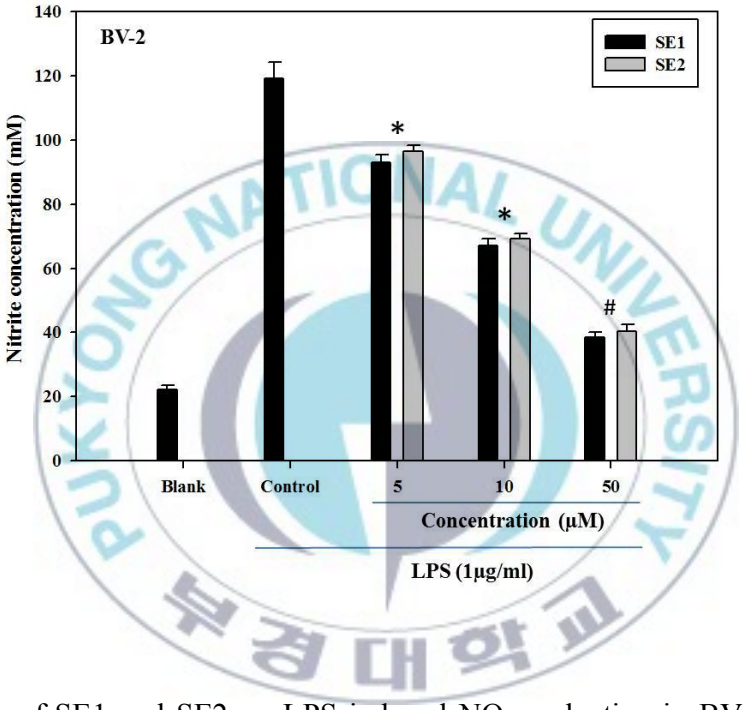


Figure 5: Effects of SE1 and SE2 on LPS induced NO production in BV-2 cells. BV-2 cells cultured in phenol red and serum free media were pre-treated with different concentrations (5, 10 and 50 μM) of SE1 and SE2 for 1 h, following stimulation of LPS (1 μg/ml). Conditioned medium was collected after 48 h and NO concentrations were measured using the Griess reaction. The nitrite (stable oxidation product of NO) levels of the media were calculated compared to standard nitrite curve.The data indicate the mean ± S.E.M of three independent experiments. * $p< 0.05$ and # $p <0.01$ compared to control.

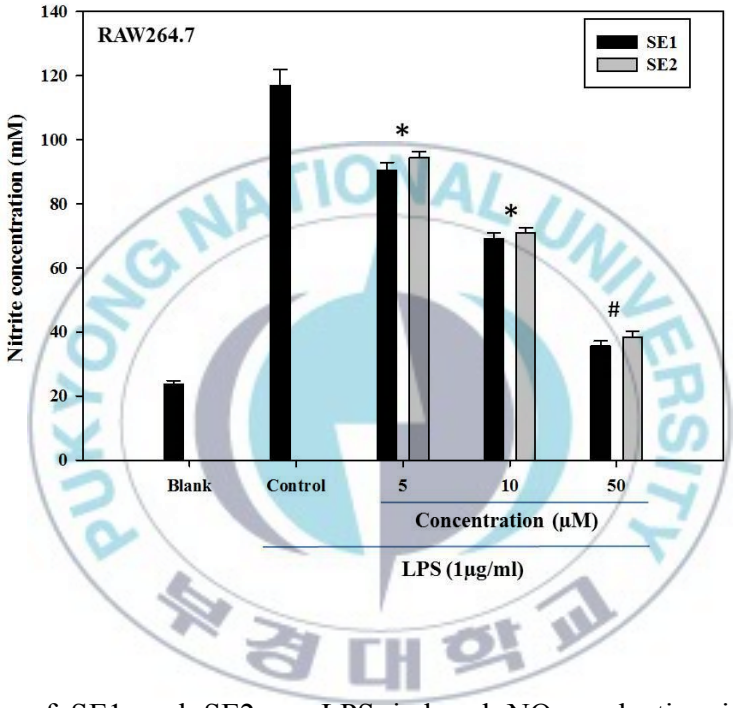


Figure 6: Effects of SE1 and SE2 on LPS induced NO production in RAW264.7 cells. RAW264.7 cells cultured in phenol red and serum free media were pre-treated with different concentrations (5, 10 and 50 μM) of SE1 and SE2 for 1 h, following stimulation of LPS (1 μg/ml). Conditioned medium was collected after 48 h and NO concentrations were measured using the Griess reaction. The nitrite (stable oxidation product of NO) levels of the media were calculated compared to standard nitrite curve.The data indicate the mean ± S.E.M of three independent experiments. * $p< 0.05$ and # $p <0.01$ compared to control.

3.3. SE1 and SE2 inhibited theLPS induced iNOS expression in BV-2 and RAW264.7 cells

Activated microglial cells and macrophages are one of the most important types of effector cells in mediating central nerve system (CNS) inflammatory responses. After stimulation of these cells with LPS, expression of iNOS is induced and it mediates the production of NO which ultimately results in neuronal damage by NO itself and its more-toxic metabolite, peroxynitrite ($ONOO^-$) (Dringen, 2005; Li et al., 2005).

The effects of SE1 and SE2 on protein and gene expression levels of iNOS were investigated in BV-2 and RAW264.7 cells. In the Western blot analysis, it was clear that LPS treatment sufficiently increased iNOS protein expression compared to the non-treated groups in both cell lines (Figures 7 and 8). This stimulated iNOS protein expression levels were evidently decreased with SE1 and SE2 treatment (5, 10 and 50 μg/ml) for 18 h. RT-PCR analysis was conducted to find the effects of SE1 and SE2 on m-RNA expression level of iNOS gene on both cell types, and as shown in Figures 7 and 8 the SE1 and SE2 treatment for 18 h inhibited the LPS stimulated expression of iNOS mRNA. Collective analysis of this data showed that the inhibition of NO production is a resultant of down regulation of iNOS protein and m-RNA expression by SE1 and SE2 treatment in LPS stimulated BV-2 and RAW264.7 cells. The inhibition of NO production and iNOS protein expression in BV-2 and RAW264.7 cells further confirms the anti-inflammatory effect of SE1 and SE2.

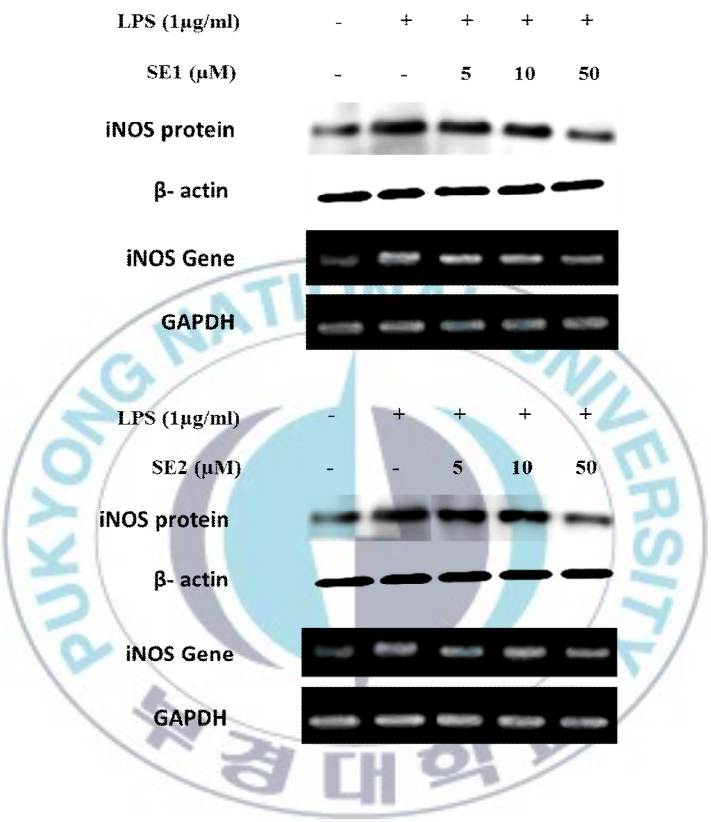


Figure 7: SE1 and SE2 inhibited LPS-induced iNOS protein and mRNA expressions in BV-2 cells. BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml) for 18 h. Cell lysates were extracted, and protein and gene levels of iNOS were analyzed by Western blotting and RT-PCR respectively. β-Actin and GAPDH expressions were used as an internal control for protein and loading in Western blot and RT-PCR respectively.

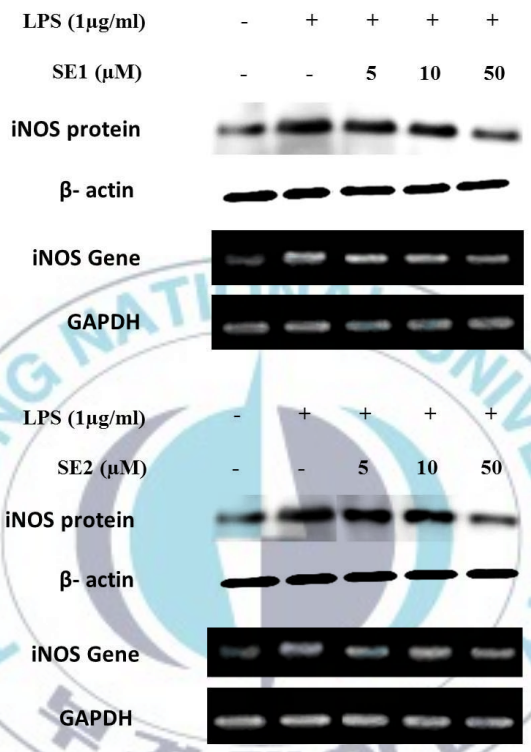


Figure 8:SE1 and SE2 inhibited LPS-induced iNOS protein and mRNA expressions in RAW264.7 cells. RAW264.7 cells were pretreated with SE1 and SE2(5, 10 and 50 μM)for 1 h, and then stimulated with LPS (1 μg/ml) for 18 h. Cell lysates were extracted, and protein and gene levels of iNOS were analyzed by Western blotting and RT-PCR respectively. β-Actin and GAPDH expressions were used as an internal control for protein and loading in Western blot and RT-PCR respectively.

3.4. SE1 and SE2 inhibited the LPS induced PGE_2 production in BV-2 and RAW264.7 cells

Prostaglandins are another type of pro-inflammatory mediators, which generated through the cyclooxygenase pathway (Tzeng et al., 2005).The inducible cyclooxygenase, COX-2 drives the onset of inflammation through the production of pro-inflammatory prostaglandin E_2. Thereforethe potential effects of SE1 and SE2were determined on inhibition of the production of PEG_2, the amount of released PGE_2 was measured in LPS stimulated BV-2 and RAW264.7 cells using anti-PGE_2-coated ELISA plates. The PEG_2concentrations were markedly increased in BV-2 and RAW264.7 cells as consequence of LPS challenge. SE1 and SE2 treatment significantly ($P < 0.05$) inhibited this stimulated levels of PGE_2 at all treated concentrations in a dose dependent manner (5, 10 and 50 μg/ml) in both cell lines(Figures 9 and 10).

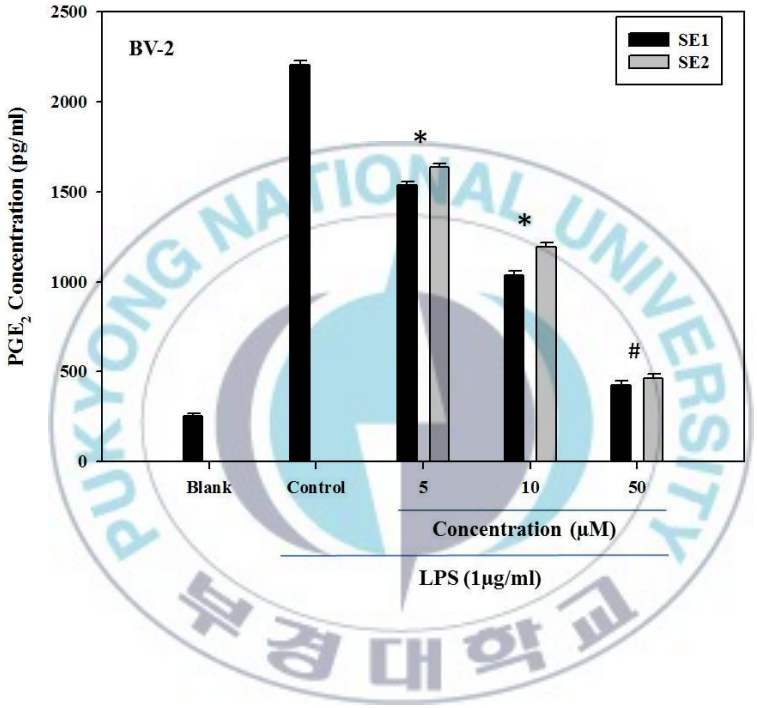


Figure 9:SE1 and SE2 inhibited LPS-induced PGE_2 production in BV-2 cells.BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1μg/ml). Culture media were collected after 18 h in order to measure PGE_2 concentrations by the enzyme immunoassay. The data indicate the mean ± S.E.M of three independent experiments. * $p< 0.05$ and # $p <0.01$ compared to control.

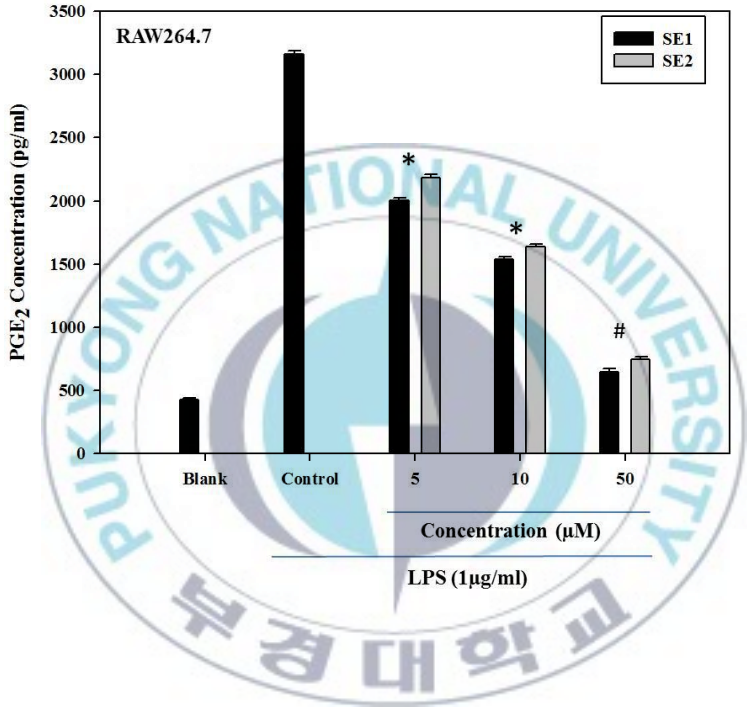


Figure 10:SE1 and SE2 inhibited LPS-induced PGE_2 production in RAW264.7 cells.RAW264.7 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1μg/ml). Culture media was collected after 18 h in order to measure PGE_2 concentrations by the enzyme immunoassay. The data indicate the mean ± S.E.M of three independent experiments. * $p< 0.05$ and # $p <0.01$ compared to control.

3.5. SE1 and SE2 inhibited the LPS induced COX-2 expression in BV-2 and RAW264.7 cells

Accumulating evidence has shown that COX-2 plays a crucial role in inflammation and pathogenesis in brain inflammation (Scali et al., 2003). PEG2 is the resultant product of the COX-2 enzyme activity, which mediates inflammatory responses. Therefore, to investigate the underlying processes of PEG2 inhibition by SE1 and SE2 treatment expression of COX-2 protein and COX-2 genein BV-2 and RAW264.7 cells were analyzed after SE1 nad SE2 treatment through Western blotting and RT-PCR analysis respectively. Figures 11 and 12 show the COX-2 protein and mRNA expressions after 18 h treatment with SE1 and SE2 in LPS stimulated BV-2 and RAW264.7 cells respectively. It was very clear that both protein and mRNA expressions were inhibited by the treatment of SE1 and SE2 in both cell lines. This is in agreement with the results of SE1 and SE2atternuated decrease in PGE_2 production.

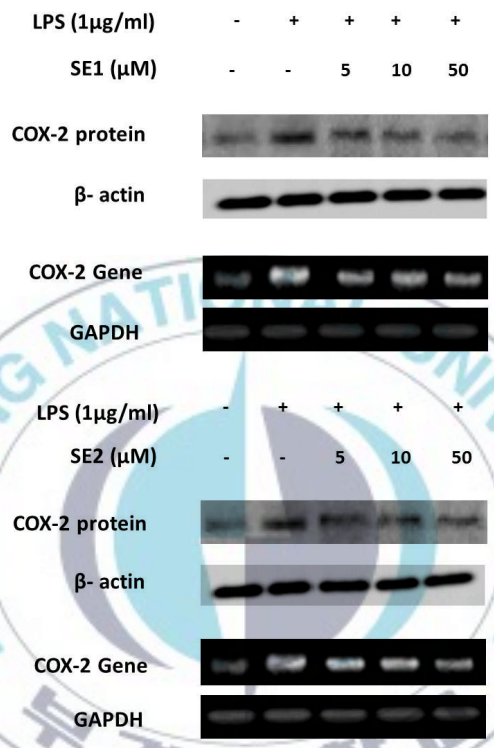


Figure 11: SE1 and SE2 inhibited LPS-induced COX-2 protein and mRNA expressions in BV-2 cells. BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml) for 18 h. Cell lysates were extracted, and protein and gene levels of COX-2 were analyzed by Western blotting and RT-PCR respectively. β-Actin and GAPDH expressions were used as an internal control for protein and loading in Western blot and RT-PCR respectively.

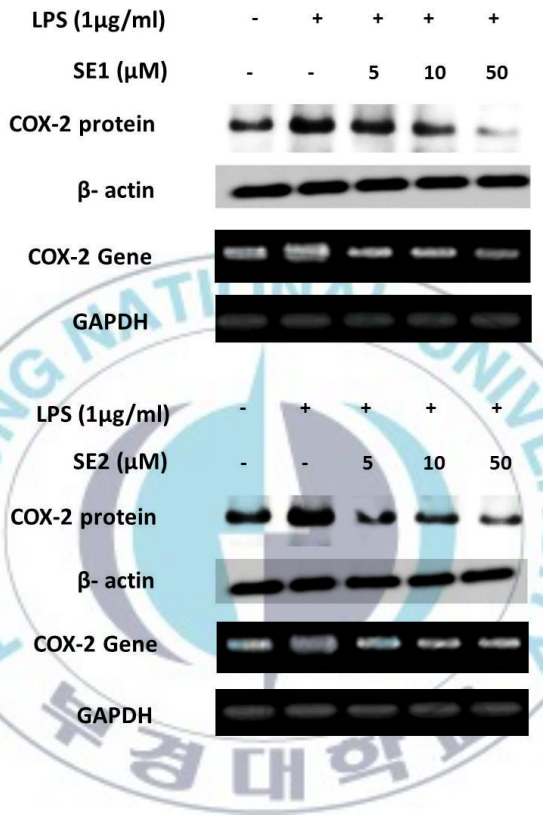


Figure 12:SE1 and SE2 inhibited LPS-induced COX-2 protein and mRNA expressions in RAW264.7 cells. RAW264.7 cells were pretreated with SE1 and SE2(5, 10 and 50 μM)for 1 h, and then stimulated with LPS (1 μg/ml) for 18 h. Cell lysates were extracted, and protein and gene levels of COX-2 were analyzed by Western blotting and RT-PCR respectively. β-Actin and GAPDH expressions were used as an internal control for protein and loading in Western blot and RT-PCR respectively.

3.6. SE1 and SE2inhibited the LPS induced pro-inflammatory cytokines in BV-2 and RAW264.7 cells

Pro-inflammatory cytokines are key components of inflammation. In the CNS, proinflammatory cytokines produced from activated microglia or infiltered macrophages is involved in pathogenesis and/or the exacerbation of brain inflammation. Therefore, the effects of SE1 and SE2 on the production levels of major inflammatory cytokines TNF-α, IL-1β and IL-6 were investigated by ELISA using conditioned media of LPS challanged BV-2 and RAW264.7 cells. LPS stimulation have significantly increased the production of pro-inflammatory cytokines compaired to non-stimulated blank groups. LPS induced production of TNF-α, IL-1βand IL-6 showed a significant ($p < 0.05$) concentration dependentdecrease [Figures 13-18 (a)] following the treatment of SE1 and SE2 (10, 50 and 100 μg/ml). To strengthen this result, transcriptional levels of TNF-α, IL-1β and IL-6 were analyzed by RT-PCR. The results obtained were parallel with the above data where it showed a dose dependant decrease of TNF-α, IL-1β and IL-6 mRNA expression levels when treated with SE1 and SE2 for 18 h [Figures 13-18 (b)]. These results strongly suggest that SE1 and SE2 treatments are effective in suppressing the production of pro-inflammatory cytokines at their transcriptional level in LPS induced BV-2 and RAW264.7 cells.

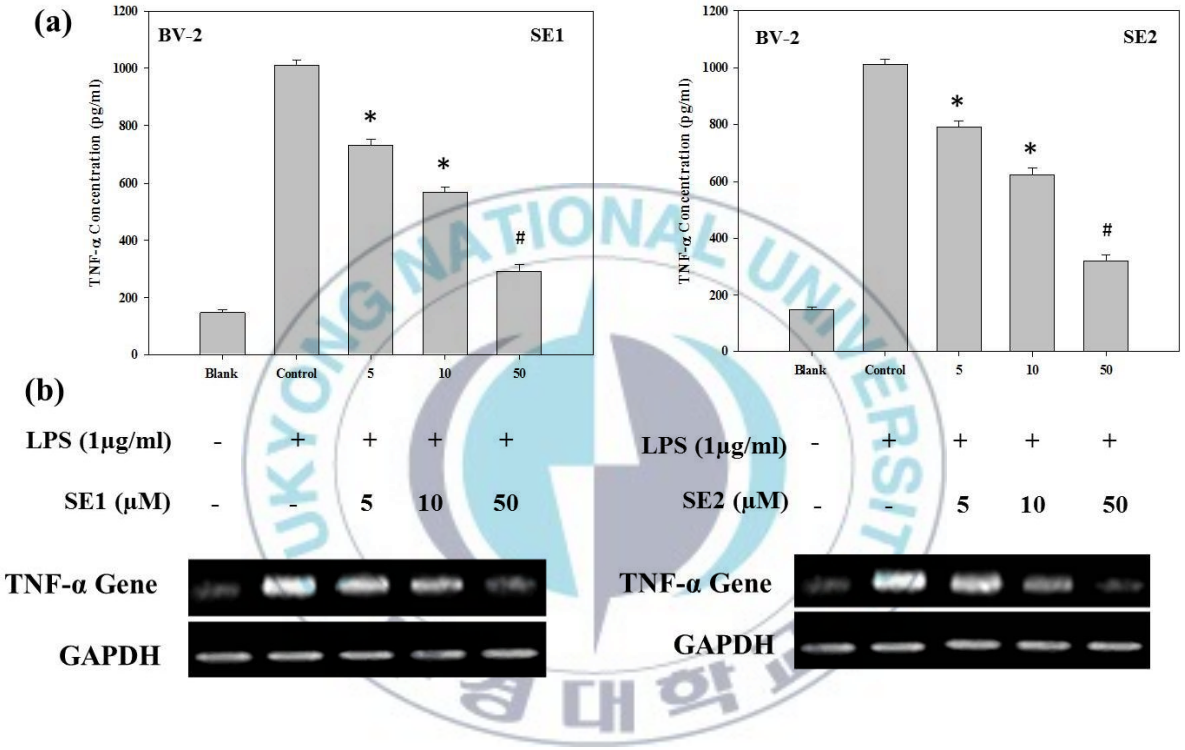


Figure 13: SE1 and SE2 inhibited the production of LPS-induced TNF-α in BV-2 cells. (a) BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure TNF-α concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (bBV-2 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of TNF-α were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

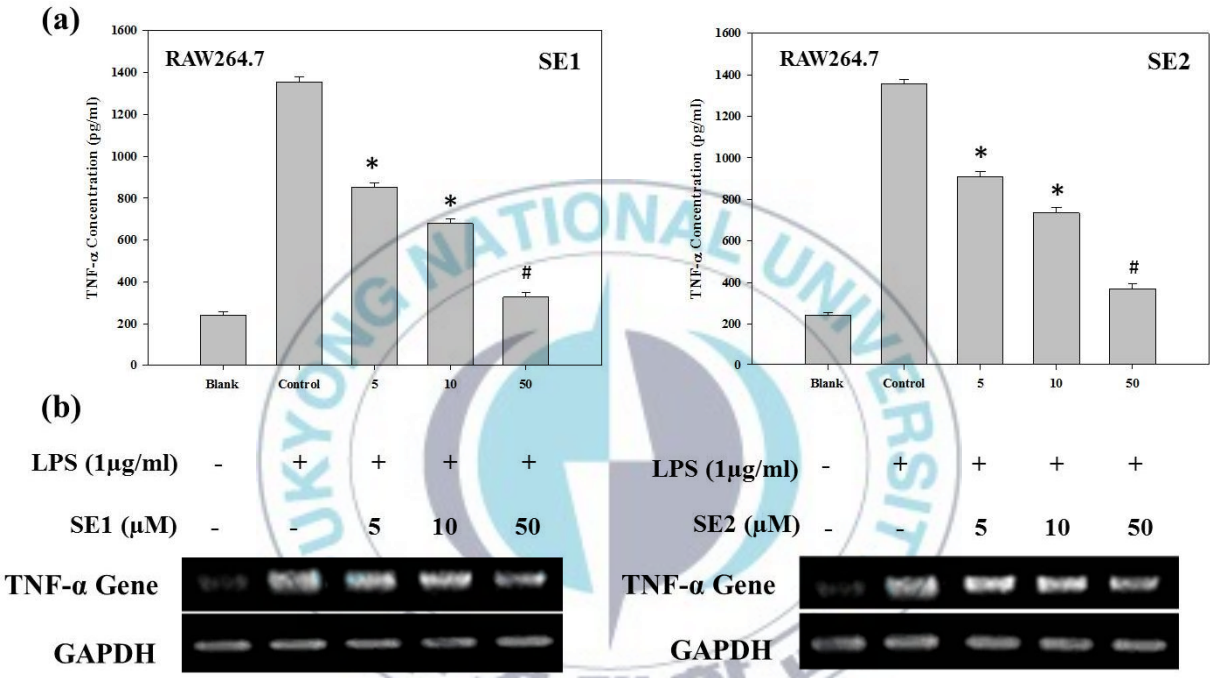


Figure 14: SE1 and SE2 inhibited the production of LPS-induced TNF-α in RAW264.7 cells. (a) RAW264.7 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure TNF-α concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (b)RAW264.7 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of TNF-α were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

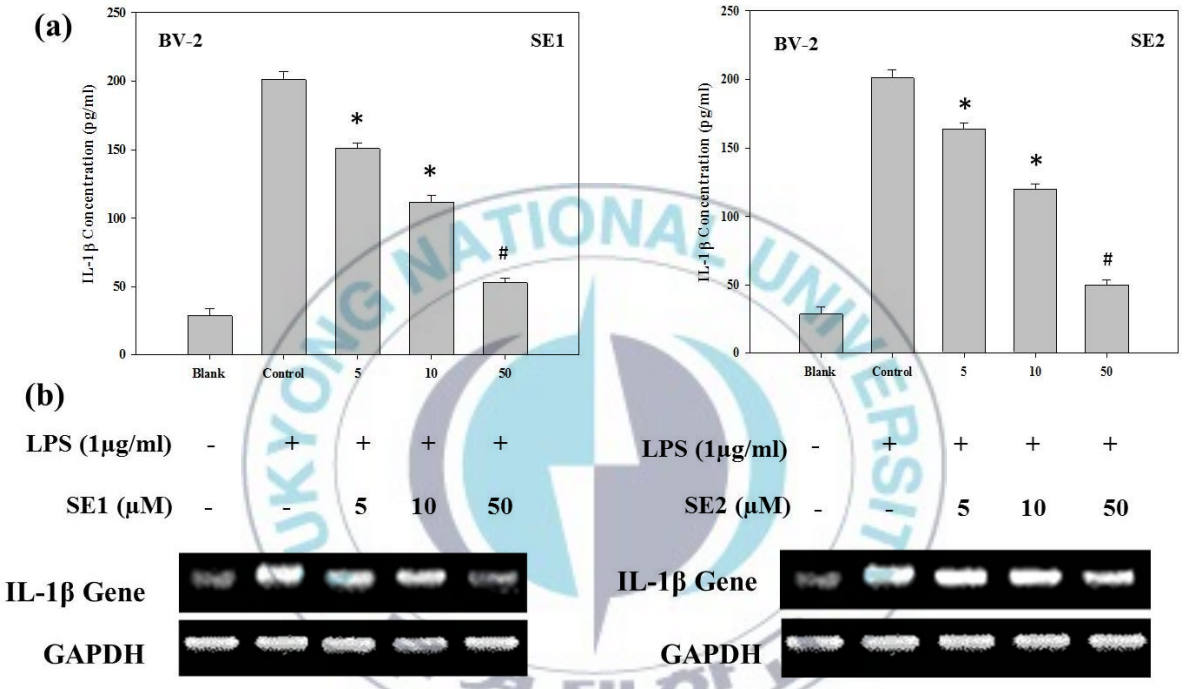


Figure 15: SE1 and SE2 inhibited the production of LPS-induced IL-1β in BV-2 cells. (a) BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure IL-1β concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (bBV-2 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of IL-1β were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

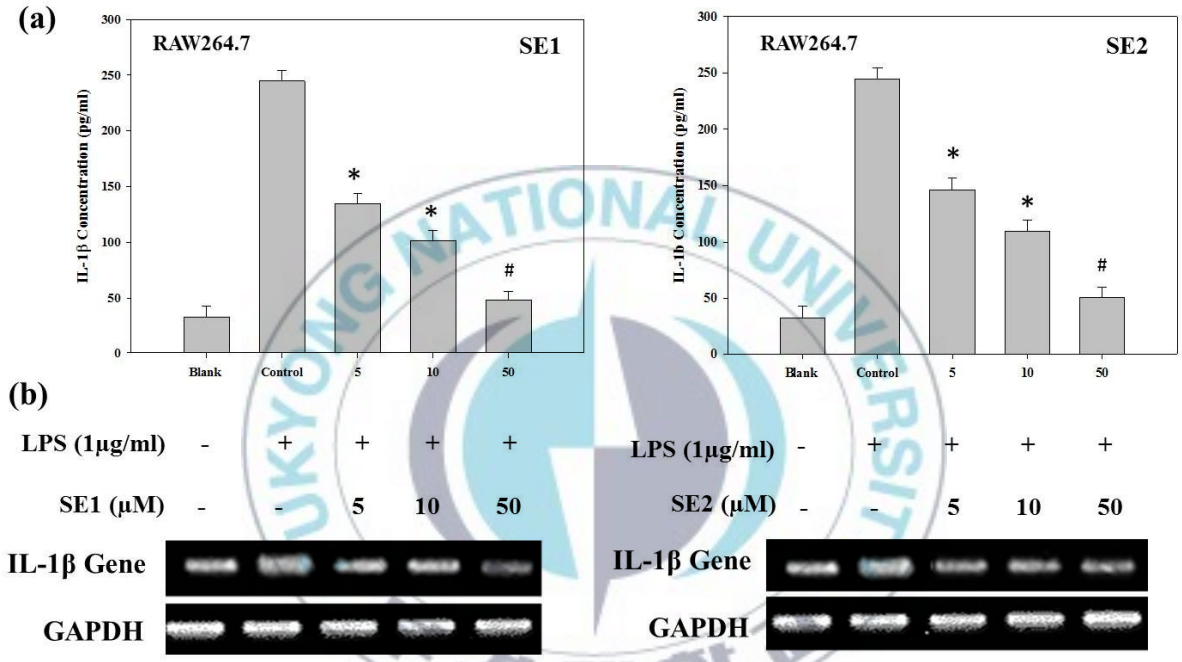


Figure 16: SE1 and SE2 inhibited the production of LPS-induced IL-1β in RAW264.7 cells. (a) RAW264.7 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure IL-1β concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (bRAW264.7 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of IL-1β were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

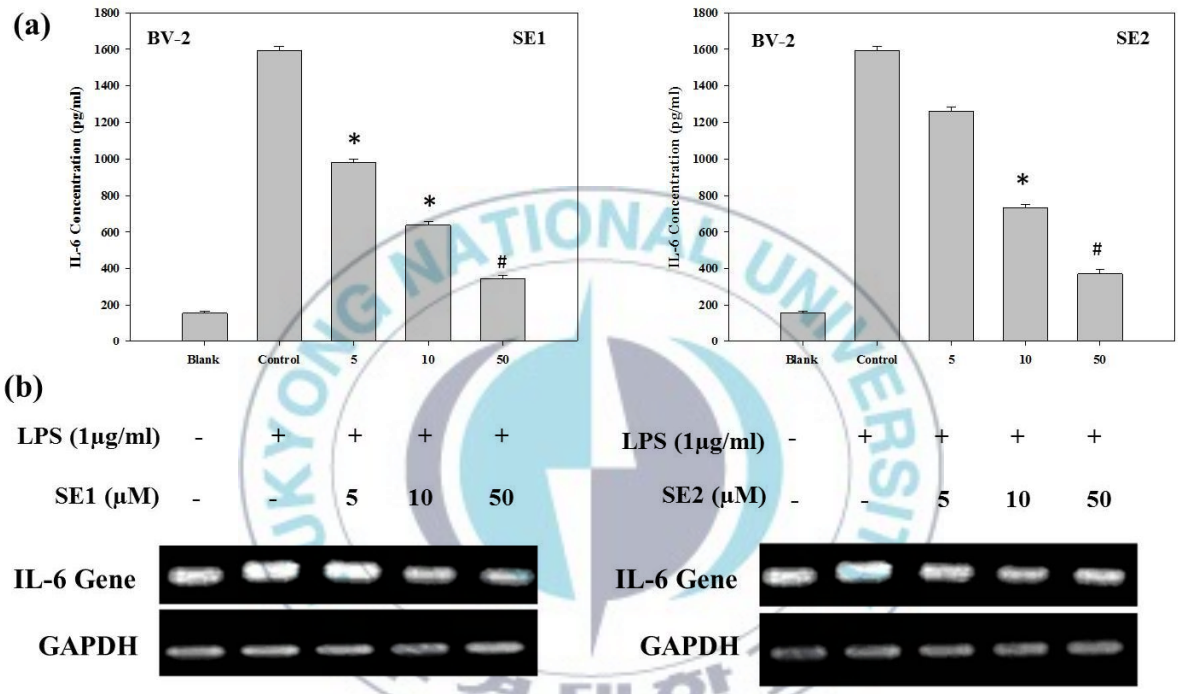


Figure 17: SE1 and SE2 inhibited the production of LPS-induced IL-6 in BV-2 cells. (a) BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure IL-6 concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (bBV-2 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of IL-6were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

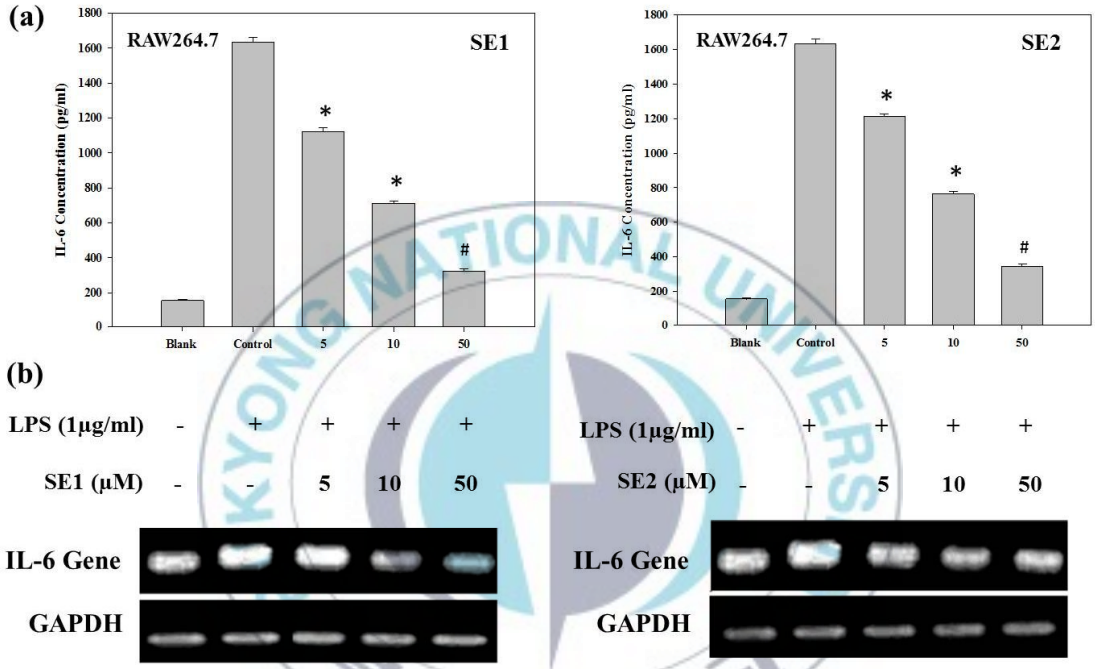
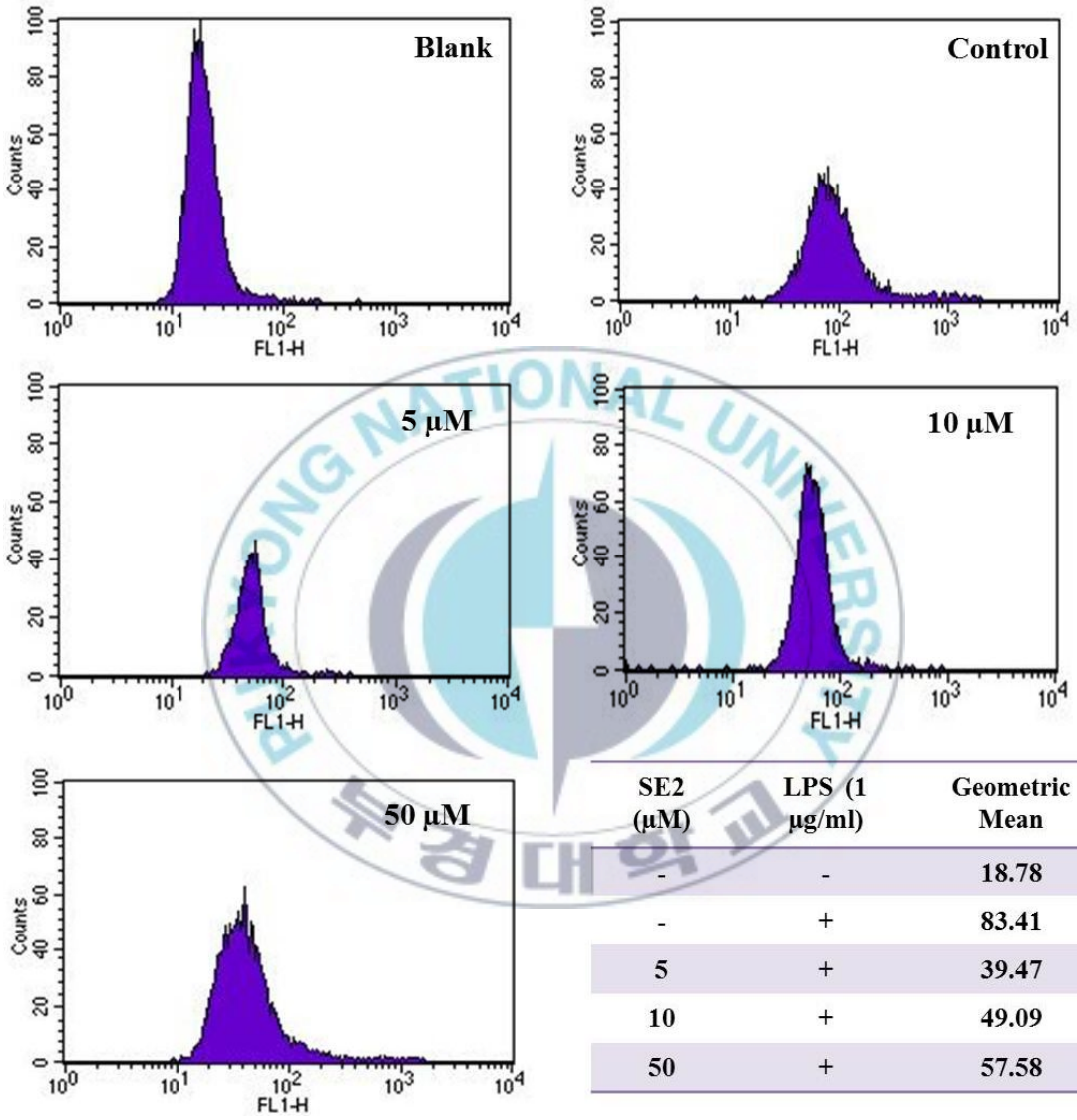


Figure 18: SE1 and SE2 inhibited the production of LPS-induced IL-6 in RAW264.7 cells. (a) RAW264.7 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1 h, and then stimulated with LPS (1 μg/ml). Culture media were collected after 18 h in order to measure IL-6 concentration using ELISA. The data indicate the mean ± S.E.M of three independent experiments. * $p < 0.05$ and #$p < 0.01$ compared to control. (b)RAW264.7 cells were pretreated with SE1 and SE2 for 1 h, following LPS stimulation for 18 h. Total RNAs were isolated, and mRNA levels of IL-6were measured by RT-PCR. GAPDH expression was used as an internal control for RT-PCR.

3.7. SE1 and SE2inhibited the LPS induced ROS production in BV-2 and RAW264.7 cells

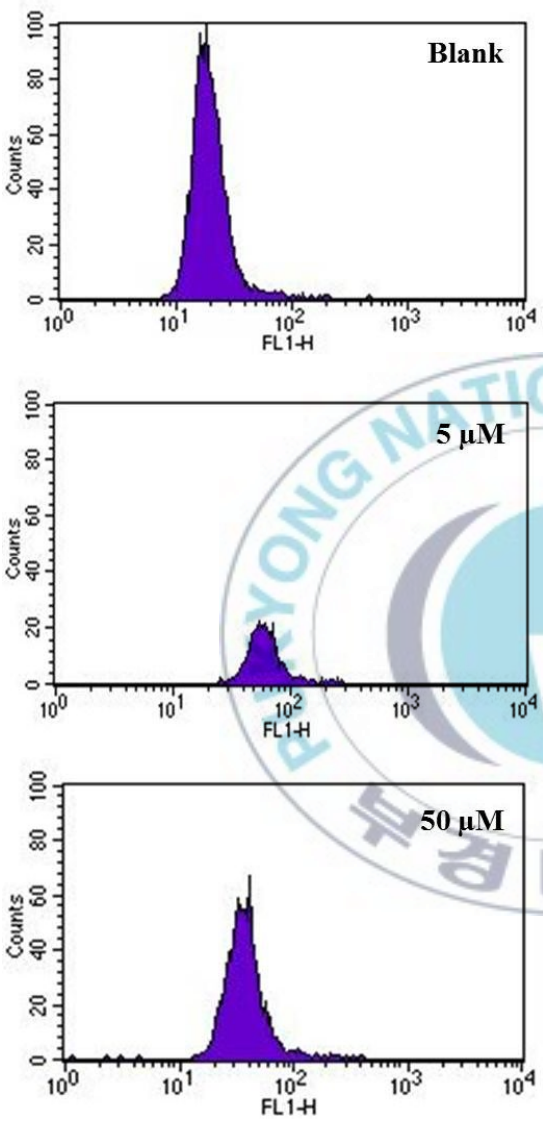
In order to investigate the effects of SE1 and SE2 on LPS-induced ROS production, DCFH-DA was applied in order to detect cell oxygen burst. As illustrated in Figures 19-22, LPS challenged BV-2 cells rapidly increased intracellular ROS level compaired to non stimulated cells. Preincubation with SE1 and SE2 (5, 10 and 50 μM) for 1 h attenuated the ROS generation in a dose-dependent manner in both LPSactivated microglia.

The rapid production of reactive oxygen species is an important inflammatory response in human macrophages (Ko et al., 2007). During inflammatory process ROS involved in signal transduction and gne activation. Excessive generation of ROS stimulate the production of pro-inflammatory cytokines and chemokines via activation of NF-κB (Conner and Grisham, 1996). After stimulation of microglial and macrophage cells with LPS, production of reactive oxygen species is accompanied by the activation of NADPH oxidase that results in neuronal damage (Dringen, 2005; Li et al., 2005). It has been reported that LPS rapidly induces the generation of ROS especially H_2O_2 in macrophages (Hsu and Wen, 2002; Lu and Wahl, 2005).



SE2 (µM)	LPS (1 µg/ml)	Geometric Mean
-	-	18.78
-	+	83.41
5	+	39.47
10	+	49.09
50	+	57.58

Figure 19: Intracellular ROS scavanging abilitiy of SE1 in BV-2 cell line. Cells were labeled with oxidation sensitive dye, DCFH-DA (1 h) and treated with different concentrations of SE1 (5, 10 and 50 µM) for another 1h following the stimulation with LPS (1µg/ml). the levels of intracellular ROS were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average H2DCF-DA fluorecence intensities.



SE1 (µM)	LPS (1 µg/ml)	Geometric Mean
-	-	18.78
-	+	83.41
5	+	38.16
10	+	39.47
50	+	51.85

Figure 20: Intracellular ROS scavanging abilitiy of SE2 in BV-2 cell line. Cells were labeled with oxidation sensitive dye, DCFH-DA (1 h) and treated with different concentrations of SE2 (5, 10 and 50 µM) for another 1h following the stimulation with LPS (1µg/ml). the levels of intracellular ROS were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average H2DCF-DA fluorecence intensities.

3.8. SE1 and SE2 supressed the MAPK molecules in LPS challanged BV-2 and RAW264.7 cells

The p38, JNK and extracellular signal-regulated kinase (ERK) are among the most important signaling pathways that are thought to control the NF-κB activation and synthesis of proinflammatory mediators in activated macrophages (Jung et al., 2008;Jung et al., 2009a; Jung et al., 2009b) and microglia (Ock et al., 2009; Panet al., 2009). Aberrant expression of MAPKs or activation of NF-κB induced microglia is directly correlated with pathogenic events in CNS (Panet al., 2009).

To find out more information about the pathways that may probably involve in above explained inhibitions of iNOS, COX-2 and pro-inflammatory cytokines by SE1 and SE2, phosphorylation of MAPK molecules; p38MAPK, JNK and ERK, were analyzed by Western blotting. LPS induce a rapid phosphorylation of ERK, JNK and p38 MAPK, and it has been reported that this induction of prhosphorylation is done within 10-30 min of LPS treatment (Jung et al., 2009a; Yun et al., 2008). Therefore, cells for western blots were collected after 1 h of SE1 nad SE2 treatment followed by 15 min of LPS (1μg/ml) stimulation. Phosphorylation of ERK, p38 and JNK were inhibited by SCEA-F treatment. This inhibitory action was dose dependent on JNK and p38 phosporylation [Figures 25-28 (a)]where the inhibition of ERK phosphorelation was not prominently seen in both cell lines. Therefore, these results indicated that inhibition of pro-inflammatory mediators and cytokines with their respective enzymes were mediated via inhibition of signal transduction through MAP kinases mediated pathways; JNK and p38 in LPS stimulated RAW264.7 cells. These results coinside with the previous findings by Ock et al, 2009 and Pan et al, 2009.

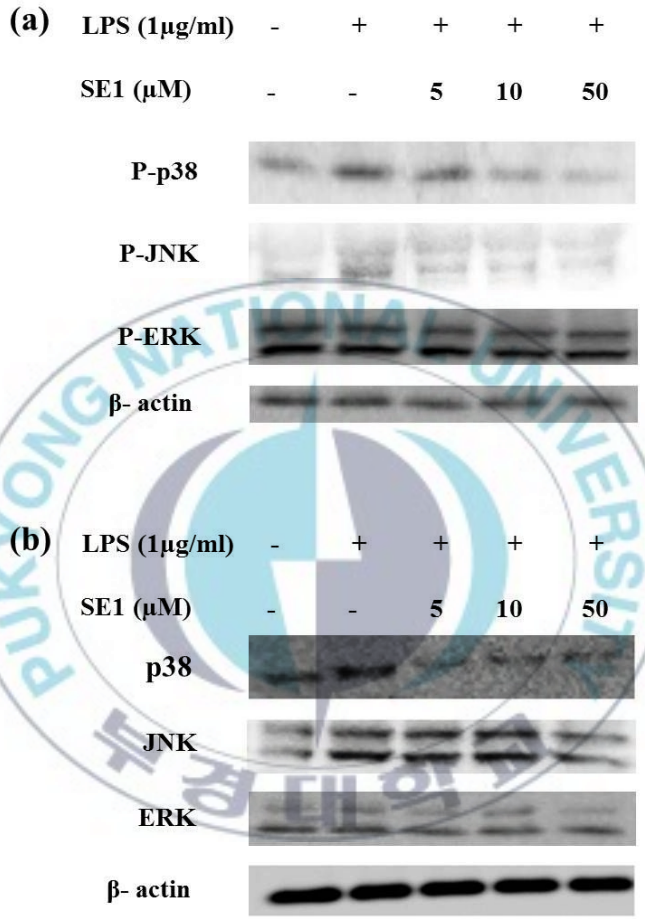


Figure 21: SE1 suppressed the LPS induced MAP kinase activities in BV-2 cells. To analyze total and phosphorylated forms of three MAPK molecules, ERK, JNK and p38MAPK protein expression was assessed by Western blotting. (a) to analyze phosphorelation of MAPK molecules cellular extracts were prepared from BV-2 cells pretreated with SE1 (5, 10 and 50 μg/ml) for 1 h, and then stimulated with LPS (1 μg/ml) for 30 min. (b) Total forms of MAPK molecules were analysed from cell extracts which were LPS stimulated for 18 h after 1 h treatment of SE1.

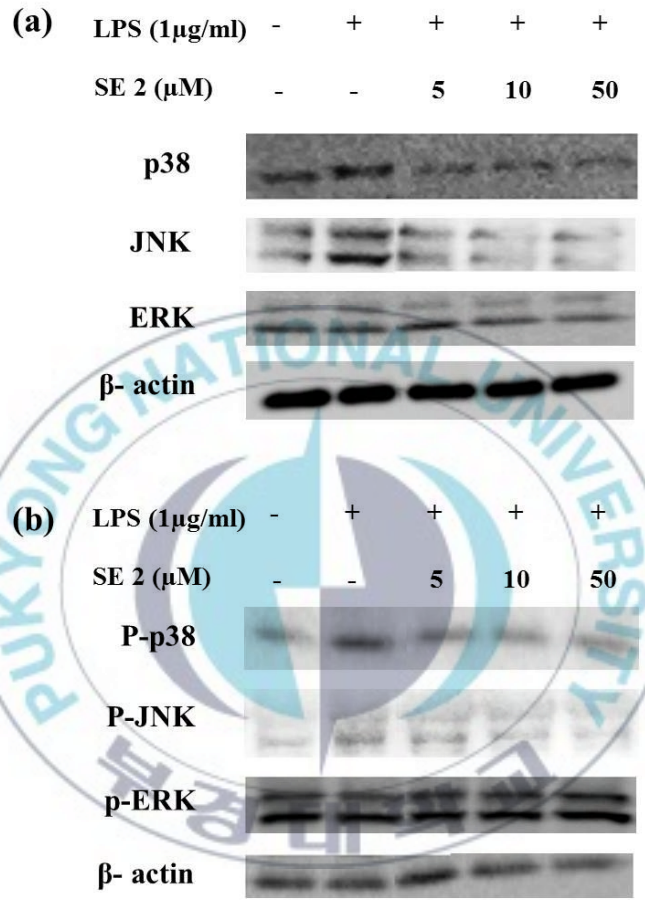


Figure 22: SE1 and SE2 suppressed the LPS induced MAP kinase activities in BV-2 cells. To analyze total and phosphorylated forms of three MAPK molecules, ERK, JNK and p38MAPK protein expression was assessed by Western blotting. (a) to analyze phosphorelation of MAPK molecules cellular extracts were prepared from BV-2 cells pretreated with SE1 and SE2 (5, 10 and 50 µg/ml) for 1 h, and then stimulated with LPS (1 µg/ml) for 30 min. (b) Total forms of MAPK molecules were analysed from cell extracts which were LPS stimulated for 18 h after 1 h treatment of SE2.

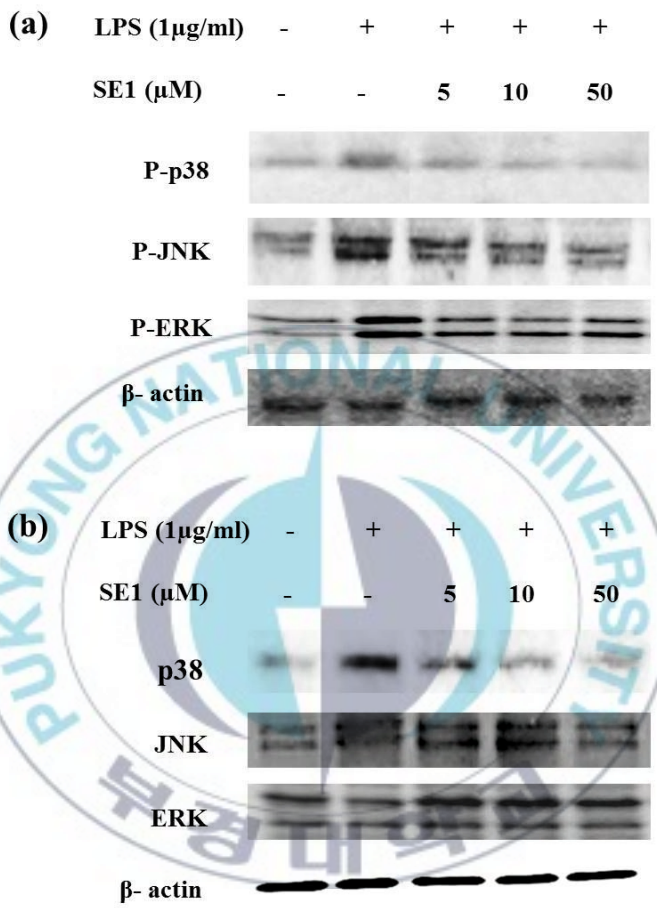


Figure 23: SE1 suppressed the LPS induced MAP kinase activities in RAW264.7 cells. To analyze total and phosphorylated forms of three MAPK molecules, ERK, JNK and p38MAPK protein expression was assessed by Western blotting. (a) to analyze phosphorelation of MAPK molecules cellular extracts were prepared fromRAW264.7 cells pretreated with SE1 (5, 10 and 50 µg/ml) for 1 h, and then stimulated with LPS (1 µg/ml) for 30 min. (b) Total forms of MAPK molecules were analysed from cell extracts which were LPS stimulated for 18 h after 1 h treatment of SE1.

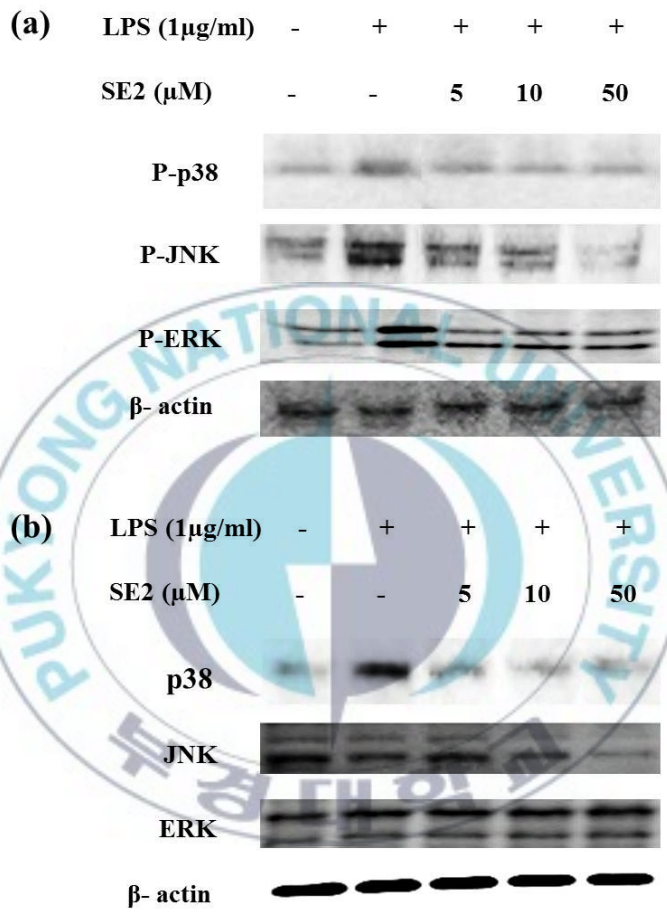


Figure 24: SE2 suppressed the LPS induced MAP kinase activities in RAW264.7 cells. To analyze total and phosphorylated forms of three MAPK molecules, ERK, JNK and p38MAPK protein expression was assessed by Western blotting. (a) to analyze phosphorelation of MAPK molecules cellular extracts were prepared fromRAW264.7 cells pretreated with SE2 (5, 10 and 50 µg/ml) for 1 h, and then stimulated with LPS (1 µg/ml) for 30 min. (b) Total forms of MAPK molecules were analysed from cell extracts which were LPS stimulated for 18 h after 1 h treatment of SE2.

3.9. SE1 and SE2 blocked the NF-κB activation in LPS challanged BV-2 and RAW264.7 cells

NF-κB has a seminal role in inflammation related pathogenesis, because it activates pro-inflammatory genes encoding iNOS, COX-2, TNF-α , IL-1β , and IL-6 (Makarov, 2000). It is activated by phosphorylation, ubiquitination, and subsequent proteolytic degradation of the IκB protein by activated IκB kinase (IKK) (Lee et al., 2000). The liberated NF-κB translocates to the nucleus and binds as a transcription factor to κB motifs in the promoters of target genes, leading to their transcription. Investigations of the effects of SE1 and SE2 on NF-κB pathway in BV-2 and RAW264.7 cell lines shows that the both compounds exert their anti-inflammatory actions through supreeion of the activation of members of NF-κB pathway, IKKα/β, IκB, NF-κB p65 and NF-κB p50. For clear understanding of the effects of SE1 and SE2 on NF-κB pathway, cytosolic and nuclear fractions were separately extracted from LPS challanged cells. Nuclear localization of the NF-κB molecule (consisiting of NF-κBp65 and NF-κBp50 subunits) is greatly responsible for the trasnscription of inflammatory genes. In figures 23 and 24 it was clearly shown that LPS treatment induces the nuclear translocation of NF-κBp65 and NF-κBp50 subunits when compared to non LPS treated blank group in both cell lines. And this translocation of NF-κBp65 and NF-κBp50 subunits were dose dependantly inhibited by the treatment of SE1 and SE2 in both cell types. The nuclear translocation and the DNA binding of the NF-κB transcription factor is preceded by the degradation of IκBα which prevents the nuclear localization of NF-κB. Therfore we checked the protein levels of IκB to clarify the nuclear localization of NF-κB. As shown in Figures 23 and 24 the IκBα protein levels in the cytoplasm is decreased by the LPS treatment indicating the IκBα degradation leading to NF-κB nuclear translocation.This decreased levels of IκBα in the cytoplasm is markedly increased by the treatment of SE1 and SE2 in LPS challanged BV-2 and RAW264.7 cell lines. And this IκBα degradation may probably decreased through SE1 and SE2 mediated inhibition of IKKα/β, which induces the degradation of IκBα. These results suggested SE1 and SE2 in LPS induced BV-2 and RAW264.7 cell lines supress the signalling cascade of NF-κB pathway and nuclear translocation of NF-κB and thereby inhibit the DNA expression of pro-inflammatory genes.

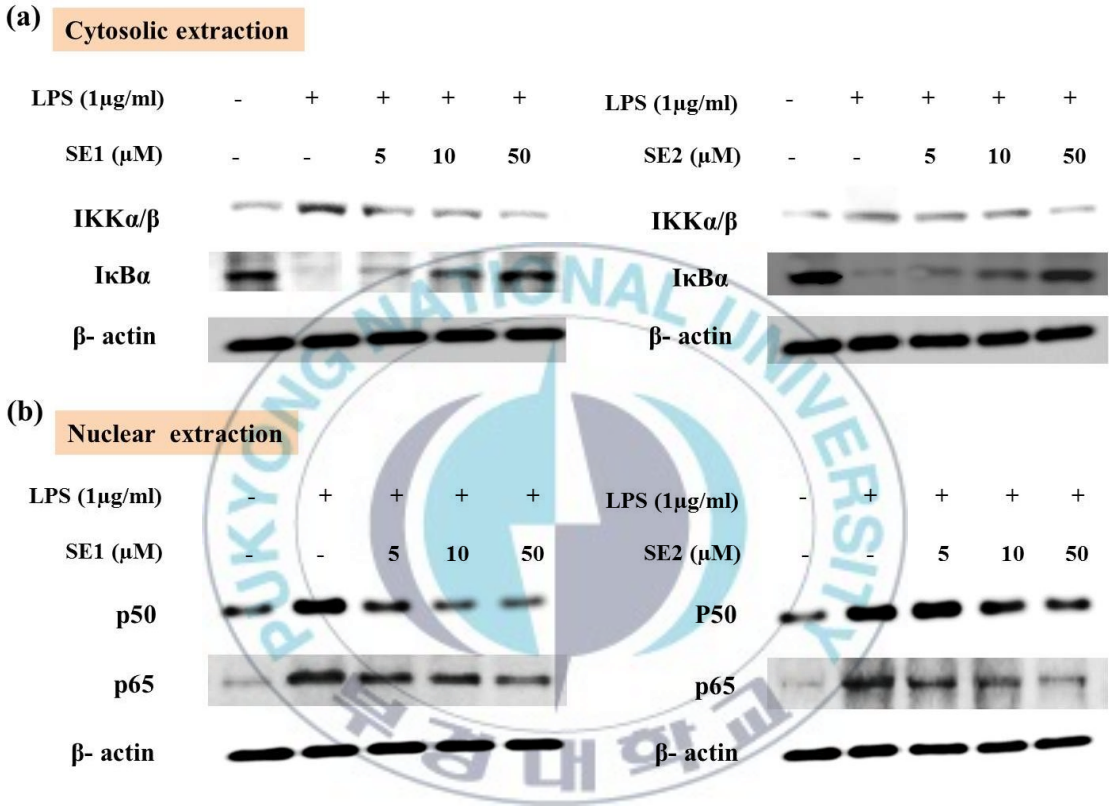


Figure 25: SE1 and SE2 supressed LPS induced IKKα/β expression, IκBα degradation and NF-κB activation in BV-2 cells. BV-2 cells were pretreated with SE1 and SE2 (5, 10 and 50 μM) for 1h followed by stimulation with LPS (1μg/ml). The cells were harvested in 1h to prepare cytosolic and and nuclear extracts for the detection of IKKα/β, IκBα, NF-κB p65 and NF-κB p50 by Western blotting.

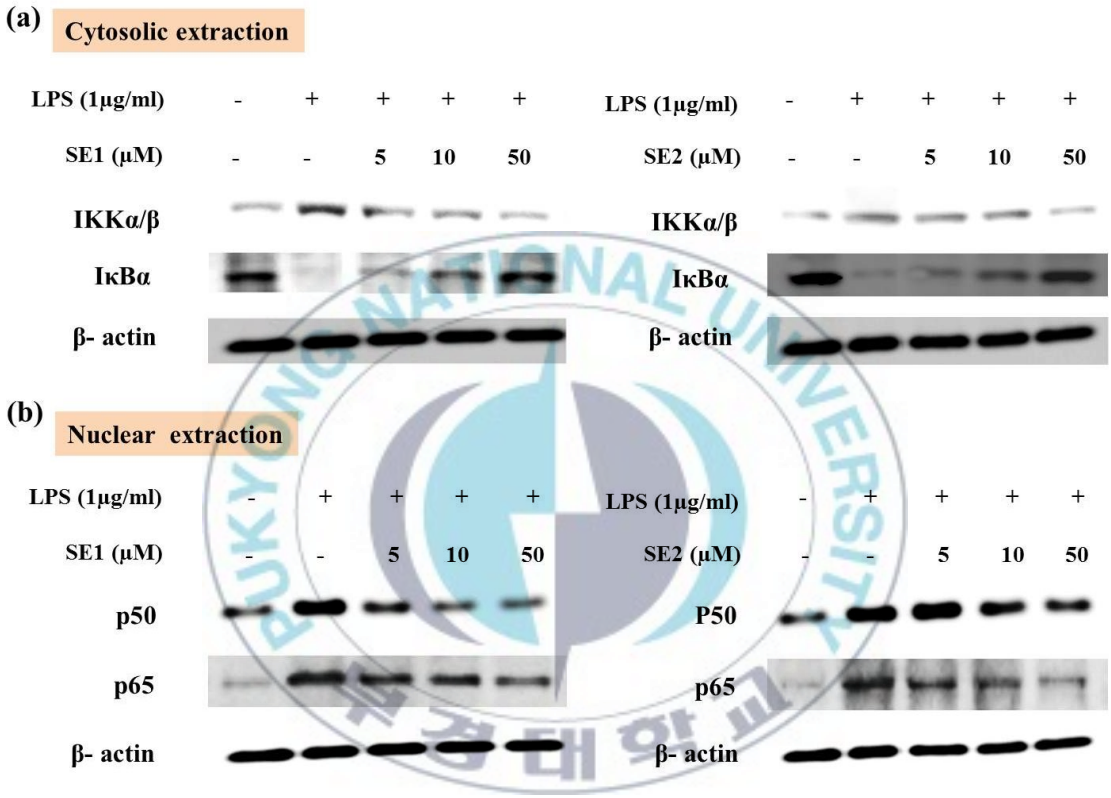


Figure 26: SE1 and SE2 supressed LPS induced IKKα/β expression, IκBα degradation and NF-κB activation in RAW264.7 cells. RAW264.7 cells were pretreated with SE1 and SE2 (5, 10 and 50 µM) for 1h followed by stimulation with LPS (1µg/ml). The cells were harvested in 1h to prepare cytosolic and and nuclear extracts for the detection of IKKα/β, IκBα, NF-κB p65 and NF-κB p50 by Western blotting.

4. Summary

This study was conducted to find out the neuroinflammatory effects of natural compounds SE1 and SE2 isolated from sea horse *Hippocampus Kuda*Bleeler on the BV-2 microglial cell line and RAW264.7macropahge cell line as microglia and macrophages are the major cell types responsible for neuroinflammatory responses.

Nontoxic concentrations of SE1 and SE2 were selected through cytotoxicity assessment for further cellular studies. Moreover, it was found that the two compounds are potent inhibitors of production of major pro-inflammatory mediators NO, PEG_2, pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and ROS. In addition, enzyme and gene expression levels of iNOS and COX-2, which are responsible for production of NO and PEG_2 respectively; also suppressed by the treatment of SE1 and SE2 in both cell lines. Gene expression levels of cytokines are also inhibited. Next efforts have taken to understand the underlying molecular signaling pathways responsible for these inhibitions. For that, the effects of SE1 and SE2 on major inflammatory pathways NF-κB and MAPK were studied. SE1 and SE2 showed inhibitory effects on IKKα/β and up regulated the expressions of IκBα following the blockage of nuclear translocation of NF-κB (p50 and p65) which is the major transcriptional factor responsible for expressing inflammation related genes. In MAPK pathways, only JNK and p38MAPK phosphorylation were suppressed by the treatment of SE1 and SE2 in both cell lines. In careful assessment of the data obtained showed a slightly higher activity of SE1 over SE2, which was not significant.

On the basis of collective finding of the study it could be concluded that SE1 and SE2 attenuated the LPS-induced gene and protein expression of iNOS, COX-2 and pro-inflammatory cytokines via blocking NF-κB, JNK and p38 MAPK pathways in BV-2 and RAW264.7 cell lines. Thus it could be suggested that SE1and SE2 possess potential in neuro-inflammation therapy.

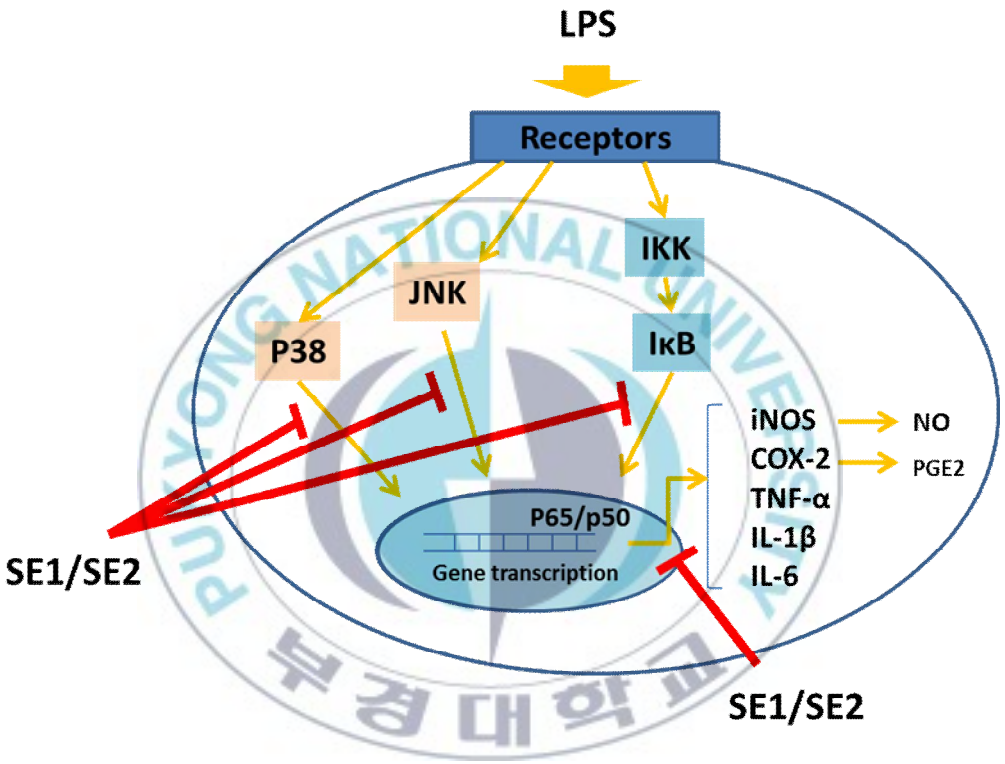


Figure 27: Scematic diagram of the inhibitory actions of SE1 and SE2 on LPS induced BV-2 and RAW264.7 cells.

References

Adachi, M., Fukuda, M. nad Nishida, E. 2000. Nuclear export of MAP kinase (ERK) involves a MAP kinase (MEK) dependant active transport mechanism. Journal of cell biology, 148:849-856.

Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W. and Rossi, F.M. 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nature reviews neuroscience, 10:1538–1543.

Bandeira-Melo, C., Serra, M.F., Diaz, B.L., Cordeiro, R.S.B., Silva, P.M.R., Lenzi, H.L., Bakhle, Y. S., Serhan, C.N. and Martins, M.A. 2000. Cyclooxygenase-2-derived prostaglandin E2 and lipoxin A4 accelerate resolution of allergic oedema in *Angiostrongyluscostaricensis*-infected rats: relationship with concurrent eosinophilia. Journal of immunology,164:1029–1036.

Block, M.L., Zecca, L. and Hong, J.S. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature reviews; neuroscience, 8:57-69.

Castrillo, A., de Las, H. B., Hortelano, S., Rodriguez, B., Villar, A., and Bosca, L. (2001) Inhibition of the nuclear factor kappa B (NFκ B) pathway by tetracyclic kaurenediterpenes in macrophages. Specific effects on NF-κB-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK.The journal of biological chemistry, 276:15854 15860

Chen, C.Y., Peng, W.H., Tsai, K.D., and Hsu, S.L. 2007. Luteolin suppresses inflammation-associated gene expression by blocking NF-κB and AP-1 activation pathway in mouse alveolar macrophages. Life sciences, 81:1602–1614.

Cho, B.P., Song, D.Y., Sugama, S., Shin, D.H., Shimizu, Y., Kim, S.S., Kim, Y.S. and Joh, T.H. 2001. Pathological dynamics of activated microglia following medial forebrain bundle transection. Glia,53:92–102.

Coker, R.K., Laurent, G. J. 1998. Pulmonary fibrosis: Cytokine in the balance. European respiratory journal, 11:1218-1221.

Conner, E.M. and Grisham, M.B. 1996. Inflammation, free radicals and antioxidants. Nutrition, 12:274-277.

Cuadros, M.A. and Navascues, J. 1998. The origin and differentiation of microglial cells during development.Progress in neurobiology, 56:173–189.

Cunningham, C., Wilcokson, D.C., Campion, S., Lunnon, K. and Perry, V.H. 2005. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegenaration. Journal of neuroscience, 25:9275-9284.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L. and Gan, W.B. 2005. ATP mediates rapid microglial response to local brain injury *in vivo*. Nature Neuroscience,8:752–758.

Dringen, R., 2005. Oxidative and antioxidative potential of brain microglial cells.Antioxidants and Redox Signalling, 7:1223–1233.

Ekdahl, C.T., Kokaia. Z. and Lindvall, O. 2009. Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience, 158:1021–1029.

Faure, E., Equils, O., Sieling, P. A., Thomas, L., Zhang, F. X., Kirschning, C. J., Polentarutti, N., Muzio, M. and Arditi, M. 2000. Bacterial lipopolysaccharide activates NF-kappaB through toll like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. The journal of biological chemistry, 275:11058–11063.

Fujiwara, N. and Kobayashi, K. 2005. Macrophages in Inflammation.Current drug Targets - inflammation & allergy,4:281-286.

Gibbons, H. M. and Dragunow, M. 2006. Microglia induce neural cell death via a proximity-dependent mechanism involving nitric oxide. Brain research, 1084:1–15.

Gilmore, T.D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 25, 6680–6684.

Gilroy, D.W., Colville-Nash, P.R., Willis, D., Chivers, J., Paul-Clark, M.J. and Willoughby, D.A. 1999. Inducible cyclooxygenase may have antiinflammatory properties. Nature medicine, 5:698–701.

Gouwy, M. Struyf, S., Proost, P. and Damme, V.J. 2005. Synergy in cytokine and chemokine networks amplifies the inflammatory response. Cytokine and growth factor reviews, 16:561–80.

Hanada, T., Yoshimura, A. 2002.Survey, Regulation of cytokine signaling and inflammation.Cytokine growth factor reviews, 13:413–421.

Hanisch, U.K. and Kettenmann, H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature reviews neuroscience, 10:1387–1394.

Hansen, M. B., Nielson, S. E., Berg, K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of immunological methods, 119:203-210.

Hayden, M.S., West and A.P., Ghosh, S. 2006. NF-kB and the immune response.Oncogene 25, 6758–6780.

Himaya, S.W.A., Ryu, B., Qian, Z.J. and Kim, S.K. 2010. Sea cucumber, Stichopusjaponicus ethyl acetate fraction modulates the lipopolysaccharide induced iNOS and COX-2 via MAPK signaling pathway in murine macrophages. Environmental toxicology and pharmacology, doi:10.1016/j.etap.2010.03.019

Hsu, H.Y. and Wen, M.H., 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. The journal of biological chemistry, 277:22131–22139.

Ianaro, A., Ialenti, A., Maffia, P., Pisano, B. and Di Rosa, M. 2001. Role of cyclopentenone prostaglandins in rat carrageenin pleurisy.FEBS letters, 508:61–66.

Jung, H. W., Seo, U. K., Kim, J. H., Leem, K. H. and Park, Y. K. 2009a. Flower extract of *Panaxnotoginseng*attenuates lipopolysaccharide-induced inflammatory response via

blocking of NF-κBsignaling pathway in murine macrophages. Journal of ethnopharmacology, 122:313–319.

Jung, H. W., Yoon, C. H., Park, K. M. Han, H. S. and Park, Y. K. 2009b. Hexane fraction of ZingiberisRhizomacrudus extract inhibits the production of nitric oxide and proinflammatory cytokines in LPS-stimulated BV2 microgilial cells via the NF-kappaB pathway. Food and chemical toxicology, 47:1190-1197.

Jung, W. K., Choi, I., Lee, D. Y., Yea, S. S., Choi, Y. H., Kim, M. M., Park, S. G., Seo, S. K., Lee, S. W., Lee, C. M., Park, Y. M. and Choi, I. W. 2008. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-κB pathways. The international journal of biochemistry and cell biology,40:2572-2582.

Karin, M. and Greten, F.R. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nature reviews immunology, 5:749–759.

Kasahara, T.; Matsushima, K. 2001.Macrophage signaling, apoptosis, lectins and leukocyte trafficking. Trends in immunology, 22:593-594.

Kawai.T. and Akira. S. 2007. Signaling to NF-kB by Toll-like receptors.Trends in molecular medicine, 13:460-469.

Keifer, J. A., Guttridge, D. C., Ashburner, B. P., and Baldwin, A. S. Jr. 2001.Inhibition of NFκ B activity by thalidomide through suppression of I κ B kinase activity.The journal of biological chemistry,276:22382 22387.

Kim, S.S., Oh, O.J. and Min, H.Y. 2003.Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide stimulated mouse macrophage RAW264.7 cells. Life sciences, 73:337-348.

Ko, H.C., Wang,Y.H., Liou, K.T., Chen, C.M., Chen, C.H., Wang, W.Y., Chang, S., Hou, Y.C., Chen, K.T., Chen, C.F. and Shen, Y.C. 2007. Anti-inflammatory effects and mechanisms

of the ethanol extract of Evodiarutaecarpa and its bioactive components on neutrophils and microglial cells. European journal of pharmacology, 555:211 –217.

Kreutzberg, G.W. 1996. Microglia: a sensor for pathological events in the CNS. Trends in neuroscience,19:312-318.

Krieger, M. 2007. The other side of scavenger receptors: pattern recognition for host defense. Current openion in lipidology, 8:275–280.

Kulinsky, V. I. 2007.Biochemical Aspects of Inflammation.Biochemistry (Moscow), 72:595-607.

Lawrence, T., Gilroy, D.W., Colville-Nash, P.R., Willoughby, D.A. 2001.Possible new role for NF-kappaB in the resolution of inflammation. Nature medicine, 7:1291–1297.

Lawrence, T., Willoughby, D. A., Gilroy, D. W. 2002.Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature reviews immunology, 2:787-795.

Lee, S.J., Bai, S.K., Lee, K.S., Namkoong, S., Na, H.J., Ha, K.S., Han, J.A., Yim, S.V., Chang,K., Kwon, Y.G., Lee, S.K. and Kim, Y.M. 2003.Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing IκB kinase-dependent NF-κB activation. Molecules and cells, 16:97-105.

Li, J., Baud, O., Vartanian, T., Volpe, J.J., Rosenberg, P.A., 2005.Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. Proceedings of national academic science U.S.A. 102:9936–9941.

Li, Y., Qian, Z.J. and Kim, S.K. 2008.Cathepsin B inhibitory activities of three new phthalate derivatives isolated from seahorse, Hippocampus KudaBleeler. Bioorganic & Medicinal Chemistry Letters, 18:6130–6134

Li, Y.H., Yan, Z.Q., Jensen, J.S., Tullus, K. and Branner, A., 2000. Activation of nuclear factor-κB and induction of inducible nitric oxide synthase by Ureaplasmaurealyticum in macrophages. Infection and immunity, 68:7087–7093.

Libby P. 2002. Inflammation in atherosclerosis. Nature, 420:868–874.

Libby, M. D. P. 2007. Inflammatory Mechanisms: The molecular basis of inflammation and disease.Nutrition Reviews, 65, S140–S146.

Ling, Z., Gayle, D.A., Ma, S.Y., Lipton, J.W. Tong, C.W., Hong, J.S. and Carvey, P.M. 2002. *In utero* bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain. Movement disorders, 17:116–124.

Lu, Y. and Wahl, L.M., 2005. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. Journal of Immunology, 175:5423–5429.

Makarov, S. S. 2000. NFκB as a therapeutic target in chronic inflammation: recent advances. Molecular medicine today,6:441 448.

Marin-Teva, J. L., Dusart. I., Colin, C., Gervais, A., Rooijen, N. and Mallat, M. 2004. Microglia promote the death of developing purkinje cells. Neuron,41:535–547.

Minghetti, L and Levi, G. 1998. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Progress in neurobiology, 54:99–125.

Morgan, S. C., Taylor, D. L. &Pocock, J. M. 2004. Microglia release activators of neuronal proliferation mediated by activation of mitogen-activated protein kinase, phosphatidylinositol-3-kinase/Akt and delta-Notch signalling cascades. Journal of neurochemistry,90:89–101.

Moss, D. W. & Bates, T. E. 2001. Activation of murine microglial cell lines by lipopolysaccharide and interferon-γ causes NO-mediated decreases in mitochondrial and cellular function. European journal of neuroscience,13:529–538.

Nakajima, K. and Kohsaka, S. 2004. Microglia: Neuroprotective and neurotrophic cells in the central nervous system. Current drug targets - cardiovascular and haematologicaldisorders, 4:65-84

Nimmerjahn, A., Kirchhoff, F. &Helmchen, F. 2005. Resting microglial cells are highly surveillants of brain parenchyma *in vivo*. Science308, 1314–1318.

Ock, J., Kim, S. and Suk, K. 2009. Anti-inflammatory effects of a fluorovinyloxyacetamide compound KT-15087 in microglia cells. Pharmacological research,59:414-422.

Pan, X.D., Chen, X.C., Zhu, Y.G., Chen, L.M., Zhang, J., Huang, T.W., Ye, Q.Y. and Huang.H.P. 2009.Tripchlorolide Protects Neuronal Cells from Microglia-Mediated b-Amyloid Neurotoxicity Through Inhibiting NF-κB and JNK Signaling. Glia, 57:1227–1238.

Perry, V.H. 1994. Modulation of microglia phenotype.Neuropathology and applied neurobiology,20:167-177.

Perry,V., Cunningham, C.and Holmes, C. 2007. Systemic infections and inflammation affect chronic neurodegenaration. Nature reviews immunology,7:161-167.

Polazzi, E. and Contestabile, A. 2002. Reciprocal interactions between microglia and neurons: from survival to neuropathology. Reviews in Neuroscience,13:221–242.

Rajapakse, N., Kim, M. M., Mendis, E., Kim. S. K. 2008. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolyssacharide stimulated raw cells by carboxybutyrylated glucosamine takes place via down regulation of mitogen activated protein kianse mediated nuclear factor-κbsignaling. Immunology, 123:348-357.

Ryu, B. Qian, Z.J. and Kim, S.K. 2010. SHP-1, a novel peptide isolated from seahorse inhibits collagen release through the suppression of collagenases 1 and 3, nitric oxide products regulated by NF-kB/p38 kinase. Peptides, 31:79–87

Scali, C. Giovannini, M. G. Prosperi, C. Bellucci, A. Pepeu, G. and Casamenti, F. 2003. The selective cyclooxygenase-2 inhibitor rofecoxib suppresses brain inflammation and protects cholinergic neurons from excitotoxic degeneration in vivo. Neuroscience, 117:909-19.

Sebban, H., Courtois, G. 2006. NF-κB and inflammation in genetic disease. Biochemical pharmacology, 7:1153-1160.

Srinivasan, B., Johnson, T.E., Lad, R. and Xing, C. 2009. Structure-activity relationship Studies of chalcone leading to 3-Hydroxy-4,3□,4□,5□-tetramethoxychalcone and its analogues

as potent nuclear factor KB inhibitors and their anticancer activities. Journal of medicinal chemistry, 52:7228–7235.

Tak, P.P. and Firestein, G.S. 2001. NF-kB: a key role in inflammatory diseases. The journal of clinical investigation, 107:7–11.

Takeda, K. and Akira, S. 2005. Toll-like receptors in innate immunity. International Immunology, 17:1–14.

Tzeng, S.F., Hsiao, H.Y. and Mak, O.T. 2005. Prostaglandins and Cyclooxygenases in Glial Cells During Brain Inflammation. Current Drug Targets - Inflammation & Allergy,4:335-340.

Wu, M. and Gu, Z. 2007. Screening of Bioactive Compounds from Moutan Cortex and Their Anti-Inflammatory Activities in Rat Synoviocytes.eCAM, 6:57–63.

Xiao, C. and Ghosh, S. 2005. NF-kB, an evolutionarily conserved mediator of immune and inflammatory responses. Advances in experimental medicine and biology,560:41–55.

Yunbiao, L. and Wahl, L.M. 2005. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2 and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide activated human primary monocytes. Journal of immunology, 175:5423-5429.

Zaodung Ling, Z., Zhu, Y., Tong, C.W., Snyder, J.A., Lipton, J.W. and Carvey, P.M. 2006. Progressive dopamine neuron loss following supra-nigral lipopolysaccharide (LPS) infusion into rats exposed to LPS prenatally. Experimenatal neurology, 199:499–512.

Zhang, N., Xu, B., Mou, C., Yang, W., Wei, J., Lu, L., et al. 2003. Molecular profile of the unique species of traditional Chinese medicine, Chinese seahorse (Hippocampus kudaBleeker). FEBS letters, 550:124–34.

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