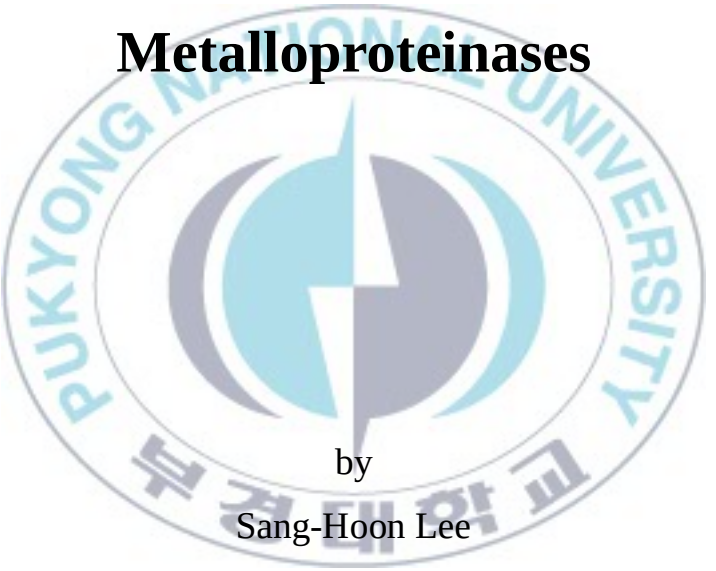
Thesis for the Degree of Doctor of philosophy

# Anti-Inflammatory Mechanisms of Phlorotannins derived from *Eisenia bicyclis* and Their Inhibitory Effects on Matrix Metalloproteinases




by

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February 2010

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# 대황으로부터 분리한 Phlorotannins의 염증 저해기전 및 기질금속단백질분해효소 저해효과

Advisor: Prof. Se-Kwon Kim

by

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February 2010

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A Dissertation

by

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# Anti-Inflammatory Mechanisms of Phlorotannins derived from *Eisenia bicyclis* and Their Inhibitory Effects on Matrix Metalloproteinases

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Abstract

*Eisenia bicyclis* (Kjellman. *E. bicyclis*) Setchell is a perennial brown alga, belonging to the family Laminariaceae. In present study, antioxidant, anti-inflammatory and matrix Metalloproteinases (MMPS) inhibitory effects of *E. bicyclis* were investigated. The antioxidant effects of methanolic extract of *E. bicyclis* and its organic solvent soluble fractions including dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and water ($H_2O$) fractions were measured through their free radical scavenging activities in non-cellular and cellular systems *in vitro*. Several antioxidant assays, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide anion and peroxynitrite radicals scavenging activities using the electron spin resonance spectrometry (ESR) technique and intracellular reactive oxygen species (ROS) by 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) method in mouse macrophages cell line (RAW 264.7 cell) were evaluated. The antioxidant activities of the each fractions were in the order of ethyl acetate (EtOAc) > *n*-butanol (*n*-BuOH) > dichloromethane ($CH_2Cl_2$) > and water ($H_2O$) fraction. Moreover, the order of total poly-phenolics

contents of each fraction showed the same order in accordance with the radical scavenging activities. Five phlorotannins were isolated and characterized from EtOAc fraction of *E. bicyclis*, which showed strongest antioxidant activity among the fractions. According to comprehensive spectral analysis of MS and NMR data, phloroglucinol, Fucofuroeckol-A (FF), Dioxinodehydroeckol (DD), Eckol (EK) and Dieckol (DE) were isolated. DD and FF were isolated from *E. bicyclis* for the first time, and furthermore, no biological reports of FF have been published up to now. In cellular and non-cellular oxidative systems, EK and FF showed significant activities compared to other phlorotannins.

To evaluate the anti-inflammatory effects of phlorotannins derived from *E. bicyclis* on RAW264.7 mouse macrophage, FF and EK, which have no cytotoxicity and previous reports regarding anti-inflammatory activity, were selected for further study. To identify the effects of FF and EK on inflammation, mediated by reactive oxygen species (ROS), The anti-inflammatory effects of these phlorotannins on lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophage was investigated and their anti-inflammatory mechanisms were elucidated. The results showed that FF and EK suppress LPS-induced production of nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) dose-dependently. Enzyme-linked immunosorbent (ELISA) and cytometric bead array assay (CBA) clearly demonstrated that FF and EK significantly reduced the productions of pro-inflammatory cytokines such as, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1. Moreover, these phlorotannins reduced nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) activation. These results strongly suggest that the inhibitory effects of FF and EK on LPS-induced NO and $PGE_2$ production might be due to the suppression of NF-κB and MAPKs signaling pathway. Moreover, above intracellular antioxidant activities of FF and EK supports that their anti-inflammatory effects might be attributable to scavenging ROS in

RAW264.7 mouse macrophage.

Consequently, we evaluated the inhibitory effects of FF and EK on matrix metalloproteinase (MMP) -2 and 9 in HT1080 human fibrosarcoma cell line. In MMPs inhibitory assay, FF and EK showed strong direct inhibition on both MMP-2 and 9 dose-dependently. FF and EK also inhibited protein expression of MMP-2 and 9. Especially, FF stimulates the expression of tissue inhibitor of matrix metalloproteinase (TIMP) -2 and this may cause inhibition on MMPs activities via direct binding. Moreover, FF and EK suppressed the cell migration, and cell invasion in 3D culture model on HT1080 cells.

Therefore, these results suggested that FF and EK have remarkable antioxidant activities and strong potential as valuable natural anti-inflammatory and cancer chemopreventive agents to develop nutraceuticals and pharmaceuticals.

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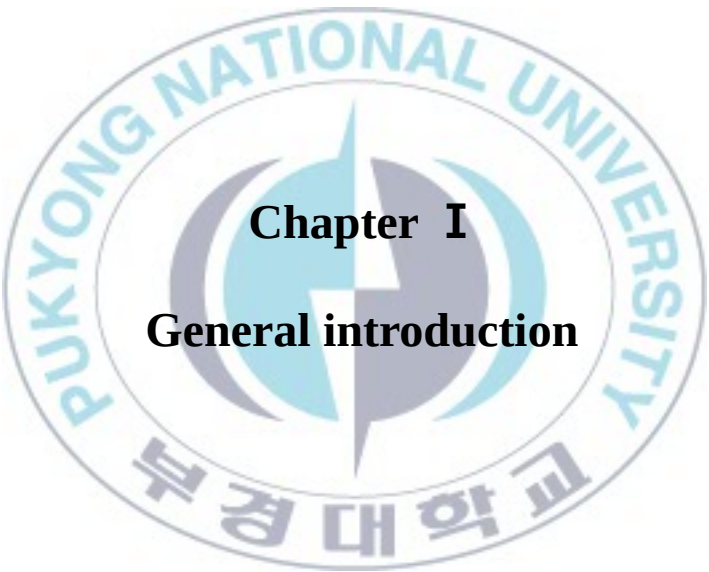
# List of abbreviations

| | |
|---|---|
| AP-1 | activator protein-1 |
| BHT | butylated hydroxytoluene |
| CBA | cytometric bead array |
| COX | cyclooxygenase |
| DCFH-DA | 2', 7'-dichlorofluorescin diacetate |
| DD | dioxinodehydroeckol |
| DE | dieckol |
| DMPO | 5,5-dimethyl-1-pyrroline-*N*-oxide |
| DMSO | dimethylsulfoxide |
| ECM | extracelluar matirixes |
| EK | eckol |
| ELISA | enzyme-linked immunosorbent assay |
| EMSA | electrophoretic Mobility Shift Assay |
| eNOS | endothelial nitric oxide synthase |
| ERK | extracellular signal-regulated kinase |
| ESR | electron spin resonance |
| FACS | fluorescence activated cell sorter |
| FBS | fetal bovine serum |
| FF | fucofuroeckol-A |
| FITC | fluorescein isothiocyanate |
| IL-1 | interleukin -1 |
| IL-6 | interleukin -6 |
| iNOS | inducible nitric oxide synthase |

| | |
|---|---|
| JNK | c-Jun N-terminal kinase |
| LPS | lipopolysaccharides |
| MAPK | mitogen-activated protein kinases |
| MCP-1 | chemokines monocyte chemotactic protein-1 |
| MMP | matrix metalloproteinase |
| MPO | Myeloperoxidase |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide |
| NF-κB | nuclear transcription factor kappa-B |
| NO | nitric oxide |
| $PGE_2$ | prostaglandin $E_2$ |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcription-polymerase chain reaction |
| SOD | superoxide disumutase |
| TIMP | tissue inhibitors of metalloproteinase |
| TNF-α | tumor necrosis factor-α |




# Chapter Ⅰ

# General introduction

# General Introduction

Free radicals are produced by endogenous factors, such as normal respiration, and exogenous factors, such as metabolism of foreign materials, smoking and UV radiation (Pryor, 1986; Robinson et al., 1997). Inspired molecular oxygen reacts readily with free radicals to generate reactive oxygen species including superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$), and reactive nitrogen species (RNS) including peroxynitrite ($ONOO^-$), formed by the reaction of nitric oxide ($NO\cdot$) and superoxide anion in the body (Sawa et al., 2000; Yildirim et al., 2000). These ROS and RNS can cause oxidative damage of several components, such as lipid, protein, nucleic acid and DNA, and induce inflammation or lesion on various organs. Furthermore, they are involved in regulation of gene expression, regulation of signal transduction, regulation of immune system and aging in our body. Also, these reactive species are associated with various degenerative diseases including cancer, aging, arteriosclerosis, rheumatoid arthritis and allergy. Therefore, research on free radicals, such as ROS and RNS, has been increasing the enormous importance of antioxidants materials in pharmaceuticals and nutraceuticals. Especially, both ROS and RNS are involved in the redox regulation of cell functions. They have an important role in inflammatory response as a major upstream component controlling signaling cascade, stimulation of adhesion and chemoattractant production. Inflammation is a complex process regulated by a cascade of various pro-inflammatory cytokines, growth factor, NO and prostaglandins produced by activated macrophages. There are many reports that certain types of inflammatory injury are mediated by reactive oxygen metablolites and administration of specific antioxidants. Inflammation has attracted great attention because of their implications in causing various human

diseases including cancer, neurodegenerative disorders, arthritis, diabetes, pulmonary diseases, cardiovascular diseases, as well as aging. Moreover, there are many reports that ROS have been demonstrated to be involved in the progression of tumor-induced angiogenesis and chronic inflammation plays a multifaceted role in carcinogenesis. Carcinogenesis is a multi-step process that includes tumor initiation, promotion, and progression. In particular, matrix metalloproteinases (MMPs) play an important role in cancer metastasis, such as tumor migration or invasion. MMPs are a family of zinc-containing endopeptidase and degrade specific components of extracelluar matirixes (ECMs), which has long been considered in association with both normal tissue remodeling, pathologic conditions and tumor metastasis. Furthermore, ROS and pro-inflammatory cytokines have been demonstrated to be involved in the progression of tumor-induced angiogenesis because MMP expression is regulated by the intracellular redox state (Inoue et al., 2001; Yoon et al., 2002) In these respects, there are enormous efforts to developing antioxidants from various natural resources, which can be potential candidates for anti-inflammatory and cancer chemopreventive materials during last few decades. *Eisenia bicyclis* (Kjellman. *E. bicyclis*) Setchell is a perennial brown alga, belonging to the family Laminariaceae. It is frequently used as a foodstuff, along with *Ecklonia cava* and *Ecklonia stolonifera*. *E. bicyclis* has been researched many beneficial bioactivities including inflammation, hyaluronidase and diabetic complication inhibitory activities and bioactive components, such as phlorotannins, polysaccharides, pyropheophytin, tripeptides and oxylipin (Kojima et al., 1993; Kousaka et al., 2003; Noda et al., 1989; Okada et al., 2004; Shibata et al., 2002; Whitaker and Carlson, 1975).

The aim of the present study is to isolate new phlorotannins from *E. bicyclis* and investigate their antioxidant, anti-inflammatory and anti-cancer activities for develop ing natural antioxidant materials against free radicals and oxidative stress related diseases

# Chapter Ⅱ

# Isolations of phlorotannins derived from brown alga *Eisenia bicyclis* and their antioxidative activities.

## 1. Introduction

Free radicals are produced by endogenous factors, such as normal respiration, and exogenous factors, such as metabolism of foreign materials, smoking and UV radiation (Pryor, 1986; Robinson et al., 1997). Inspired molecular oxygen reacts readily with free radicals to generate reactive oxygen species (Berenbaum, 2000)including superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$), and reactive nitrogen species (RNS) including peroxynitrite ($ONOO^-$), formed by the reaction of nitric oxide ($NO\cdot$) and superoxide anion in the body (Sawa et al., 2000; Yildirim et al., 2000). These ROS and RNS can cause oxidative damage of several components, such as lipid, protein, nucleic acid and DNA, and induce inflammation or lesion on various organs (Beckman et al., 1990). Also, these reactive species are associated with various degenerative diseases including cancer, aging, arteriosclerosis, rheumatoid arthritis and allergy (Dreher and Junod, 1996; Griffiths and Lunec, 1996; Sohal, 2002; Squadrito and Pryor, 1998).

Therefore, antioxidants are important for protection against oxidative stress in our body and have attracted great attention among the researchers during last few decades. Usually, many synthetic antioxidants, such as butylated hydroxyanisole (Ganesan et al., 2008), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are commonly used as antioxidants to scavenging free radicals in food and biological systems (Winata and Lorenz, 1996). Even though these synthetic antioxidants are effective and cheap compared to natural antioxidant products, there are great attentions to develop the natural antioxidants due to the potential health risks of synthetic antioxidants as a food additive. α-Tocopherol, vitamin E, is the most widely used natural antioxidant as an effective agent in stabilization of lipid-containing foods, and abundant in whole wheat, rice germ, and vegetable oils. However, the chemical instability and poor solubility of α-tocopherol probably result

in the loss of significant amounts and limitation of its application in food. Recently, there are great interests in searching the powerful and nontoxic natural antioxidants from marine resources to prevent oxidative stress and aging-induced disorders. Numerous crude extracts, peptides, and pure compounds obtained from marine resource were reported to have antioxidant and radical scavenging activities (Byun et al., 2009; Duan et al., 2006; Ganesan et al., 2008; Kang et al., 2004; Kuda et al., 2005; Lim et al., 2002; Zou et al., 2008).

*Eisenia bicyclis* (Kjellman. *E. bicyclis*) Setchell is a perennial brown alga, belonging to the family Laminariaceae. This species is distributed widely in Korea and Japan. Especially, it is abundantly produced in Ulleung Island in South Korea. It is frequently used as a foodstuff, along with *Ecklonia cava* and *Ecklonia stolonifera. E. bicyclis* has been researched many beneficial bioactivities including inflammation, hyaluronidase and diabetic complication inhibitory activities and bioactive components, such as phlorotannins, polysaccharides, pyropheophytin, tripeptides and oxylipin (Kojima et al., 1993; Kousaka et al., 2003; Noda et al., 1989; Okada et al., 2004; Shibata et al., 2002; Whitaker and Carlson, 1975). Among these derivatives, phlorotannins, a class of compounds with polymerized phloroglucinol units, have shown strong antioxidant activities and various therapeutic perspectives, such as anti-inflammation (Jung et al., 2009), anti-diabetes (Dellabella et al., 2005), antioxidation (Rosa et al., 2007), radiation protection (Zhang et al., 2008), anti-cancer (Hashida et al., 2008), as well as anti-allergic activities (Le et al., 2009). Nakamura *et al.* reported the isolation of eckol and dieckol from *Eisenia bicyclis* and described the antioxidant activity of these phlorotannins (Nakamura et al., 1996). Okada *et al.* had isolated phlorotannins such as 1- (3',5'-dihydroxyphenoxy)-7-(2'',4'',6''-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin, Eckol and dieckol from *Eisenia bicyclis* and described their potentials for effective treatment of Diabetic Complications (Okada et al., 2004). However, there has been minimal research regarding the antioxidant and radical scavenging activities of phlorotannins,

and little study has been done in cellular system. Moreover, no more available reports have been published about new phlorotannins derived from *E. bicyclis.*

Therefore, the aim of the present study is to isolate new phlorotannins, and investigate their antioxidant activities derived from *E. bicyclis* using various *in vitro* free radical scavenging activity assays in different cellular and non-cellular oxidative systems for developing natural antioxidant materials against free radicals and oxidative stress related diseases.

## 2. Materials and Methods

### 2.1. Materials and chemicals

*E. bicyclis* was purchased from Ullengdomall (Ulleng Island, Korea) in March 2008. The samples ground with grinder and the alga powders were stored in a freezer at -20℃ until use. DPPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), $FeSO_4$, DCFH-DA were purchased from Sigma Chemical Co. (St. Louis, MO). Human fetal lung fibroblasts cell line mouse macrophages cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA). Cell culture medium and all the other materials required for culturing were obtained from Gibco BRL, Life Technologies (U.S.A.). Dihydrorhodamine 123 (DHR 123) was purchased from Molecular Probes (Eugene, OR, USA), and the authentic peroxynitrite was obtained from Cayman Chemicals (Ann Arbor, MI, USA). DL-glyceraldehyde dimer, and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

### 2.2. Extraction, isolation and purification of phlorotannins from *E. bicyclis*

The lyophilized powder of *E. bicyclis* (3.8 kg) was extracted with 10 L of hot methanol for three times. The MeOH extract (624.3 g) was partitioned with organic solvents to yield dichloromethane ($CH_2Cl_2$, 170.5 g), EtOAc (90.4 g) and *n*-butanol (*n*-BuOH, 100.8 g) fractions, in addition to an $H_2O$ layer (262.6 g) (Figure 1). The $^1$H- and $^{13}$C-NMR spectra were determined using a JEOL JNM ECP-400 spectrometer (400 MHz for $^1$H-NMR and 100 MHz for $^{13}$C-NMR) in dimethylsulfoxide (DMSO)-$d_6$. The chemical shifts were referenced to residual solvent peaks (2.49 ppm for $^1$H-NMR and 39.50 ppm for $^{13}$C-NMR). Column

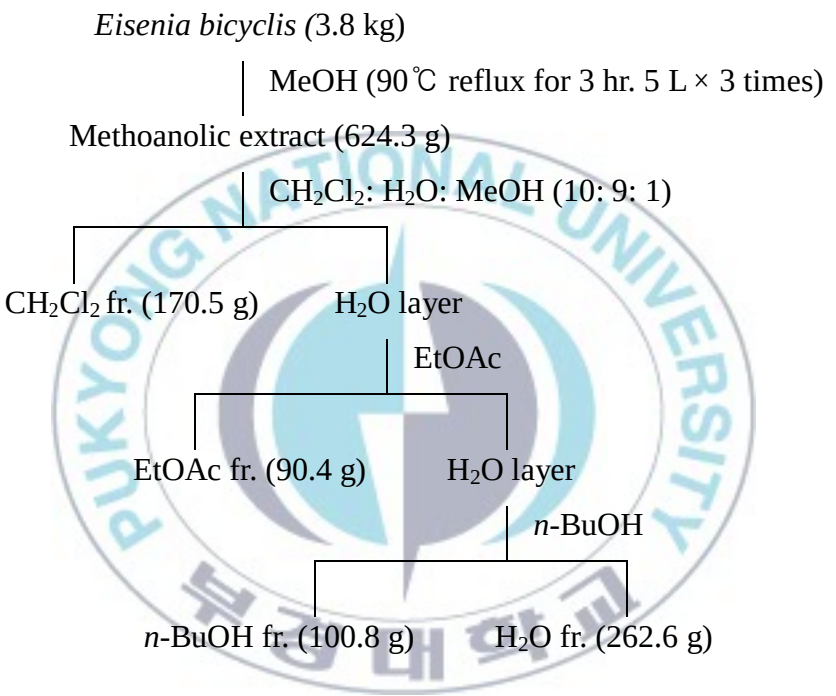



Fig. 1. Extraction and fractionation procedure of *Eisenia bicyclis.*

chromatography was performed using silica (Si) gel 60 (70~230 mesh, Merck, Germany), and TLC was conducted on precoated Merck Kieselgel 60 F254 plates (20 x 20 cm, 0.25 mm), using 50% $H_2SO_4$ as a spray reagent.

### 2.3. Quantification of total phenol contents

The total phenolic contents were determined via a modified version of Folin-Ciocalteu's method, using gallic acid as a standard (Singleton and Rossi, 1965). A 0.1 ml aliquot of the extract solution was mixed with 1 ml of Folin-Ciocalteu reagent [previously diluted with water 1:1 (v/v) and 2 ml of 20% sodium carbonate ($Na_2CO_3$) solution]. The mixed solution was maintained at room temperature for 45 min, followed by 10 min of centrifugation at 5000 g. The absorbance of the supernatant was measured at 730 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). The total phenol contents of the fractions were expressed as a percentage (wt %) compared to the weight of the dried extract or fractions

### 2.4. DPPH radical scavenging activity assay

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (Nanjo et al., 1996). A 30 $\mu$L sample solution (or ethanol itself as control) was added to 30 $\mu$L of DPPH (60 $\mu$M) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 $\mu$L quartz capillary tube, and the scavenging activity of samples on DPPH radical was measured using a JESFA ESR spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions are as follows: magnetic field, 336.5 (5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 30 s. DPPH radical scavenging ability was calculated according to the following equation : DPPH scavenging activity (%) = (1

- *A*/*A*0) × 100. *A* and *A*0 were the relative peak heights of radical signals with and without sample, respectively. The percentage of scavenging activity was plotted against the sample concentration to obtain the $IC_{50}$.

### 2.5. Hydroxyl radical scavenging activity assay

Hydroxyl radicals were generated by ironcatalyzed Fenton Haber-Weiss reaction, and the generated hydroxyl radicals rapidly reacted with nitrone spin trap DMPO. The resultant DMPO-OH adducts were detected with an ESR spectrometer (Rosen and Rauckman, 1984). The sample solution (20 $\mu$L) was mixed with DMPO (0.3 M, 20 $\mu$L), $FeSO_4$ (10 mM, 20 $\mu$L), and $H_2O_2$ (10 mM, 20 $\mu$L) in a phosphate buffer solution (pH 7.4) and then transferred into a 100 $\mu$L quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions: magnetic field, 336.5 (5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 200; sweep time, 4 min. The scavenging activity was calculated as follows: *Hydroxyl radical* scavenging activity (%) = (1 - *A*/*A*0) × 100, in which *A* and *A*0 were the relative peak heights of radical signals with and without sample, respectively.

### 2.6. Superoxide radical scavenging activity assay

Superoxide radicals were generated by a UV-irradiated riboflavin/EDTA system (Guo et al., 1999). The reaction mixture containing 0.3 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO, and the indicated concentrations of tested samples was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was transferred to a 100 $\mu$L quartz capillary tube of the ESR spectrometer for measurement. Experimental conditions: magnetic field, 336.5 (5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 1 min.

Superoxide radical scavenging ability was calculated as follows: Superoxide radical scavenging activity (%) = (1 - *A*/*A*0) × 100, in which *A* and *A*0 were the relative peak heights of radical signals with and without sample, respectively.

### 2.7. Peroxynitrite scavenging activity assay

Peroxynitrite scavenging activity was assessed according to a modified Kooy's method (Kooy et al., 1994). Highly fluorescent rhodamine 123, which rapidly oxidizes from non-fluorescent DHR 123 in the presence of peroxynitrite, is monitored. In brief, the rhodamine buffer (pH 7.4) used in this assay consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM diethylenetriaminepentaacetic acid (DTPA). The final DHR 123 concentration was 5 μM. The buffer in this assay was prepared before use and maintained on ice. The samples were dissolved in 10% dimethyl sulfoxide (DMSO) at concentrations of 12.5–100 μg/mL for the extracts/fractions. The fluorescence intensity of the oxidized DHR 123 was evaluated using GENios microplate reader (Tecan Austria GmbH, Austria) at excitation and emission wavelengths of 480 and 530 nm, respectively. All values were expressed as means ± standard deviation (SD) from three experiments.

### 2. 8. Cell cultures

Mouse macrophages (RAW 264.7) cell line was maintained in Dulbecco's modification of eagle's medium (DMEM, GIBCO, New York, USA) supplemented with 100 μg/ml penicillin–streptomycin, 10% fetal bovine serum (FBS) and human leukemic cell line (HL-60) cell lines were cultured in Roswell Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, 2 mM

glutamine, and 100 U/ml penicillin–streptomycin at 37 ℃ in a humidified incubator under 5% $CO_2$. Confluent cultures were washed twice with PBS and then collected with scraper. Collected cells were resuspended in medium and seeded to cell culture dish or well plates.

### 2.9. Cell viability assay

Cytotoxicity levels of the samples on RAW 264.7 and HL60 were measured using MTT (3- (4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen *et al* with slight modifications (Hansen et al., 1989). RAW 264.7 cells were cultured in 96-well plates at a density of $1 \times 10^5$ cells/well. After 24 h, cells were washed with fresh medium and treated with various concentrations of samples. After incubation for 24 h, cells were washed two times with PBS and 100 μL of MTT solution (1 mg/ml) was added to each well for 3 h. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) were added to solubilize the formed formazan salt. Amount of formazan salt was determined by measuring the OD at 540 nm using UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the non-treated group ((OD of non-treatment group – OD of treatment group) / OD of non-treatment group x 100). The data were expressed as means of at least three independent experiments. Each value was expressed as the mean ± SD of triplicate experiments

### 2.10. Myeloperoxidase activity

Amount of myeloperoxidase (MPO) released by HL60 was determined by O-dianisidine method with modification (Bradley et al., 1982). HL60 cells were cultured in RPMI-1640 without phenol red and FBS and seeded into 96-well plates. Cells were pre-incubated with various concentrations of samples for 30 min

followed by stimulation with TNF-α (0.05 μg/mL) at 37 ℃ for 30 min. Then cells were treated with the assay mixture containing 0.05 ml of 1 mM $H_2O_2$ in 0.1 M phosphate buffer (pH 6.0) and 50 μL of 20 mM O-dianisidine (freshly prepared) in DW. The amount of MPO released was measured spectrophotometrically at 460 nm and relative MPO activity was plotted as an absorbance value compared to the non-treated blank group.

### 2.11. Measurement of intracellular reactive oxygen species (ROS)-scavenging activities using DCFH-DA labeling

For analysis of intracellular ROS formation, the redox-sensitive fluorescent probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA) was used. RAW264.7 cell lines were incubated with 50 μM DCFH-DA for 30 min at 37 ℃ in the dark. After washing the cells with PBS three times, cells were incubated with different samples (100 μg/mL) for 1 h and 500 μM $H_2O_2$ was added to the cells for 2 h. The levels of intracellular ROS in the collected cells were determined using FACS Calibur™ flow cytometer (488 nm excitation, 530 nm emission) equipped with CELLQUEST analysis software (Becton Dickinson, Mountain View, CA, USA). For each treatment, 10,000 cells were counted and the results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

### 2.12. Statistical analysis

Each value was expressed as means ± S.E.M. (n = 3). The statistical significance of difference was analyzed by Student's t-test using SPSS (Chicago, IL, USA).

## 3. Results and discussion

It is well known the free radicals and reactive oxygen or nitrogen species including DPPH radical, hydroxyl radical, superoxide anion and peroxynitrite play a crucial role in etiology of various diseases (Beckman et al., 1990). It has been implicated in diseases such as cancer, Alzheimer's disease, rheumatoid arthritis and atherosclerosis (Squadrito and Pryor, 1998). Electron spin resonance (ESR) spectroscopy is a technique for evaluating chemical species such as free radical or inorganic complexes possessing a transition metal ion. In recent years, it has been widely used as a valuable method to measure free radical levels in radical scavenging activity assay due to its convenience and high sensitivity (Sachindra et al., 2007). In the present study, we investigated the scavenging activities of the MeOH extract and its solvent partitioned fractions derived from *E. bicyclis* towards DPPH, superoxide anion, hydroxyl and peroxynitrite radicals. Furthermore, we isolated 5 phlorotannins including phloroglucinol, fucofuroeckol-A, dioxinodehydroeckol, eckol and dieckol from EtOAc fractioin which showed most strong anti-oxidative activity among the other fractions (Table 5), and examined their antioxidative activities. Among these phlorotannins, fucofuroeckol-A and dioxinodehydroeckol were first isolated from *E. bicyclis* and showed strong antioxidative activities in different cellular and non-cellular oxidative systems.

### 3.1. Total phenolic contents of the MeOH extract and its solvent fractions

Phenolic components are commonly found in plants and have been reported to have various bioactivities such as antioxidant activities. According to previous studies, marine algae and its polyphenols have antioxidant properties (Kuda et al., 2005; Lim et al., 2002; YAN et al., 1999). The major active polyphenols in algae extract are phlorotannins (YAN et al., 1999). The amounts of the total phenolics in the MeOH

extracts and its solvent soluble fractions of *E. bicyclis* are shown in Table 1. The highest amount of total phenolic was exhibited in EtOAc fraction (68.8 %), followed by *n*-BuOH fr. (35.3 %), MeOH ex. (34.2 %), $CH_2Cl_2$ fr. (13.4 %) and $H_2O$ fr. (7.8 %). These results revealed that the order of the amounts of total phenolics is similar to that of their antioxidant activities (Table 2.). Several studies have been shown close relationship between antioxidant activities and the amount of total phenolics (Negro et al., 2003; Ramandeep and Geoffrey, 2005).

### 3.2. DPPH radical scavenging activity of the MeOH extract and its solvent fractions

DPPH radical is a stable free radical, which has been extensively used for screening the antioxidative activities of the antioxidant (Antolovich et al., 2002). As summarized in Table 2, the scavenging activity of the MeOH extract and its solvent soluble fractions on DPPH radical increased in the order of EtOAc fr. > *n*-BuOH fr. > $CH_2Cl_2$ fr. > MeOH ex. > $H_2O$ fr. and were $IC_{50}$ values of 27.0, 29.8, 44.1, 239.6 and >500 μg/mL, respectively. These results indicated that the EtOAc fraction of the MeOH extract from *E. bicyclis* showed the strongest scavenging activity on DPPH radical. The *n*-BuOH fraction also exhibited a noticeable scavenging activity. On the other hands, the $H_2O$ fraction showed no activity.

### 3.3. Hydroxyl radical scavenging activity MeOH extract and its solvent fractions

Hydroxyl radical (·OH) is the shortest existed and highly reactive ROS, which can react rapidly with biological molecules and attack cellular molecules including hepatic tissue (Hippeli and Elstner, 1997). It was generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) and trapped by a stable radical 5,5-dimethyl-

Table 1. Total phenol contents of the methanolic extract of *E. bicyclis* and its solvent soluble fractions

| Samples | Total phenol contents (%)[a] |
|---|---|
| MeOH ex. | 34.2 |
| $CH_2Cl_2$ fr. | 13.4 |
| EtOAc fr. | 68.8 |
| *n*-BuOH fr. | 35.3 |
| $H_2O$ fr. | 7.8 |

[a]Data were expressed as percentage (wt %) compared to weight of dried extract or fractions.

Table 2. Radical scavenging effects of methanolic extract and its solvent fractions of *E. bicyclis.*

| | $IC_{50}$ (μg/mL ± SD) | | | |
|---|---|---|---|---|
| Samples | DPPH | Hydroxyl | Superoxide | ONOO- |
| MeOH ex. | 239.6 | 29.5 | 301.1 | 17.2 ± 0.6 |
| $CH_2Cl_2$ fr. | 44.1 | 36.6 | 302.7 | 36.9 ± 0.5 |
| EtOAc fr. | 27.0 | 22.0 | 14.6 | 5.4 ± 0.8 |
| *n*-BuOH fr. | 29.8 | 24.8 | 246.6 | 9.4 ± 0.7 |
| $H_2O$ fr. | > 500 | 101.5 | 325.8 | 72.4 ± 1.2 |

pyrrolidine-1-oxyl (DMPO), forming nitroxide adducts as detected by ESR spectrometer (Makino et al., 1991). As shown in Table 2, extract and fractions exhibited potent or moderate scavenging activity against hydroxyl radical. The order of $IC_{50}$ values for those extract and fractions was showed as EtOAc fr. (22.0 μg/ml) > *n*-BuOH fr. (24.8 μg/ml) > MeOH ex. (29.5 μg/mL) > $CH_2Cl_2$ fr. (36.6 μg/mL) > $H_2O$ fr. (101.5 μg/mL). Among fractions, the EtOAc and *n*-BuOH fractions more potently inhibited hydroxyl radical. In contrast, the $H_2O$ fraction exhibited weak scavenging activity.

### 3.4. Superoxide anion scavenging activity of the MeOH extract and its solvent fractions

Superoxide anion is one of the precursors of the single oxygen and hydroxyl radicals, even though it is a weak oxidant, it indirectly initiates lipid peroxidation, and also the presence of superoxide anion can magnify the cellular damage because it consequentially increased other free radicals and oxidizing agents (Zou et al., 2008). Superoxide anion scavenging activity in the presence of the extract and fractions are showed in Table 2. The extract and fractions revealed the possessed potential scavenging activity in order of EtOAc fr. > *n*-BuOH fr. > MeOH ex. > $CH_2Cl_2$ fr. > $H_2O$ fr. and were $IC_{50}$ values of 14.6, 246.6, 301.1, 302.7 and 325.8 μg/mL, respectively. EtOAc fraction showed the highest activity regarding to the lowest $H_2O$ fraction.

### 3.5. Peroxynitrite scavenging activity of the MeOH extract and its solvent fractions

Peroxynitrite ($ONOO^-$) occurs as the result of a reaction of superoxide and nitric oxide and causes cytotoxicity (Squadrito and Pryor, 1998). Moreover, the need for a

strong $ONOO^-$scavenger is clear due to the absence of any enzyme that might exert protective effects against damage induced by $ONOO^-$. As shown in Table 2, $IC_{50}$ values were found to be 17.2 ± 0.6, 36.9 ± 0.5, 5.4 ± 0.8, 9.4 ± 0.7 and 72.4 ± 1.2 μg/mL, for MeOH ex., $CH_2Cl_2$ fr., EtOAc fr., *n*-BuOH fr. and $H_2O$ fr., respectively. Evidently, the order of the peroxynitrite scavenging activity was EtOAc fr. > *n*-BuOH fr. > MeOH ex. > $CH_2Cl_2$ fr. > $H_2O$ fr. Among fractions, the EtOAc and *n*-BuOH fractions exhibited potent scavenging activities against peroxynitrite. However, the $H_2O$ fraction exerted moderate scavenging activity.

### 3.6. Viability of MeOH extract and its fractions in RAW264.7cell lines

The viabilities of the tested extract and fractions were carried out on RAW264.7 cell lines for evaluating the endocellular action of antioxidation. The results showed that the tested extract and fractions performed no cytotoxic effects even at the highest concentration of 100 μg/mL, and a remarkable difference could not be found between tested extract and fractions and control as described in figure 2.

### 3.7. Effects of MeOH extract and its solvent fractions in cellular reactive oxygen species (ROS)

To evaluate the direct radical scavenging effects of MeOH extract and its solvent fractions in cellular systems, dichlorofluorescin diacetate (DCFH-DA) was used as a substrate for measuring intracellular ROS production in neutrophils. DCFH-DA freely penetrates into cells and hydrolyzed to dichlorofluorescin (DCFH) by intracellular esterases. This non-fluorescent DCFH-DA dye is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular ROS. RAW264.7 was selected for investigating effects of phlorotannins on intracellular production of ROS and MeOH extract and its solvent fractions didn’t show any cytotoxic effects on

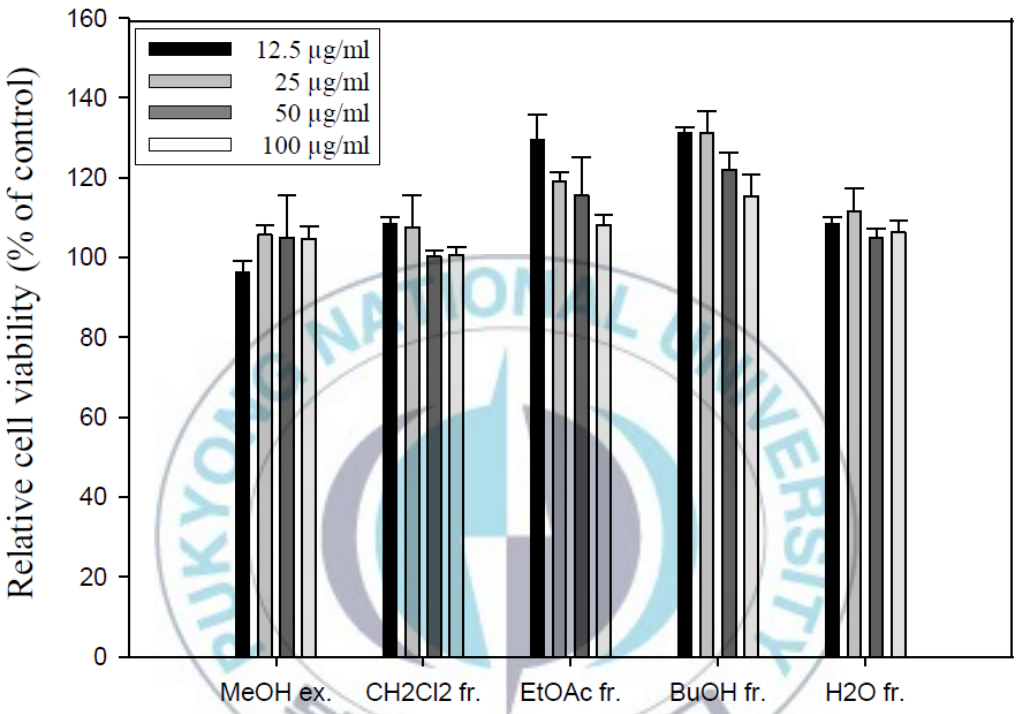



Fig. 2. Effects of methanolic extract and its solvent soluble fractions of *E. bicyclis* on cell viability in Raw 246.7 cells. Values are expressed as the mean ± SD of triplicate experiments.

RAW264.7 cell line up to concentration of 100 μg/mL for 24 h (Figure 3). As shown in figure 3, average DCF fluorescence of control, which was treated with 500 μM $H_2O_2$, increased around two times (23.6) compared to that of blank ($H_2O_2$ non-treated) after 2 h using flow cytometry. However, pre-treatment with MeOH extract and its solvent fractions decreased the average DCF fluorescence compared to control. This result indicates that MeOH extract and its solvent fractions exert considerable intracellular radical scavenging activities in RAW264.7. While MeOH extract and $H_2O$ fraction showed moderate radical scavenging activities, EtOAc fraction exhibited the strongest radical scavenging activities (13.7) and it was the same results with other radical scavenging assay. This result seems reasonable to assume that radical scavenging activity of EtOAc fractions, which contains highest total phenol content (68.8%) among the others, is caused by its phlorotannins. Therefore, we can suggest that EtOAc fraction is a potent antioxidant material that can protect radical-mediated oxidation of cellular biomolecules and contains various antioxidative phenolic compounds such as phlorotannins.

### 3.8. Isolation of phlorotannins from *E. bicyclis*

Several reports suggest that marine red and brown algae have phenols and polyphenols as secondary metabolites and these are responsible their biological activities (Kim et al., 2006; Okada et al., 2004). The previous reports on *E. bicyclis* have revealed that it contains plenty of phlorotannin derivatives with various bioactivities, such as anti-inflammation (Jung et al., 2009), anti-diabetes (Dellabella et al., 2005), antioxidation (Rosa et al., 2007) and anti-cancer (Hashida et al., 2008). However, most of reports exhibited activities of MeOH extract or solvent fractions of *E. bicyclis* and there has been a few research regarding the antioxidant and radical scavenging activities of isolated phlorotannins. Moreover, no more available reports have been published about new phlorotannin derived from *E. bicyclis.* Hence, this made us to isolate new phlorotannins from *E. bicyclis* for evaluating its antioxidant

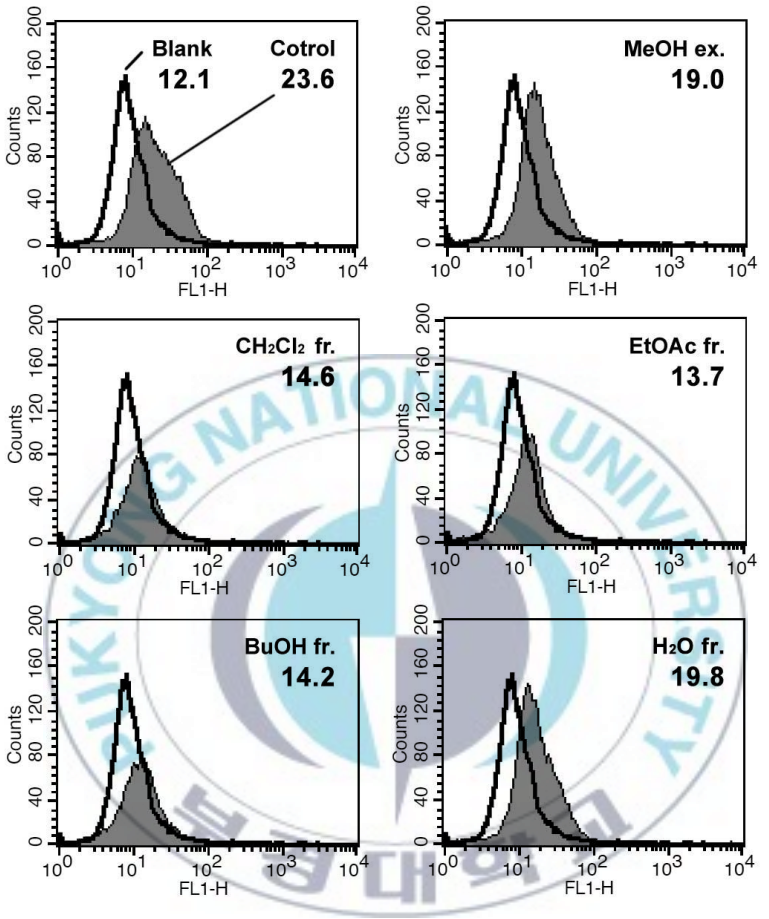



Fig. 3. Cellular radical scavenging activities of methanolic extract and its solvent soluble fractions of *E. bicyclis* on RAW 264.7. Cells were labeled with oxidation sensitive dye, DCFH-DA and treated each sample for 1 h. After washing the cells with PBS for three times, 500 μM $H_2O_2$ was added to cells (blank: - $H_2O_2$, Control: + $H_2O_2$). The levels of intracellular ROS in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

effects in cell with various oxidative systems. The EtOAc fraction showed strong antioxidant activities in tested model system. Thus the EtOAc fraction (90.4 g) was chromatographed on a Sephadex LH-20 column using MeOH as solvent to yield 5 subfractions. The fraction 1 (27 g) was chromatographed on a RP-18 column eluting with aqueous MeOH (20% to MeOH) to obtained 6 subfractions [Fr. 1-1 to Fr. 1-6]. The fraction 1-3 (12 g) was chromatographed with aqueous MeOH (20% to MeOH) to obtained 6 fractions [Fr.1-3-1 to Fr.1-3-6]. The fraction 1-3-1 (300 mg) was purified by Sephadex LH-20 with MeOH to yield compound **1** (250 mg). The fraction 1-3-3 (750 mg) was further separated by Sephadex LH-20 with MeOH to give compound **2** and **3** (70 mg and 40 mg, respectively). The fraction 1-3-4 (370 mg) was chromatographed over the Sephadex LH-20 column with MeOH to obtain compound 4 (150 mg). The fraction 1-3-5 (160 mg) was purified by Sephadex LH-20 with MeOH to yield compound **5** (30 mg) (Figure 4).

### 3.9. Spectroscopic properties of compounds 1-5 isolated from the EtOAc fraction (Table 3)

Compound **1** (phloroglucinol)

: The compound was identified by TLC with an authentic sample.

Compound **2** (fucofuroeckol-A, FF): Pale brown powder, $C_{24}H_{14}O_{11}$. $^{1}$H-NMR (400 MHz, DMSO-$d_6$) δ: 10.05 (1H, s, 14-OH), 9.88 (1H, s, 4-OH), 9.76 (1H, s, 10-OH), 9.44 (1H, s, 2-OH), 9.18 (2H, s, 3', 5'-OH), 8.22 (1H, s, 8-OH), 6.71 (1H, s, H-13), 6.47 (1H, d, *J*=1.1 Hz, H-11), 6.29 (1H, s, H-3), 6.25 (1H, d, *J*=1.5 Hz, H-9), 5.83 (1H, s, H-4'), 5.76 (2H, d, *J*=1.5 Hz, H-2', 6'). $^{13}$C-NMR (100 MHz, DMSO-$d_6$) δ: 160.7 (C-1'), 158.8 (C-3', 5'), 158.3 (C-11a), 157.6 (C-10), 150.5 (C-12a), 150.2 (C-8), 146.9 (C-2), 144.4 (C-14), 142.0 (C-4), 136.8 (C-15a), 133.6 (C-5a), 126.1 (C-14a), 122.6 (C-4a), 122.4 (C-1), 103.1 (C-6), 102.4 (C-7), 98.2 (C-3), 98.0 (C-9),

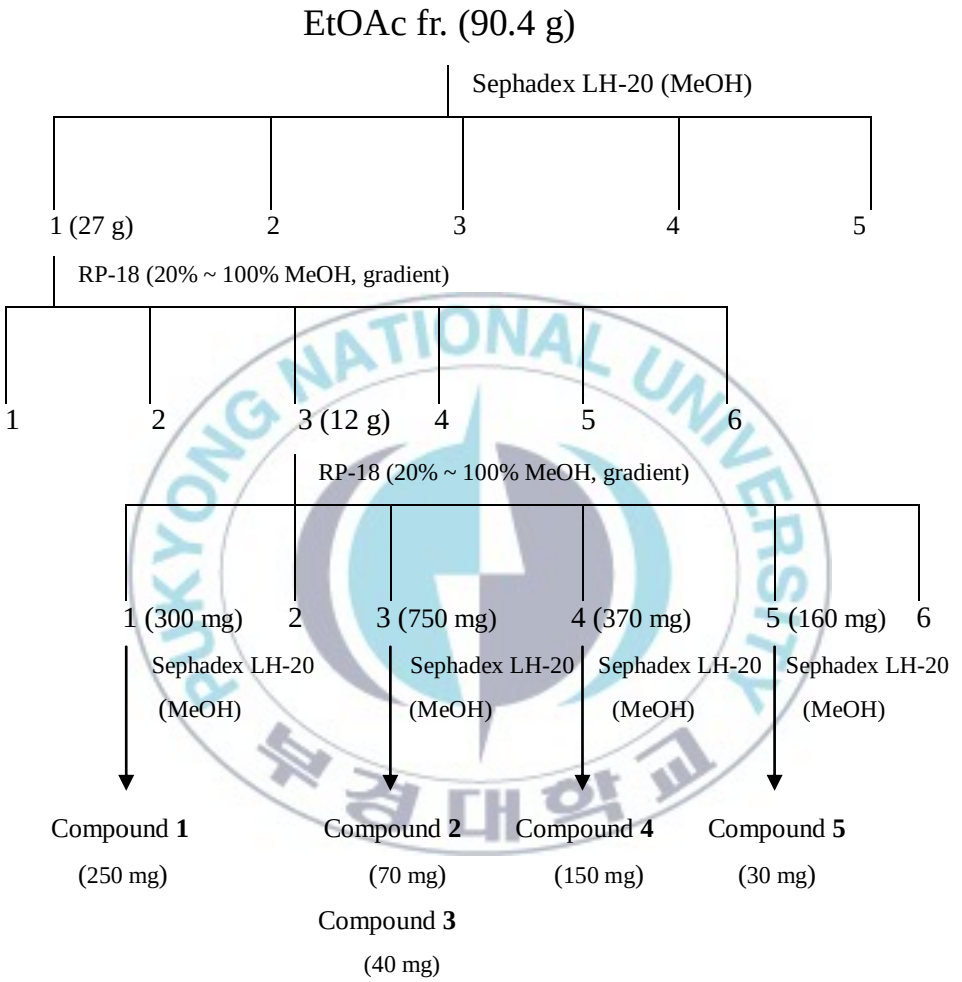



Fig. 4. Isolation of compounds **1-5** from the EtOAc fraction of *Eisenia bicyclis*

Table 3. $^{13}$C NMR data for isolated phlorotannins (2-5) in DMSO-$d_6$.

| C | Compound 2 | Compound 3 | Compound 4 | Compound 5 |
|---|---|---|---|---|
| **1** | 122.4 | 146.1 | 122.1 | 122.9 |
| **2** | 146.9 | 98.8 | 146.1 | 145.6 |
| **3** | 98.2 | 153.3 | 98.3 | 97.9 |
| **4** | 142.0 | 93.9 | 141.8 | 141.5 |
| **4a** | 122.6 | 142.1 | 123.2 | 122.9 |
| **5a** | 133.6 | 125.9 | 142.4 | 142.2 |
| **6** | 103.1 | 140.1 | 93.5 | 93.6 |
| **7** | 102.4 | 97.6 | 154.2 | 152.6 |
| **7a** | | 137.2 | | |
| **8** | 150.2 | | 97.9 | 98.3 |
| **8a** | | 122.7 | | |
| **9** | 98.0 | 146.0 | 146.0 | 146.0 |
| **9a** | | | 123.9 | 122.3 |
| **10** | 157.6 | 98.8 | | |
| **10a** | | | 137.2 | 136.8 |
| **11** | 90.5 | 153 | | |
| **11a** | 158.3 | | | |
| **12** | | 93.9 | | |
| **12a** | 150.5 | 141.7 | | |
| **13** | 94.6 | | | |
| **13a** | | 122.5 | | |
| **13b** | | 131.6 | | |
| **14** | 144.4 | | | |
| **14a** | 126.1 | 122.3 | | |
| **15a** | 136.8 | | | |
| **1'** | 160.7 | | 160.3 | 160.4 |
| **2'** | 93.7 | | 93.6 | 93.5 |
| **3'** | 158.8 | | 158.8 | 158.4 |
| **4'** | 96.3 | | 96.2 | 96.0 |
| **5'** | 158.8 | | 158.8 | 158.4 |
| **6'** | 93.7 | | 93.6 | 93.5 |
| **1''** | | | 122.2 | |
| **2''** | | | 145.9 | |
| **3''** | | | 98.2 | |
| **4''** | | | 141.9 | |
| **4a''** | | | 123.1 | |
| **5a''** | | | 142.6 | |
| **6''** | | | 93.9 | |
| **7''** | | | 153.1 | |
| **8''** | | | 98.5 | |
| **9''** | | | 146.1 | |
| **9a''** | | | 122.6 | |
| **10a''** | | | 137.1 | |
| **1'''** | | | 155.9 | |
| **2'''** | | | 94.5 | |
| **3'''** | | | 151.2 | |
| **4'''** | | | 124.2 | |
| **5'''** | | | 151.2 | |
| **6'''** | | | 94.5 | |

96.3 (C-4'), 94.6 (C-13), 93.7 (C-2', 6'), 90.5 (C-11).

Compound **3** (dioxinodehydroeckol, DD): Pale brown powder, $C_{18}H_{10}O_9$. $^1$H-NMR (400 MHz, DMSO-$d_6$) δ: 9.77 (1H, s, 1-OH), 9.64 (1H, s, 9-OH), 9.60 (1H, s, 6-OH), 9.27 (1H, s, 3-OH), 9.26 (1H, s, 11-OH), 6.10 (1H, s, H-7), 6.04 (1H, d, *J*=2.7 Hz, H-2), 6.01 (1H, d, *J*= 2.7 Hz, H-10), 5.84 (1H, d, *J*=2.7 Hz, H-4), 5.82 (1H, d, *J*=2.7 Hz, H-12). $^{13}$C-NMR (100 MHz, DMSO-$d_6$) δ:153.3 (C-3), 153.0 (C-11), 146.1 (C-1), 146.0 (C-9), 142.1 (C-4a), 141.7 (C-12a), 140.1 (C-6), 137.2 (C-7a), 131.6 (C-13b), 125.9 (C-5a), 122.7 (C-8a), 122.5 (C-13a), 122.3 (C-14a), 98.8 (C-2, 10), 97.6 (C-7), 93.9 (C-4, 12).

Compound **4** (dieckol, DE): Pale brown powder, $C_{36}H_{22}O_{18}$. $^1$H-NMR (400 MHz, DMSO-$d_6$) δ: 9.71 (1H, s, OH-9), 9.61 (1H, s, 9''-OH), 9.51 (1H, s, 4''-OH), 9.46 (1H, s, 4-OH), 9.36 (2H, s, 3''', 5'''-OH), 9.28 (1H, s, 2''-OH), 9.23 (1H, s, 2-OH), 9.22 (1H, s, 7''-OH), 9.15 (2H, s, 3'-, 5'-OH), 6.02 (1H, d, *J* = 2.7 Hz, H-8), 5.98 (1H, d, *J* = 2.7 Hz, H-8''), 5.95 (1H, s,H-2''', 6'''), 5.82 (1H, d, *J* = 2.7 Hz, H-6), 5.81 (1H, d, *J* = 2.7 Hz, H-6''), 5.80 (1H, t, *J* = 2.0 Hz, H-4'), 5.78 (2H, d, *J* = 2.0 Hz, H-2', 6'). $^{13}$C-NMR (100 MHz, DMSO-$d_6$) δ: 160.3 (C-1'), 158.8 (C-3', 5'), 155.9 (C-1'''), 154.2 (C-7), 153.1 (C-7''), 151.2 (C-3''', 5'''), 146.1 (C-2), 146.1 (C-9''), 146.0 (C-9), 145.9 (C-2''), 142.6 (C-5a''), 142.4 (C-5a), 141.9 (C-4''), 141.8 (C-4), 137.2 (C-10a), 137.1 (C-10a''), 125.3 (C-4'''), 123.9 (C-9a), 123.2 (C-4a), 123.1 (C-4a''), 122.6 (C-9a''), 122.2 (C-1''), 122.1 (C-1), 98.5 (C-8''), 98.3 (C-3), 98.2 (C-3''), 97.9 (C-8), 96.2 (C-4'), 94.5 (C-2''', 6'''), 93.9 (C-6''), 93.6 (C-2', 6'), 93.5 (C-6).

Compound **5** (eckol, EK): Pale brown powder, $C_{18}H_{12}O_9$. $^1$H-NMR (400 MHz, DMSO-$d_6$) δ: 9.54 (1H, s, 9-OH), 9.45 (1H, s, 4-OH), 9.21 (2H, s, 2-, 7-OH), 9.16 (2H, s, 3'-, 5'-OH), 6.14 (1H, s, H-3), 5.96 (1H, d, *J* = 2.8 Hz, H-8), 5.80 (1H, d, J = 1.7 Hz, H-4'), 5.78 (1H, d, *J* = 2.8 Hz, H-6), 5.72 (2H, *J* = 1.7 Hz, H-2', 6'). $^{13}$C-

NMR (100 MHz, DMSO-$d_6$) δ: 160.4 (C-1'), 158.4 (C-3', 5'), 152.6 (C-7), 146.0 (C-9), 145.6 (C-2), 142. (C-4a), 141.5 (C-4), 136.8 (C-10a), 123.3 (C-5a), 122.9 (C-1), 122.3 (C-9a), 98.3 (C-8), 97.9 (C-3), 96.0 (C-4'), 93.6 (C-6), 93.5 (C-2', 6').

### 3.10. Identification of compounds isolated from *E. bicyclis*

FF (fucofuroeckol-A) was isolated as pale brown powder. The molecular formula of FF was deduced as $C_{24}H_{14}O_{11}$ based on the NMR spectra data (Figures 5-6). The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of contained signals characteristic of five aromatic protons, *i.e.* $AB_2$ system signals at δ 6.71 (1H, s) and 6.47 (1H, d, *J*=1.10 Hz), and δ 6.25 (1H, d, *J*=1.46 Hz) and 5.76 (2H, d, *J*=1.46 Hz), and two singlet at δ 6.29 (1H, s) and 5.83 (1H, s) as well as eight phenolic hydroxyl signals at δ 10.05, 9.88, 9.76, 9.44, 9.18 and 8.22. The carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectrum of FF indicated the presence of seven methanes, fifteen *O*-bearing aromatic carbons and two quaternary carbons (δ 103.1, 102.4). This compound is composed of four benzene rings which are linked together via a furanoid, a 1,4 dioxin structure and a phenyl ether bridge. The chemical shifts of C-5a, C-13, C-14 and C-14a (δ 133.6, 94.6, 144.4 and 126.1, respectively) were very close to those of the corresponding signals of eckol, and those of the other two carbons [C-6 (δ 103.1) and C-12a (δ 150.5)] were different from the corresponding signals. FF has to be an eckol substituted by phloroglucinol via a biaryl bond at C-9. The elimination of one molecule of water from the hydroxyl groups at C-2 of the phloroglucinol and C-8 of the eckol unit must then have led to formation of the furan ring. This result indicates that this compound contained an eckol moiety, and that C-12a or C-13 can be modified or can exist close to the modification. FF was first isolated as free form from natural sources from *E. bicyclis*, although hepta-acetate of FF has been previously isolated from *Eisenia arborea* (Glombitza et al., 1985).

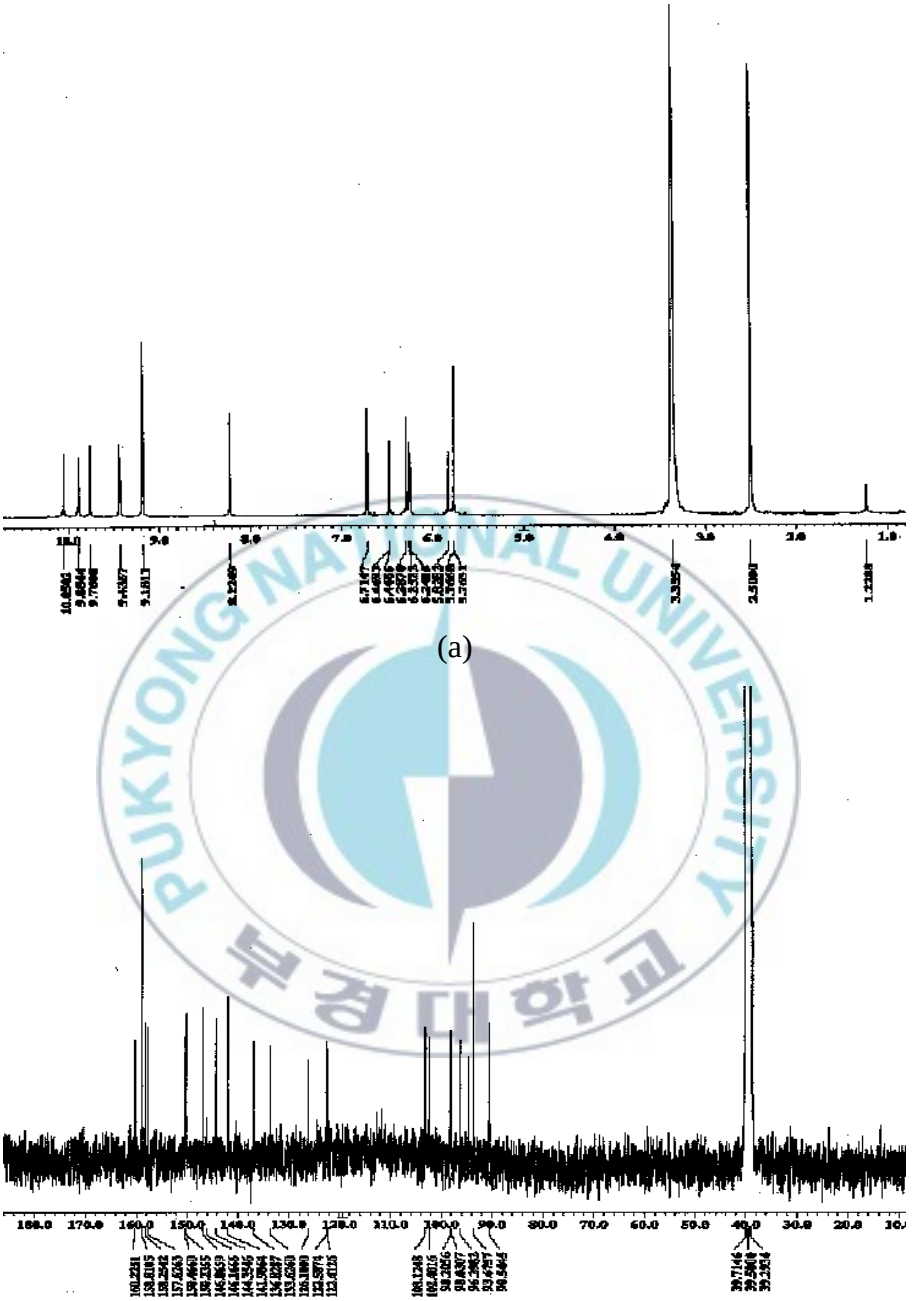
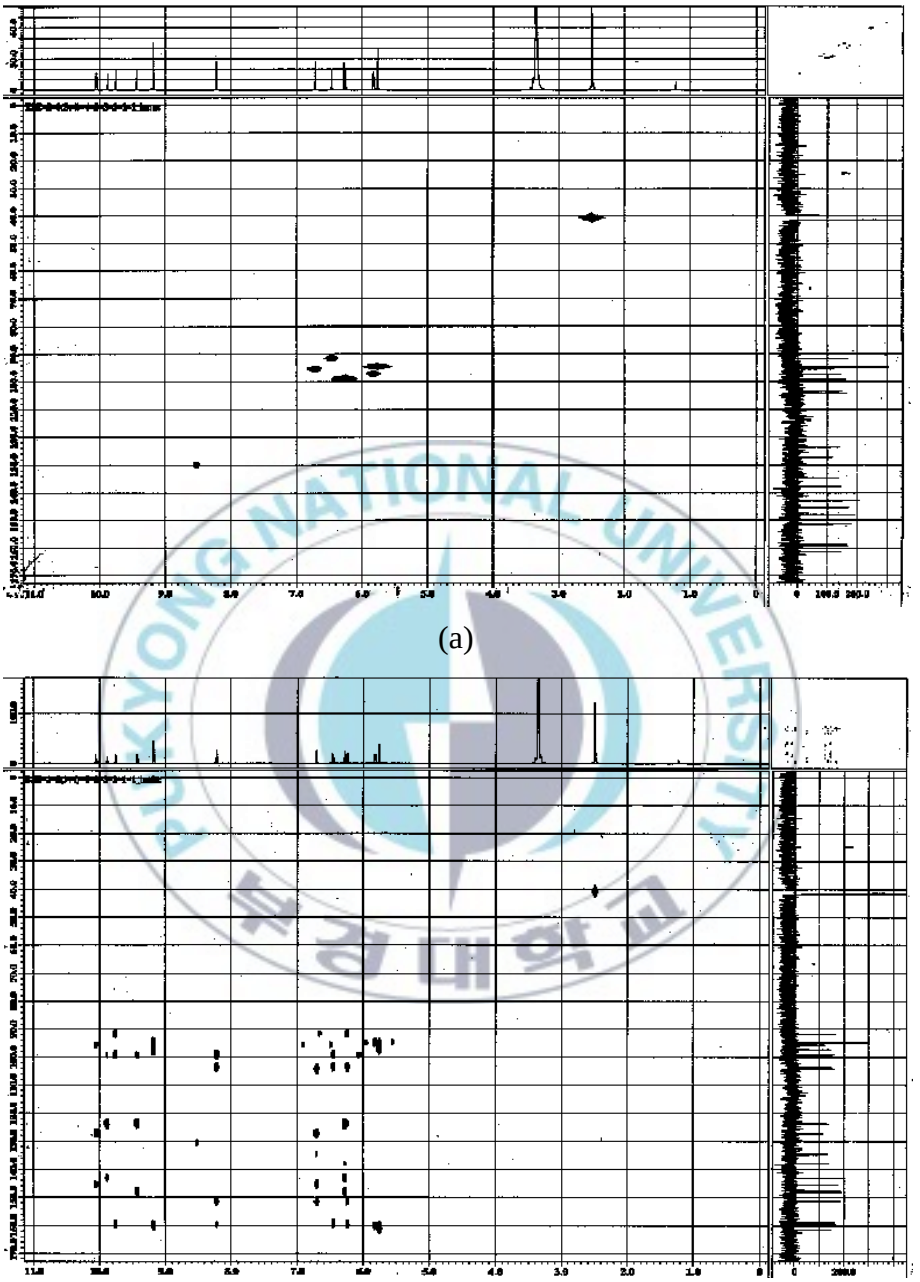


Fig. 5. $^{1}$H-NMR spectrum (a) and $^{13}$C-NMR spectrum (b) of fucofuroeckol-A in DMSO-$d_6$.



(a)

(b)

Fig. 6. HMQC (a) and HMBC (b) spectrum of fucofuroeckol-A in DMSO-$d_6$.

DD (dioxinodehydroeckol) was isolated as pale brown powder. The molecular formula of 3 was deduced as $C_{18}H_{10}O_9$ based on the NMR spectra data (Data not shown). The carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectrum of DD indicated the presence of five non-substituted and thirteen *O*-bearing aromatic carbons, whereas the proton nuclear magnetic resonance ($^{1}$H-NMR) spectrum contained signals characteristic of five aromatic protons, *i.e.* two AB systems at δ 6.04 (1H, *J*=2.7 Hz) and 5.82 (1H, *J*=2.7 Hz), and δ 6.01 (1H, *J*=2.7 Hz) and 5.84 (1H, *J*=2.7 Hz), and a singlet at 6.10 (1H) as well as five singlets indicating phenolic hydroxy protons at δ 9.77, 9.64, 9.60, 9.27 and 9.26. In the HMBC spectrum, each cross peak between δ 9.77 and C-1 (146.1), C-2 (98.8), and C-14a (122.3), and between δ 9.27 and C-2 (98.9), C-3 (153.3), and C-4 (δ 93.9) indicated the presence of the hydroxyl group at C-1 and C-3, respectively. Similarly, each cross peak between δ 9.26 and C-10 (98.8), C-11 (153.0), and C-12 (93.9), and between δ 9.64 and C-8a (122.7), C-9 (146.0), and C-10 (98.8) designated the existence of the hydroxyl groups at C-11 and C-9, respectively. Each cross peak between δ 9.60 and C-5a (125.9), C-6 (140.1) and C-7 (97.6), established the presence of the hydroxyl group at C-6. Consequently, the structure of DD was established as dioxinodehydroeckol by comparison of reported spectral data (Kang et al., 2003). This compound was isolated from *E. bicyclis* for the first time.

### 3.11. Radical scavenging activity of the phlorotannnins derived from *E. bicyclis*

As summarized in Table 4, the scavenging activity of the 4 phlorotannins derived from *E. bicyclis* on DPPH, hydroxyl, superoxide, alkyl and Peroxynitrite radical scavenging acitivities were investigated. Most of phlorotannins exhibited remarkable antioxidant activities compared to those of EtOAc fraction, not in hydroxyl radicals, and this may contributable for synergic effects of unknown components in EtOAc fraction. In summarizing all antioxidant activities, EK showed best strongest

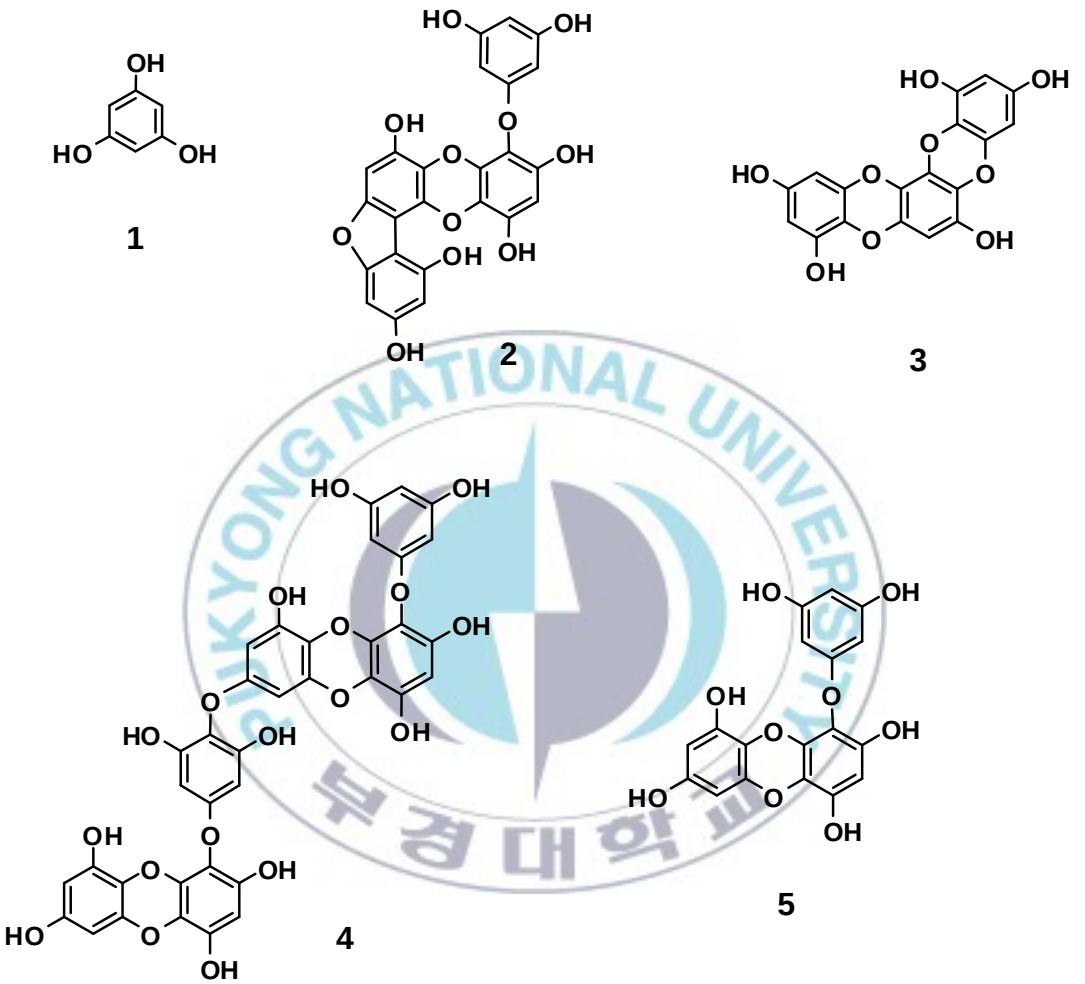



Fig. 7. Structures of the compounds **1**-**5** isolated from *Eisenia bicyclis*.

(Compound **1**, phloroglucinol; Compound **2**, fucofuroeckol-A (FF); Compound **3**, dioxinodehydroeckol (DD); Compound **4**, dieckol (DE); Compound **5**, eckol (EK).

Table 4. Radical scavenging effects of phlorotannins derived from *E. bicyclis*

| | $IC_{50}$ (μM ± SD) | | | | |
|---|---|---|---|---|---|
| Samples | DPPH | Hydroxyl | Superoxide | Alkyl | ONOO- |
| Fucofuroeckol-A (FF) | 3.52 ± 0.23 | 29.5 ± 0.75 | 137.25 ± 8.89 | 66.25 ± 0.58 | 1.28 ± 0.03 |
| Dioxino-dehydroeckol (DD) | 3.02 ± 0.06 | 56.5 ± 0.51 | 264.13 ± 5.49 | 92.5 ± 0.62 | 2.50 ± 0.07 |
| Eckol (EK) | 1.60 ± 0.06 | 260 ± 0.54 | 19.17 ± 0.66 | 148.75 ± 1.5 | 1.54 ± 0.06 |
| Dieckol (DE) | 3.42 ± 0.06 | 65.6 ± 0.9 | 114.86 ± 2.16 | 107.5 ± 0.47 | 1.81 ± 0.07 |

antioxidant activities among the tested phlorotannins, however it exhibited lower radical scavenging activities against hydroxyl and alkyl radical. It may be due to structural differences and unique bonding with corresponding receptors of each phlorotannin regarding their number of hydroxyl and O-bridge linkages.

### 3.12. Cellular reactive oxygen species (ROS) determination of the phlorotannnins derived from *E. bicyclis* using DCFH-DA assay

To evaluate the intracellular radical scavenging effects of isolated phlorotannins from *E. bicyclis*, Raw264.7 cells were pre-incubated with phlorotannins with different concentrations for 1 h. As shown in figures 8-11, all isolated phlorotannins exhibited strong radical scavenging activities in dose-dependent manner compared to that of EtOAc fraction and pre-incubation with 100 μM of each sample decreased average DCF fluorescence with the similar value of $H_2O_2$ non treated blank. Furthermore, pre-incubation with 100 μM of FF decreased average DCF fluorescence up to 3.2 which is over 2 times lower than that of blank. This result suggests that isolated phlorotannins have remarkable potential as a natural antioxidant compound that can protect radical-mediated oxidation of cellular biomolecules, and FF which was isolated from *E. bicyclis* for the first time in this study has strong radical scavenging activity in cellular system.

### 3.13. Effects of phlorotannnins derived from *E. bicyclis* on MPO activities

Neutrophils play an important role in defense against invading pathogens by producing superoxide radicals and converting them into more reactive secondary oxidants such as $H_2O_2$ (Jackson and Das, 1990). Myeloperoxidase (EC 1.11.1.7), the most abundant heme peroxidase in neutrophil granulocytes, has been considered as a microbial enzyme centrally liked to the inspecific immune defense system and

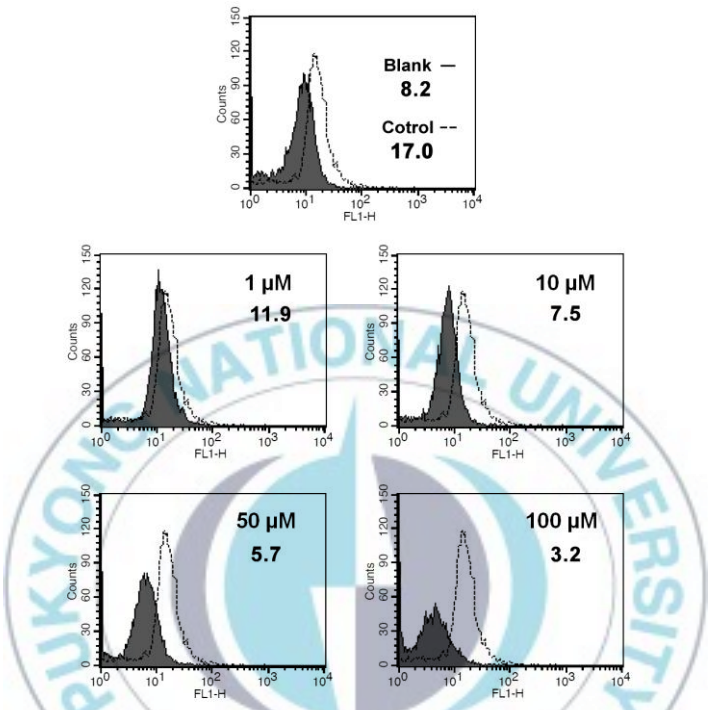



Fig. 8. Cellular radical scavenging activities of FF (Fucofuroeckol-A) derived from *E. bicyclis* on RAW 264.7 macrophages. Cells were labeled with oxidation sensitive dye, DCFH-DA and treated with different concentrations of FF for 1 h. After washing the cells with PBS for three times, 500 μM $H_2O_2$ was added to cells (blank: - $H_2O_2$, Control: + $H_2O_2$). The levels of intracellular ROS in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

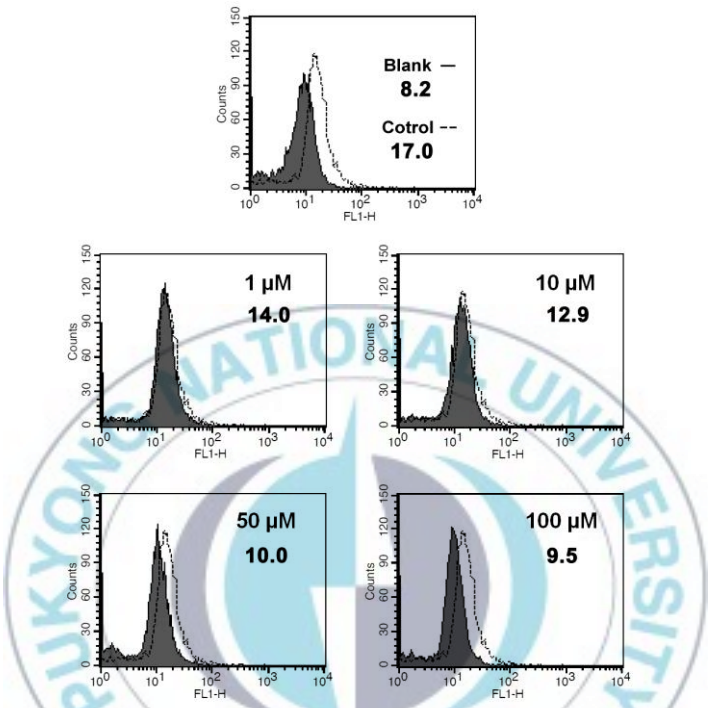



Fig. 9. Cellular radical scavenging activities of DD (Dioxinodehydroeckol) derived from *E. bicyclis* on RAW 264.7 macrophages. Cells were labeled with oxidation sensitive dye, DCFH-DA and treated with different concentrations of DD for 1 h. After washing the cells with PBS for three times, 500 μM $H_2O_2$ was added to cells (blank: - $H_2O_2$, Control: + $H_2O_2$). The levels of intracellular ROS in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

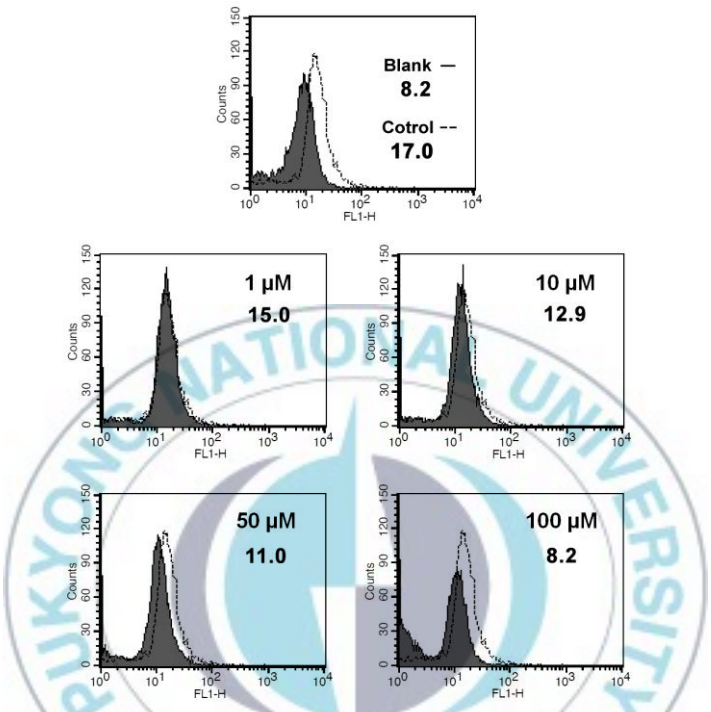



Fig. 10. Cellular radical scavenging activities of EK (Eckol) derived from *E. bicyclis* on RAW 264.7 macrophages. Cells were labeled with oxidation sensitive dye, DCFH-DA and treated with different concentrations of EK for 1 h. After washing the cells with PBS for three times, 500 μM $H_2O_2$ was added to cells (blank: - $H_2O_2$, Control: + $H_2O_2$). The levels of intracellular ROS in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

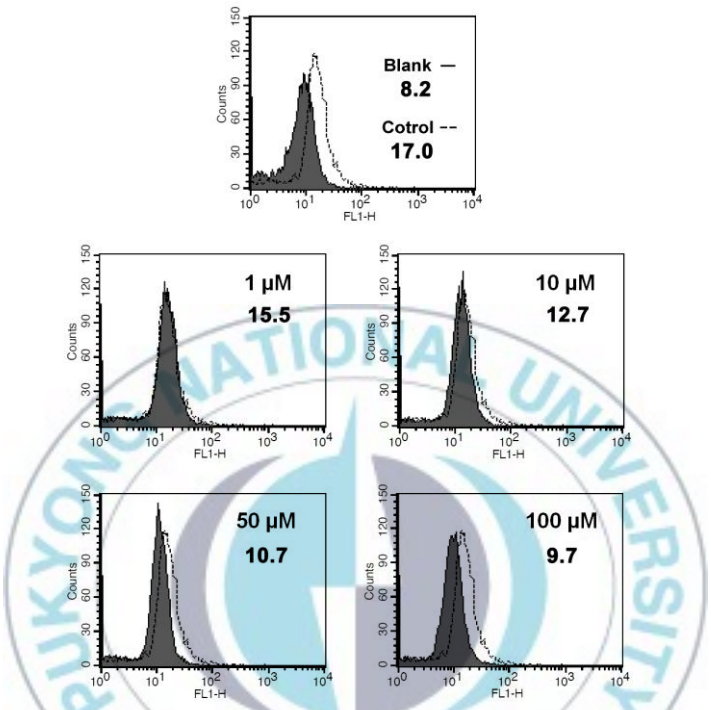



Fig. 11. Cellular radical scavenging activities of DE (Dieckol) derived from *E. bicyclis* on RAW 264.7 macrophages. Cells were labeled with oxidation sensitive dye, DCFH-DA and treated with different concentrations of DE for 1 h. After washing the cells with PBS for three times, 500 μM $H_2O_2$ was added to cells (blank: - $H_2O_2$, Control: + $H_2O_2$). The levels of intracellular ROS in the collected cells were determined using flow cytometer (488 nm excitation, 530 nm emission). The results were expressed as the average $H_2$DCF-Da fluorescence intensities in the cells to determine the free radical scavenging activities of the samples.

catalyzes the production of hypochlorous acid (HOCl) from $H_2O_2$. HOCl is the most powerful oxidant and contributes to both microbial killing and subsequent oxidative injury of host tissue triggering severe inflammatory disorders (Kettle et al., 1995). Therefore, inhibition of MPO activity is an important approach to control ROS-mediated oxidation of biomolecules in neutrophils. In present study, we evaluated inhibitory effects of isolated phlorotannins on MPO activity using human leukemic cell line (HL-60), which constitutively has high levels of MPO protein and mRNA by stimulation with TNF-α. As shown in figure 12, the tested phlorotannins (DD, DE, FF and EK) exhibited strong inhibitory effects on MPO activity in dose-dependent manner with MPO activity (IU/$10^6$ cells) of 0.51, 0.48, 0.49 and 0.55 at the 100 μM of each sample, respectively. Moreover, MTT assay result revealed that isolated phlorotannins didn't show any cytotoxicity to the HL-60 under the tested concentrations (Figure 13).

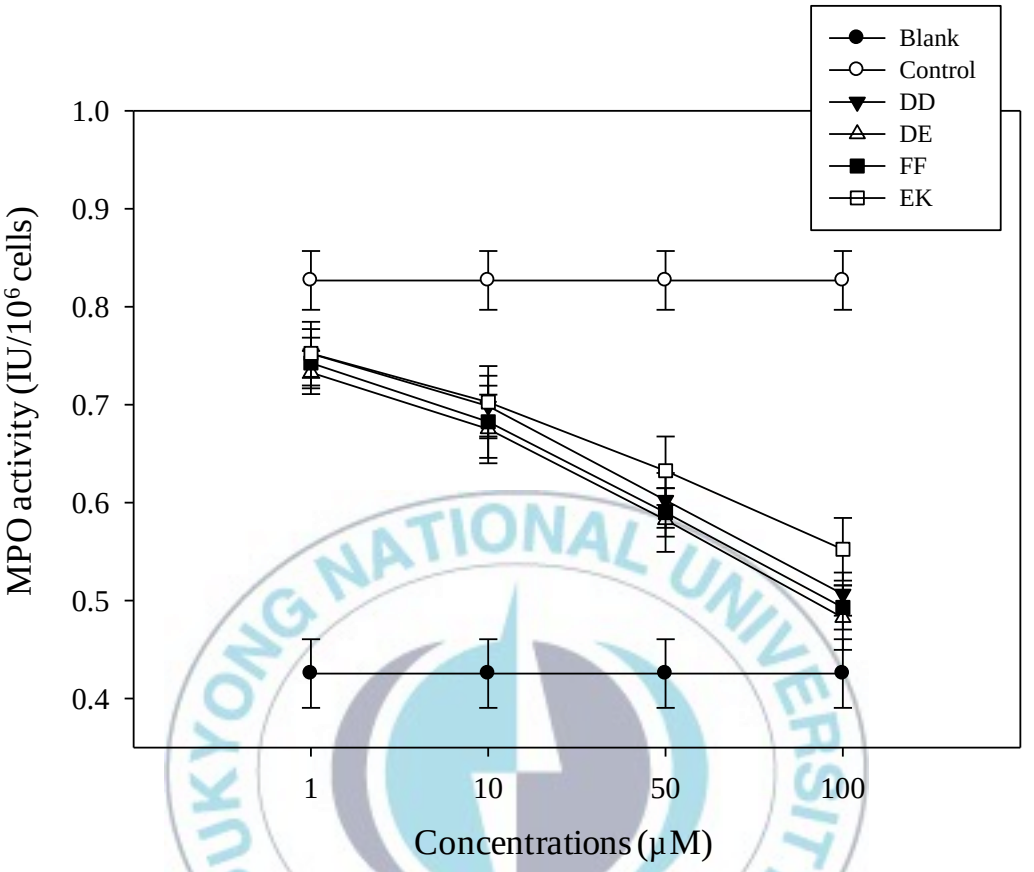



Fig. 12. The inhibitory effect of phlorotannins derived from *E. bicyclis* on myeloperoxidase activity (MPO) in HL-60 cells. Cells were pre-incubated with various concentrations of samples for 30 min followed by stimulation with TNF-α (0.05 μg/mL) at 37 ℃ for 30 min. Then cells were treated with the assay mixture containing 0.05 ml of 1 mM $H_2O_2$ in 0.1 M phosphate buffer (pH 6.0) and 50 μL of 20 mM O-dianisidine in DW. The amount of MPO released was measured spectrophotometrically at 460 nm and relative MPO activity was plotted as an absorbance value compared to the TNF- α non-treated group. Each value was expressed as the mean ± SD of triplicate experiments.

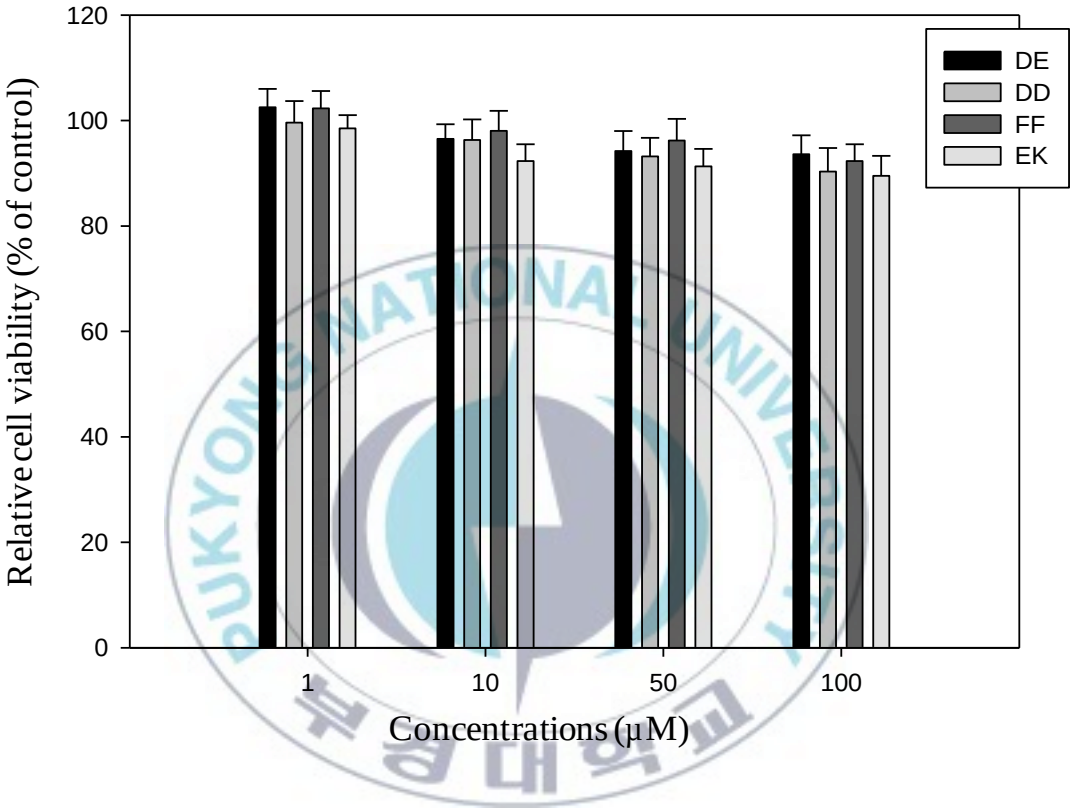



Fig. 13. Cytotoxic effects of phlorotannins derived from *E. bicyclis* on cell viability in HL-60. Cells were grown at a density of 1 x $10^5$ cells/well and different concentrations of each samples were treated. After 24h incubation, MTT solution were treated to each well and incubate for 3 h. DMSO was added to solubilize formed formazan salt and amount of formazan salt was determined by measuring the OD at 540 nm. Each value was expressed as the mean ± SD of triplicate experiments.

## 4. Summary

The present study evaluated the antioxidant activities of the methanolic extract of *E. bicyclis* and its solvent soluble fractions using a variety of cellular and non-cellular oxidative systems. Furthermore we isolated 5 phlorotannins from EtOAc fraction of *E. bicyclis* and evaluated their antioxidant activities using various antioxidative assays. Particularly, it was first report that dioxinodehydroeckol (DD) and fucofuroeckol-A (FF) were isolated from *E. bicyclis* and these phlorotannins showed strong antioxidative activities in not only cellular but also non-cellular oxidative systems except for the phloroglucinol due to the well-known in its bioactivities before.

Among tested extract and fractions, the order of antioxidant activities was exerted to be EtOAc fr. > *n*-BuOH fr. > MeOH ex. > $CH_2Cl_2$ fr. > $H_2O$ fr. through five radical scavenging assay. This order is similar to the total phenolics contents of extract and fractions. Phenolic compounds are commonly found in both edible and non-edible plants and algae. The antioxidant activity of phenolics is mainly due to their redox properties, which can play an important role in hydrogen donators, adsorbing and neutralizing free radicals, and oxygen (singlet and triplet) quenchers. They have been reported to have multiple biological effects, including antioxidant activity, anti-cancer, cardio-protection and anti-inflammation (Kahkonen et al., 1999; Rice-Evans et al., 1997; Yoon and Baek, 2005). These results suggested that *E. bicyclis* might be proposed as a functional foods, dietary supplement or healthy medicine for the preventions or treatment of various diseases. However, the compounds responsible for the antioxidant activities of its extract and fractions are unclear. Therefore, we conducted additional studies in the isolation and identification of the active components in the EtOAc fraction of *E. bicyclis* MeOH extract. In our continuous investigation on phlorotannins derived *E. bicyclis*, 5

phlorotannins were isolated from the methanol extract of *E. bicyclis* as described, phloroglucinol, fucofuroeckol-A (FF), dioxinodehydroeckol (DD), Eckol (EK) and dieckol (DE). Their chemical structures were clearly elucidated on the basis of comprehensive spectral analysis of MS and NMR ($^{1}H$ and $^{13}C$) data, and comparison with the data published previously. Among the 5 phlorotannins, FF and DD were obtained from *E. bicyclis* for the first time with the high yields at 70 mg and 40 mg from the EtOAc fraction (90.4g), respectively. Among tested phlorotannins the order of antioxidant activities was exerted to be EK> FF > DE > DD through the summarization of six radical scavenging assay, especially cellular ROS scavenging activities. Regarding the bioactivities of DD, several reports were recently published about its anti-allergy (Shim et al., 2009), anti-cancer (Kong et al., 2009) and anti-inflammation (Kim et al., 2009). However, in case of FF, there was no report regarding its bioactivities. Therefore, it is the first report that evaluated antioxidant activities of FF in cellular and non-cellular oxidative systems. In this report, the antioxidant activities were assessed using a variety of antioxidant activity assay such as DPPH radical scavenging, Peroxynitrite radical scavenging, ESR method for 3 radicals scavenging, DCFH-DA assay using flow cytometry and myeloperoxidase activity, which has been widely used for assessment of inhibition on ROS-mediated oxidation of biomolecules in cellular system. According to the results described above, it is clear that most of tested phlorotannins have considerable antioxidant activities and FF and EK showed strong antioxidative activities compared others in both of cellular and non-cellular system. Regarding their structural differences, different molecular weights were not responsible to their antioxidant capacities. However, the number of hydroxyl groups present regarding their molecular weight in these compounds showed to have an important role in antioxidant activity. EK showed higher activities than others, demonstrating that the skeletons of phloroglucinal polymers with O-bridge linkages (ether linkage) play a key role in their antioxidation ability, probably due to its unique bonding with corresponding

receptors.

In conclusion, *E. bicyclis* is a very interesting resource, not only due to its limited origin (only Korea and Japan) but also due to the presence of unique phlorotannins with special natural ratio in *E. bicyclis*. Based on the above description, it could be suggested that phlorotannins derived from *E. bicyclis*, especially FF and EK have noteworthy potential for application as antioxidants in functional food, cosmetics, and pharmaceutical industries. Meanwhile, additional studies on the mechanisms and in vivo are highly warranted to achieve a better understanding of important antioxidant properties of the isolated phlorotannins from *E. bicyclis.* Furthermore to understand various bioactivities of phlorotannins derived from *E. bicyclis*, we need more various biological activities related to its antioxidant activity such as anti-inflammation and anti-cancer.

# Chapter Ⅲ

# Anti-inflammatory mechanisms of phlorotannins derived from *Eisenia bicyclis*

## 1. Introduction

Macrophages are white blood cells within tissues and play an important role in inflammatory process and host defense. Pro-inflammatory stimuli such as bacterial lipopolysaccharides (LPS), IFN-γ, pro-inflammatory cytokines and tumor necrosis factor-α (TNF-α) induce activation of macrophages to produce inflammatory mediators (Berenbaum, 2000; Zhang and Ghosh, 2000). LPS is a major component of the outer membrane of Gram-negative bacteria consisting of lipid and polysaccharide. LPS acts as the prototypical endotoxin, when Gram-negative bacteria multiply in the host, and induce strong cellular responses such as TNF-α, interleukin (IL) -1, prostagrandins and nitric oxide (NO) from normal animal inflammatory immune system (Triantafilou and Triantafilou, 2005). Therefore, LPS induced inflammation of macrophages is a suitable model for the mechanism study of anti-inflammatory materials by various factors released from activated macrophages.

Inflammation is a complex process regulated by a cascade of various pro-inflammatory cytokines, growth factor, NO and prostaglandins produced by activated macrophages. One of the most significant mechanism of inflammation is the production of NO by inducible nitric oxide synthase (iNOS). NO is, a key vertebrate biological messenger, playing an important regulatory roles in various biological process (Xie et al., 1992). Increased NO mediates various biological functions such as nonspecific host defense, antimicrobial defense, and antitumor activities, as well as pathological process which include the pathogenesis of septic shock and organ destruction in some inflammatory and autoimmune diseases (Petros et al., 1991). NO, produced from the oxidation of terminal guanidine of L-argine by endothelial NOS (eNOS), is a potent powerful vasodilator and possesses various vasoprotective effects such as inhibition of platelet aggregation, suppression of adhesion of leukocytes or monocytes on the endothelial surfaces, and inhibition of

proliferation and migration of vascular smooth muscle cells (Ozaki et al., 2002). However, excessive NO production cause the pathogenesis of inflammatory tissue injury and several disease (Petros et al., 1991; Vodovotz et al., 1996) and the transcriptionally expressed iNOS is responsible for this excessive production of NO In activated macrophages. Therefore, the inhibition of NO overexpression by blocking iNOS expression offers promising strategy for the treatment of various inflammatory disorders.

Prostaglandin $E_2$ ($PGE_2$) is lipid mediator that synthesized from arachidonic acid by cyclooxygenase (COX) in response to cell specific trauma, stimuli or signaling molecules, and a hormone-like prostaglandin that participates in various regulatory functions such as the contraction and relaxation of muscle, the dilation and constriction of blood vessels, control of blood pressure, as well as inflammatory effects . Inhibition of $PGE_2$ synthesis by inhibition of COX has been an important anti-inflammatory strategy for treatment of various inflammatory disorders. Two major isozymes of COX are currently known: COX-1, a constitutive COX, and COX-2, an isoform induced in response to many stimuli and inflammation (Vane et al., 1994). Even though both isozymes act in the same manner, COX-1 catalyzes the production of prostaglandins for normal physiological functions and found in most mammalian cells (O'neill and Ford-Hutchinson, 1993). COX-2, however, is induced in macrophages and endothelial cells by pro-inflammatory mediators such as TNF- α, IL-1β, and LPS. Induced COX-2 catalyze the synthesis of high amount of $PGE_2$ and responsible for the pathogenesis of inflammation (Futaki et al., 1997; Hammond et al., 1999).

TNF-α, IL-1β, and IL-6 is often used as a marker for systemic activation of proinflammatory cytokines. These cytokines lead to secondary immune response such as proliferation of T and B cells, activation of macrophages for phagocytosis, and killing of microorganism (Lee et al., 2005). TNF-α is a cytokine involved in systemic inflammation and the earliest cytokine produced in large amounts in

response to LPS (Levine et al., 1990). TNF-α plays an important role in the regulation of immune cells and also able to induce apoptotic cell death, inflammation, and inhibit tumorigenesis and viral replication. It has been also reported that TNF-α production is crucial for the induction of NO synthesis in LPS stimulated macrophages (Jun et al., 1995). Several reports suggest that anti-TNF-α therapy provides protection against the LPS-induced cytotoxicity and inflammation (Beutler and Kruys, 1995; Tak et al., 2005). IL-1β is a proinflammatory cytokine protein which encoded by the IL-1β gene and produced by activated macrophages as a proprotein. IL-1β is an important mediator of the inflammatory response and involved in a variety of biological activities including cell proliferation, differentiation and apoptosis. IL-6 is secreted by T cells and macrophages to stimulate immune response to trauma, burns and tissue damage leading to inflammation. IL-6 has long been regarded as a proinflammatory cytokine induced by LPS along with TNF-α and IL-1. IL-6, however, has both proinflammatory and anti-inflammatory properties.

Nuclear transcription factor kappa-B (NF-κB) plays an important role in immune and acute phase inflammatory responses, and cell survival (Li and Verma, 2002). The activity of NF-κB is regulated by its interaction with an inhibitor protein called I-κB family of proteins. This interaction appears to block the nuclear localization of NF-κB by inhibition of dissociation of NF-κB with I-κB. NF-κB can be activated through a variety of stimuli including bacterial products (LPS), certain viral gene products, UV irradiation, B or T cell activation, proinflammatory cytokines, and by other physiological and nonphysiological stimuli. The NF-κB protein translocates to the nucleus and binds to specific elements in the promoter region of target gene to activate the transcription of various inflammatory mediators, such as iNOS or COX-2 (Crinelli et al., 2000; Xie et al., 1994).

Mitogen-activated protein kinases (MAPKs) plays important role in also regulating key proinflammatory pathways following stimulation with LPS (Zhao et al., 2005).

The three major MAPKs proteins, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are thought to play different roles in inflammatory diseases in differentities (Morel and Berenbaum, 2004). Therefore, participation of MAPKs has been frequently implicated in occurrence of diseases and expression of immune or inflammatory responses.

*Eisenia bicyclis* (Kjellman, *E. bicyclis*) Setchell is a perennial brown alga, belonging to the family Laminariaceae. This species is distributed widely in Korea and Japan. Especially, it is abundantly produced in Ulleung Island in South Korea. It is frequently used as a foodstuff, along with *Ecklonia cava* and *Ecklonia stolonifera.* *E. bicyclis* has been researched many beneficial bioactivities including inflammation, hyaluronidase and diabetic complication inhibitory activities and bioactive components, such as phlorotannins, polysaccharides, pyropheophytin, tripeptides and oxylipin (Kojima et al., 1993; Kousaka et al., 2003; Noda et al., 1989; Okada et al., 2004; Shibata et al., 2002; Whitaker and Carlson, 1975). In previous chapter, we evaluate Methanolic extract of *E. bicyclis* and its solvent soluble fractions using a variety of cellular and non-cellular oxidative systems and isolated 5 phlorotannins from EtOAc fraction of *E. bicyclis* and evaluated their antioxidant activities using various antioxidative assays.

There are many reports that certain types of inflammatiory injury are mediated by reactive oxygen metablolites and administration of specific antioxidants, such as superoxide disumutase (SOD) and/or caltalase, are effective at attenuating the tissue inflammation and injury observed in experimental models of ischemia and reperfusion, arthritis, chronic gut inflammation, and immune complex-induced pulmonary (Clark et al., 1988; Granger et al., 1981; Hultqvist et al., 2009; Johnson and Ward, 1981). These reports suggest that antioxidants, which can scavenge reactive oxygen species (ROS), are effective in ROS mediated inflammation.

In this study, we investigated the inhibitory effects of fucofuroeckol-A (FF) and eckol (EK) derived from *E. bicyclis* on endotoxin-stimulated pro-inflammatory

enzymes such as iNOS and COX-2, which reduce iNOS-derived NO and COX-2-derived $PGE_2$ production on the RAW264.7 cells. Furthermore, we studied various intracellular signaling pathways such as NF-κB activity, inflammatory cytokine expression, mitogen-activated protein kinases (MAPKs) which are proposed in response to LPS stimulation on RAW264.7 cells.

## 2. Materials and method

### 2.1. Materials and chemicals

Fucofuroeckol-A (FF) and eckol (EK) were isolated from EtOAc fraction of *E. bicyclis* as described in chapter 1. LPS from Escherichia coli serotype 0111:B4 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma. Specific antibodies against iNOS, COX-2, p65, I-κB, ERK, JNK, p38, phosphorylated (p)-ERK, p-p38, p-JNK and p-I-κBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All Enzyme-linked immunosorbent assay (ELISA) kits for cytokines were purchased from Amersham Pharmacia Biosciences (NJ, USA) and mouse inflammation cytometric bead array kit were purchased from BD Biosciences (CA, USA). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

### 2.2. Cell culture

RAW 264.7 mouse macrophages cell line was maintained in Dulbecco's modification of eagle's medium (DMEM, GIBCO, New York, USA) supplemented with 100 μg/mL penicillin–streptomycin, 10% fetal bovine serum (FBS) at 37 ℃ in a humidified incubator under 5% $CO_2$. Confluent cultures were washed twice with PBS and then collected with scraper. Collected cells were resuspended in DMEM and seeded to cell culture dish or well plates.

### 2.3. Cell viability assay

Cytotoxicity levels of the samples on RAW 264.7 macrophages were measured using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as

described by Hansen *et al* with slight modifications (Hansen et al., 1989). RAW 264.7 cells were cultured in 96-well plates at a density of $1 \times 10^5$ cells/well. After 24 h, cells were washed with fresh medium and treated with various concentrations of samples. After incubation for 24 h, cells were washed two times with PBS and 100 μL of MTT solution (1 mg/ml) was added to each well for 3 h. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) were added to solubilize the formed formazan salt. Amount of formazan salt was determined by measuring the OD at 540 nm using UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the non-treated group ((OD of non-treatment group – OD of treatment group) / OD of non-treatment group x 100). The data were expressed as means of at least three independent experiments. Each value was expressed as the mean ± SD of triplicate experiments

**2.4. Nitrite assay**

The concentrations of NO in culture supernatants were determined as nitrite, a major stable product of NO, using the Griess reagent (1% sulfanilic acid and 0.2 mmol/L N-[1-naphthyl] ethylenediamine-HCl in 2.5% $H_3PO_4$). Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 24 h. 100 μL of each culture supernatants were mixed with same volume of the Griess reagent. Nitrite levels of each sample were determined colorimetrically at 540 nm using ELISA microplate reader (Tecan Austria GmbH, Groedig, Austria). The standard curve of Nitrite concentrations were calculated with sodium nitrite. Each value was expressed as the mean ± SD of triplicate experiments

**2.5. Determination of TNF-α and IL-1β using Enzyme-liked immunosorbent assay**

The levels of TNF-α and IL-1β were determined using Biotrak™ ELISA kits (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instruction. Briefly, Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for indicated time. Following incubation, 50 μL of medium was added to wells of antibody coated 96-well plates. 50 μL of biotinylated antibody reagent was added and incubated for 3 h at RT. Reaction mixture was removed from each well and washed 4 times with washing buffer. Subsequently, 100 μL of streptavidin-HRP conjugated was added and incubate for 30 min at RT. After same washing step as above, 100 μL of TMB substrate solution was added and incubated for 30 min at RT. After adding 50 μL of stop solution, optical density was measured at 450nm using ELISA microplate reader (Tecan Austria GmbH, Groedig, Austria).

### 2.6. Determination of prostaglandin $E_2$ ($PGE_2$) using prostaglandin $E_2$ direct assay kit

The levels of $PGE_2$ were determined using Biotrak™ direct assay kits (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instruction. Briefly, Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 24 h. Following incubation, 50 μL of medium were added to wells of antibody coated 96-well plates. 50 μL of mouse anti $PGE_2$ and 50 μL of diluted conjugate were added and incubated for 1 h at RT on well plate shaker. Reaction mixture was removed from each well and washed 4 times with washing buffer. Subsequently, 150 μL of TMB enzyme substrate was added into each well and incubated for 30 min at RT on microplate shaker. After adding 100 μl of stop solution, optical density was measured at 450nm using ELISA microplate reader (Tecan Austria GmbH, Groedig,

Austria).

### 2.7. Determination of IL-6 and MCP-1 using cytometric bead array (CBA) method

The levels of IL-6 and MCP-1 were determined using BD™ CBA Mouse Inflammation kit (BD Biosciences, San Diego, CA, USA) with flow cytometry according to the manufacturer's instruction. Briefly, Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 24 h. Following incubation, 50 μL of medium and 50 μL of capture bead mixture were added to 5 ml tube and treated with 50 μL PE detection reagent. After following incubation for 2 h, reaction mixture was washed with 1 ml of wash buffer and centrifuged for 5 min at 5,000 rpm. Finally, capture bead were resuspended with 300 μL of wash buffer and analyzed with flow cytometery (BD Biosciences, San Diego, CA, USA) calibrated with cytometer setup bead procedure. The results were analyzed using BD™ CBA software (BD Biosciences, San Diego, CA, USA).

### 2.8. Western blot

RAW 264.7 cells were treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 24 h. To obtain the cytoplasmic protein, cells were harvested and washed 2 times with PBS. Collected cells were resuspended with lysis buffer (pH 7.5 50 mM Tris-HCl, 0.4 % Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/mL leupeptin, 3 mM NaF and 1 mM DTT) and incubated at 4 ℃ for 20 min. Cell lysates were centrifuged at 12,000 x *g* for 10 min and protein concentrations of supernatants were determined with lowry method using bovine serum albumin as a standard. To separate nuclear

extracts, CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich Co., MO, USA) was used following manufacturer's instructions. Proteins (20-40 μg) were diluted in 5 x protein loading buffer (10% SDS, 100 mM each dithitheritol, glycerol, bromophenol blue, and tris-HCl) and denatured at 100 ℃ for 10 min. Proteins extracts were separated on 10 or 12 % SDS-polyacrylamide gels and transferred to nitro cellulose membrane (Hybond ECL nitrocellulose membrane, Amersham biosciences, UK). Transferred protein blots were blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween20 for 1 h at RT. Membrane was washed 3 times with Tris-buffered saline containing 0.1% Tween20 and incubated with primary antigodies (1:500 dilution) for 1 h at RT. After 3 times wash with Tris-buffered saline containing 0.1% Tween20, membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 1 h at RT. Following 4 times washing with Tris-buffered saline containing 0.1% Tween20, membrane was developed with chemiluminescence reagent (ECL Reagent, Amersham biosciences, UK). Blot bands were visualized using LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

### 2.9. RT-PCR

RAW 264.7 cells were treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 12 h. Cells were harvested and washed 2 times with PBS. The total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA). Chloroform was added to the cell lysate and centrifuged at 13,000 x *g* for 12 min and supernatant was collected. Same volume of Isopropanol was added RNA pellet was collected following centrifugation. After washing with 70% ethanol, extracted RNA was dissolved in diethylpyrocarbonate-treated RNase free water and incubation for 10 min at 60℃. RNA concentrations were quantified by measuring optical density at 260 nm using microplate reader (Tecan Austria GmbH, Groedig,

Austria). The 1 μg of RNA obtained from the cells was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) at 42℃ for 45 min to produce the cDNAs. RT-generated cDNAs was used as a template to amplify iNOS, COX-2, IL-1β, TNF-α and G3DPH genes in a PCR mixture containing dNTPs, taq DNA polymerase (Promega, Madison, WI, USA). PCR was performed using Whatman thermocycler (Biometra, Kent, UK) with selective upstream and downstream primers for the mouse iNOS (5'-ATGTCCGAAGCAAACATCAC-3' and 5'-TAATGTCCAGGAAGTAGGTG-3'), COX-2 (5'-CAGCAAATCCTTGCTGTTC-C-3' and 5'-TGGGCAAAGAATGCAAACATC-3'), IL-1β (5'-ATGGCAACTGTT-CCTGAACTCAACT-3' and 5'-TTTCCTTTCTTAGATATGGACAGGAC-3'), IL-6 (5'-AGTTGCCTTCTTGGGACTGA-3' and 5'-CAGAATTGCCATTGCACAAC-3'), TNF-α (5'-ATGAGCACAGAAAGCATGATC- 3' and 5'-TACAGGCTTGTC-ACTCGAATT-3') and G3DPH (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' and 5'-CATGTAGGCCATGAGGTCCACCAC -3'). The amplified DNA was analyzed by 2% agarose gel electrophoresis and visualized by ethiduim bromide staining. The G3DPH was used as a internal control for sample loading and mRNA integrity.

### 2.10. Electrophoretic Mobility Shift Assay (EMSA)

RAW264.7 cells were pretreated with indicated concentrations of FF and EK for 2 h before stimulation with LPS (1 μg/mL) for another 2 h. EMSA for NF-κB/p65 was performed by employing Lightshift™ Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Nuclear extracts were prepared using CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich Co., MO, USA) according to the manufacturer's instructions. Briefly, DNA was biotin-labeled using the Biotin 3′ End DNA Labeling kit (Pierce, Rockford, IL, USA). In 50 $\mu$l of reaction buffer, 10 pmol of double-stranded NF-κB oligonucleotide ( 5'-

AGTTGAGGGGACTTTCCCAGGC-3'; 3'-TCAACTCCCCTGAAAG-GGTCCG-5' )was incubated in 10 $\mu$l of 5x TdT buffer, 5 $\mu$l of unlabeled control oligo (100 nM), 5 $\mu$l of biotin-11-dUTP (0.5 $\mu$M), 5 $\mu$l of diluted TdT (2 U/$\mu$l) and 25 $\mu$l of ultrapure water at 37℃ for 30 min. The reaction was stopped with 2.5 $\mu$l of 0.2 M EDTA and treated with 50 $\mu$l of chloroform : isoamyl alcohol (24:1) to extract labled DNA. Following centrifugation at 15,000 x *g* for 2 min, The top aqueous phase containing the labeled DNA was used for further binding reaction. The binding reactions contained 3-5 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% NP-40, and 2.5% glycerol), 50 ng of poly(dI-dC), and 30 fM biotin end-labeled DNA. The binding reactions were incubated for 20 min at RT in a final volume of 20 μL. the reaction mixture was subjected to gel electrophoresis on 5% polyacrylamide gel and transferred to a nylon membrane (Biodyne® Precut Nylon Membranes, Pierce, Rockford, IL, USA). DNA was cross-linked to the membrane using UV cross-linker (Bio-Link crosslinker, Vilber Lourmat, France) at 120 mJ/cm$^2$ and spectral peak was detected at 312 nm using LAS3000 image analyzer (Fujifilm Life Science, Tokyo, Japan).

### 2.11. NF-κB/p65 nuclear localization image using Confocal Laser Scanning Microscopy

The NF-κB p65 nuclear localization was detected by indirect immunofluorescence assays using confocal microscopy. RAW 264.7 cells were cultured directly on glass coverslips in 24-well plates for 24 h and treated with different concentrations of sample for 1 h before incubation with LPS (1 μg/mL) for 2 h. After incubation with LPS, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and blocked with 1.5% normal donkey serum. Polyclonal antibodies to NF-κB/p65 (1 μg/well) were applied for 1 h followed by 1 h of

incubation with fluorescein isothiocyanate (FITC)-onjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laborities, Inc., West Grove, PA). After washing with PBS, the coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL), and the fluorescence was visualized using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

### 2.12. Statistical analysis

Each value was expressed as means ± S.E.M. (n = 3). The statistical significance of differences was analyzed by Student's t-test using SPSS (Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Cell cytotoxic effects of FF and EK on RAW264.7 cell line

The viabilities of the phlorotannins were carried out on RAW264.7 cell line for evaluating the anti-inflammation assay experiments. The results showed that FF and EK performed no cytotoxic effects even at the highest concentration of 100 μM, and a remarkable difference could not be found between tested phlorotannins and control as described in figure 14. DD, however, showed remarkable cytotoxicity on RAW264.7 cells at over concentration 50 μM (data not shown). Kong et al reports that DD has potential inhibitory effect on growth of MCF-7 human breast cancer cell lines and can be used as a valuable chemopreventive agent (Kong et al., 2009). Even though DE has no cytotoxic effect on RAW264.7 cells (data not shown), its anti-inflammatory activity on murine BV2 microglia has already studied by Jung et al (Jung et al., 2009). So, we used only FF and EK for further anti-inflammatory study on RAW264.7 cell line.

### 3.2. Effects of FF and EK on NO production in LPS-stimulated RAW264.7 cell line

To examine the inhibitory effects of FF and EK on LPS-stimulated NO production in RAW264.7 cells, we measured nitrite released into the culture medium using the Griess reagent. The amount of produced NO was determined by the amount of nitrite, a stable metabolite of NO. RAW264.7 cells were treated with various concentrations of FF and EK ( 1, 10, 50, or 100 μM) for 1 h before the addition of LPS (1 μg/mL). Pretreatment with different doses of FF and EK led to a significant reduction in the formation of NO in a dose-dependent manner, as measured in the supernatants 24 h following LPS stimulation (Figures 15 and 16). According to the NO detection assay,

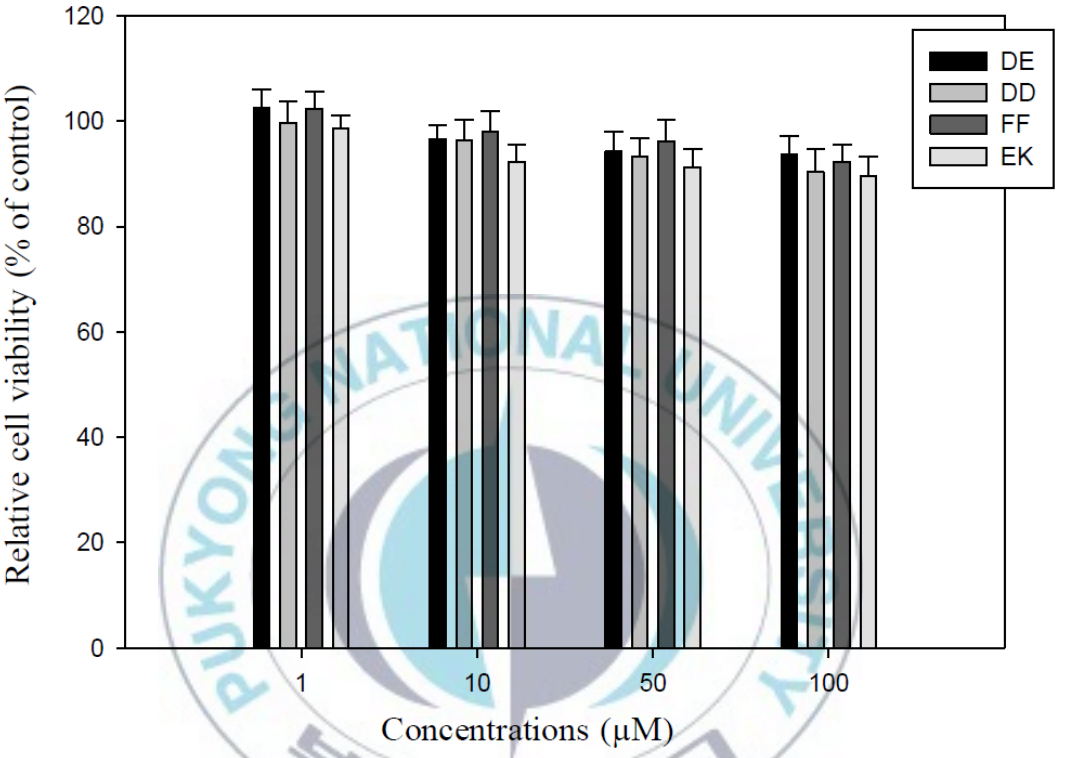



Fig. 14. Cytotoxic effects of phlorotannins derived from *E. bicyclis* on cell viability in Raw 246.7 macrophages. Cells were grown at a density of 1 x $10^5$ cells/well and different concentrations of each samples were treated. After 24h incubation, MTT solution were treated to each well and incubate for 3 h. DMSO was added to solubilize formed formazan salt and amount of formazan salt was determined by measuring the OD at 540 nm. Each value was expressed as the mean ± SD of triplicate experiments.

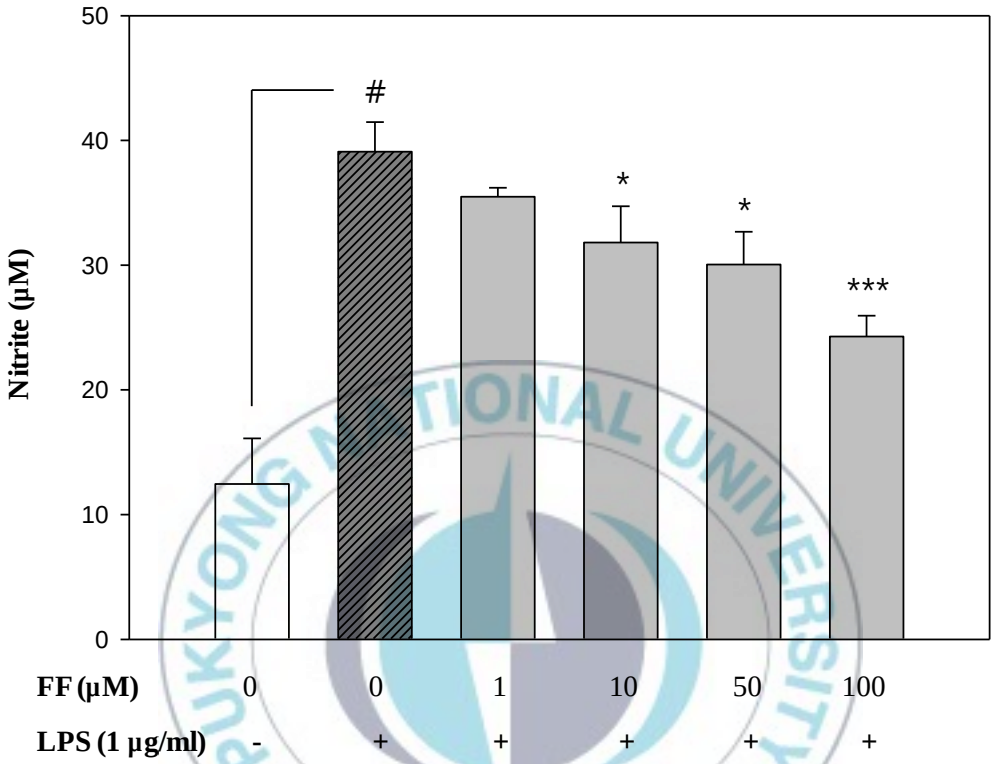



Fig. 15. Effect of FF on LPS-induced NO production in RAW 264.7 macrophages. Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of FF for 1 h before incubation with LPS (1 μg/mL) for 24 h. Collected culture supernatants were analyzed for nitrite production using the Griess reagent. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

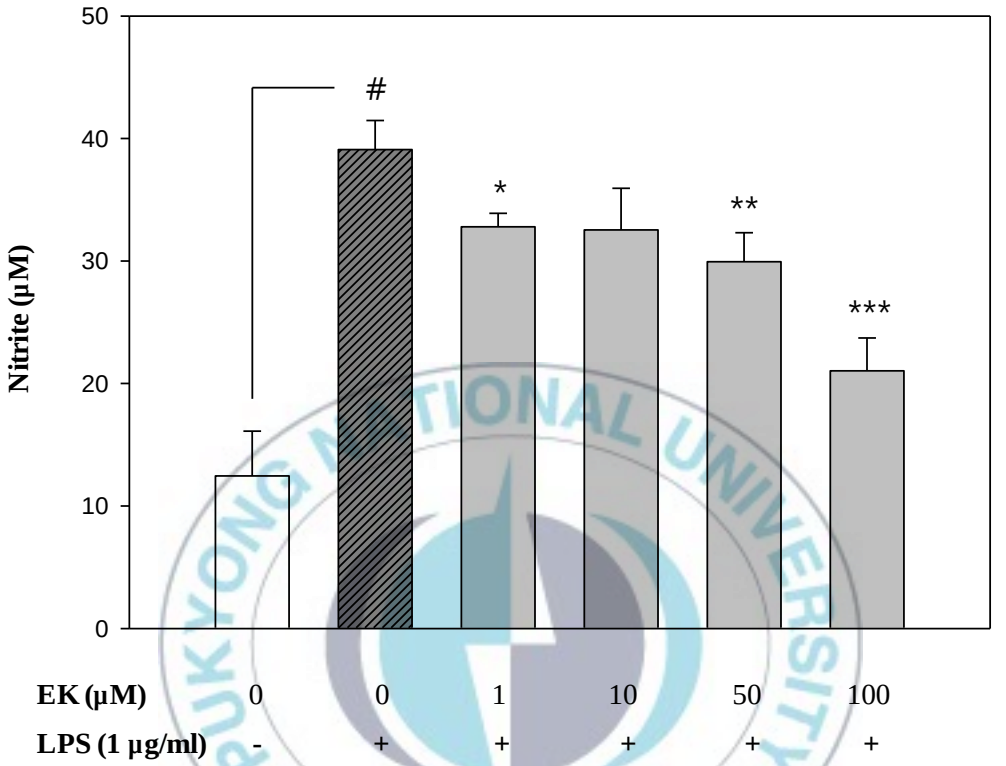



Fig. 16. Effect of EK on LPS-induced NO production in RAW 264.7 macrophages. Cells were grown at a density of 1 x $10^5$ cells/well and treated with different concentrations of EK for 1 h before incubation with LPS (1 μg/mL) for 24 h. Collected culture supernatants were analyzed for nitrite production using the Griess reagent. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

NO was significantly increased to 3.13 times the basal level after 24 h of LPS stimulation, and this increase was inhibited by FF and EK treatment with the relative NO production of 62.1 and 53.8% compared to non-treated control, respectively.

### 3.3. Effects of FF and EK on productions of TNF-α, IL-6 and MCP-1 in LPS-stimulated RAW264.7 cell line

We attempted to determine the potential effects of FF and EK on the production of pro-inflammatory cytokines, such as TNF-α and IL-6, and chemokines monocyte chemotactic protein-1 (MCP-1). RAW264.7 cells were incubated with FF and EK (1, 10, 50 and 100 μM) in the presence or absence of LPS (1 μg/mL) for 24 h, and the cytokine levels were measured in the culture media by ELISA and CBA kit. As shown in figures 17-20, the TNF-α and IL-6 levels were increased significantly in the culture media of LPS-stimulated RAW264.7 cells, and these increases were significantly decreased in a concentration-dependent manner by treatment with FF and EK. The results indicate that FF and EK negatively regulate the accumulation of pro-inflammatory cytokines at the transcriptional level. Furthermore, MCP-1 levels were also increased significantly in the culture media of LPS-stimulated RAW264.7 cells and these increases were remarkably decreased in dose-dependent manner (Figures 21 and 22). MCP-1 is a chemotatic factor plays a role in recruitment of monocytes to sites of inflammation and injury, and stimulate endothelial cells (Rhodes et al., 1997). Monocytes stimulate the expressions of TNF- $\alpha$ and IL-1$\beta$ and this cause the release of a variety of chemotatic factors including MCP-1, which can then induce the migration of monocytes resulting in inflammation. It is also widely accepted that many types of active factors are induced and regulate each other during an immune reaction (Ding et al., 2009). Therefore MCP-1 could be a potential marker to assess inflammation. This result indicates that FF and EK reduced the production of MCP-1 via suppressing inflammatory cytokines and reducing the

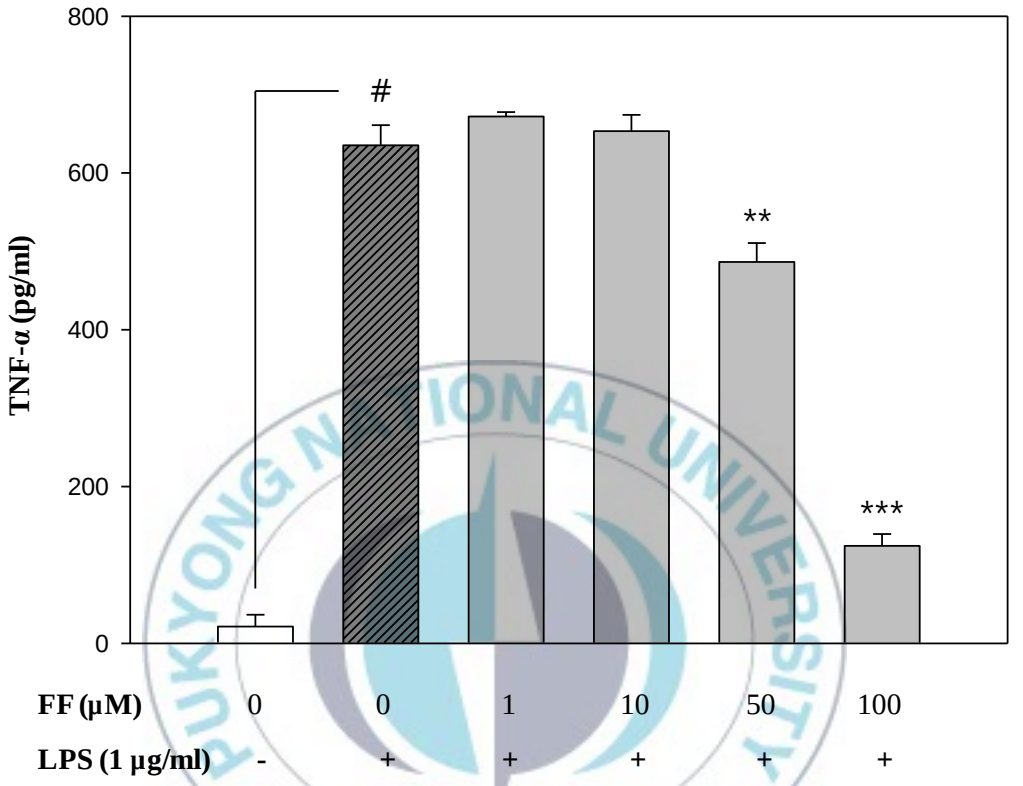



Fig. 17. Effect of FF on LPS-induced production of TNF-α in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 12 h, The TNF-α concentration was measured in culturemedia using a commercial ELISA kit. Each value was expressed as the mean ± SD of triplicate experiments. *,P < 0.05, **,P < 0.01 and ***,P < 0.001 as compared with LPS-treated cells (1 μg/mL). $^{\#}$,P < 0.001 as compared with LPS non-treated cells.

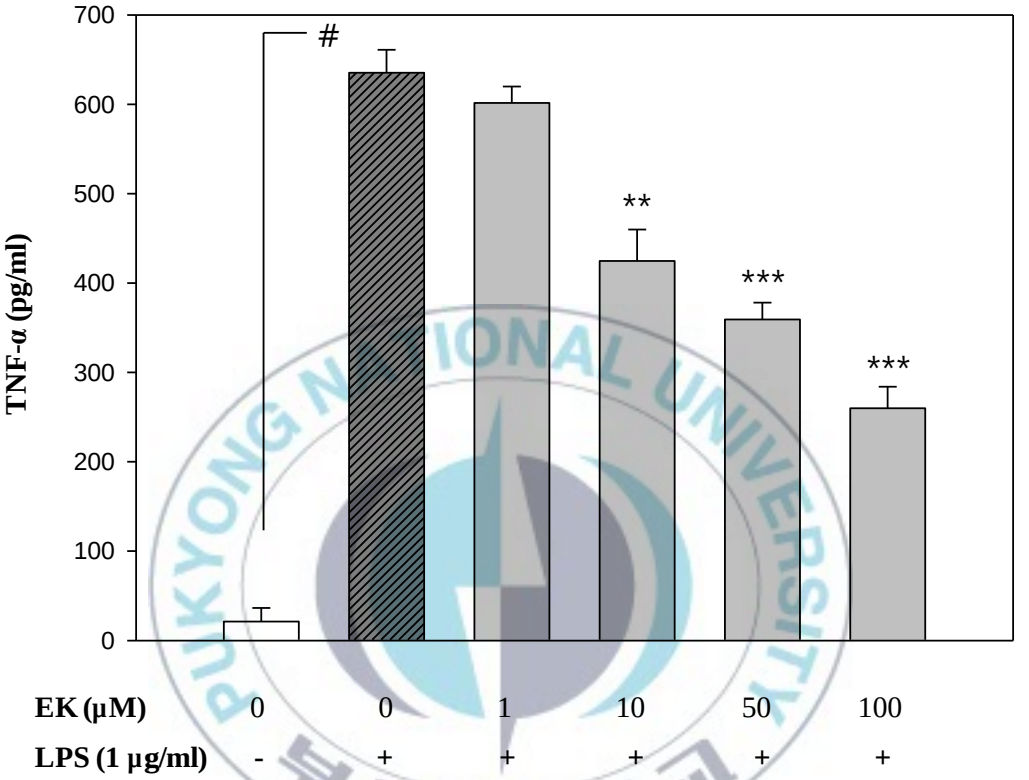



Fig. 18. Effect of EK on LPS-induced production of TNF-α in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 12 h, The TNF-α concentration was measured in culturemedia using a commercial ELISA kit. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

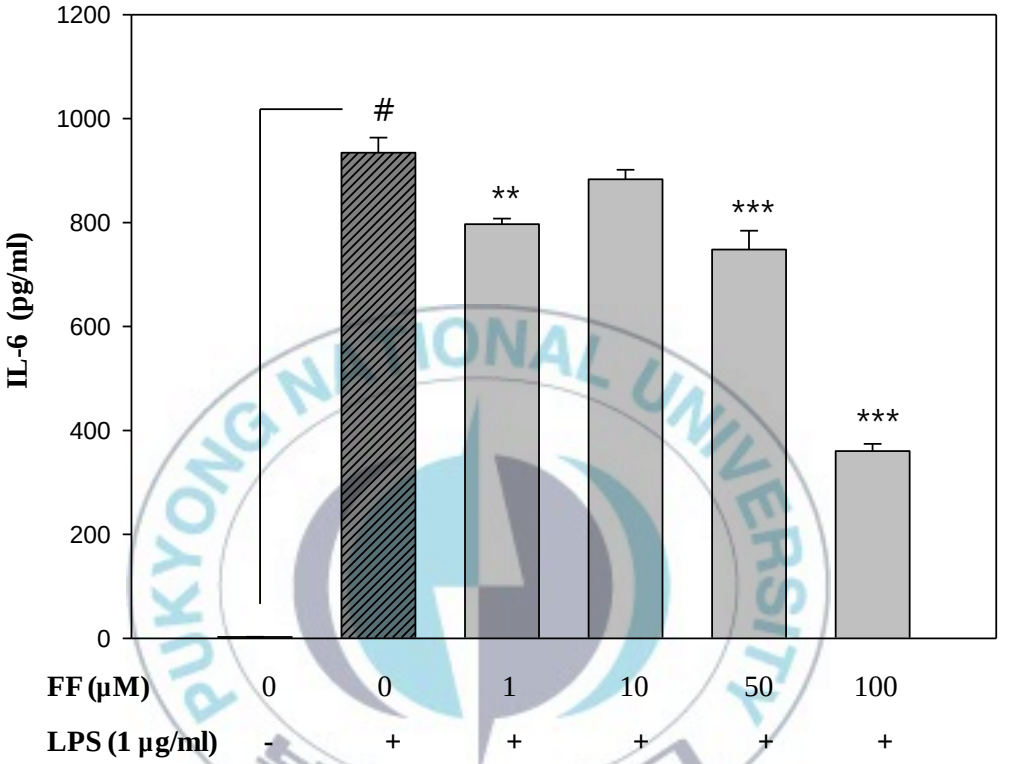



Fig. 19. Effect of FF on LPS-induced production of IL-6 in RAW264.7. Cells were grown at 5 x $10^{5}$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The IL-6 concentration was measured in culturemedia using a commercial CBA (cytometric bead array) kit using flow cytometery. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

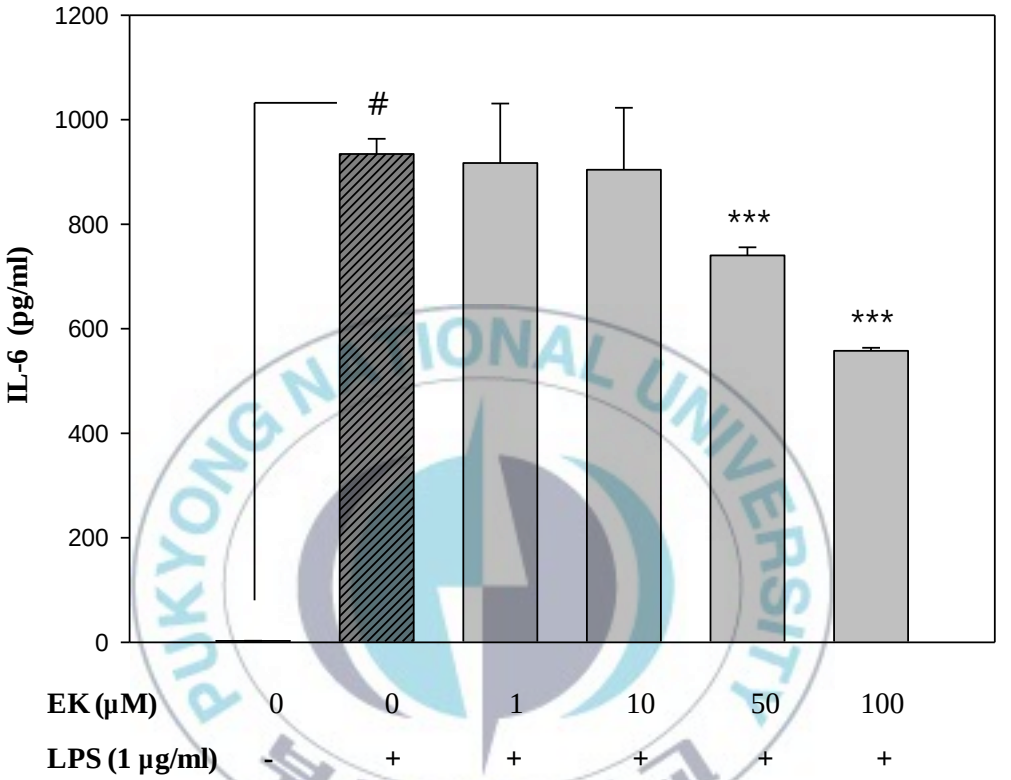



Fig. 20. Effect of EK on LPS-induced production of IL-6 in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The IL-6 concentration was measured in culturemedia using a commercial CBA (cytometric bead array) kit using flow cytometery. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

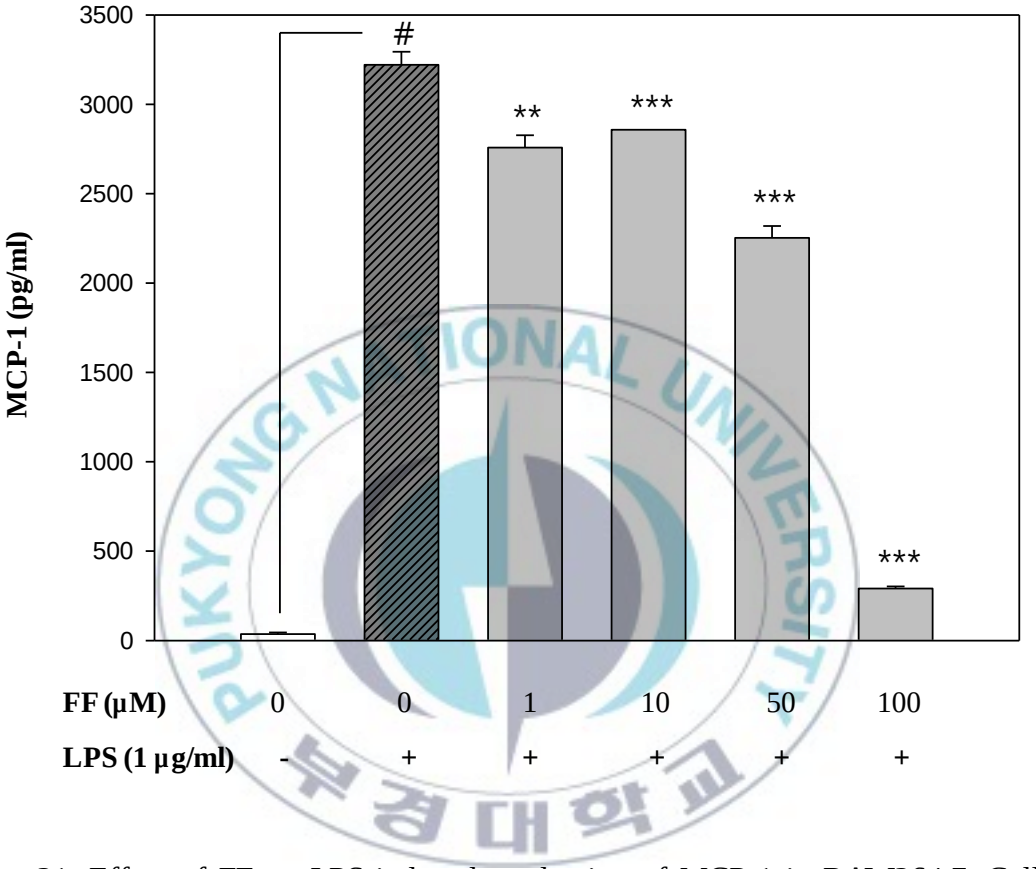



Fig. 21. Effect of FF on LPS-induced production of MCP-1 in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The MCP-1 concentration was measured in culturemedia using a commercial CBA (cytometric bead array) kit using flow cytometery. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

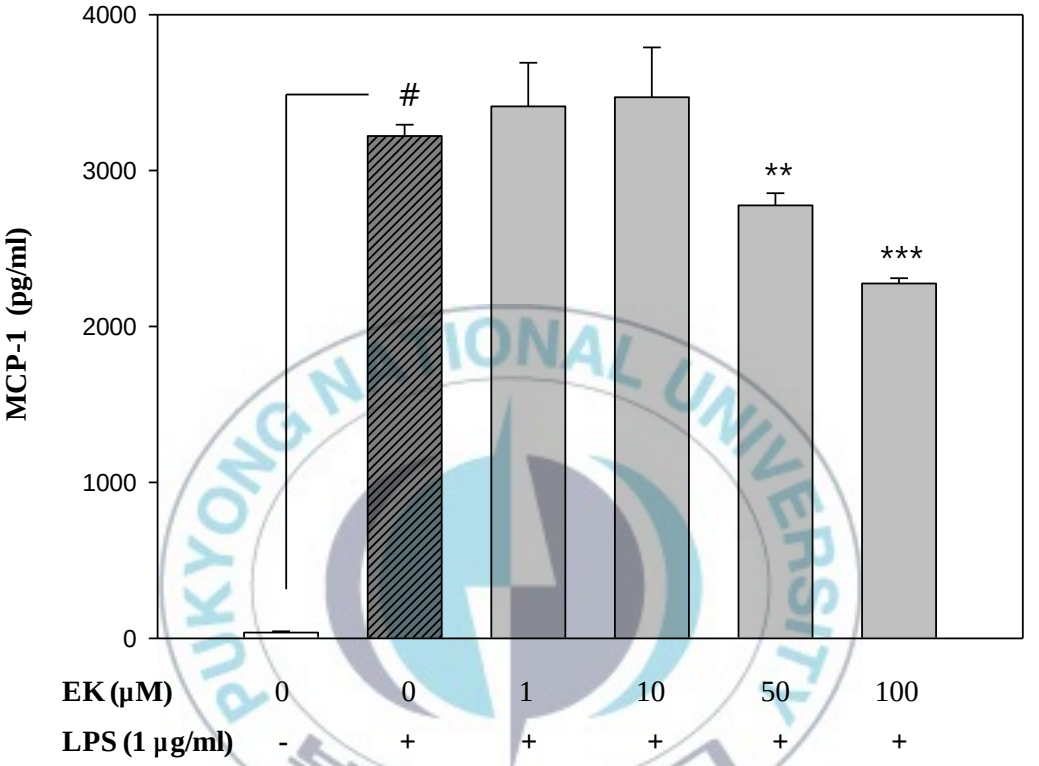



Fig. 22. Effect of EK on LPS-induced production of MCP-1 in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The MCP-1 concentration was measured in culturemedia using a commercial CBA (cytometric bead array) kit using flow cytometery. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

inflammation resulted from the implantation of biomaterials

### 3.4. Effects of FF and EK on productions of prostaglandin $E_2$ ($PGE_2$) in LPS-stimulated RAW264.7 cell line

$PGE_2$ represents the most important inflammatory product of COX-2 activity and, thus, it was quantified in the supernatant. To assess whether FF and EK could inhibit production of LPS-induced $PGE_2$ in RAW264.7, the cells were pretreated with dieckol for 2 h and then stimulated with 1 μg/mL. After incubation for 24 h, the cell culture medium was harvested, and the production of $PGE_2$ was measured using ELISA. Pretreatment of the cells with FF and EK (1, 10, 50 and 100 μM) and LPS resulted in a significant dose-dependent reduction in $PGE_2$ production (Figures 23 and 24). Non-steroidal anti-inflammatory drug (NSAID), such as Ketoprofen, has been shown to have therapeutic potential in the treatment of inflammatory disease via the inhibition of the COX, which is responsible for the biosynthesis of prostaglandins (Offenbacher et al., 1990). Ketoprofen, however, has side effects such as, gastrointestinal ulcers and kidney damage, due to concomitant inhibition of COX-1 (Whelton and Hamilton, 1991). Even though, 10 μM of Ketoprofen showed strong inhibitory activity on productions of prostaglandin $E_2$ with 97.6% $PGE_2$ inhibition in this study (data not shown), the search for non-toxic inflammatory drug from natural bioresources is of great interest among researcher. As shown in figure 23 and 24, pretreatment with FF and EK significantly suppresses the expression of LPS-stimulated pro-inflammatory mediators dose-dependently. At the concentrations of 100 μM FF showed strong suppression activity against production of $PGE_2$ with 4.3% of relative production compared to control

### 3.5. Effects of FF and EK on productions of iNOS and COX-2 in LPS-stimulated RAW264.7 cell line

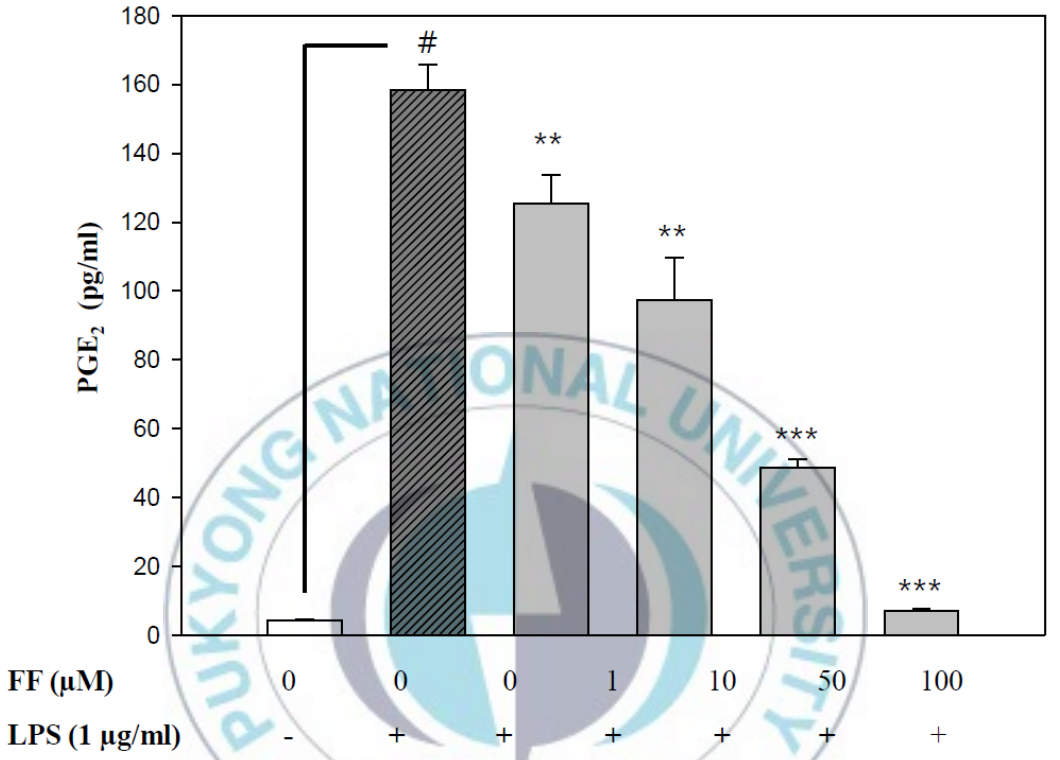



Fig. 23. Effect of FF on LPS-induced production of $PGE_2$ in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The IL-1β concentration was measured in culturemedia using a commercial ELISA kit. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

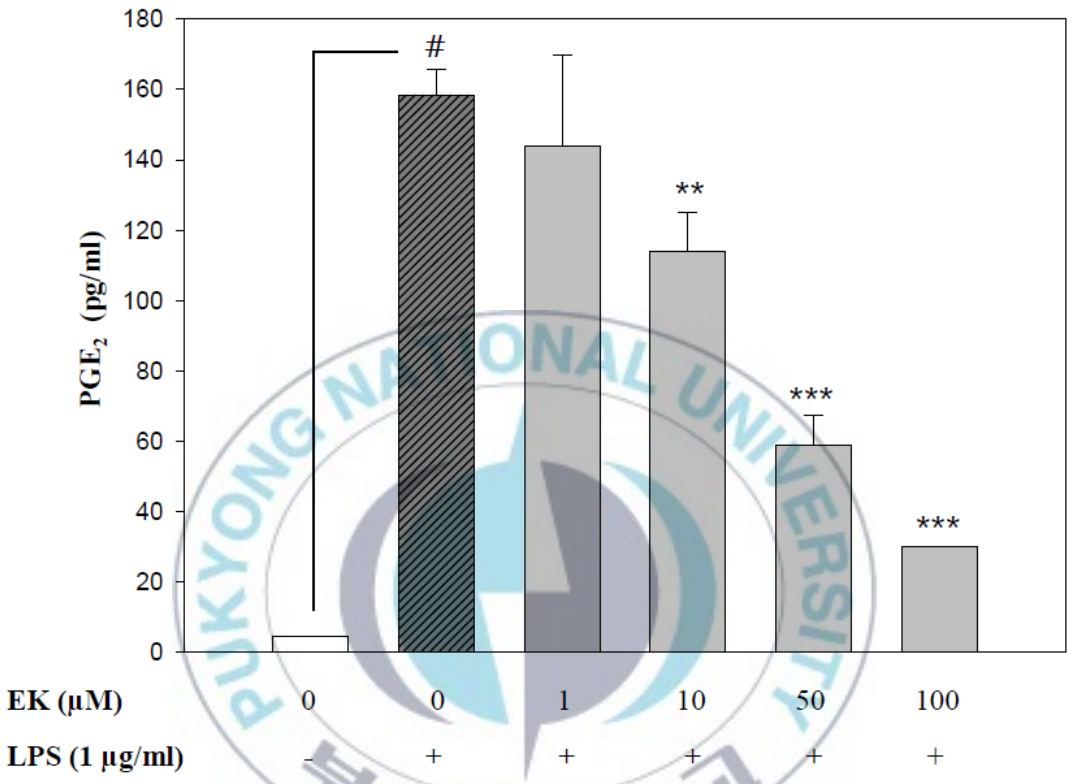



Fig. 24. Effect of EK on LPS-induced production of $PGE_2$ in RAW264.7. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, The IL-1β concentration was measured in culturemedia using a commercial ELISA kit. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with LPS-treated cells (1 μg/mL). $^{\#}$,$P < 0.001$ as compared with LPS non-treated cells.

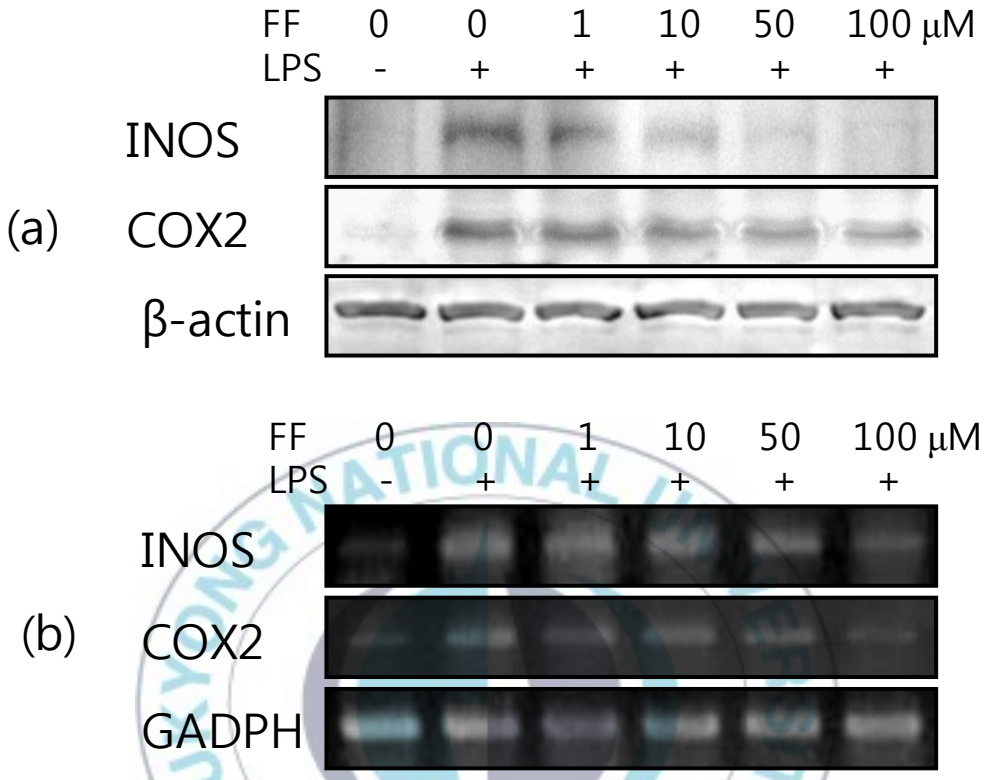



Fig. 25. Effect of FF on LPS-induced protein (a) and mRNA (b) expressions of iNOS and COX-2 in RAW 264.7 macrophages. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 12h (RT-PCR) and 24 h (Western blot), (a) Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for INOS and COX-2. β-actin was used as an internal control. (b) The levels of INOS and COX-2 mRNA were determined using RT-PCR Analysis and GAPDH was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

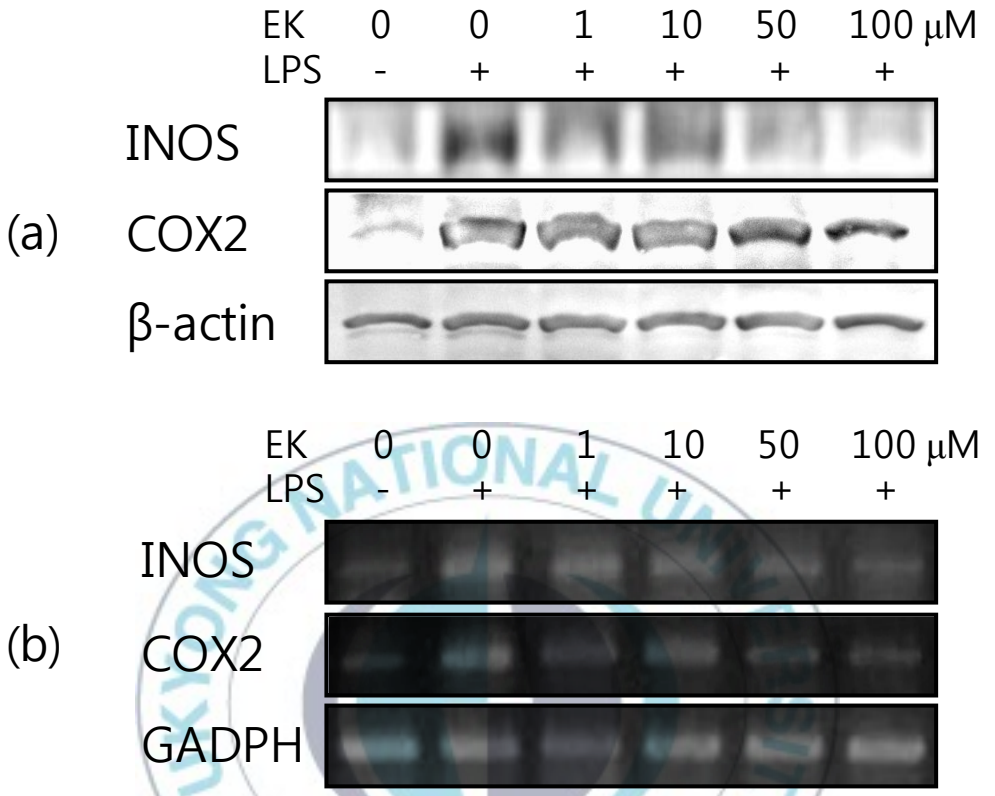



Fig. 26. Effects of EK on LPS-induced protein (a) and mRNA (b) expressions of iNOS and COX-2 in RAW 264.7 macrophages. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 12h (RT-PCR) and 24 h (Western blot), (a) Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for INOS and COX-2. β-actin was used as an internal control. (b) The levels of INOS and COX-2 mRNA were determined using RT-PCR Analysis and GAPDH was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

To confirm whether the inhibition of NO and PGE2 production is due to a decreased level of iNOS and COX-2, the effects of FF and EK on the level of iNOS and COX-2 protein and mRNA was determined by Western blot analysis and RT-PCR, respectively. The expressions of iNOS and COX-2 proteins were barely detectable in unstimulated in RAW264.7 cell line, but markedly increased after 24 h of LPS (1 μg/mL) treatment. However, FF and EK significantly attenuated iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 (Figures 25a and 26a). The effects of FF and EK on iNOS and COX-2 mRNA expression were also evaluated (Figure 25b and 26b). RT-PCR analysis also showed that iNOS and COX-2 mRNA expression correlated with their protein levels. These results indicate that LPS exposure increased the expression of iNOS and COX-2 mRNA and protein, but treatment with FF and EK significantly suppressed the induction of LPS-stimulated mediators through transcriptional inhibition.

### 3.6. Effects of FF and EK on productions of TNF-α, IL-6 and IL-1β in LPS-stimulated RAW264.7 cell line

We attempted to determine the potential effects of FF and EK on the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β. RAW264.7 cells were incubated with different concentrations of FF and EK (1, 10, 50 and 100 μM) in the presence or absence of LPS (1 μg/mL) for 24 h, and the cytokine levels were measured in cytoplasmic extract using western blot assay. As shown in figures 27a and 28b, the TNF-α, IL-6 and IL-1β levels were increased in the culture media of LPS-stimulated RAW264.7 cells, and were significantly decreased in a dose-dependent manner by treatment with FF and EK. In a parallel experiment, we performed RT-PCR to determine whether FF and EK inhibit the expression of these cytokines at a transcriptional level. As shown in figures 27b and 28b, treatment of various concentrations of FF and EK 2 h before LPS treatment resulted in a dose-

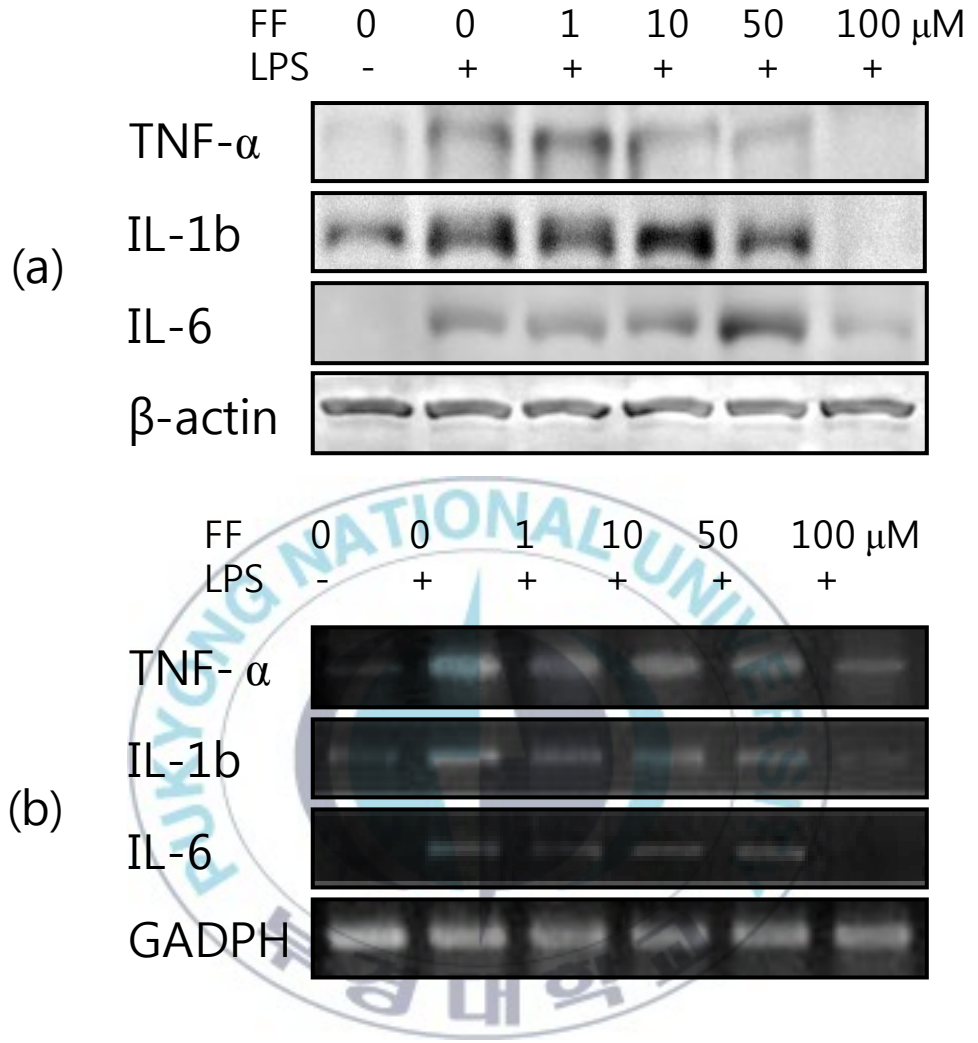



Fig. 27. Effects of FF on LPS-induced protein (a) and mRNA (b) expressions of TNF-α, IL-1β and IL-6 in RAW 264.7 macrophages. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 12h (RT-PCR) and 24 h (Western blot), (a) Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for INOS and COX-2. β-actin was used as an internal control. (b) The levels of INOS and COX-2 mRNA were determined using RT-PCR Analysis and GAPDH was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

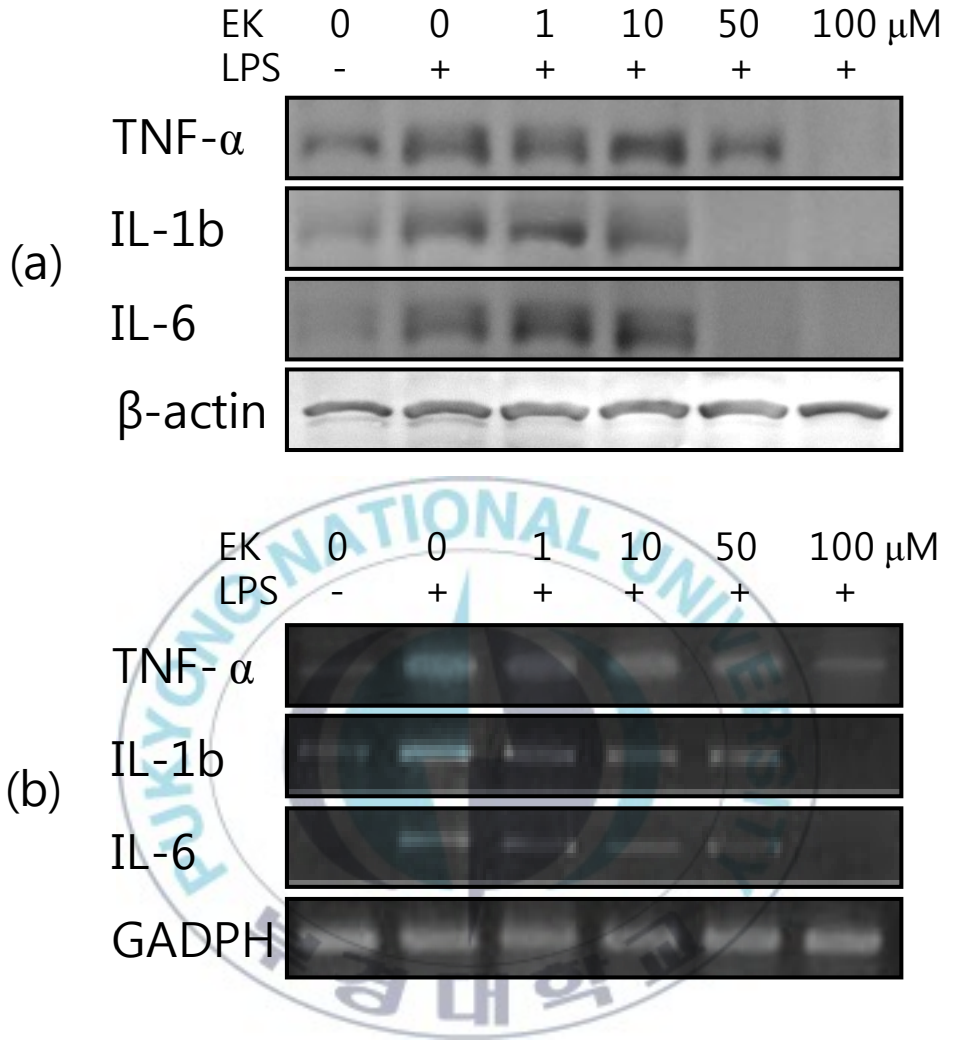



Fig. 28. Effect of EK on LPS-induced protein (a) and mRNA (b) expression of TNF-α, IL-1β and IL-6 in RAW 264.7 macrophages. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 12h (RT-PCR) and 24 h (Western blot), (a) Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for TNF-α, IL-1β and IL-6. β-actin was used as an internal control. (b) The levels of TNF-α, IL-1β and IL-6 mRNA were determined using RT-PCR Analysis and GAPDH was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using

dependent decrease in IL-1β and TNF-α mRNA. The results suggest that dieckol negatively regulates the accumulation of pro-inflammatory cytokines at the transcriptional level.

### 3.7. Effects of FF and EK on LPS-induced Nuclear translocation of NF-κB and I-κBα.

NF-κB is one of the principal factors for COX-2 and iNOS expression mediated by LPS or proinflammatory cytokines. We investigated the effect of FF and EK on LPS-induced NF-κB p65 nuclear translocation as measured by Western blot analysis, because translocation of NF-κB to the nucleus has been shown to be required for NF-κB-dependent transcription following LPS stimulation. As shown in figures 29 and 30, significant levels of NF-κB p65 was localized to the nucleus at 1 h after LPS treatment. The p65 protein decreased in the nucleus of cells exposed to LPS in combination with FF and EK, which verified that these phlorotannins inhibited nuclear translocation of p65 protein. The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by the increasing of phosphorylated-inhibitory factor-κBα (p-I-κBα). To determine whether the inhibitions of NF-κB DNA binding by FF and EK are related to p-I-κBα, cytoplasmic levels of p-I-κBα were examined by Western blot analysis (Figures 29 and 30). Pretreatment of RAW264.7 cells with FF and EK blocked LPS-induced p-I-κBα. The decrease of p-I-κBα protein in RAW264.7 cells provides strong evidence that FF and EK inhibited the activation of NF-κB. To clearly understand the influences of FF and EK on the NF-κB p65 nuclear translocation, the NF-κB p65 nucleus shift situation in RAW264.7 cells were determined by immunofluorescence analysis (Figure 31). After fixation, the cells were stained with anti-p65 antibody and observed at 400 x magnification. Confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasmic compartment (Figure 31), and nuclear accumulation of NF-κB

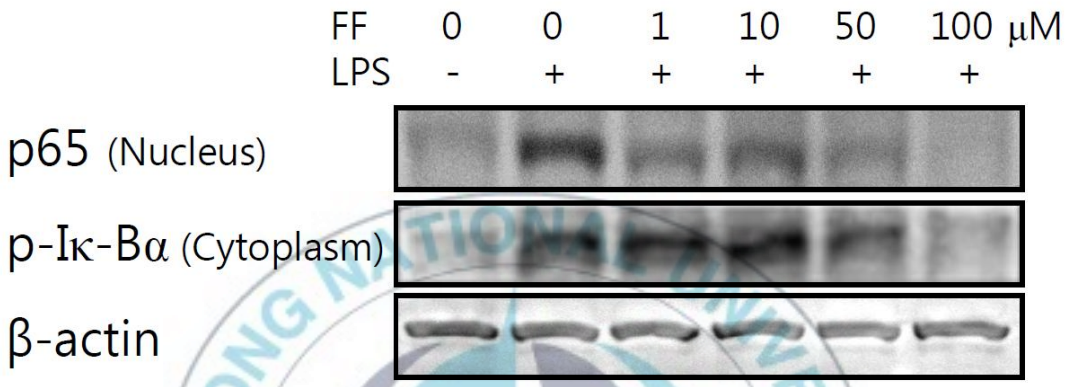



Fig. 29. Effects of FF on NF-κB translocation and p-Iκ-Bα expression in LPS-induced RAW 264.7 macrophages. Western blot analysis showing the effect of FF treatment on expression of p65 and p-Iκ-Bα in cytoplasmic and nuclear extracts. Cells were grown at 5 x $10^{5}$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for p65 and p-Iκ-Bα. β-Actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

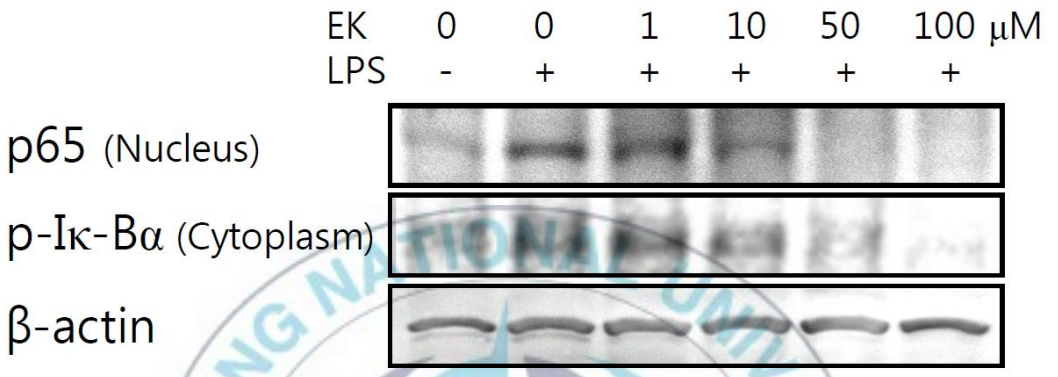



Fig. 30. Effects of EK on NF-κB translocation and p-Iκ-Bα expression in LPS-induced RAW 264.7 macrophages. Western blot analysis showing the effect of FF treatment on expression of p65 and p-Iκ-Bα in cytoplasmic and nuclear extracts. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for p65 and p-Iκ-Bα. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

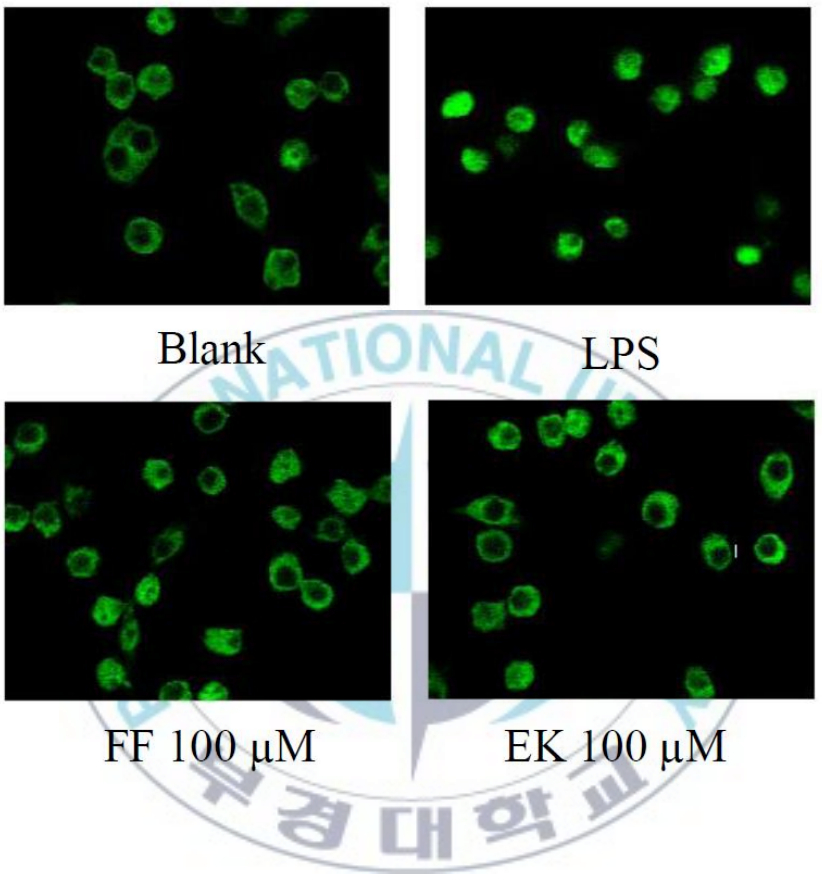



Fig. 31. Effects of FF and EK on NF-κB nuclear localization in LPS-stimulated RAW 264.7 macrophage. Cells were pretreated with the 100 μM of FF and EK for 1 h before stimulation with LPS (1 μg/mL) for 2 h. The p65 protein localization in cells was determined with an anti-p65 antibody and a FITC-labeled anti-rabbit IgG antibody using confocal laser scanning microscopy.

p65 was strongly induced after stimulation of RAW264.7 with LPS. The LPS-induced translocation of NF-κB p65 was completely abolished by pretreatment of the cells with FF and EK. The results showed that FF and EK inhibited the translocation of NF-κB p65. To characterize further the inhibitory mechanisms of FF and EK on expression of iNOS and COX-2, NF-κB DNA-binding activity was determined by an electrophoretic mobility shift assay (Figure 32). LPS treatment caused a significant increase in the DNA-binding activity of NF-κB. In contrast, the treatments of EK markedly suppressed the induced activity of NF-κB by LPS. However, FF didn't showed significant suppression of NF-κB DNA-binding acitivity. NF-κB, signal transducer, is one of the most important transcription factors and expressed ubiquitously and regulates the expression of many genes, most of which encode proteins that play an important role in the processes of immune response and inflammation. In most cells, the NF-κB dimmers, complex of p65 and p50, are retained by an inhibitor (Iκ-B) in the cytoplasm of non-stimulated cells. Following various stimuli such as ROS and pro-inflammatory cytokines, Iκ-B is phosphorylated by the IKK kinase complex, polyubiquitinated and degraded. Then, the nuclear localization of NF-κB is induced and activate the nuclear translocation of the transcription factor and the induction of its target genes which encode pro-inflammatory molecules as well as pro or anti-apoptotic proteins (Wang et al., 1999). NF-κB transactivation can be suppressed by inhibiting its DNA bionding or inhibiting phosphorylation of Iκ-B via the NIK/IKK signaling pathway. These results clearly showed that the inhibition of NF-κB DNA binding activation by EK may be the mechanism responsible for the suppression of NO, PGE2, and pro-inflammatory cytokines in RAW264.7 macrophages. Even though FF didn't inhibit the NF-κB DNA-binding activity, FF showed inhibitory effect on translocation of NF-κB p65 and it may be responsible for its the suppression of NO, PGE2, and pro-inflammatory cytokines in RAW264.7 macrophages.

### 3.8. Effects of FF and EK on the phosphorylation of MAPKs in LPS-stimulated RAW264.7 cell line.

The subsequent experiments were designed to elucidate the signaling cascades, which turn on the expression of iNOS and COX-2 gene in RAW264.7 cells in response to stimulation by LPS. Evidence has accumulated that the mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. They play a critical role for the activation of NF-κB. Moreover, MAP kinase has been known to be important for the expression of iNOS and COX-2 expression. To investigate whether the inhibition of NF-κB activation by FF and EK is mediated through the MAP kinase pathway, we examined the effect of these phlorotannnins on the LPS-induced phosphorylation of ERK-1/2, JNK, and p38 kinase in RAW264.7 cells using Western blot analyses (Figures 33 and 34). In our study, we confirmed that ERK-1/2, JNK, and p38 kinase were phosphorylated by stimulation with LPS. As shown in Figures 33 and 34, Both FF and EK (100 μg/mL) remarkably inhibited JNK and p38 kinase activation, whereas phosphorylation of ERK-1/2 was not affected by FF and EK treatment. These results suggest that phosphorylation of JNK and p38 is involved in the inhibitory effect of FF and EK on LPS-induced iNOS and COX-2 expression in RAW264.7 cells.

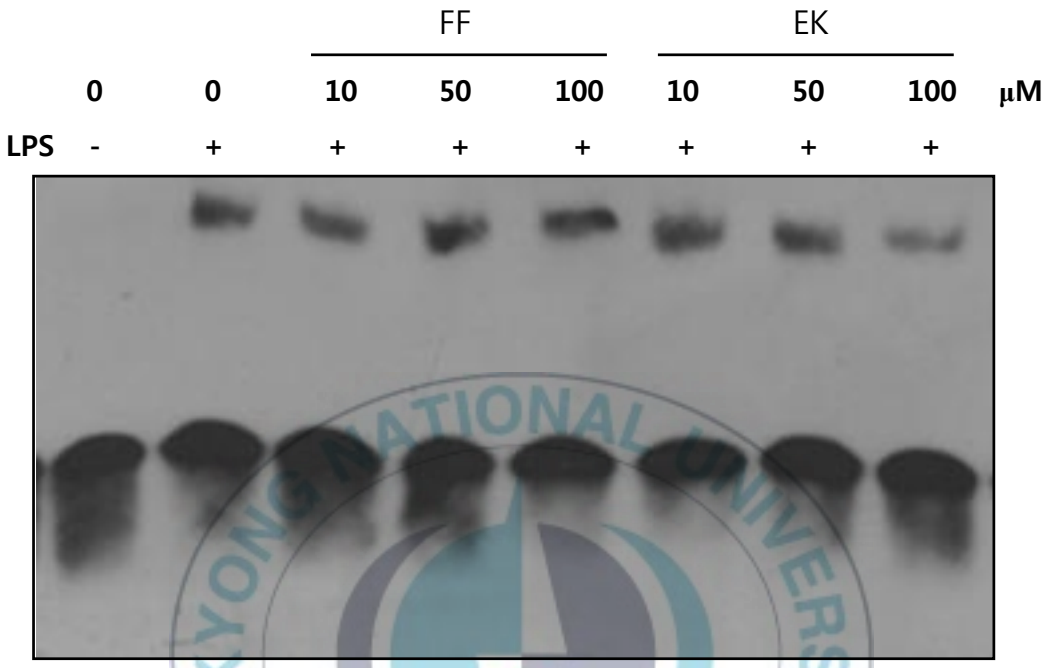



Fig. 32. Effects of FF and EK on NF-κB/p65 binding activities using an electrophoretic mobility shift assay (EMSA). RAW264.7 cells were pretreated with indicated concentrations of FF and EK for 2 h before stimulation with LPS (1 μg/mL) for another 2 h. Nucleus DNA was biotin-labeled using the Biotin 3′ End DNA Labeling kit The binding reactions were incubated for 20 min at RT in a final volume of 20 μL. The reaction mixture was subjected to gel electrophoresis on 5% polyacrylamide gel and transferred to a nylon membrane. DNA was cross-linked to the membrane using UV cross-linker at 120 mJ/cm$^2$ and spectral peak was detected at 312 nm using image analyzer.

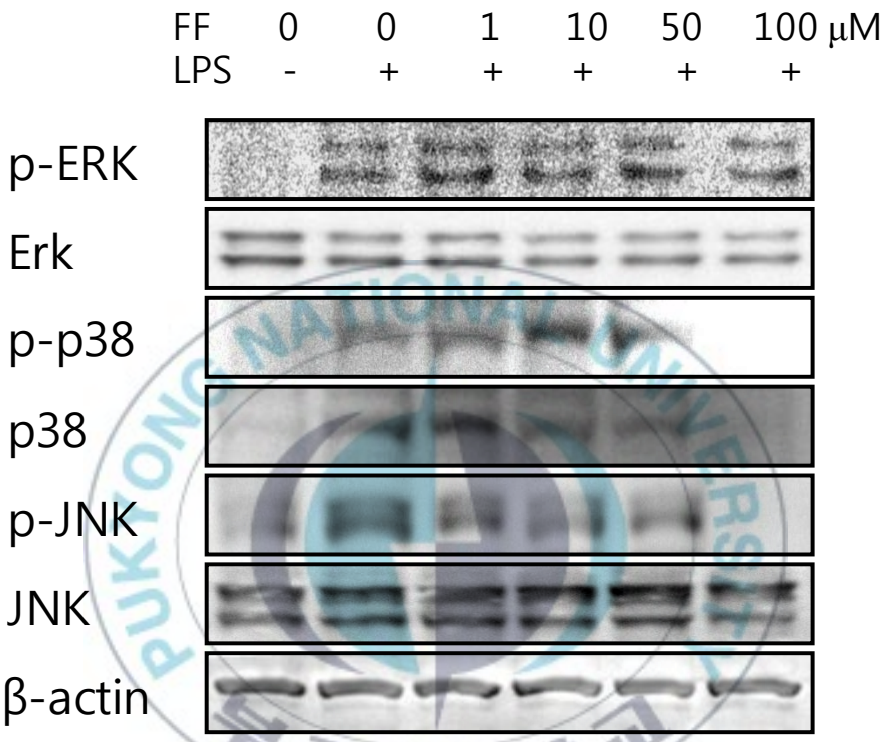



Fig. 33. Effect of FF on LPS-induced protein expression of MAPKs in RAW 264.7 macrophages. Cells were grown at 5 x $10^{5}$ cell/well and treated with various concentrations of FF at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for ERK, p38 and JNK including their posphorylated forms. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

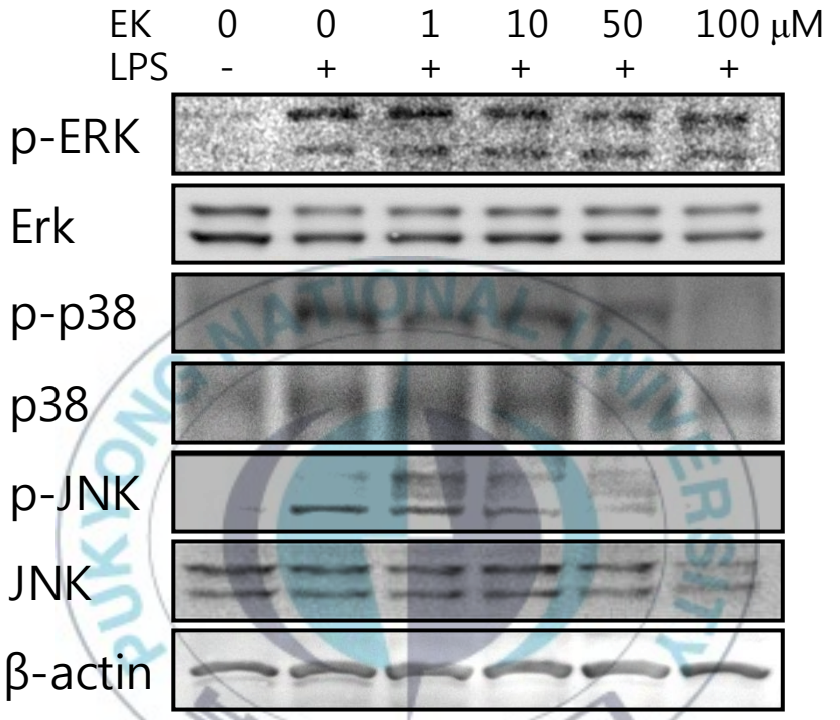



Fig. 34. Effect of EK on LPS-induced protein expression of MAPKs in RAW 264.7 macrophages. Cells were grown at 5 x $10^{5}$ cell/well and treated with various concentrations of EK at 2 h before LPS (1 μg/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for ERK, p38 and JNK including their posphorylated forms. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

## 4. Summary

In the present study, we evaluated FF and EK, which are phlorotannins derivatives of *E. bicyclis* having biological effects on the production of inflammatory mediators in LPS stimulated RAW264.7 macrophages. We investigated the effects of FF and EK on the productions of NO, $PGE_2$, expression of iNOS and COX-2, cytokines (TNF-α, IL-1β, IL-6) and chemokines (MCP-1). These results clearly indicate that FF and EK are effective inhibitors of LPS-induced cytokines, chemokines and expression of iNOS and COX-2 through the blockages of NF-κB and MAPK pathway in RAW264.7 cells. Furthermore, regarding their strong antioxidant activities we evaluated in chapter Ⅱ FF and EK inhibit the production of pro-inflammatory cytokines via scavenging ROS. Therefore, FF and EK may have beneficial effects in the treatment of ROS and inflammatory reaction. Marine algae are generally considered attractive source that can be used as a new chemopreventive agents and have wide range of bioactive components. Among their components which are responsible for their various bioactivities, polyphenols such as phlorotannins are pharmacologically prominent compounds, which are found in the form of organic polymers with polymerized phloroglucinol units. They have shown strong antioxidant activities and various therapeutic perspectives. EK is one of the major phlorotannins isolated from Ecklonia species and exhibits a variety of biological and pharmacological activities such as free radical scavenging activity (Heo et al., 2009), bactericidal activity (Nagayama et al., 2002), antiplasmin inhibiting activity (Fukuyama et al., 1989), anti-allergic activity (Sugiura et al., 2007) and matrix metalloproteinase (Joe et al., 2006) inhibitory activity, and anti-allergic activity (Sugiura et al., 2007). On the other hand, there are no biological reports about FF and its biological activity in this paper is the first report. In the present study, we report that FF and EK decreases NO and $PGE_2$ overproduction in LPS

activated RAW264.7 macrophages. Furthermore, we found that FF and EK attenuated the mRNA and protein expressions of iNOS and COX-2. These results indicate that their actions occur at the transcriptional level. This study also evaluate the suppressive effects of these phlorotannins on the production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and pro-inflammatory chemokine (MCP-1). Suppression of these cytokines and chemokine is important for the prevention of various diseases related to inflammation.

In this study, we described the anti-inflammatory mechanisms of FF and EK, which are based on the inhibition of LPS-mediated activation of NF-κB signaling pathway. Our results clearly indicate that FF and EK showed remarkable anti-inflammatory activities dose dependently without any cytotoxic effect, provide their strong potential as a promising therapeutic candidates for inflammatory diseases. NF-κB, which is well-known as a critical regulator of various inflammatory genes, plays an important role in cellular proliferation and immune and inflammatory responses. It has been shown that NF-κB activation is a factor critical to the expression of various cytokines and enzymes. Inducers of NF-κB activation include pro-inflammatory cytokines, growth factors, microbial infections, endotoxin, and oxidant stress (Gerondakis et al., 1998). It has been reported that NF-κB binding to its binding sites plays an important role in the LPS-induced upregulation of the iNOS and COX-2 genes (Lee et al., 2003). The activity of NF-κB is regulated by its interaction with an inhibitor protein called I-κB family of proteins and this interaction appears to block the nuclear localization of NF-κB. LPS induced NF-κB activation through increasing nuclear p65 protein associated with decreased cytosolic I-κB protein, followed by the translocation of NF-κB into the nucleus to activate genes with NF-κB binding to its sites. NF-κB is an important transcription factor for iNOS, COX-2, and pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in LPS-stimulated RAW 264.7. Because the NF-κB binding activity is known to regulate the expression of pro-inflammatory mediators, cytokines and chemokines, we performed EMSA to

ensure the possibility that FF and EK inhibits NF-κB activity. This result suggested that FF and EK treatment inhibit the degradation of I-κB to p-I-κB, and NF-κB activation induced by LPS in RAW264.7. However, FF didn't exhibit inhibitory activity on NF-κB DNA binding in RAW264.7.

During inflammatory processes, ROS involved in signal transduction and gene activation, and can contribute to host cell and organ damage (Adler et al., 1999). Excessive generation of ROS stimulate the productions of pro-inflammatory cytokines and chemokines via activation of NF-κB. It has been reported that anti-inflammatory effects of antioxidants are due to increasing antioxidant enzyme and their ROS scavenging activities (Conner and Grisham, 1996). Moreover, changes in intracellular ROS can regulate the redox signal transduction pathway, leading to modulation of NF-κB activity. Scavenging of ROS by antioxidants could inhibit the NF-κB dependent production of pro-inflammatory mediators (Victor et al., 2005).

ROS, including superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide, may have multiple roles in the pathogenesis of a number of diseases. In the previous chapter Ⅱ, we demonstrated that FF and EK have an intracellular ROS scavenging activity in RAW264.7 cells, and these results suggest another possible mechanism responsible for their inhibitory effect of on NF-κB activation. Thus, potential inhibition of ROS generation by FF and EK may reduce inflammation by inhibition of NF-κB-dependent cytokines, iNOS and COX-2 expression.

Various intracellular signaling pathways such as MAPKs are involved in the modulation of NF-κB activity and inflammatory cytokine expression. It has been reported that activation of MAPK significantly regulate the iNOS and COX-2 expression by controlling the activation of NF-κB (Moon et al., 2007). It is possible that anti-inflammatory mechanisms are related to MAPKs or the inhibition of NO and $PGE_2$ production. Therefore, we investigated the effects of FF and EK on phosphorylation of ERK-1/2, JNK, and p38 kinase in LPS-stimulated RAW 264.7

cells. Interestingly, the phosphorylation of JUN and p38 kinase in response to LPS ware decreased by FF and EK treatment, however, no significant changes by these phlorotannins in the LPS-induced phosphorylation of ERK-1/2 was observed. Hence, these results suggest that JNK and p38, but not ERK-1/2 is involved in the inhibitory effect of FF and EK on LPS-induced iNOS and COX-2 expression and NF-κB activation. In conclusion, the results obtained in this study indicate that FF and EK derived from *E. bicyclis* treatment of RAW264.7 cells results in decreased proinflammatory cytokines and mediator following LPS stimulation. These phlorotannins significantly inhibited the release of NO, $PGE_2$, TNF-α, IL-1β and IL-6 in a dose-dependent manner, and it acts at the transcription level. The anti-inflammatory properties of FF and EK are mediated by down-regulation of NF-κB, JNK and p38 kinase activations, and/or inhibition of ROS generation in RAW264.7 cells. These studies indicate that phlorotannins derived from *E. bicyclis* appears to have the potential to target JNK and p38 kinase, NF-κB, and inhibit iNOS and COX-2 expression in inflammatory diseases.

# Chapter Ⅳ

# Inhibitory effects of phlorotannins derived from brown alga *Eisenia bicyclis* on MMP-2 and -9 expressions in HT1080 cell line

## 1. Introduction

In biological system oxidative stress is a general term used to identify the level of oxidative damage in cellular biomolecules caused by the oxygen and its reactive oxygen species (ROS) or free radicals. ROS, such as superoxide anions, hydrogen peroxide and hydroxyl radicals, generated from various aerobic cells during normal metabolism and have physiological roles as secondary mediators in multiple cell signaling pathways (Finkel and Holbrook, 2000; Nathan, 2003). Equilibrium between oxidant formation and endogenous antioxidant defense mechanism exists to protect a cell, tissue, or organ against oxidation by ROS. However, ROS levels can increase dramatically due to the excessive production of active oxygen or imbalance of redox system in human body, and this cause significant damage to cellular components such as DNA, lipids, and proteins by a variety of different mechanism (Diplock et al., 2007). ROS and free radicals are very unstable due to unpaired electron and excessive generation of ROS cause cell or tissue injury leading to cell death (Kang et al., 2005). There are a number of direct or indirect involvement of ROS with oxidative process of cellular components and human diseases such as cancer, inflammation, arthritis, Alzheimer's, diabetes and aging (Calabrese et al., 2005; Je et al., 2004; Vajragupta et al., 2000). Furthermore, ROS have been demonstrated to be involved in the progression of tumor-induced angiogenesis because matrix metalloproteinase (MMP) expression is regulated by the intracellular redox state (Inoue et al., 2001; Yoon et al., 2002). Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidase and degrade specific components of extracelluar matirixes (ECMs), which has long been considered in association with both normal tissue remodeling and pathologic conditions. MMPs share certain biochemical properties, yet each has distinct substrate specificity and up to date several mammalian enzymes have been identified ranging from well-characterized enzymes such as collagenase, stremolysin, gelatinase and more recently described

membrane type MMPs

The MMPs are categorized simplistically into three major functional groups, in part based on substrate specificity. The interstitial collagenases (MMP-1, -8, and -13), that preferentially have affinities toward collagen types I, II, and III, the stromelysins (MMP-3, -10, and -11) with specificity for laminin, fibronectin, and proteoglycans, and the gelatinases (MMP-2 and -9), which most effectively cleave type IV and V collagen are the main three groups (Nelson et al., 2000). Regulation of gene expression of most MMPs is controlled by two major transcription factors, NF-κB and AP-1 (Benbow and Brinckerhoff, 1997). MMP promoter contains an AP-1 binding consensus site at -79 upstream from the starter site and further upstream there is a cluster of regulatory elements including another AP-1 binding site and NF-κB binding site. However depending on the type of MMP, these transcription factors affect differentially to regulate their gene expressions. Under normal physiological conditions, MMP transcripts are generally expressed at low levels, but these levels rise rapidly when tissues are locally induced to undergo remodeling events such as inflammation, wound healing, cancer, and arthritis (Coussens et al., 2002). The expressions of most MMPs are transcriptionally regulated by growth factors, hormones, cytokines, cell–matrix, cell–cell interactions and cellular transformation. These enzymes act primarily on the cell surface or in the extracellular space and the activities are controlled by a combination of zymogen activation and inhibition by endogenous inhibitors like $\alpha_2$-macroglobulin and the tissue inhibitors of metalloproteinases (TIMPs). While $\alpha_2$-macroglobulin and related inhibitors are primarily the regulators in the fluid phase, TIMPs are considered to be the key inhibitors in tissue. Mammalian TIMPs are two-domain molecules, having N-terminal domains of about 125 amino acids and a smaller C-terminal domain of about 65 residues, each domain being stabilized by three disulfide bonds (Williamson et al., 1990). N-terminal domains are capable, in isolation, of forming a stable native molecule which has inhibitory activity against MMPs (Huang et al.,

1996; Murphy et al., 1991; Willenbrock et al., 1993). Currently, four TIMPs (TIMP-1 to TIMP-4) have been identified in vertebrates and homologous inhibitors have also been found in *Drosophila* (Pohar et al., 1999) and *Caenorhabditis elegans*. Like matrixins, the expression of TIMPs in the tissue is also controlled during tissue remodeling and physiological conditions to maintain a balance in the metabolism of the extracellular matrix (Das et al., 1997; Gomez et al., 1997). Disruption of this balance may result in diseases associated with uncontrolled turnover of matrix, such as arthritis, cancer, cardiovascular diseases, nephritis, neurological disorders, tissue ulceration and fibrosis. Carcinogenesis is a multi-step process that includes tumor initiation, promotion, and progression. In particular, cancer metastasis, such as tumor migration or invasion, is responsible for the deaths of cancer patients (Saha et al., 2001). Zeng et al suggested that both suppression of the expression of MMP and stimulation of TIMP inhibit the migration and invasion potential of tumor cells (Zeng et al., 2006).

Therefore, inhibition of MMP activities and stimulation of TIMP expression in the extracellular space has been extensively studied as an approach to inhibit metastasis. At present, several MMP inhibitors are under clinical trials and most of these MMP inhibitors are synthetic peptides, chemically modified tetracyclines, bisphosphonates or compounds isolated from natural sources. However, most of these drugs are reported to exert side effects such as, musculoskeletal pain in tendons and joints (Nelson et al., 2000).

In previous chapters, we evaluated the antioxidant and anti-inflammatory activities of phlorotannins derived from *E. bicyclis.* There are many reports that ROS have been demonstrated to be involved in the progression of tumor-induced angiogenesis and chronic inflammation plays a multifaceted role in carcinogenesis. Chronic inflammation represents a major pathologic basis for the majority of human malignancies. The role of inflammation in carcinogenesis has first been proposed by Rudolf Virchow in 1863, when he noticed the presence of leukocytes in neoplastic

tissues (Balkwill and Mantovani, 2001). Since the Virchow's early observation that linked inflammation and cancer, accumulating data have supported that tumors can originate at the sites of infection or chronic inflammation (Mueller and Fusenig, 2004). Approximately, 25% of all cancers are somehow associated with chronic infection and inflammation (Hussain and Harris, 2007). Although inflammation acts as an adaptive host defense against infection or injury and is primarily a self-limiting process, inadequate resolution of inflammatory responses often leads to various chronic ailments including cancer (Jackson and Evers, 2006; Schottenfeld and Beebe-Dimmer, 2006). Therefore, in this study we carried out a detailed study to investigate the inhibitory effects of fucofuroeckol-A (FF) and eckol (EK) derived from *E. bicyclis on* MMP-2 and 9 activation in HT1080, which express MMP-2 and -9 at a high level (Oba et al., 2002) and expressions for developing safe and effective chemoprevention materials.

## 2. Method and materials

### 2.1. Materials and chemicals

Fucofuroeckol-A (FF) and eckol (EK) were isolated from EtOAc fraction of *E. bicyclis* as described in chapter Ⅱ. Human fibrosarcoma cell line (HT1080) was obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS), and other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (Kousaka et al.). Primary and secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (CA,USA) and Amersham Pharmacia Biosciences (NJ,USA), respectively. MMP-2 and 9 fluorometric assay kit, and MMP-2 and 9 enzymes were purchased from Anaspec (CA, USA). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

### 2.2. Cell culture

Human fibrosarcoma (HT1080) cell line was maintained in Dulbecco's modification of eagle's medium (DMEM, GIBCO, New York, USA) supplemented with 100 μg/mL penicillin–streptomycin, 10% fetal bovine serum (FBS) at 37 ℃ in a humidified incubator under 5% $CO_2$. Confluent cultures were washed twice with PBS and then collected with scraper. Collected cells were resuspended in DMEM and seeded to cell culture dish or well plates.

### 2.3. Cell viability assay

Cytotoxicity levels of the samples on HT1080 were measured using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen *et al* with slight modifications (Hansen et al., 1989). HT1080 cells were cultured in 96-well plates at a density of $1 \times 10^5$ cells/well. After 24 h, cells were washed with fresh medium and treated with various concentrations of samples. After incubation for 24 h, cells were washed two times with PBS and 100 μL of MTT solution (1 mg/ml) was added to each well for 3 h. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) were added to solubilize the formed formazan salt. Amount of formazan salt was determined by measuring the OD at 540 nm using UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the non-treated group ((OD of non-treatment group – OD of treatment group) / OD of non-treatment group x 100). The data were expressed as means of at least three independent experiments. Each value was expressed as the mean ± SD of triplicate experiments

### 2.4. In vitro wound migration assay

A cell migration assay was performed as previously described (Lee et al., 2006). HT1080 Cells were grown at density of 5 x $10^5$ cell/well in a 24-well plate and confluent monolayer were wounded with 1 mm width tip. Then cells, which have injury line, incubated with treatment of various concentrations of samples in serum-free medium. After 0 and 24h incubatons, the migrations of wounded cells were photographed under a phase contrast microscope (Carl Zeiss, Jena, Germany).

### 2.5. In vitro three-dimensional (3D) culture of HT1080 cell line

The 3D culture model was established as described previously (Hotary et al., 2003). Briefly, HT1080 cell line ($1.5\times10^3$) were suspended with a neutralized solution of

type IV collagen (200 μg/mL) and 1/5 volume of a 5×DMEM. The cell suspension containing various final concentrations of samples was added to 24-well plates and kept at 37℃ until gelled. The plates were then incubated at 37℃ for 36 h. The results were observed on microscope (Carl Zeiss, Jena, Germany).

### 2.6. MMP-2 and 9 enzyme inhibitory assay

Relative enzymatic activities of MMP-9 levels in HT1080 cells were performed using commercial MMP-2 and 9 assay kit according to the manufacturer's instruction. Briefly, 50μl of assay buffer solution containing MMP-2 and 9, activated by 1mM APMA for 2 h, was treated with various concentrations of each sample for 15 min at 37℃. Subsequently, 50μl of substrate solution was added and incubated for 1 h at 37℃ in the dark. Finally 50μl of stop solution was added and the fluorescence intensity (Ex/Em = 340 nm/490) was measured using ELISA reader (Tecan Austria GmbH, Groedig, Austria). Each value was expressed as the mean ± SD of triplicate experiments.

### 2.7. Western blot analysis

HT1080 cells were treated with different concentrations of sample for 1 h before incubation with PMA (1 μg/mL) for 24 h. To obtain the cytoplasmic protein, cells were harvested and washed 2 times with PBS. Collected cells were resuspended with lysis buffer (50 mM Tris-HCl (pH 7.5), 0.4 % Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/mL leupeptin, 3 mM NaF and 1 mM DTT) and incubated at 4 ℃ for 20 min. Cell lysates were centrifuged at 12,000 x *g* for 10 min and protein concentrations of supernatants were determined with lowry method using bovine serum albumin as a standard. Proteins extracts were separated on 10 or 12 % SDS-polyacrylamide gels and transferred to nitro cellulose

membrane (Hybond ECL nitrocellulose membrane, Amersham biosciences, UK). Transferred protein blots were blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween20 for 1 h at RT. Membrane was washed 3 times with Tris-buffered saline containing 0.1% Tween20 and incubated with primary antigodies (1:500 dilution) for 1 h at RT. After 3 times wash with Tris-buffered saline containing 0.1% Tween20, membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 1 h at RT. Following 4 times washing with Tris-buffered saline containing 0.1% Tween20, membrane was developed with chemiluminescence reagent (ECL Reagent, Amersham biosciences, UK). Blot bands were visualized using LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

### 2.8. Statistical analysis

Each value was expressed as means ± S.E.M. (n = 3). The statistical significance of differences was analyzed by Student's t-test using SPSS (Chicago, IL, USA).

## 3. Results and discussion

Recently, much attention has been given to the use of marine algae as a anti-inflammation (Jung et al., 2009), anti-diabetes (Dellabella et al., 2005), antioxidation (Rosa et al., 2007), radiation protection (Zhang et al., 2008), anti-cancer (Hashida et al., 2008), as well as anti-allergic activities (Le et al., 2009) due to the presence of diverse natural products with unique structures possibly caused by extreme marine environment. However, these biological activities remain unclear in human, and much more efforts are needed to evaluate their exact biological activity mechanism in our body. In this study, the inhibitory effect of fucofuroeckol-A (FF) and eckol (EK) derived from *E. bicyclis* on the expression and activation of MMP-2 and 9 were evaluated in HT1080 cell line.

These phlorotannins did not exert any cytotoxic effect on HT1080 cell line and this was supported by the fact that marine phlorotannins have long been utilized as functional foods. The broad specificity of MMP-2 and 9 may play a role in the regulation of various cellular activities. MMP-2 and 9, secreted endopeptidases, are able to hydrolyze several components of the extracellular matrix including basement membrane collagen IV and has been associated with tumor invasion and metastasis (Aimes and Quigley, 1995; Liotta et al., 1991). To examine whether phlorotannins inhibit MMP-2 and 9 expression and activation in HT1080 cell line, MMPs inhibitory assay, *in vitro* wound healing assay, in vitro 3D culture assay and western blot assay were carried out. We observed that PMA treatment could stimulate MMPs expression and activation in HT1080. Moreover, this observation was consistent with previous report that MMPs are secreted in a latent form that is activated by a sequential cleavage of the N-terminal propeptide domain (Sato et al., 1994). In this study, for the first time we found that FF and EK could inhibit activation of pro-MMPs.

### 3.1. Cell cytotoxic effects of FF and EK on HT1080 cell line

The viabilities of the phlorotannins were carried out on HT1080 cell line for evaluating the MMP inhibitory assay experiments. The results showed that FF and EK performed no cytotoxic effects even at the highest concentration of 100 μM, and a remarkable difference could not be found between tested phlorotannins and control as described in figure 35.

### 3.2. In vitro effects of FF and EK on the migration of HT1080 cell line

HT1080 cells were treated with phlorotannins derived from *E. bicyclis* in the concentration range 0–100 μM during subsequent experiments. To investigate whether these phlorotannins inhibit tumor cell migration, wound migration assays were performed in HT1080 cells. Treatment with 0-100 μM of both FF and EK strongly reduced the migration of cells to the wounded surface in dose dependent manner (Figures 36 and 37). Especially, FF suppressed the migration of HT1080 cells across the wounded space more strongly compared to EK in the concentration of 100 μM). These results indicate that FF and EK may be used for suppressing HT1080cell metastasis and migration.

### 3.3 Effects of FF and EK on the 3D culture in HT1080 cell line

The behavior and morphology of cells cultured in a 3D system is quite different from that observed in the 2D system (Hotary et al., 2003). To investigate the effects of FF and EK on HT1080 cell line, we seeded the same density of cells suspended in various final concentrations of the FF and EK into a 3D collagen gel. As shown in figure 38 and 39, After 36 h the control (without sample treatment) stretched out in the gel and formed many branched and elongated structures in type IV collagen

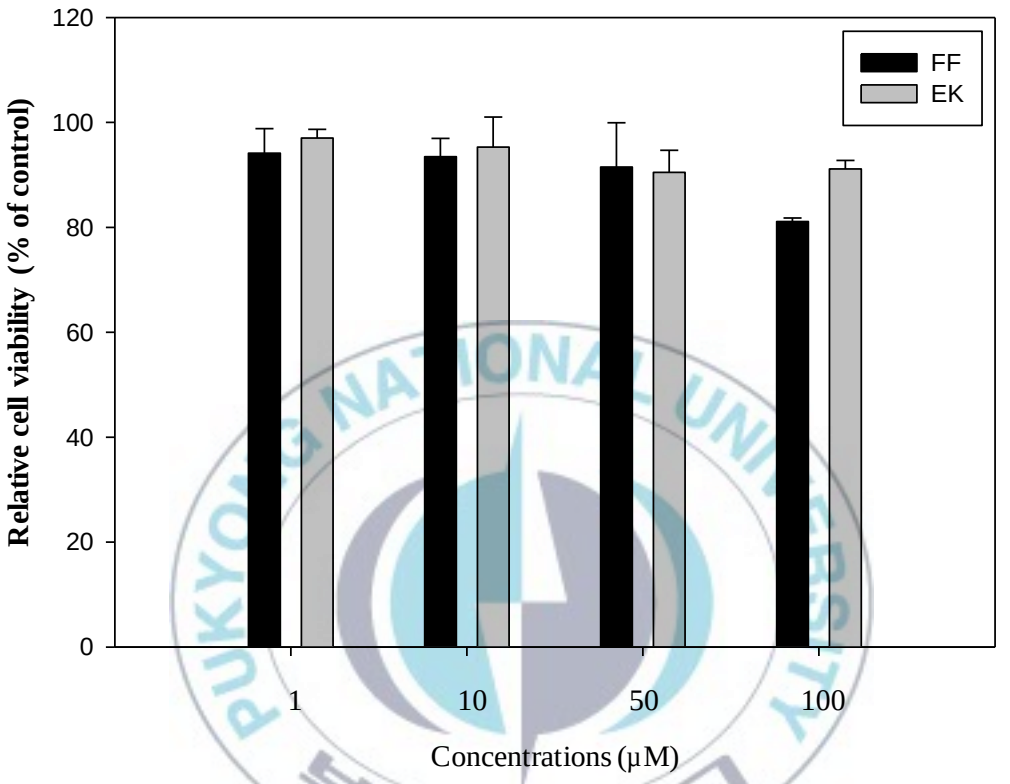



Fig. 35. Cytotoxic effects of FF and EK derived from *E. bicyclis* on cell viabilities of HT1080 human fibrosarcoma cells. Cells were grown at a density of 1 x $10^5$ cells/well and different concentrations of each sample were treated. After 24h incubation, MTT solution were treated to each well and incubate for 3 h. DMSO was added to solubilize formed formazan salt and amount of formazan salt was determined by measuring the OD at 540 nm. Each value was expressed as the mean ± SD of triplicate experiments.

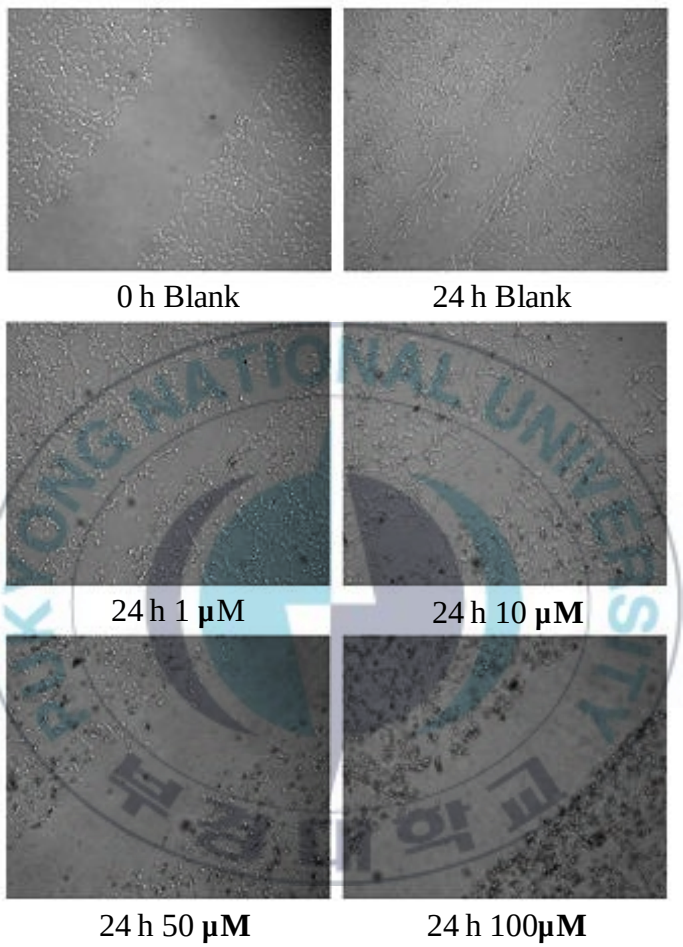



Fig. 36. Effect of FF on migration of HT1080 human fibrosarcoma cells. Cells were grown at density of 5 x $10^{5}$ cell/well in a 24-well plate and confluent monolayer were wounded and then incubated with treatment of various concentrations of FF in serum-free medium. After 0 and 24h incubations, the migrations of wounded cells were photographed under a phase contrast microscope.

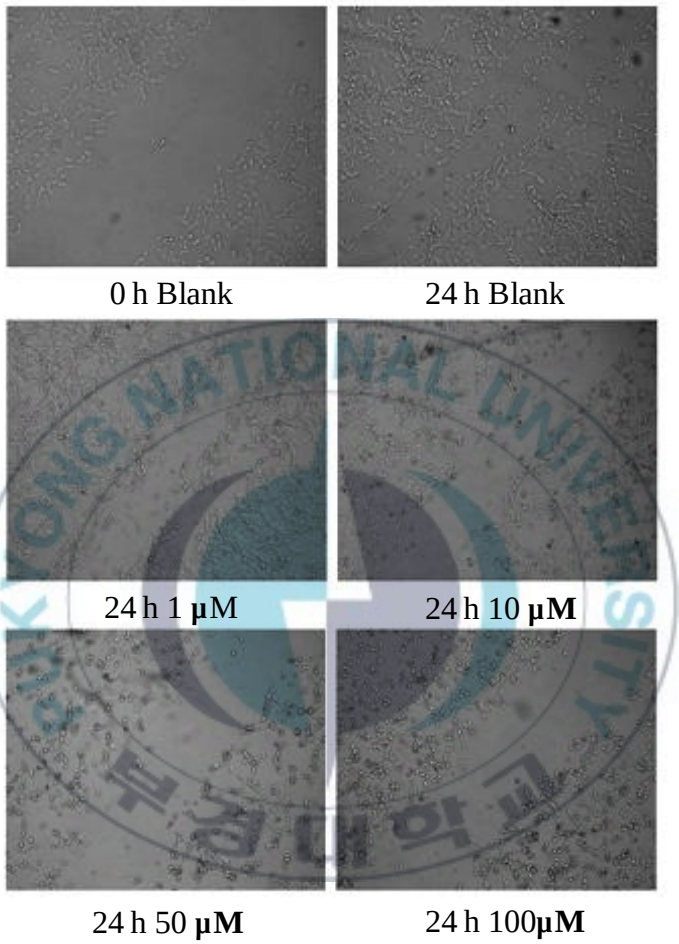



Fig. 37. Effect of EK on migration of HT1080 human fibrosarcoma cells. Cells were grown at density of 5 x $10^5$ cell/well in a 24-well plate and confluent monolayer were wounded and then incubated with treatment of various concentrations of EK in serum-free medium. After 0 and 24h incubations, the migrations of wounded cells were photographed under a phase contrast microscope.

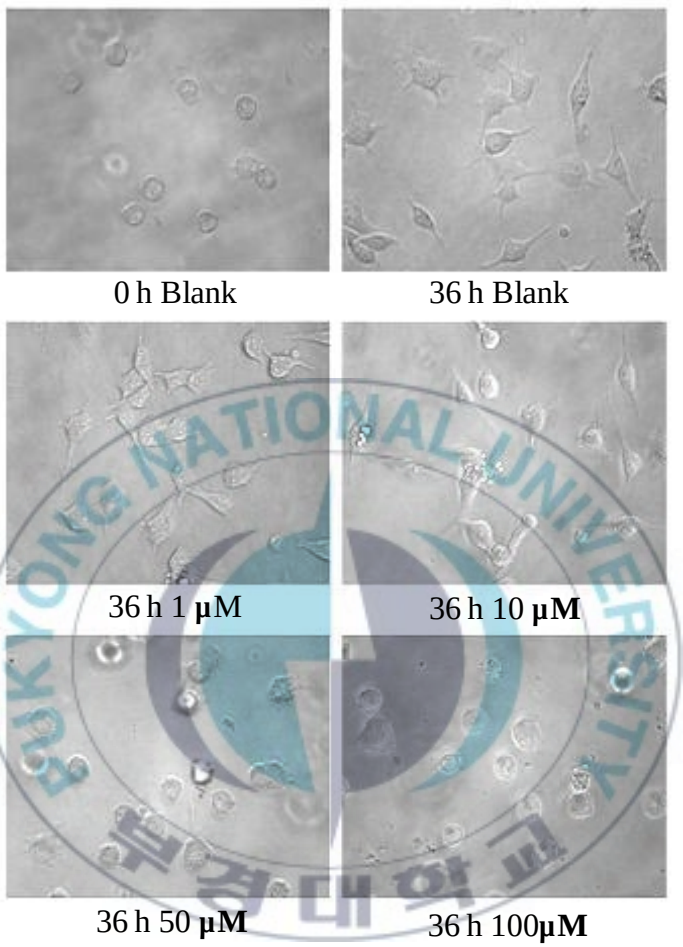



Fig. 38. Effect of FF on cell invasion in the HT1080 3D culture. Cells ($1.5\times10^3$) were suspended with a neutralized solution of type IV collagen (200 μg/mL) and 1/5 volume of a 5×DMEM. The cell suspension containing various concentrations of FF was added to 24-well plates and kept at 37℃ until gelled. The plates were then incubated at 37℃ for 36 h. The 3D culture images were observed using phase contrast microscope.

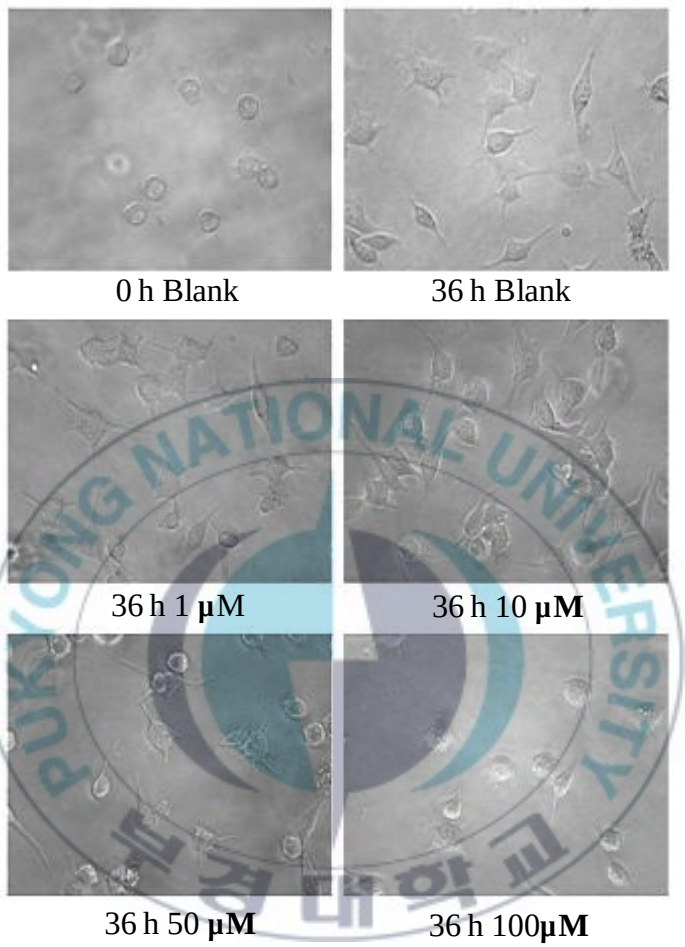



Fig. 39. Effect of EK on cell invasion in the HT1080 3D culture. Cells ($1.5\times10^3$) were suspended with a neutralized solution of type IV collagen (200 μg/mL) and 1/5 volume of a 5×DMEM. The cell suspension containing various concentrations of EK was added to 24-well plates and kept at 37℃ until gelled. The plates were then incubated at 37℃ for 36 h. The 3D culture images were observed using phase contrast microscope.

matrix. In contrast, the branched structures of the cells treated with 10 μM concentration of FF and EK were reduced and when the concentration increased to 100 μM, the branched structure disappeared, and interestingly the cells change to a spherical shape. At the concentration of 100 μM, FF inhibited completely the branched and elongated formation of HT1080 cells and its shape is almost similar with that of 0 h control group. The extracellular matrix protein degradation is a critical step during tumor invasion and migration. Although degradation of matrix protein is achieved by several MMPs, especially MMP-2 and -9 play most important roles due to their specific enzymatic activities on degradation of type IV collagen (Zeng et al., 1999). Therefore, inhibitions of MMP-2 and -9 activities and expressions, cause inhibition of tumor migration and invasion capacity. These results suggest that the FF and EK influenced morphology and increasing cell invasion in 3D culture condition and these seem to be related to inhibition of MMPs activities and expression.

### 3.4 Effects of FF and EK on MMP-2 and -9 activities.

Based on the above results, further studies were carried out to examine the direct inhibitory effect of FF and EK on MMP-2 and -9 using fluorometric enzyme inhibitory assay kit. Both FF and EK exhibited remarkable inhibitory effects on MMP-2 and 9 dose-dependently (Figures 40 and 41). Phlorotannins are the secondary metabolites of polyphenol mainly produced by brown and red algae, which can be complex with macromolecules through hydrophobic interactions and through formation of hydrogen, covalent, or ionic bonds. It has been reported that phlorotannins derivatives were isolated from EtOAc fraction and are responsible for the biological activities of EC (Kang et al., 2005; Kim et al., 2006) and red wine polyphenolic compounds strongly inhibit MMP activity via direct inhibition. As shown in figure 40, treatment with FF and EK inhibited MMP-2 activity

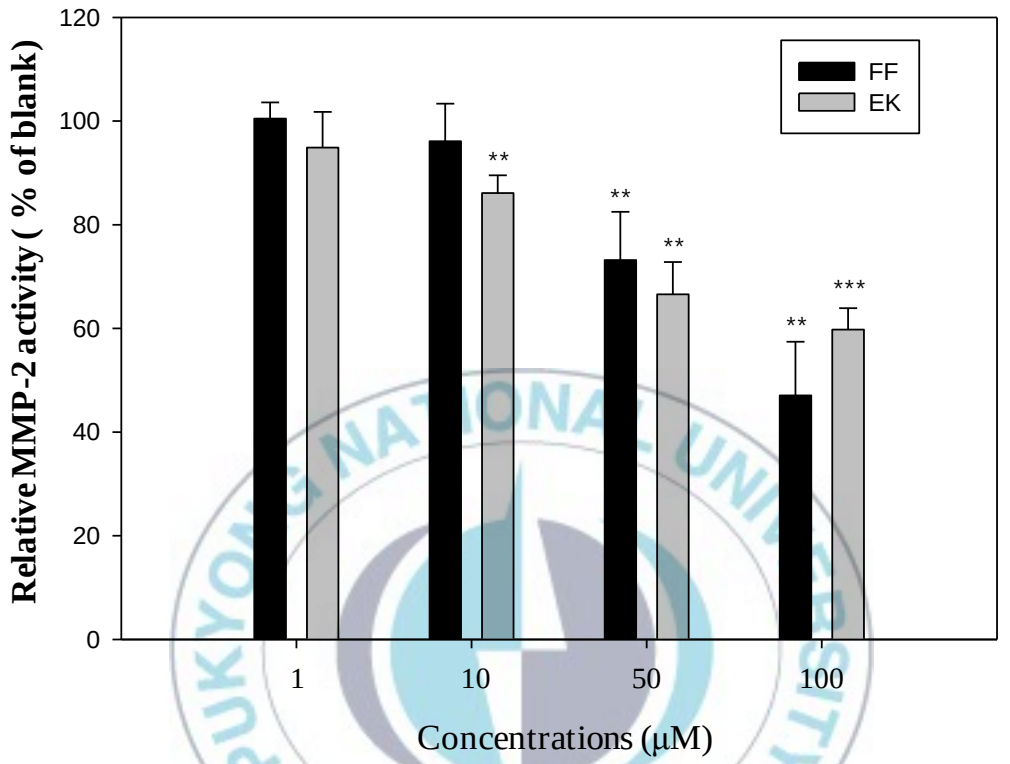



Fig. 40. Inhibitory effects of FF and EK on MMP-2. Relative enzymatic activities of MMP-2 levels in HT1080 cells were performed using commercial MMP-2 assay kit according to the manufacturer's instruction. Briefly, 50μl of assay buffer solution containing MMP-2, activated by 1mM APMA, was treated with various concentrations of each sample for 15 min at 37℃. Subsequently, 50μl of MMP-2 substrate solution was added and incubated for 1 h at 37℃ in the dark. Finally 50μl of stop solution was added and the fluorescence intensity (Ex/Em = 340 nm/490) was measured using ELISA reader. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with control groups.

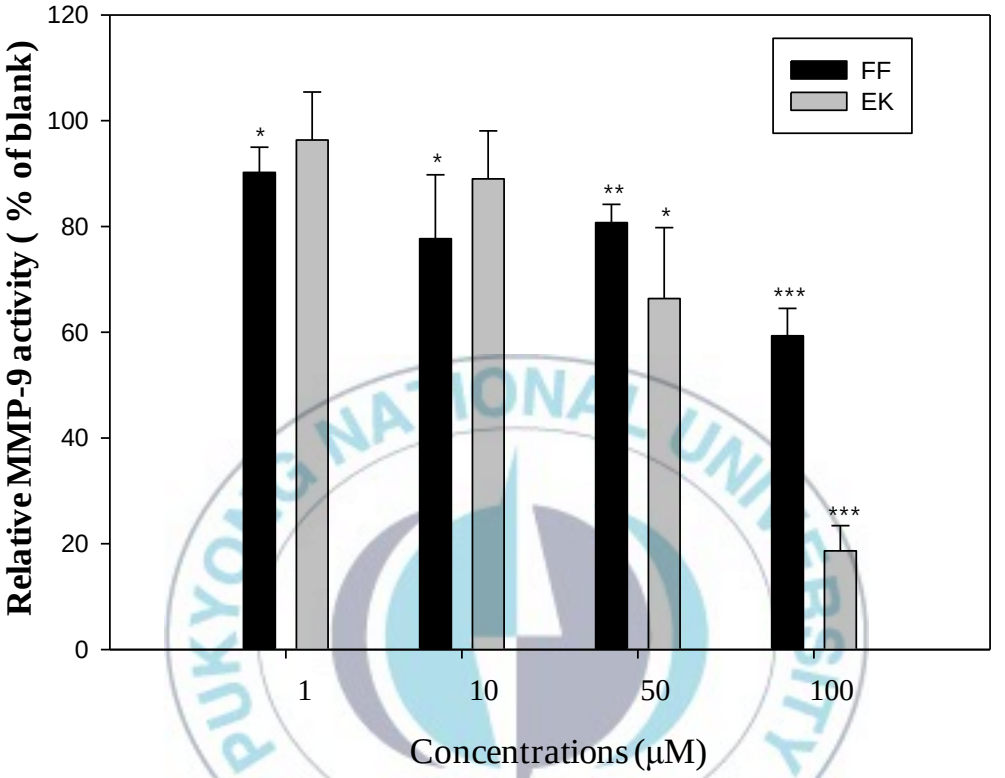



Fig. 41. Inhibitory effects of FF and EK on MMP-9. Relative enzymatic activities of MMP-9 levels in HT1080 cells were performed using commercial MMP-9 assay kit according to the manufacturer's instruction. Briefly, 50μl of assay buffer solution containing MMP-2, activated by 1mM APMA, was treated with various concentrations of each sample for 15 min at 37℃. Subsequently, 50μl of MMP-9 substrate solution was added and incubated for 1 h at 37℃ in the dark. Finally 50μl of stop solution was added and the fluorescence intensity (Ex/Em = 340 nm/490) was measured using ELISA reader. Each value was expressed as the mean ± SD of triplicate experiments. *,$P < 0.05$, **,$P < 0.01$ and ***,$P < 0.001$ as compared with control groups.

significantly compared with blank group. MMP-2 activity was clearly inhibited by FF and EK at the concentration of 100 μM with 47.1 ($P < 0.01$) and 59.7 ($P < 0.001$) % relative MMP-2 activities, respectively. While FF showed more strong MMP-2 inhibitory activity compared to EK, EK exerted more strong MMP-9 inhibitory activity with the 18.7% relative MMP-9 activity at the concentration of 100 μM (Fig. 41). These results indicate that FF and EK directly inhibit the MMP-2 and 9 activities through the hydrophobic interactions to the MMP biomolecules.

### 3.5 Effects of FF and EK on the protein levels of MMP-2 and 9 on HT1080 cell line

As discussed above result, we exhibited direct effects of FF and EK on MMP-2 and 9. While it is clear that FF and EK showed direct inhibitory activities on MMPs, effects of FF and EK on expression of MMP-2 and 9 are needed to be studied. To investigate whether FF and EK inhibit protein expression levels of MMP-2 and -9, Western blot assay was performed. As shown in figures 42 and 43, while PMA activated cells increased the expression of MMPs compared to that of blank group, protein expressions for MMP-2 and -9 were clearly inhibited in FF and EK treated cells and down-regulation of MMP-2 and -9 expressions was observed in a dose-dependent manner. Further as shown in above results, the MMP-2 and -9 proteins expression were inhibited by FF and EK in a similar manner with direct MMP-2 and 9 inhibitory activities results. Meanwhile according to MTT cell viability assays, it was clear that these results were not due to cytotoxic influence of phlorotannins.

### 3.6 Effects of FF and EK on the protein levels of TIMP-1 and 2 on HT1080 cell line

In order to test whether TIMP-1 and 2, the natural MMPs inhibitors, are altered in

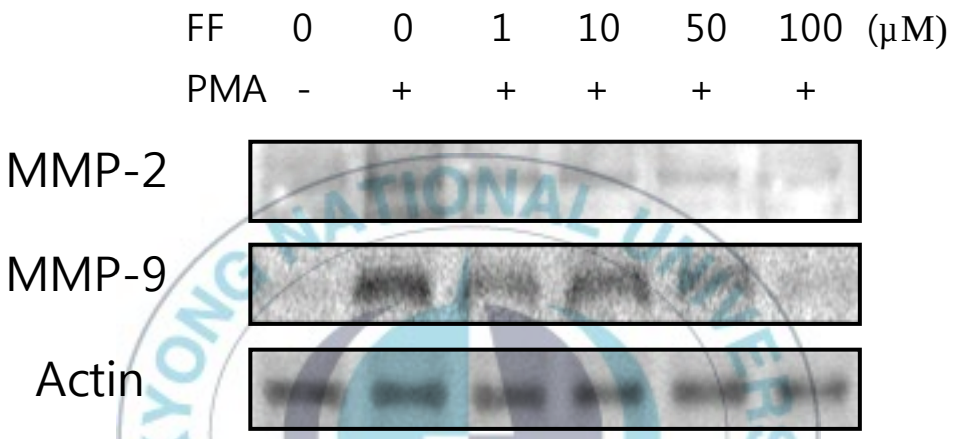



Fig. 42. Effects of FF on PMA-induced protein expression of MMP-2 and 9 in HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for MMP-2 and MMP-9. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

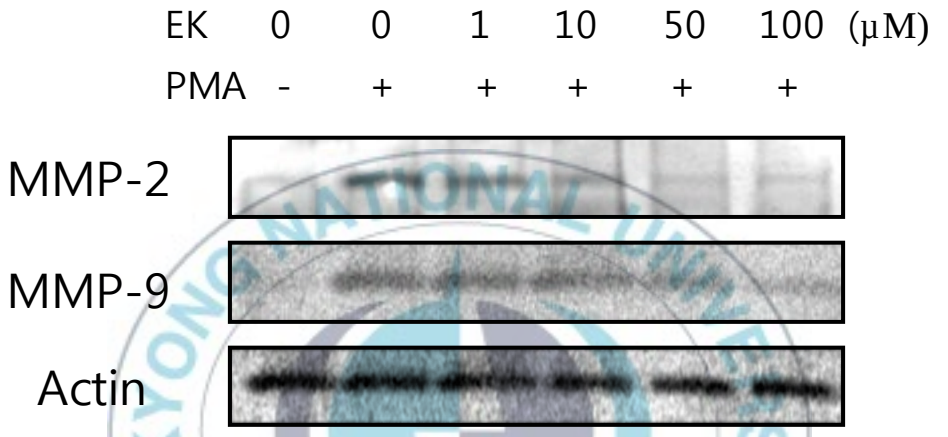



Fig. 43. Effects of EK on PMA-induced protein expression of MMP-2 and 9 in HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of EK at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for MMP-2 and MMP-9. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

the presence of phlorotannins, their protein expression levels were assessed using western blot assay. Except of TIMP-2 expression level influenced by FF, no any significant differences in TIMP-1 and 2 protein levels were observed even at the concentration of 100 μM (Figures 44 and 45). However, FF showed considerable inhibitory effect on TIMP-2 protein expression dose-dependently (Fig. 44). The tissue inhibitors of matrix metalloproteinase (TIMP)s are natural inhibitors of MMPs and act by tightly binding the MMP in a 1:1 stoichiometric ratio. This interaction occurs through an MMP binding domain within the N-terminal region of the protein (1–126 amino acids referred to as N-TIMP) (Huang et al., 1997; Murphy et al., 1991). These results indicate that the incubation of FF can stimulate the TIMP-2 expression and this cause inhibition on MMPs activities via direct binding of TIMP-2 to MMPs.

### 3.7 Effects of FF and EK on transcriptional regulation of NF-κB and C-fos on HT1080 cell line

Moreover, western blot analysis carried out to evaluate the effects of FF and EK on the expression of NF-κB (p65 and p50) and C-fos. As shown in figures 46 and 47, FF and EK suppressed expressions of NF-κB (p65 and p50) and C-fos dose-dependently. NF-κB is a key component necessary for the expression of pro-inflammatory cytokines and many immunoregulatory molecules (Baeuerle and Baltimore, 1988; Baeuerle and Henkel, 1994) and also its activity has been associated with the regulation of protein levels of MMP-9 (Bond et al., 1998). Therefore, this suppression of NF-κB subsequently leads to MMPS inhibition. Furthermore, MMPs gene expressions are known to be regulated via AP-1 transcription factor that binds to promoter of all MMPs (Kim and Kim, 2006). Therefore, we investigated whether FF and EK exert any inhibitory effect on AP-1. For that we tested the gene expression of C-fos, a part of AP-1 transcription factor in the presence of FF and EK. FF showed remarkable inhibitory effect on the

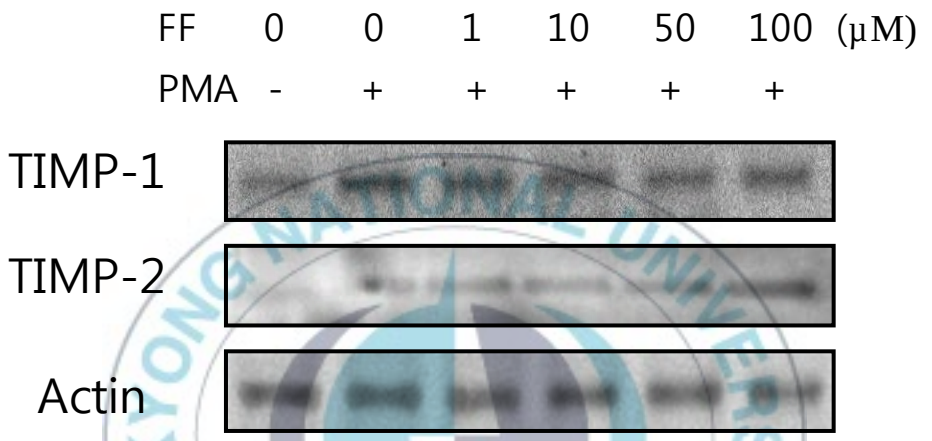



Fig. 44. Effects of FF on PMA-induced protein expression of TIMP-1 and 2 in HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for TIMP-1 and TIMP-2. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

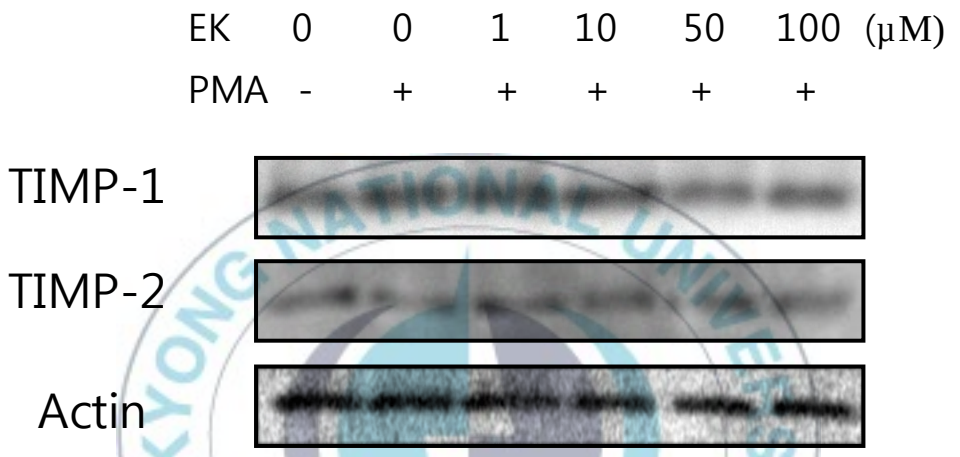



Fig. 45. Effects of EK on PMA-induced protein expression of MMP-1 and 2 in HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^{5}$ cell/well and treated with various concentrations of EK at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for MMP-2 and MMP-9. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

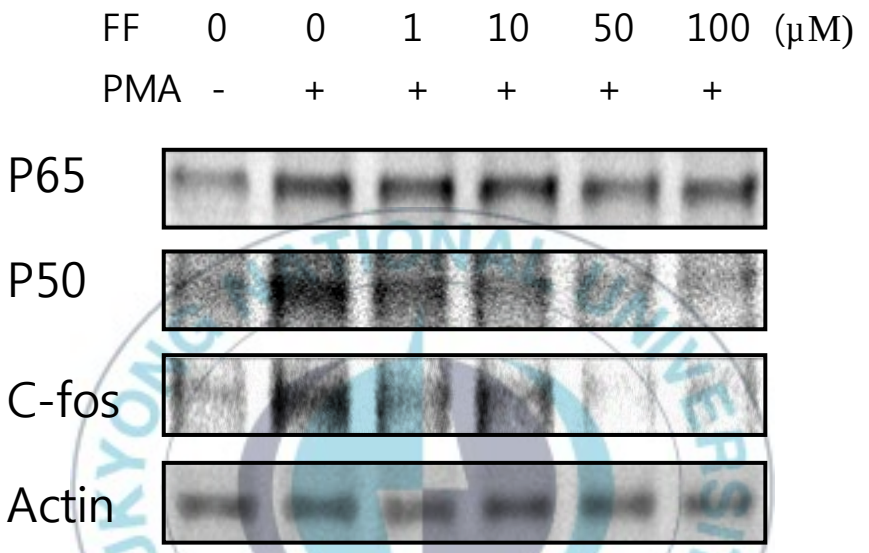



Fig. 46. Effect of FF on protein expression of NF-κB and AP-1 in PMA-induced HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of FF at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for p65, p50, C-fos and c-jun. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

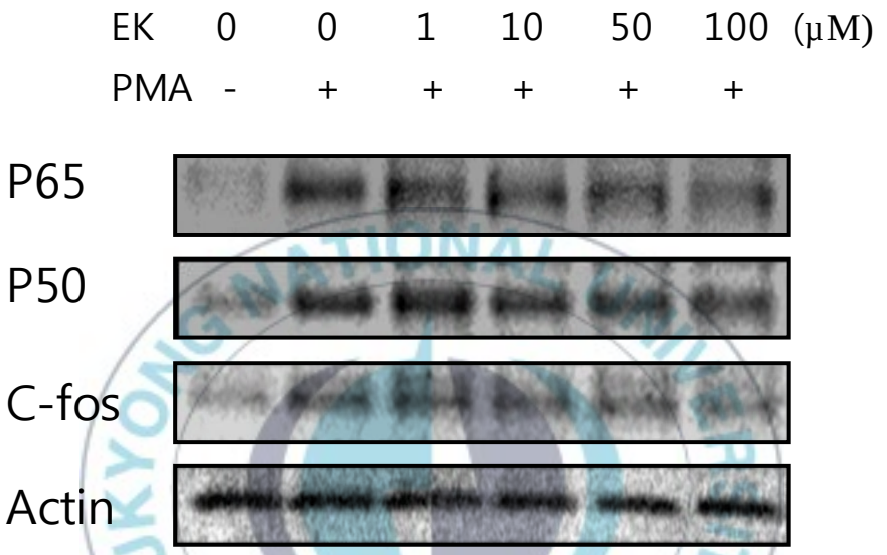



Fig. 47. Effect of EK on protein expression of NF-κB and AP-1 in PMA-induced HT1080 human fibrosarcoma cells. Cells were grown at 5 x $10^5$ cell/well and treated with various concentrations of UN at 2 h before PMA (10 ng/mL) treatment. After incubation for 24 h, Cell lysates were collected and subjected to SDS-PAGE and Western blots using antibodies specific for p65, p50, c-fos and c-jun. β-actin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager.

expression of C-fos on HT1080 cell line dose-dependently and suppressed this expression completely at the concentration of 100 μM (Figure 46). EK, however, didn't show any significant inhibitory effects on the expression of C-fos even at the concentration of 100 μM (Figure 47).

**4. Summary**

Several studies have been focused on the cancer chemopreventive agents from natural resources for nutraceuticals and pharmaceuticals. In this regard, a promising strategy is the development of inhibitors of MMPs(Brown and Giavazzi, 1995). MMPs are a family of zinc-containing endopeptidase which degrade specific components of extracelluar matirixes and have been demonstrated to play a crucial role in pathological conditions such as rheumatoid arthritis and cancer (Anderson et al., 1995; Kim et al., 1998). Carcinogenesis, which include multi-step such as tumor invasion and metastatic spread, involve the breakdown of the basement membrane and loss of cell adhesion. Thus, MMPs are critical for entry of tumor cells into lymphatic and blood vessels and the colonization of distant organs (Rabbani, 1998). Excessive production of MMPs, which are due to imbalance between MMPs and natural MMP inhibitors, are capable of cleaving most components of basement membrane and play a substantial role in the pathogenesis of various chronic diseases (Nagase and Woessner Jr, 1999). MMPs inhibitors are capable of regulating activities of MMPs at several biochemical pathways or directly inhibit the activity of enzyme. Expression of MMPs and TIMPs has been correlated with the increased invasions of various human cancers such as of the breast, colon, stomach and kidney(Davies et al., 1993; Lein et al., 2000)

In this study, we evaluated effects of FF and EK on MMP-2 and-9 expression and activities using PMA induced HT1080 cells, which is commonly used as a model for MMP studies to assess the effect of FF and EK on MMP-2 and -9 expressions. At the promoter sites of MMPs genes, there are several binding sites for transcriptional factors that regulate gene expressions. MMPs are highly regulated at the levels of both gene expression and protein activation. It has been reported that transcriptional regulation of MMP gene is mediated by an AP-1 regulatory element in their proximal promoter region (Fini et al., 1998). MMP-9 transcription is known to be

regulated mainly via AP-1 (Moon et al., 2004; Rajapakse et al., 2006). However, transcriptions of MMPs can be regulated by NF-κB via different signaling pathways under inflammatory or other pathology conditions. Even though AP-1 and NF-κB transcriptional factors are regulated by different mechanisms, they appear to be activated simultaneously by the same multitude of stimuli (Fan et al., 2002). FF and EK inhibited NF-κB gene expressions with a similar dose-dependent manner observed in MMP-2 and -9 gene expressions, and also FF and EK showed significant effects on AP-1 expressions significantly. Furthermore, FF and EK exhibited strong direct inhibitory activities against MMP-2 and -9 in enzyme assay. Phlorotannins are secondary metabolites of polyphenols and can be complexed with macromolecules through hydrophobic interactions and formation of hydrogen, covalent or ionic bonds (Lee et al., 2008). Therefore, it is clear that FF and EK blockade both transcriptions of NF-κB and AP-1, and inhibit the MMP-2 and -9 directly. These results may due to unique chemical structure of these phlorotannins. Up to date, MMP inhibitors under clinical trial were synthetic inhibitors, and their side-effects led to failure of the trials (Overall and Lopez-Otin, 2002). Therefore, development of non-toxic natural MMP inhibitors from marine resources is great interest among researchers at present. In our continuous investigation of effects of on MMPs and cell morphology, our data indicated that FF and EK have potential inhibitory activities on cancer metastasis. Further study is needed to clarify the relationship between activities and structural features of the tested phlorotannins. The structure-activity relationship (SAR) could be described according to the unique structures of FF and EK. The numbers of hydroxyl groups and O-bridge linkages (ether linkage) are important factors on inhibitory activities of FF and EK against MMP-2 and -9. Summarizing our data, we could conclude that FF and EK act as inhibitors of MMP-2 and -9 expressions by down-regulation of NF-κB and, directly inhibit activities of MMP-2 and -9. Furthermore, previous results in chapter Ⅱ

support the possibilities that FF and EK may reduce ROS involved progression of tumor metastasis.

In conclusion, our findings provide the first experimental evidence that FF and EK inhibit activation and expression of MMP-2 in HT1080 cell line. Therefore, these results suggest that FF and EK derived from *E. bicyclis* may play an important role in the prevention and treatment of MMP-2 and 9 mediated several health problems such as metastasis.


# Part Ⅴ

# Conclusions

The purpose of this study is to isolate phlorotannins from *Eisenia bicyclis* (Kjellman. *E. bicyclis*), which are responsible for its various biological activities, and investigate their antioxidant activities various *in vitro* free radical scavenging activity assays in different cellular and non-cellular oxidative systems. Furthermore,

Isolated phlorotannins such as fucofuroeckol-A (FF) and eckol (EK), which have no cytotoxicities and strong ROS scavenging activities among the phlorotannins, were investigated on their anti-inflammatory and matrix metalloproteinase (MMP) -2 and -9 inhibitory effects, and inhibitory mechanisms to evaluate their potentials as natural nutraceuticals and pharmaceuticals. The summarized results obtained from each chapter are as follows:

- The antioxidant activities of the each fractions from MeOH extract of *E. bicyclis* were in the order of ethyl acetate (EtOAc) > *n*-butanol (*n*-BuOH) > dichloromethane ($CH_2Cl_2$) > and water ($H_2O$) fraction. Moreover, the order of total poly-phenolics contents of each fraction showed the same order in accordance with the radical scavenging activities.
- Phloroglucinol, Fucofuroeckol-A (FF), Dioxinodehydroeckol (DD), Dieckol (DE), Eckol (EK) were isolated from EtOAc fraction and showed strong antioxidant activities in non-cellular and cellular system. Among these phlorotannins, FF and DD were first isolated from *E. bicyclis* and it is the first report of biological activities of FF.
- To evaluate the effects of phlorotannins derived from *E. bicyclis* on inflammation in lipopolysaccharide induced RAW264.7 mouse macrophage, FF and EK, which have no cytotoxicity and no previous reports regarding anti-inflammatory activity, were selected.
- FF and EK suppress LPS-induced production of nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in dose-dependent manner.

- FF and EK significantly reduced the productions of pro-inflammatory cytokines such as, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-**1.**
- FF and *EK* reduced nuclear factor κB (NF-κB) nuclear localization and mitogen-activated protein kinases (MAPKs) activation, such as JNK and p38. Furthermore, EK inhibits the DNA binding activity of NF-κB. These results suggested that the inhibitory effects of FF and EK on LPS-induced NO and $PGE_2$ production are due to the suppression of NF-κB and MAPKs signaling pathway. Moreover, intracellular antioxidant activities of FF and EK supports that their anti-inflammatory effects might be attributable to scavenging ROS in RAW264.7 mouse macrophage.
- FF and EK suppressed the tumor cell migration and invasion in 3D culture model on HT1080 and exhibited significant direct inhibition on MMP-2 and -9 activities.
- FF and EK inhibit expressions of MMP-2/9 via down regulation of NF-κB and AP-1in PMA-induced HT1080 human fibrosarcoma. Moreover, FF stimulate the expression of tissue inhibitor of matrix metalloproteinase (TIMP) -2 dose-dependently, and this may cause inhibition on MMPs activities via direct binding

Therefore, these results suggested that FF and EK have remarkable ROS scavenging activities in both cellular and non-cellular oxidative systems, and strong anti-inflammatory and MMP inhibitory effects on the basis of their antioxidant activities. In addition, the present study provides a possible inhibitory mechanisms (Figure 48) and potentials of FF and EK as valuable natural anti-inflammatory and chemopreventive agents for cancer to develop nutraceuticals and pharmaceuticals.

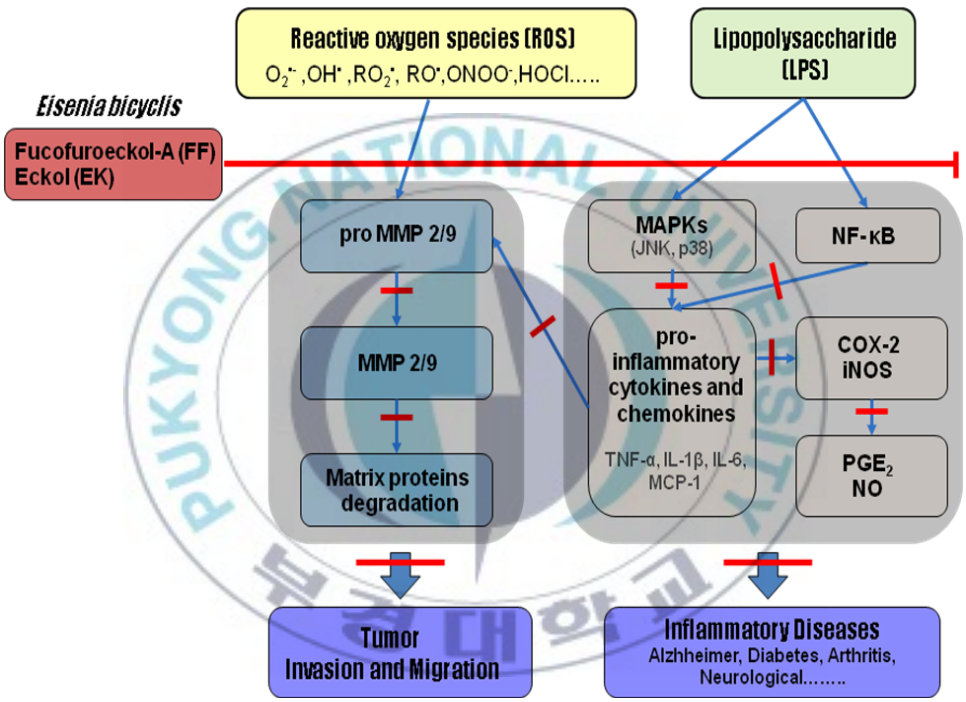



Fig. 48. Suggested models of inhibitory mechanisms of FF and EK derived from *Eisenia bicyclis* on inflammation and tumor metastasis.

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# Anti-Inflammatory Mechanisms of Phlorotannins derived from *Eisenia bicyclis* and Their Inhibitory Effects on Matrix Metalloproteinases

# 대황으로부터 분리한 Phlorotannins 의 염증저해기전 및 기질금속단백질분해효소 저해효과

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## 요 약

대황은 주로 한국 및 일본에 서식하는 갈조류에 속하는 식용 해조류이다. 지금까지 대황의 추출물로부터 항산화 활성 및 염증저해효과에 관한 연구는 이미 이루어져 있으나 그 정확한 저해 메카니즘과 추출물의 주성분인 phlorotannin 의 분리정제를 통한 저해효과에 관한 연구는 거의 이루어 지지 않았다. 따라서 본 연구에서는 대황으로부터 phloroglucinol, fucofuroeckol-A, dioxinodehydroeckol, eckol 그리고 dieckol 을 분리 정제하여 그 구조를 동정하고 이들의 항산화 효과를 검증하였다. 또한 대황으로부터 처음 분리된 fucofuroeckol-A 과 eckol 의 염증억제효과 및 그 메커니즘과, 활성산소 및 염증 등으로 인하여 유발되어 암의 진행에 관여하는 기질금속단백질분해효소에 대한 억제효과를 처음으로 확인하였다.

Fucofuroeckol-A 과 eckol 은 lipopolysaccharide 로 인하여 염증이 유도된 쥐의 대식세포 (RAW264.7)에서 농도 의존적으로 nitric oxide 및 prostaglandin $E_2$ 와 같은 염증매개물질의 발현을 억제하는 것으로 나타났으며, 이러한 억제효과는 nuclear factor kappa B (NF-κB) 및 Mitogen-activated protein kinases (MAPKs) 활성경로의 저해를 통하여 전염증 매개인자인 tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β 그리고 monocyte chemoattractant protein (MCP)-1 일어나는 것을 확인하였다. 또한 fucofuroeckol-A 과 eckol 은 활성산소 및 염증에 의해 유도되는 기질금속단백질분해효소의 발현 및 활성을 저해하여 암세포의 이동 및 활성화를 억제하는 것을 확인하였다. 본 연구결과를 통하여 대황에서 처음으로 분리한 fucofuroeckol-A 와, eckol 의 염증저해 메카니즘을 구명하였으며 또한 암세포 증식억제효과를 검증함으로써 향후 염증억제 및 암예방 기능성 소재로서의 대황의 활용가능성을 확인할 수 있었다

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1993년에 부경대학교에 화학과에 입학하고 16년이란 시간이 지났습니다. 짧으나마 그 동안의 제 인생을 뒤돌아볼 때, 뚜렷한 목표없이 방황하던 시절 생화학 실험실은 내 인생의 큰 전환점이 아니었나 생각됩니다. 연구를 좋아했다기 보다는 실험실이라는 공동체가 좋았던 그 시절, 힘들고 어려울 때 서로 도와가며 같이 열심히 연구했던 선후배님과 교수님으로부터 많은 것을 경험하고 배울 수 있었습니다. 제 인생에 있어 가장 힘들고 보람찬 박사과정을 마무리하는 지금, 다시금 공부를 할 수 있는 기회와 아낌없는 도움을 주신 많은 분들에게 진심으로 감사 말씀을 전하고자 합니다.

우선 다시금 저에게 학문의 길을 열어주시고 뒤늦게 공부하는 부족한 저에게 생활비까지 걱정해주시며 연구자로서의 길을 가르쳐 주신 김세권 교수님께 무한한 감사를 드립니다. 어디서도 얻을 수 없을 것 같은 많은 것을 경험하고 배울 수 있었습니다. 제 인생에 처음으로 목표를 가질 수 있게 이끌어 주신 은혜, 앞으로 부족한 제자가 되지 않도록 열심히 노력하는 모습으로 갚도록 하겠습니다.

박사학위 과정을 무사히 마칠 수 있도록 많은 도움과 연구자로서의 희망을 주신 안창범 교수님, 김용태 선배님, 변희국 선배님, 전유진 선배님, 박표잠 선배님, 박선배 교수님, 정원교 선배님, 제재영 후배님께 감사드리며 앞으로도 연구자로서 최선을 다하는 모습 보여드릴 수 있도록 노력하겠습니다. 또한 지금의 생화학실험실이 있기까지 노력하시고 선후배로서 항상 따뜻한 힘이 되어 주신 이배진 선배님, 김원석 선배님, 노호석 선배님, 이민수 선배님, 장지태 선배님, 김규형 선배님, 최정호 선배님, 백호철 동기님, 김희주 후배님, 장민정 후배님께 깊은 감사의 말씀을 드립니다. 이번 학위논문을 무사히 마무리 할 수 있도록 학문적 조언과 지원을 아끼지 않으시고 언제나 따뜻이 맞아주셨던 김문무 교수님께 깊은 감사를 드리며 바쁜 와중에도 실험과 관련하여 많은 도움을 준 최일환 교수님, 윤나영 박사님, 천충길 후배님, 강경화 선생님, 이다영 선생님께 고마운

마음을 전합니다. 학위과정 동안 실험실에서 힘들고 어려운 시절을 동거동락했던 Li-Yong 박사님, 공창숙 박사님, 엄태길 후배님, 조영숙 후배님, 류보미 후배님, 김정애 후배님, 안별님 후배님, 김영상 후배님을 비롯하여 머나먼 타국에서 학업중인 여러 외국인 실험실원들에게 깊은 감사의 말씀 드립니다. 또한 지면관계상 일일이 열거하지는 못하였지만 나의 사랑하는 친구들과 여러 지인들에게도 고마움을 전합니다.

항상 장손인 큰아들이 잘 되기를 뒤에서 묵묵히 소원하시는 부모님께 지금이 있기까지 보살펴 주신 은혜에 머리 숙여 감사 드립니다. 더불어 부족한 사위를 언제나 자식처럼 아껴주신 장인 장모님께도 깊은 감사 드립니다. 그 동안 부족한 형을 응원했던 사랑하는 동생 상무, 재수씨, 조카 준석이와 수민이, 처제 가족, 처남에게도 고마움을 전합니다.

끝으로 두 아이를 키우느라 힘든 가사생활에도 불구하고 언제나 진심으로 따뜻한 내조를 아끼지 않았던 아내 진경에게 표현할 수 없는 고마움과 사랑의 마음을 전합니다. 이제 5살이 되는 자칭 공주인 큰딸 서영이와 태어난 지 한달 만에 2살이 된 둘째딸 지민이에게 열심히 노력하여 얻은 작은 결실인 이 논문을 아내와 함께 바칩니다.