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# Therapeutic Potential of Ethanolic Extract of *Ecklonia cava* on Inhibition of Inflammation

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**Abstract** Chronic inflammation has been known to have a close relationship with several diseases including periodontitis, colitis, hepatitis and arthritis. Recently anti-inflammatory agents have been developed from marine natural resources. In this study, *Ecklonia cava* (EC) was found to have anti-inflammatory effect. Ethanolic extract of EC belonging to brown algae exhibited an excellent inhibitory effect on the production of inflammatory mediators such as tumor necrosis factor-α, interleukin-1β, interleukin-6 and prostaglandin $E_2$ by RAW264.7 cells. Furthermore, in reporter gene assay and western blot analysis, EC extract exerted anti-inflammatory effect via inactivation of NF-κB transcription factor that regulates the expression of these inflammatory mediators in macrophages. In addition, EC extract inhibited the activity of matrix metalloproteinase that play an important role in chronic inflammation. These results suggest that EC extract may provide a pharmaceutical potential in inhibiting chronic inflammation.

**Key words:** *Ecklonia cava,* RAW264.7, NF-κB, interleukin, TNF-α, prostaglandin $E_2$

## Introduction

Chronic inflammation has been known to have a close relationship with several diseases such as colitis, arthritis, atherosclerosis, periodontal disease, asthma and multiple sclerosis [2]. In this inflammation, NF-κB has been known to play the key role in the immune system [17]. NF-κB was reported to regulate the expression of inflammatory mediators such as cytokines and prostaglandins such as including tumor necrosis factor-α, interleukin-1β, interleukin-6 and prostaglandin $E_2$ [3]. These cytokines have been known to induce matrix metalloproteinases (MMPs) that degrade extracellular matrix such as collagen and fibronectin [16]. Cytokine such as tumour necrosis factor-α activates NF-κB by the phosphorylation of IκB by IκB kinase (IKK) that results in its ubiquitylation and degradation by the proteasome. The freed NF-κB dimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes that play an important role in inflammation [14]. Therefore, we screened the marine algae that can inhibit activation of NF-κB and finally found that *Ecklonia cava* (EC) extract was effective in inhibiting activation of NF-κB, leading to anti-inflammatoy effect. EC inhabits in the subtidal regions of Jeju Island in Korea had an excellent efficacy to inhibit generation of reactive oxygen species. In addition to radical scavenging activity, extracts of several *Ecklonia* species have been reported to possess number of other important biological activities such as antiplasmin inhibiting activity [6], HIV-1 reverse transcriptase and protease inhibiting activity [1] and tyrosinase inhibitory activity [19]. Recently, several researches have reported that *Ecklonia* species exhibit radical scavenging activity [12]. It is expected that high content of phlorotannins present in *Ecklonia* species is responsible for above bioactivities. However, the effect of EC extract on anti-inflammation remains obscure. Therefore, we investigated the effects of EC extract on anti-inflammation through inactivation of NF-κB transcription factor in macrophages.

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## Materials and Methods

### Materials

Dulbecco's Modified Eagle's Medium (DMEM), penicillin /strepto- mycin/amphotericin (10,000 U/ml, 10,000 μg/ml, and 2,500 μg/ml, respectively) and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (USA). RAW264.7 cells were obtained from American Type of Culture Collection (Manassas, VA, USA). MTT reagent and all the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA). Aspirin and dexamethasone were used as positive controls for inhibition of prostaglandin $E_2$ and interleukins production, respectively and were purchased from Sigma (USA). PMA (phorbol 12-myristate 13-acetate) was used to stimulate expression of matrix metalloproteinase and was obtained from Sigma (USA).

### Preparation of EC extract

The brown seaweed, EC was collected along Jeju Island coast of Korea. Fresh EC was washed three times with tap water to remove salt, epiphytes and sand attached to the surface of the samples and stored at -20℃. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried EC powder (1 kg) was extracted three times with 10 L of EtOH (1:10 w:v) at room temperature for 24 h while stirring, filtered using Whatman No. 41 and evaporated in vacuum. The w/w yield of extracts was about 9.5%. For the bioassay, the fractions were dissolved in dimethylsulfoxide (DMSO) and distilled water according their solubility, respectively.

### Cell culture

Cell lines were separately grown as monolayers in T-75 tissue culture flasks (Nunc, Denmark) at 5% $CO_2$ and 37°C humidified atmosphere using appropriate media supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin-streptomycin. DMEM was used as the culture medium for RAW 264.7 cells. Cells were passaged 3 times a week by treating with scraper used for experiments after 5 passages.

### MTT assay

The cytotoxic levels of *EC* extract on RAW264.7 cells were measured using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously [10]. The cells were grown in 96-well plates at a density of $5 \times 10^3$ cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of EC extract. After 48 h of incubation, cells were rewashed and 20 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control (OD of treated cells − OD of blank / OD of control − OD of blank×100) and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $P<0.05$ was considered significant.

### Enzyme immunoassay of TNF-α, IL-1β and IL-6

Inflammatory mediators such as TNF-α, IL-1β and IL-6 were measured by solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay according to the manufacturer's protocol (Code RPN 2751, RPN 2718 and RPN2708, Amersham Pharmacia Biosciences, NJ, USA) without prior extraction or purification. Standard curves from 10 - 5000, 10 - 200 and 10 - 200 pg/ml were used in order to evaluate the concentrations of TNF-α, IL-1β and IL-6, respectively. Results were calculated by using nonlinear regression of a four-parameters logistic model.

### Enzyme immunoassay of PGE2

$PGE_2$ was measured by enzyme immunoassay without prior extraction or purification according to the manufacturer's protocol (Code RPN 222, Amersham Pharmacia Biosciences, NJ, USA). A concentration range of 2.5 to 320 pg/well was used to construct the standard curve. $PGE_2$ concentrations in the samples were calculated by using nonlinear regression of a four parameters logistic model.

### Transfection and reporter gene assay

RAW264.7 cells were seeded in 24-well plates and incubated at 37°C. At about 70-80% of confluency,

cells were washed with DMEM and incubated with DMEM without serum and antibiotics for 5 h. Then the cells were transfected with DNA mixture containing 1 μg of a reporter construct including NF-κB binding site sequence and 300 ng of pcDNA 3.1 which adjusts a total amount of DNA per well by LipofectamineTM reagent (Invitrogen, USA) as instructed by the manufacturer. After 72 h of incubation, cells were lysed and luciferase activity was measured using a luminometer (Tecan Austria GmbH, Austria). The luciferase activity was normalized to transfection efficiency by β-galactosidase activity using o-nitrophenyl β-galactopyranoside as a substrate. β-galactosidase staining was carried out by method as described by Lin et al.. Data were expressed as mean ± standard error of the mean ($n$ = 3).

### Extraction of nuclear protein

After appropriate treatment of the cells with EC extract in the absence or presence of TNF-α, nuclear protein was extracted. Briefly, the cells were harvested with 1 ml of ice-cold PBS and centrifuged for 1 min at 5000 rpm at 4°C. The cell pellet was lysed with 0.4 ml of buffer A containing 10mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol (DTT), and 1mM phenylmethylsulfonyl fluoride (PMSF), for 15 min on ice. Then, 25m l of 10% Nonidet P-40 solution was added and the samples were vortexed for 15 s before centrifuging at 15000 rpm for 5 min at 4°C. The pellet was washed once with 0.5 ml of buffer A and resuspended in 50m l of buffer B, which was composed of 20mM HEPES, pH 7.9, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, and 1mM PMSF. The lysed nuclei were left on ice for 30 min and then centrifuged at 15000 rpm for 5 min at 4°C. The nuclear protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA, U.S.A.). Nuclear extracts were stored at -80°C until use.

### Western blot analysis

Western blotting was performed according to standard procedures. Briefly, RAW264.7 cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.4% Nonidet P-40, 120 mM NaCl, 1.5mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/ml leupeptin, 3 mM NaF and 1 mM DTT at 4°C for 30 min. Cell lysates (10 μg) were resolved on a 4-20% Novex® gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane, and blocked with 10% skim milk. p50 monoclonal antibody (Cat. No. sc-8414,Santa cruz Biotechnology, Inc., USA) were used to detect p50 using chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. Western blot bands were quantified using ImageMaster software (Amersham Pharmacia Biosciences, NJ, USA).

### Casein digestion assay

Agarose solution (1%) was prepared in collagenase buffer (50 mM Tris/HCl, 10 mM $CaCl_2$, 0.15 M NaCl, 7.8 pH) with 0.15% casein and allowed to solidify in wells of 6-well plate (3 ml/well) for 1 h at room temperature. Different concentrations of EC extract (1 μl) was incubated with 10 μl of bacterial collagenase-1 (0.1 mg/ml) in 89 μl of collagenase buffer for 1 h. The reaction products (10 μl) were loaded onto paper disks placed on gelatin-agarose gel and incubated for 18 h at 37°C. The degree of gelatin digestion in agarose gel was visualized by Coomassie Blue staining after removal of the paper disks. Following destaining, the area of light translucent zone over blue background was determined to estimate collagenase activity.

### Statistical analysis

Comparisons of all data were performed using two-tailed, unpaired Student's $t$-test. A $P$ value less than 0.05 was considered statistically significant. Data are expressed as means ± SE.

## Results

### Effect of EC extract on cytotoxicity of RAW 264.7 Cells

In order to investigate the cytotoxic effect of EC extract, MTT assay was carried out to investigate cytotoxity of EC extract. As shown in Fig. 1, EC extract did not have cytotoxic effect below 100 μg/ml for 24 h. Therefore, we teseted anti-inflammatory effect of EC extract with concentrations from 10 to 100 μg/ml.

### Effect of EC extract on expression of Inflammatory Mediators

Immunoassays were performed to examine the inhibitory effect of EC extract on expression of inflammatory mediators including TNF-α, IL-1β, IL-6

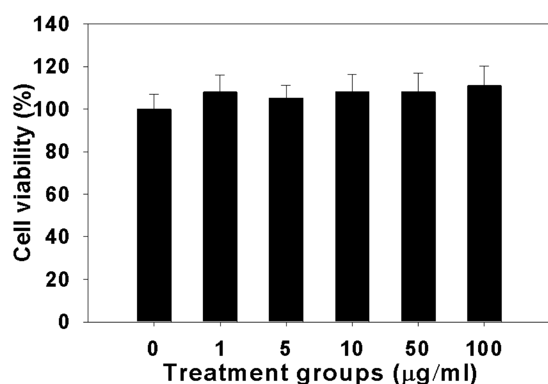


**Fig. 1.** EC extract exhibited no cytotoxicity in RAW244.7 cells. Cells were treated with different concentrations of EC extract and cell viability was determined by MTT assay after 24 h. Data are given as means of values ± SD from three independent experiments.

and $PGE_2$. In this study, RAW264.7 cells were treated with EC extract at 10 μg/ml for 1 h before treatment of cells with 1 μg/ml of lipopolysaccharide to stimulate inflammation. After 1 day of incubation, conditioned media were collected, and then the level of inflammatory mediators was measured. As shown in Fig. 2, EC extract exhibited a dose dependent inhibitory effect on production of all inflammatory mediators such as TNF-α, IL-1β, IL-6. Treatment of EC extract decreased TNF-α level by 65% compared to blank group (Fig. 2A). There was no significant difference between treatment groups of EC extract 10 μg/ml and dexamethasone at 1 μM. As shown in Fig. 2B, the inhibitory effect of EC extract on production of IL-1β was increased in similar trend to that on TNF-α. The level of IL-6 in the presence of EC extract at 10 μM was similar to that of dexamethasone at 1 μM (Fig. 2C). The inhibitory effect of EC extract on production of $PGE_2$ was more effective than those on production of cytokines (Fig. 2D). Treatment of EC extract decreased the level of $PGE_2$ by below 38%, which was lower than that of aspirin at 1 μM used as a positive control in this experiment.

## Effects of EC extract on transcriptional activity of NF-κB in RAW264.7 Cells

The expression of these inflammatory mediators are regulated by NF-κB transcription factor. Therefore, re-

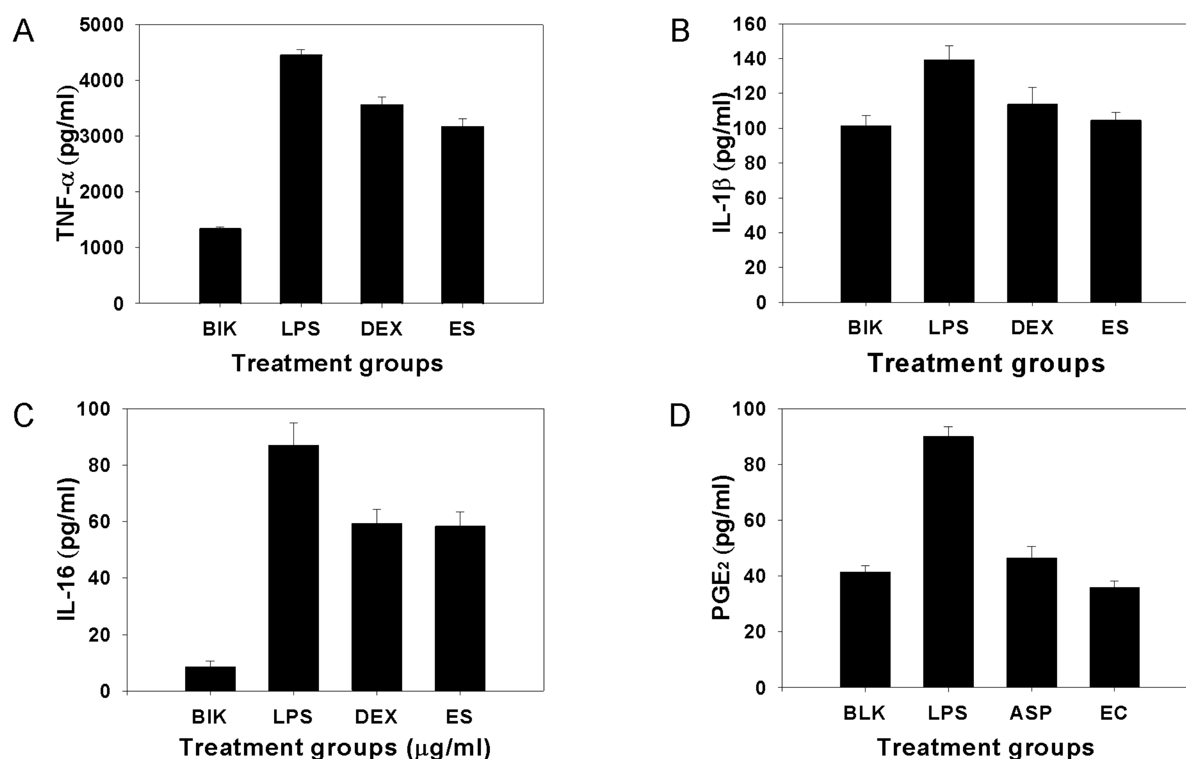


**Fig. 2.** EC extract inhibited the production of inflammatory mediators in RAW244.7 cells. Cells were treated with EC extract at different concentrations for 1 h before treatment of cells with 1 μg/ml of lipopolysaccharide to stimulate inflammation. After 1 day of incubation, conditioned media were collected, and then immunoassays for TNF-α (panel A), IL-1β (panel B), IL-6 (panel C) and $PGE_2$ (panel D) were performed. Dexamethasone at 1 μM and aspirin at 10 μM were used as positive control in cytokines and $PGE_2$, respectively. Data are given as means of values ± SD from three independent experiments.

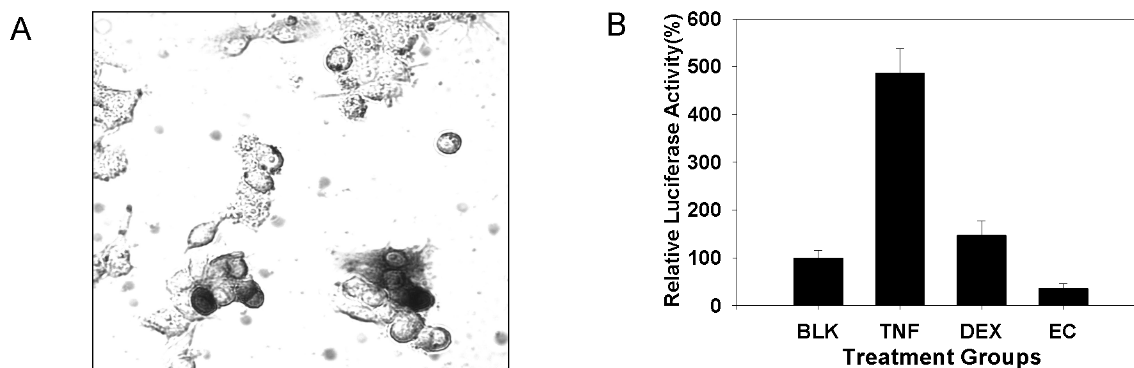


**Fig. 3.** EC extract inhibited the transcriptional activity of NF-κB in RAW264.7 cells. Cells were treated with EC extract at different concentrations for 1 h before treatment of cells with TNF-α treatment at 10 ng/ml to activate NF-κB. (A) pcDNA 3.1 vector was cotransfected to normalize the luciferase activity for all treatment groups and the transfected cells were stained for beta-galactosidase. (B) Transcriptional activity of NF-κB was measured by luciferase assay after cotransfection with NF-κB reporter vector and pcDNA 3.1 vector. Data represent the mean ± S.E. of at least three independent experiments. Level of significance was identified statistically ($P < 0.01$) using Student's $t$ test.

porter gene assay using a reporter construct including NF-κB binding DNA sequence-luciferase gene was carried out to investigate whether transcriptional activity of NF-κB is influenced EC extract in RAW264.7 cells. As shown in Fig. 3A, the RAW264.7 cells cotransfected with reporter vectors of NF-κB with pcDNA 3.1 vector showed above 30% of SA-beta gal positive cells. As shown in Fig. 3B, the transcriptional activity of NF-κB by TNF-α treatment at 10 ng/ml was greatly increased compared to the untreated blank group. Transcriptional activity of NF-κB in cells treated with EC extract was significantly decreased compared to the untreated blank group as shown Fig. 3B ($P < 0.01$). These results suggest that EC extract can inhibit production of inflammatory mediators via inhibition of transcriptional activity of NF-κB in RAW264.7 cells.

## Effect of EC extract on Protein Expression of p50 in RAW264.7 Cells

In order to directly verify the inhibitory effect of EC extract on activation of NF-κB transcription factor which is involved in inflammation, we examined the expression level of p50, a part of NF-κB transcription factor in the presence of EC extract using western blot analysis. As shown in Fig. 4, there was no significant difference between TNF-α stimulated group and treatment group with 1 μg/ml EC extract. However, treatment with EC extract above 5 μg/ml significantly decreased the expression level of p50 in the nucleus compared with TNF-α stimulated group ($P < 0.01$). Furthermore, the expression level of p50 was decreased

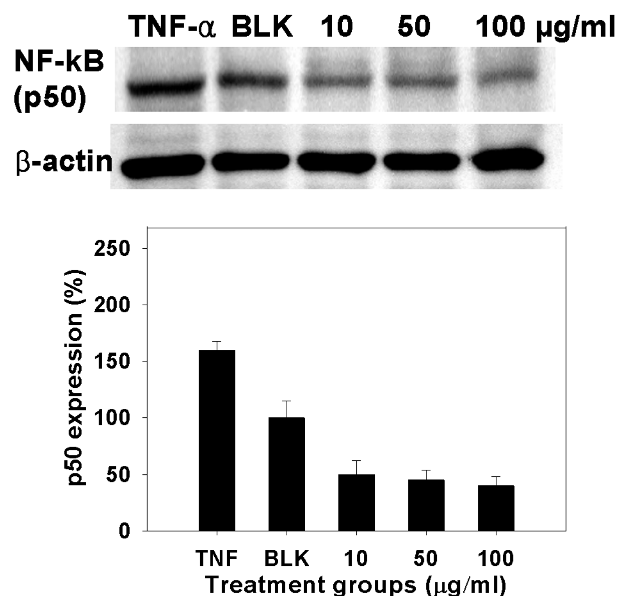


**Fig. 4.** Western blot analysis of protein expression of p50 in RAW264.7 cells after treatment with EC extract. Western blot analysis of cell lysates was performed using p50 monoclonal antibody as indicated. Lower panel represents respective relative protein expression as percentage. Values are expressed as relative protein expression using the following equation. Relative protein expression (%) = (Intensity of a band / intensity of blank bands) × 100. Expression of β-actin protein was used as the control for normalization of p50 protein.

with increment of concentration of EC extract. These results reveal that the inhibitory effect of EC extract on production of inflammatory mediators may be due to the inactivation of NF-κB transcription factor in RAW264.7 cells.

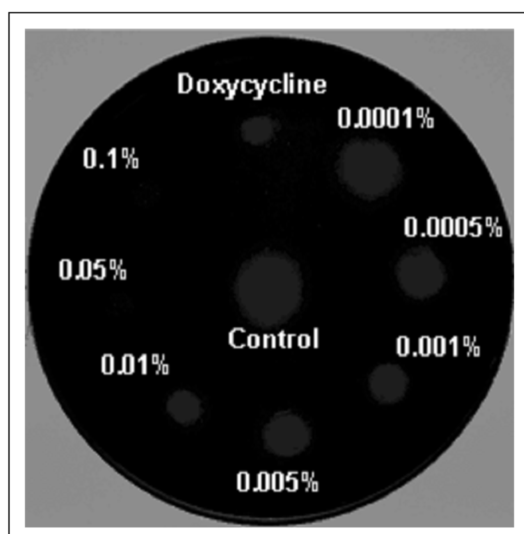


**Fig. 5.** EC extract inhibited casein digestion by bacterial collagenase-1. Bacterial collagenase-1 was reacted with 0.1% DMSO (A) as control, with doxycycline as positive control, and with different concentrations of EC extract. And then 10μl of reaction products were loaded onto paper disks placed on agarose gel containing gelatin and incubated for 18 h. Enzyme activity of remaining bacterial collagenase-1 was calculated by densiometric determination of the gelatin digested clear zone visualized by Coomassie blue staining.

### Inhibitory effect of EC extract on bacterial collagenase-1 assessed by casein digestion assay

In order to investigate the inhibitory effect of EC extract on MMPs, casein digestion assay was carried out using bacterial collagenase-1. MMP activity was compared with initial enzyme activity in control group after incubation of bacterial collagenase-1 with different concentrations of EC extract,. As shown in Fig. 5, the control group with reaction products of bacterial collagenase-1 and 0.1% of DMSO showed the highest MMP activity in the discrete zone, indicating no enzyme inhibition. In contrast, casein digestion was remarkably decreased in the presence of 20 μg/ml of doxycycline used as positive control. MMP activity in treatment of EC extract was decreased in dose-dependent manner. The clear casein digestion was observed with 5 μg/ml or higher concentrations of EC extract, indicating inhibition of more than 50% of bacterial collagenase-1 activity.

## Discussion

Recently the relationship between inflammation and cancer from epidemiological studies has been intensified with the reports that identified chronic infection and inflammation as major risk factors for various types of cancer [5]. Previous studies have reported that NF-κB in inflammation finally not only act as growth signals but also affect avoidance of apoptosis, limitless replicative potential and metastasis [7]. Therefore, NF-κB transcription factor can be a good target for development of medicine because it was widely known to play a key role in cancer development as well as inflammation. A variety of natural compounds have been reported to be able to use as potentially anti-inflammatory agents until more recent times [4].

We have screened the marine algae with anti-inflammatory effect in macrophages from marine resources, and for the first time found that EC extract can inhibit production of inflammatory mediators in macrophages via inactivation of NF-κB transcription factor. In this study, EC extract did not show any cell cytotoxicity on macrophages. This finding is supported by the fact that EC has long been utilized as a traditional food in Korea. Therefore, EC may has a therapeutic potential to prevent or treat chronic inflammation because it has been used an edible seaweed in Korea as well as anti-inflammatory effect in macrophages. EC is abundant in the subtidal regions of Jeju Island, Korea and reported to contain eisenine, biotin and laminine [14]. Previous studies on *Ecklonia* species have reported that high content of phlorotannins is responsible for some biological activities observed in these species. Recently, Kang et al. [11] reported a radical scavenging effect of extracts from *Ecklonia stolonifera* and their results suggested that phlorotannins from *Ecklonia cava* was responsible for this effect. Also, it is reported that some other brown alga contain higher amount of phlorotannins [15]. Furthermore, triphlorethanol-A and eckol , belong to phlorotannins in EC extract, have exerted an inhibitory effect on oxidative stress [13]. It has also been proposed that enzyme extracts from EC have a radical scavenging activity [9]. Based on above results, radical scavenging effects of phlorotannins from EC samples were examined in live cell system. In present study, in order to investigate whether EC can inhibit chronic inflammation, levels of main inflammatory mediators such as cytokines and $PGE_2$ synthesized by cyclooxygenase-2 were determined using immunoassay in macrophage that is the key player of the chronic in-

flammatory response. In our study, EC extract reduced the expression levels of TNF-α, IL-1β, IL-6 and $PGE_2$ in RAW264.7 cells stimulated by lipopolysaccharide, indicating that chronic inflammation can be repressed by EC extract in macrophages. This conclusion is supported by the report that proinflammatory cytokines such as TNF-α, IL-1β and IL-6 were the most potent inducers of COX-2 that are associated with mortality of colorectal cancer [18].

More recently, NF-κB not only has been known to play a vital role in the regulation of various inflammation but also control apoptotic cell death of both normal and malignant cells. Thus, it is a challenging target for anticancer and anti-inflammatory strategies It has often been described as a central mediator of the immune response, particularly because a large variety of bacteria and viruses can lead to its activation. The activation of NF-κB leads to the expression of inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules. Further evidence supporting inflammation's role in the initiation and promotion of gastrointestinal malignancy comes from the fact that constitutive expression of NF-κB has been identified in a number of gastrointestinal malignancies including hepatocellular carcinoma and colorectal cancer [10]. Therefore, in order to investigate whether EC extract could affect transcriptional activity of NF-κB in RAW264.7 cells, reporter gene assay using a reporter construct including NF-κB binding site-luciferase was performed. It was found that EC extract-treated cells repressed transcriptional activity of NF-κB enhanced by treatment of TNF-α. These results suggest that the inhibitory effect of EC extract on production of inflammatory mediators is achieved by inactivation of NF-κB transcription factor which plays a critical role in inflammation in macrophage. This is in accordance with study that an relationship between chronic inflammation and activation of NF-κB was observed in chronic liver inflammation [20]. This observation can be further confirmed by the finding that EC extract could decrease the expression level of NF-κB transcription factor which is involved in chronic inflammation. The expression level of NF-κB in nucleus was significantly decreased in dose dependent manner. These results indicate that the decrease in level of inflammatory mediators was due to inactivation of NF-κB in macrophages. Furthermore, previous research has shown that the activation of NF-κB played an important role in not only chronic inflammation by production of inflammatory mediators but also colon cancer [21, 24].

In chronic inflammation such as osteoarthritis, proinflammatory cytokines induce matrix metalloproteinases (MMPs) that release cartilage extracellular matrix [22]. Therefore, we investigated the inhibitory effects of all marine algal extracts on casein digestion by bacterial collagenase-1. Our results indicated that EC showed remarkable inhibitory effect on casein digestion. Previous studies on *Ecklonia* species have reported that high content of pholorotannins is responsible for some biological activities observed in these species. EC extract exhibited a dose dependent inhibitory effect on casein digestion by bacterial collagenase activity.

Therefore, above results support that EC can exert anti-inflammatory effect via inactivation of NF-κB transcription factor in macrophages. These findings are of considerable value if futher studies could verify that single active component purified from EC can inhibit activation of NF-κB transcription factor at a lower concentration and reduce the incidence of chronic inflammation in vivo model.

## 요 약

만성염증은 치주염, 대장염, 간염 및 관절염과 밀접한 관련성이 있다고 알려져 있다. 최근에 항염증제가 해양자원으로부터 개발되고 잇다. 본 연구에서는 감태 (EC)가 항염증효과가 있다는 것이 발견되었다. 갈조류에 속하는 감태의 에탄올 추출물이 RAW 264.7 세포에서 tumor necrosis factor-α, interleukin-1β, interleukin-6 and prostaglandin $E_2$ 와 같은 염증매개체의 생성에 탁월한 효과를 나타내었다. 더욱이 reporter gene assay 및 western blot 분석에서 감태추출물은 대식세포에서 염증매개체의 발현을 조절하는 NF-κB 전사인자의 불활성화를 통하여 항염증효과를 나타내었다. 뿐만 아니라 감태추출물은 만성염증에 중요한 역할을 하는 기질금속단백질의 활성을 억제 하였다. 이러한 결과는 감태추출물이 만성염증을 억제하는데 잠재적으로 이용될 수 있다는 것을 것을 암시하고 있다.

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