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Anti-inflammatory Effects of Enzymatic Extract from *Ecklonia cava* on TPA-induced Ear Skin Edema

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Abstract Anti-inflammatory potential of the enzymatic extract prepared by Kojizyme (ECK), a component of brown seaweeds *Ecklonia cava* (*Alariaceae*, Phaeophyta) *in vivo* was investigated. For the application of mouse ear edema model, 12-*O*-tetradecanoylphorbol acetate (TPA) was used, a topical inducer of a long-lasting inflammatory response. Our results demonstrated that ECK inhibited ear edema when topically applied to mouse ear skin. In histological evaluation, the inhibition activity of ECK on TPA-induced inflammation is similar to that of dexamethasone, although less strong. In addition, the mRNA expression levels of IL-1β, IFN-γ, TNF-α, and cyclooxygenase-2 (COX2) and the immunoreactivity to inducible nitric oxide synthase (iNOS) and COX2 expressed mainly in inflammatory cells were down-regulated by ECK. These results indicate that ECK has anti-inflammatory effects through the inhibition of Th1 cytokines and 2 inducers of inflammation in TPA-induced ear skin edema.

Keywords: anti-inflammatory effect, *Ecklonia cava*, 12-*O*-tetradecanoylphorbol acetate (TPA), ear skin edema

Introduction

Seaweeds are a widely available source of biomass over 2 million tons and are either harvested from the oceans or cultured annually for food or phycocolloid production. They have various compounds such as vitamins, minerals, natural bioactive compounds, and various functional polysaccharides. These seaweeds can be divided into 3 basic types such as brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta) seaweed by dominant pigments such as xanthophylls, fucoxanthin, phycoerythrin, and chlorophyll a and b (1). Especially, brown seaweeds are known as the resource of the fucoxanthin pigment and polysaccharides which possess many bioactive properties (2,3). For example, the brown seaweed *Ecklonia cava Alariaceae* (*E. cava*) is rich in xanthopophyll, fucoxathin, vitamins, vitamin precursors such as α-tocopherol, β-carotene, niacin, thiamin, and ascorbic acid, and polysaccharides such as fucoidan, alginates, fucans, and laminarans, which are water-soluble dietary fibers and phycocolloids. *E. cava* is common around Jeju Island of Korea and, reportedly contains richer supply of total polyphenolic compounds and sulfated polysaccharides than other brown seaweeds (4). The polyphenolic compounds of brown seaweeds have been called phlorotannins, and those of *E. cava* are the phenolic secondary metabolites eckol (a closed-chain trimer of phloroglucinol), 6,6'-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer), and triphlorethol-A, and sulfated polysaccharides with biological activities (5-7). In particular, contained polyphenols and sulfated polysaccharides in *E. cava* species alone are oxygen-radical scavengers (8), bactericidal agents (9), anti-plasmin inhibitors (10), antimutagens (11), and cell damage inhibitors (5,6). Our previous studies have demonstrated that enzymatic extracts of *E. cava* act as anti-oxidants, anticoagulants, and matrix metalloproteinase inhibitors (4,12,13). However, no other studies have reported the anti-inflammatory activities of *E. cava* extracts or their biological mechanisms.

Inflammatory response is a defense mechanism evoked by body tissues in response to injury or microbial invasion or most disease progression. In addition, the response implicates macrophages and neutrophils, which secrete a number of mediators (eicosinoids, oxidants, cytokine, and lytic enzymes) responsible for initiation, progression, and persistence of acute or chronic state of inflammation (14, 15). Since it causes discomfort to the host body, it requires a careful control and treatment.

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) which is a kind of isomers (COX1 and COX2) are most important amongst these mediators increased by pro-inflammatory cytokines in inflammatory responses (16). Normally, iNOS and COX2 produce a large amount of NO, which contributes to inflammation by increasing vascular permeability, causing edema formation and prostaglandin E_2 (PGE_2) in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced acute and chronic ear edema models, respectively (17-19). Inhibition in the release of these mediators is a potential strategy to control inflammation and is implicated in mechanism of action of a number of anti-inflammatory drugs including the representative ones like dexamethasone (20).

This study demonstrated that *E. cava* extracts prepared by Kojizyme (ECK) have anti-inflammatory activities in

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TPA-induced ear skin edema in mice. Our finding indicates that the ECK is of potential interest for the development of an agent that can easily permeate through the skin and suppress skin inflammation responses.

Materials and Methods

Reagents 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and dexamethasone were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Kojizyme was purchased from Navo Co. (Bagsvaed, Denmark). The primers were purchased from Bioneer (Daejeon, Korea) and cDNA synthesis kits were purchased from Promega (San Luis, CA, USA). Trizol reagent was purchased from Molecular Research Center Inc. (Cincinnati, OH, USA).

Mice All experiments were performed with ICR mice, ages 8 to 9 weeks, purchased from SLC, Inc. (Yokohama, Japan). The mice were housed in conventional animal facilities with a National Institutes of Health (NIH)-07-approved diet and water *ad libitum* at a constant temperature (23±1°C) and humidity (40-60%) according to the guidelines of Animal Care and Use Committee of Cheju National University.

Preparation and composition of ECK *E. cava* was collected from the coast of Jeju Island, Korea, washed with fresh water, freeze-dried, and pulverized into powder with a grinder. ECK was prepared in accordance with the method indicated in the previous study (21,22). The ECK contained rich polyphenols and crude sulfated polysaccharides with high contents of sulfate group (12,22). In addition, the previous studies reported that fucoidan represented a class of fucose-enriched sulfated polysaccharides found in the extracellular matrix of brown algae (23). Since fucoidan usually contains large amounts of fucose (more than 50%) and sulfates, this crude sulfated polysaccharide in ECK used in this study can be regarded as fucoidan (22).

Induction of the mouse ear skin edema by TPA Normally, topical application of TPA induces a long-lasting inflammatory response and offers a skin inflammation model appropriate for evaluating anti-inflammatory agents (24). To evaluate whether ECK has the topical anti-inflammatory activity in the skin inflammation model, TPA-induced mouse ear skin edema assay was carried out. ECK (100 μg/20 μL EtOH) or EtOH (20 μL) was applied to the surface of mouse ear and dexamethasone (50 μg/20 μL EtOH), a topical inhibitor of inflammation was used as positive control. After 1 hr, TPA (2.5 μg/20 μL EtOH) was directly applied to the same ear skin surface of all mice. The ear thickness was measured before treatment (a), and 6 hr after TPA treatment (b = TPA only; b' = TPA plus sample). The inhibitory activities of ECK on TPA-induced ear skin edema were calculated using caliper as follow:

Edema A is induced by TPA alone (b-a).
Edema B is induced by TPA plus sample (b'-a).

Inhibitory activity (%) = [(edema A-edema B)/edema A]×100.

Each value was the mean of individual determinations from 3 mice and statistical analysis was carried out using Student's *t*-test.

Histological analysis A histological analysis to identify anti-inflammation evaluation of ECK in TPA-induced ear skin edema in mice was performed. The ear samples were fixed in 10% neutral buffered formalin for 1 week and then washed with tap water for 5 hr. After washing, the samples were applied to tissue processing machine (Jung Histokinette 2000; Leica, Nussloch, Germany). After 18 hr, the ear samples were embedded in paraffin, molded, and sectioned on slides coated by 3-aminopropyl triethoxy silane (Sigma-Aldrich). And then, the slides were dried at about 40°C for 12 hr and were applied to haematoxylin and eosin (H&E) staining procedure. The paraffin on slides were removed and dehydrated in alcohol. Dehydrated slides were replaced by tap water and applied to haematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) solution to stain nuclear and cytoplasm, respectively. Then, representative areas were selected for qualitative light microscope analysis.

Immunohistochemical staining After incubation with normal goat or horse serum, tissue sections were allowed to react with the first antibody for 1 hr. The first antibodies used in the present study were iNOS (1:500 dilutions, Santa Cruz Biotechnology, CA, USA) and COX2 (1:200 dilutions, Santa Cruz Biotechnology). Then, sections were incubated with biotinylated anti-mouse or rabbit, IgG (Vector, Burlingame, CA, USA) followed by HRP-labeled Vectastain Elite ABC kit (Vector). HRP binding sites were detected with 3,3'-diaminbenzidine (DAB; Vector) and finally counterstained with haematoxylin.

RNA preparation The ear samples were mixed with Trizol reagent (Molecular Research Center Inc.). The addition of chloroform (Sigma-Aldrich) and incubation for 5 min at 4°C followed. The supernatants obtained after centrifugation were treated with isopropanol (Sigma-Aldrich) and the resulting RNA pellets were washed and stored at −20°C.

Reverse transcription-polymerase chain reaction (RT-PCR) The cDNA was synthesized with RNA purified from spleen cells by using a Promega A3500 kit, according to manufacturer's instructions. The PCR was performed with primers displayed in Table 1 for 40 cycles of a 5 min denaturing step at 94°C, a 1 min annealing step at 55 to 60°C and a 20 min extension phase at 72°C using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were run on a 1.5% EtBr/agarose gel and visualized by ultraviolet (UV) transillumination (Vilber Lourmat, Marne la Uallee, France).

Statistical analysis Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as means±standard error (SE). A p-value <0.05 was considered significant.

Results and Discussion

ECK decreases the TPA-induced mouse ear skin edema As shown in Fig. 1A, ECK reduced the ear thickness in TPA-induced ear skin edema similar to dexamethasone. When the inhibitory activity was evaluated by ear thickness, ECK showed potent inhibitory activity as well as

Table 1. The sequences and expected size of oligonucleotides used for RT-PCR

Oligonucleotide		Sequence	Size (bp)
IL-1β	5'-primer	5'-GCT ACC TGT GTC TTT CCC GTC G-3'	291
	3'-primer	5'-TTG TCG TTG CTT GGT TCT CCT TG-3'	
TNF-α	5'-primer	5'-GGC AGC TTC TGT CCC TTT CAC TC-3'	366
	3'-primer	5'-CAC TTG GTG GTT TGC TAC GAC G-3'	
IFN-γ	5'-primer	5'-AGG TCA ACA ACC CAC AGG TCC A-3'	397
	3'-primer	5'-CCA GAT ACA CCG CAA TCA C-3'	
COX2	5'-primer	5'-GCA AAT CCT TGC TGT TCC AAT C-3'	335
	3'-primer	5'-GGA GAA GGC TTC CCA GCT TTT G-3'	
IL-4	5'-primer	5'-ACG GAG ATG GAT GTG CCA AAC GTC-3'	361
	3'-primer	5'-CGA GTC ATC CAT TTG CAT GAT GC	
IL-10	5'-primer	5'-CAC TGC TAT GCT GCC TGC TCT T-3'	417
	3'-primer	5'-TCT TCA CCT GCT CCA CTG CCT T-3'	
GAPDH	5'-primer	5'-AAC GAC CCC TTC ATT GAC C-3'	701
	3'-primer	5'-TCA GAT GCC TGC TTC ACC-3'	

dexamethasone, the positive control (about 42 and 48%, respectively, and $p<0.05$). The optical microscopic analysis of mice' ears, 6 hr after the application of TPA, revealed marked infiltration of inflammatory cells such as neutrophils and lymphocytes (arrows) in dermis induced by TPA with the reduction of edema (Fig. 1B). In addition, it showed these events were significantly reduced after topical application of ECK (100 μg/ear) (Fig. 1C). Dexamethasone used as a reference drug also reduced these inflammatory responses markedly (Fig. 1D). Thus, ECK inhibited TPA-induced inflammatory processes, although less strong than dexamethasone. From these results, ECK decreased the ear skin thickness and the infiltration of extensive inflammatory cells in TPA-induced ear skin edema.

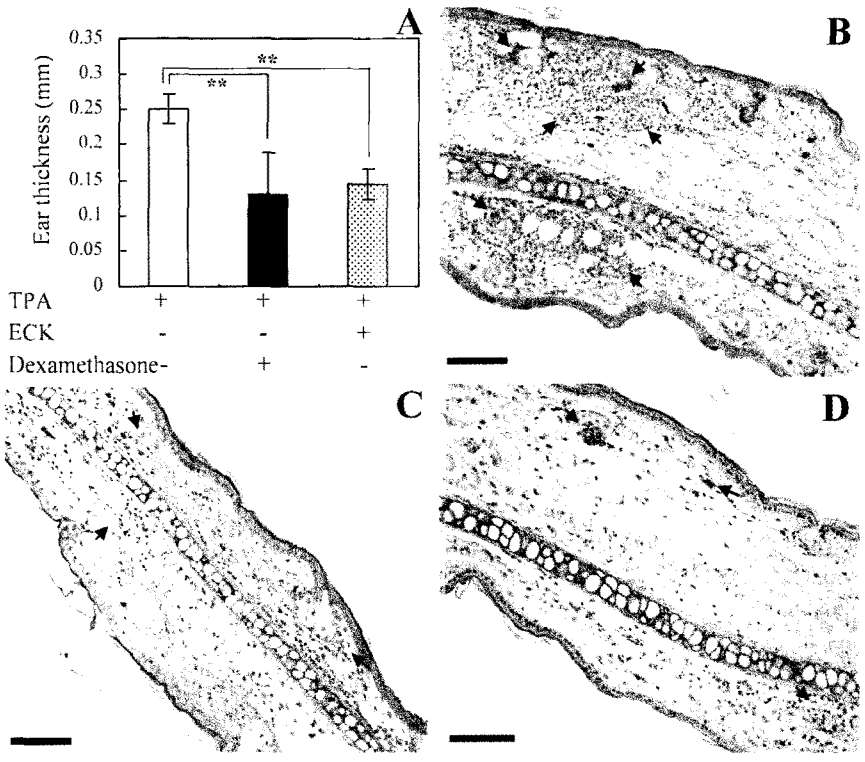

Fig. 1. Measurement of the ear thickness and histological analysis of vertical sections and on TPA-induced mouse ear skin edema. (A) Each value was the mean of individual determinations from 3 mice and statistical analysis was carried out Student's *t*-test. $^{**}p<0.05$. (B) Ears treated with TPA (2.5 μg/ear). (C) Ears treated with TPA plus ECK (100 μg/ear). (D) Ears treated with TPA plus dexamethasone (50 μg/ear). Arrows indicate the inflammatory cells of acute inflamed mass in dermis. Bars = 20.5 μm.

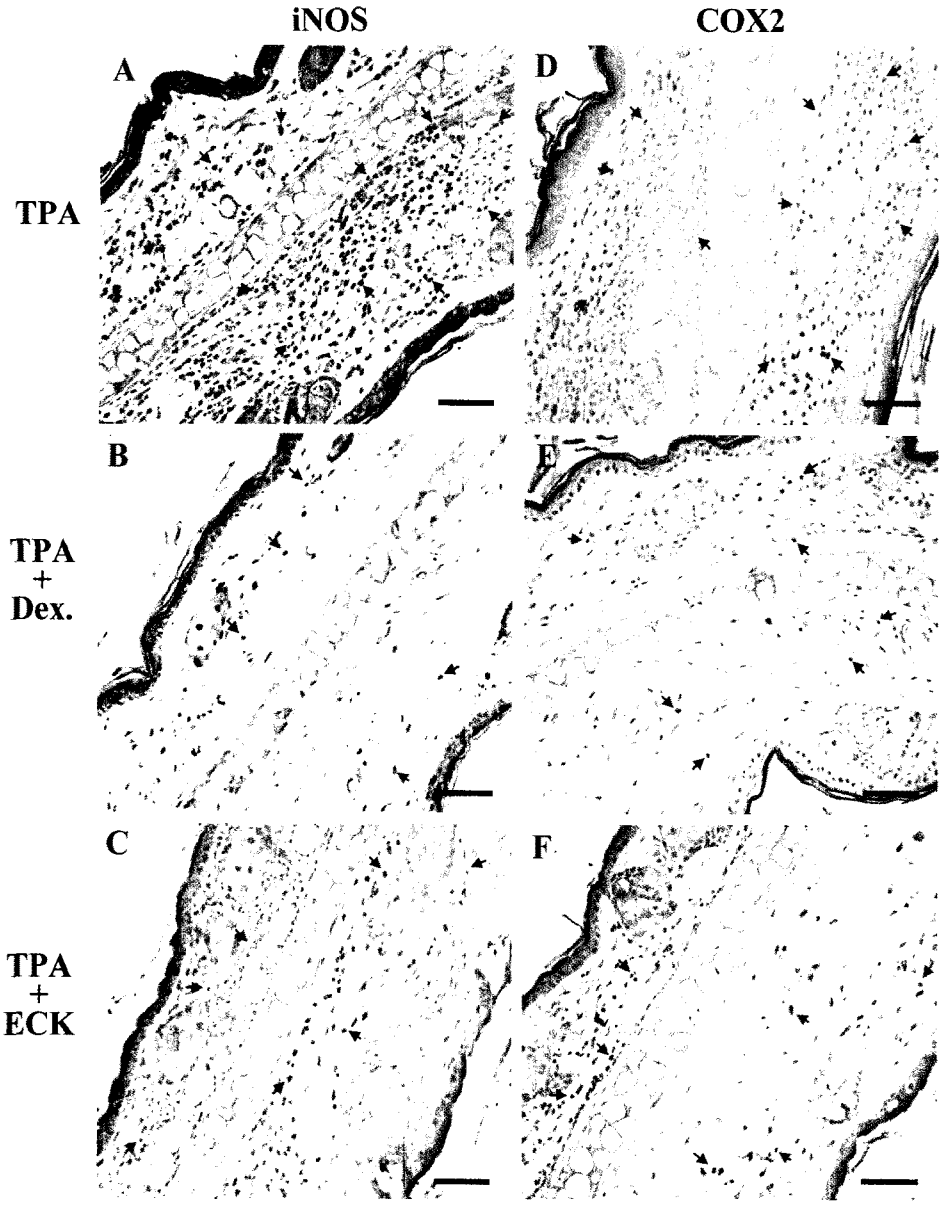

Fig. 2. Immunochemical detection for iNOS and COX2 in TPA-induced mouse ear skin edema model. (A) and (D) Ears treated with TPA (2.5 μg/ear). (B) and (E) Ears treated with TPA plus dexamethasone (50 μg/ear). (C) and (F) Ears treated with TPA plus ECK (100 μg/ear). Arrows indicate the inflammatory cells showing iNOS and COX2-positive immunoreactivity, respectively. Each value was the mean of individual determinations from 3 mice and statistical analysis was carried out Student's *t*-test. Bars=20.5 μm.

Immunohistochemical localization of iNOS and COX2 proteins in the ear samples of TPA-induced edema model The localization of iNOS and COX2 protein in the ear samples was determined by immunohistochemistry using samples from TPA-induced mouse ear skin edema models. As shown in Fig. 2A and 2D, the topical application of TPA to the ear caused the expression of iNOS and COX2 protein in inflammatory cells such as neutrophils and macrophages (arrows). In contrast, the application of ECK and dexamethasone significantly reduced the expression of iNOS and COX2 protein in neutrophils and macrophages (arrows) compared to the control (Fig. 2B, 2C, 2E, and 2F). But, the inhibitory effect of dexamethasone on the expression of iNOS and COX2 protein was slightly higher than that of ECK. This result suggested that ECK had the anti-inflammation activity due to the inhibitory effects on the cellular expression of iNOS and COX2 proteins in TPA-induced inflammation response.

ECK reduces the mRNA expression level of Th1 cytokines and COX2 in TPA-induced mouse ear edema model The specific cytokines produced by polarized Th1 and Th2 cells are the primary effectors that promote the differentiation of precursor Th cells, but these cytokines also cross-regulate the other subset's functional activities. The change pattern of cytokines can result in qualitative changes in the type of immune response (25). If ECK modulates the mRNA expression levels between Th1 (IL-1β, TNF-α, and IFN-γ) and Th2 (IL-4) cytokines or COX2, ECK offers an alternative way for inhibiting the inflammation response. For elucidating this possibility, we examined whether ECK modulates the mRNA expressions

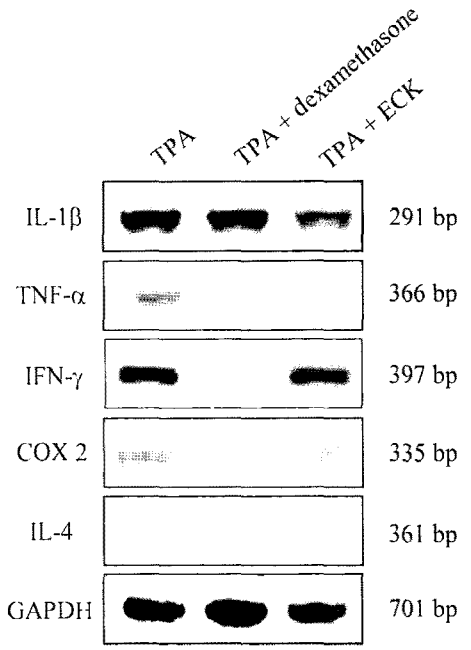

Fig. 3. Effect of ECK on the mRNA expression level of cytokines in TPA-induced ear skin edema model. TPA, ear tissue treated with 2.5 μg of TPA; TPA + dexamethasone, ear tissue treated with 2.5 μg of TPA and 50 μg of dexamethasone; TPA + ECK, ear tissue treated with 2.5 μg of TPA and 100 μg of ECK.

of Th cytokines and COX2 in TPA and/or ECK-applied ear skin tissues. As shown in Fig. 3, the mRNA expression levels of IL-1β, TNF-α, and COX2 were markedly decreased by ECK, although that of IFN-γ did not change dramatically in ECK-treated skin tissues. Furthermore, the mRNA expression level of IL-4 was up-regulated by ECK compared to those of only TPA-treated ear skin tissues. These results suggested that ECK accomplished its anti-inflammatory activity by reducing the expression levels of Th1 type cytokines and COX2.

The previous study indicated that skin leukocyte accumulation induced by TPA application is necessary for the progression of inflammatory reaction as well as for the over-expression of the enzyme COX2 (26,27). It is also reported that the application of TPA normally results in the induction of epidermal thickness and the infiltration of inflammatory cells such as leukocytes, mainly, neutrophils, and monocytes/macrophages in the dermis of mouse skin (26). Our results strongly suggest that the marked inhibition of inflammatory response by ECK on ear edema may be related to their ability to inhibit the release of IL-1β, IFN-γ, TNF-α, and COX2. In fact, a marked difference exists in the mechanisms of the inflammatory response induced by topical application of TPA in the mouse. In another study, researchers have reported that cyclooxygenase inhibitors are highly effective against the inflammation caused by TPA (28). Two major compound groups in ECK, polyphnols and fucoidans, might contribute to the anti-inflammatory effects. The polyphenols from ECK are mainly phlorotannins including eckol, dieckol, and phloroglucinol and can penetrate the skin barrier and elicit the anti-inflammatory effects. The previous study indicated that polyphenol compounds such as flavonoids, catechins induced the anti-inflammatory effects *in vivo* by modulating the expression levels of inducible enzymes such as COX1 and COX2 (29). In addition, fucoidans might also contribute to the anti-inflammatory effects. Previous studies have reported that fucoidans might function as anti-inflammatory agents in several experimental murine models by suppressing the infiltration of neutrophils and decreasing the extravasation of leukocytes (30,31). Also, the anti-inflammatory ability to prevent selectin-mediated cell-cell interactions is supported by *in vitro* experiments showing that fucoidans may indeed bind to purified and membrane-exposed P- and L-selectins (32) but not to E-selectin (33). This suggests that the inhibition capability on the ear edema might be related to the ability of polyphenol and the fucoidan (crude sulfated polysaccharide) compounds in ECK.

Consequently, our study suggests that ECK has anti-inflammatory effects by inhibiting Th1 cytokine and COX2 expressions in TPA-induced ear skin edema model. Being a natural product, ECK can be a good candidate for the therapeutic agents of inflammation-related diseases.

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