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감태 효소 추출물 및 폴리페놀 추출물의 생리활성에 관한 연구

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Biological Potential of Enzymatic and Polyphenol Extracts from *Ecklonia cava*

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요 약: 본 연구에서는 감태 효소 추출물과 그것의 폴리페놀 추출물의 화장품 원료로서의 효능을 알아보기 위하여 항산화, 항당화, 미백, 항염 효과와 관련된 실험을 실시하였다. 감태 효소 추출물과 폴리페놀 추출물은 강력한 라디컬 소거능을 가지고 있으며 BSA/Glucose 시스템에서 최종당화생성물의 형성을 저해하는 항당화 활성과 타이로시네이즈 저해를 통한 우수한 미백력을 가지고 있음을 확인하였다. 또한 두 추출물 모두 세포 내에서 PGE_2와 NO 생성 저해를 통한 항염 효과를 나타내었다. 이러한 결과를 종합해 볼 때, 감태 효소 추출물과 그 폴리페놀 추출물은 화장품 원료로서의 응용 가능성이 있을 것으로 사료된다.

Abstract: To investigate the efficacy of enzymatic extract of *Ecklonia cava* and its polyphenol extract (AG-DK) as cosmetic ingredients, the anti-oxidative effect, anti-glycation effect, anti-melanogenic effect, and anti-inflammatory effect of the extracts were evaluated *in vitro.* The enzymatic extract of *E. cava* (SC_{50} 42.9 ppm) and AG-DK (SC_{50} 6.4 ppm) showed a strong DPPH free radical scavenging activity. The anti-glycation ability of the enzymatic extract of *E. cava* and AG-DK was tested using bovine serum albumin (BSA), which inhibited the formation of advanced glycation end-products (AGEs) in the BSA/glucose system. The enzymatic extract of *E. cava* (IC_{50} 97.2 ppm) and AG-DK (IC_{50} 7 ppm) had inhibitory effects on tyrosinase activity. Moreover, the enzymatic extract of *E. cava* and AG-DK had an anti-inflammatory effect through the inhibition of nitricoxide (NO) and prostaglandin E2 (PGE_2). These findings suggest that the enzymatic extract of *E. cava* and AG-DK can be applied to skin-care products as cosmetic ingredients.

Keywords: *Ecklonia cava extracts, anti-oxidant, anti-glycation, tyrosinase inhibition, anti-inflammation*

1. Introduction

Marine plants, particularly seaweeds, have been widely used in such applications as foods, nutraceuticals, animal feeds, and cosmetics [1] and have long been used in traditional medicine, particularly in Asia.

Ecklonia cava (*E. cava*) grows plentifully in the sea surrounding Jeju Island in Korea. In previous studies, *E. cava* has been demonstrated to exhibit many beneficial bioactivities, including antioxidant, anticancer, anticoagulant, and matrix metalloproteinase inhibitory activities [2-6]. *E. cava* has been reported to contain various phlorotannins - such as eckol, 8, 8′ - bieckol, 8, 4′′ - dieckol, phlorofucofuroeckol A, triphlorethol - A, phloroglucinol,

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dioxinodehydroeckol, fucodiphlorethol G [7-10].

Although seaweeds are known to possess numerous beneficial properties, their industrial applications have been limited due to the low inclusion efficiency and high cost of manufacturing involved in chemical hydrolysis. A number of studies have examined various seaweed polysaccharide degrading enzymes, and enzymatic hydrolysis processes have been investigated for the improvement of production yields and the bioefficacy of seaweed polysaccharides [11,12]. In previous reports, the celluclast extract of *E. cava* showed higher degrees of hydrolysis, exhibited higher antioxidant activity, and contained distinctly larger amounts of phenolic compounds [13]. So in this study, we prepared enzymatic extract of *E. cava* using celluclast and its polyphenol extract (AG-DK). We investigated the efficiency of the enzymatic extract of *E. cava* and AG-DK as cosmetic ingredients. The free radical scavenging activity, anti-glycation activity, anti-melanogenesis effect, and anti-inflammatory effect of the enzymatic extract of *E. cava* and AG-DK were evaluated *in vitro*.

2. Materials and Methods

2.1. Preparation of the Extracts

Marine brown alga *E. cava* was collected from the shores of Jeju Island in Korea. In this study, *E. cava* was enzymatically hydrolyzed to prepare water-soluble extracts using carbohydrate enzyme (celluclast 1.5 L FG, Novozyme Nordisk, Bagsvaerd, Denmark), and its polyphenol extract was isolated from enzymatic extract. To briefly state the preparation procedure, 50 g of *E. cava* was homogenized with water (2 L) and mixed with 500 μL of carbohydrate enzyme. The *E. cava* enzymatic extract was adjusted to be within the optimum pH and temperature range of the carbohydrate enzyme, and enzymatic reactions were performed for 24 h. Following extraction, the extract was boiled for 10 min at 100 ℃ to inactivate the enzymes. Then, centrifugation (3,000 rpm for 20 min at 4 ℃) and ultra-filtration were performed to clarify the *E. cava* enzymatic extract and remove the residue. The *E. cava* enzymatic extract was adjusted to pH 7.0. The enzymatic extract was obtained by ultra-filtration to permeate molecular polyphenols in the supernatant and then concentrated using freeze-drying. AG-DK was then prepared by extraction with 94% ethanol and freeze-drying.

2.2. DPPH Radical Scavenging Activity

The free radical scavenging activity of two extracts, based on the scavenging activity of the stable 1, 1-diphenyl -2-picrylhydrazyl (DPPH, Sigma, USA) free radical, was determined using the method described by Fujita *et al*. [12]. First, 100 μL of sample solution was added to 100 μL of DPPH methanolic solution (0.1 mM) and incubated at room temperature for 30 min. The absorbance was then measured at 516 nm using an ELISA reader (Molecular Devices, SpectraMax 190, USA). Resveratrol (Sigma, USA) was used as a positive control.

2.3. Anti-glycation Assay

An anti-glycation assay was performed according to the methods reported by Matsuura and colleagues with a slight modification [14]. Albumin (4 mg/mL final concentration, Sigma, USA) was incubated with glucose (400 mM final concentration, Sigma, USA) in the presence of *E. cava* enzymatic extract, AG-DK, and PBS. The reaction was allowed to proceed at 60 ℃ for 48 h and then stopped by adding 20 μL of 100% (w/v) trichloroacetic acid (TCA, Sigma, USA). The TCA-added mixture was incubated at 4 ℃ for 10 min before being subjected to centrifugation at 10,000 g. The precipitate was re-dissolved with alkaline PBS (pH 10), and the relative amount of glycated BSA was immediately determined based on fluorescence intensity using a spectrofluorometer (excitation: 370, emission: 440 nm, Tecan, Infinite F500, Switzerland). Results were expressed as percentage inhibition of formation of the glycated protein. Aminoguanidine (glycation inhibitor, Sigma, USA) was used as a positive control.

2.4. Tyrosinase Activity Assay

In an aqueous solution, 0.1 M potassium phosphate

buffer (pH 6.8), 3 mM L-tyrosine solution, and 2,000 units/mL of tyrosinase (Sigma, USA) were mixed. The mixture was incubated at 37 ℃ for 10 min followed by measurement at 475 nm using an ELISA reader. The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of each sample that inhibited 50% of tyrosinase activity. The resulting data were expressed as a percentage of inhibition of tyrosinase activity.

2.5. Measurement of Cell Viability

Cell viability was determined by the methyl tetrazolium (MTT, Sigma, USA) assay [15]. Briefly, murine macrophage RAW 264.7 cells (Korea Cell Line Bank, Korea) were seeded onto a 96-well plate at 4×10^3 cells/well and incubated for 24 h. After incubation with various concentrations of the enzymatic extract of *E. cava* and AG-DK for 24 h, 50 μL of 2 mg/mL MTT, which is converted to a formazan product by metabolically active cells, was added to each well. After 3 h of incubation, the supernatant was removed, and the formazan crystals were dissolved in dimethyl sulfoxide. The optical densities at 540 nm were measured using an ELISA reader.

2.6. Measurement of Nitric Oxide

RAW 264.7 cells (2×10^5 cells/well) were seeded onto a 96-well culture plate at 37 ℃ for 24 h. The cells were treated with 1 μg/mL of lipopolysaccharide (LPS) only or with different concentrations of *E. cava* enzymatic extracts and AG-DK for 24 h. The extracellular medium containing NO was determined using a Griess reagent system kit [16]. The absorbance was measured at 540 nm after incubation for 15 min.

2.7. Measurement of PGE_2

The anti-inflammatory activities of these compounds were evaluated by determining PGE_2 production in LPS-stimulated RAW 264.7 cells. The cells were treated with 1 μg/mL of LPS only or with different concentrations of enzymatic extracts and AG-DK for 24 h. The extracellular medium containing PGE_2 was determined using a PGE_2 assay kit (R&D System, USA).

2.8. Statistics

All data are presented as means ± standard deviations (SD). Statistical differences between two groups were determined by a two-tailed Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. DPPH Radical Scavenging Activity of the *E. cava* Enzymatic Extract and AG-DK

Anti-oxidant activity of the enzymatic extract of *E. cava* and AG-DK was determined by measuring free radical scavenging activity using the DPPH test. The *E. cava* enzymatic extract and AG-DK exhibited a high free radical scavenging activity (Figure 1). The concentrations of antioxidants that scavenged free radicals by 50% (SC_{50}) of *E. cava* enzymatic extract, AG-DK, and resveratrol were 42.9 ppm, 6.4 ppm, and 6.7 ppm, respectively.

3.2. Anti-glycation Activity of the Enzymatic Extract of *E. cava* and AG-DK

The anti-glycation activity of the enzymatic extract of *E. cava* and AG-DK was tested for its ability to inhibit the formation of glycated BSA at 60 ℃ for 48 h. As shown in Figure 2, in the presence of AG-DK or aminoguanidine, the fluorescent intensity was significantly decreased, indicating less formation of glycated BSA.

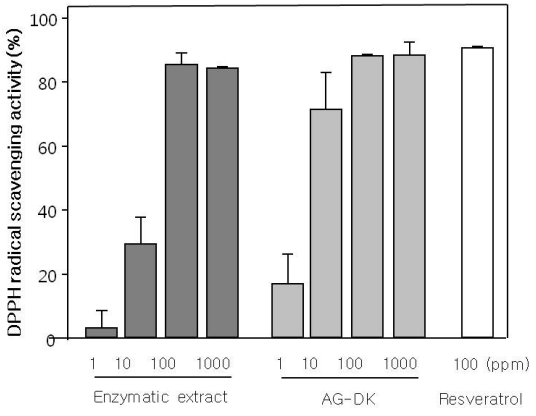

Figure 1. Free radical scavenging activity of enzymatic extract of *E. cava* and AG-DK.

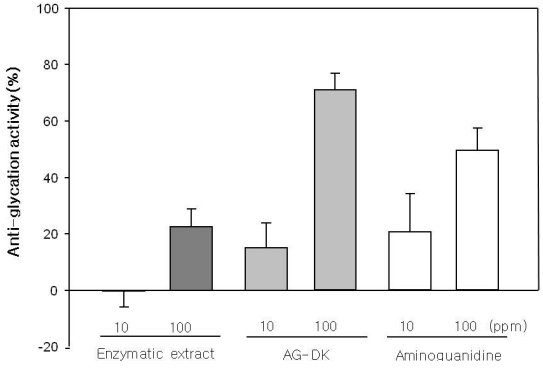

Figure 2. Anti-glycation activity of enzymatic extract of *E. cava* and AG-DK on BSA glycation by glucose.

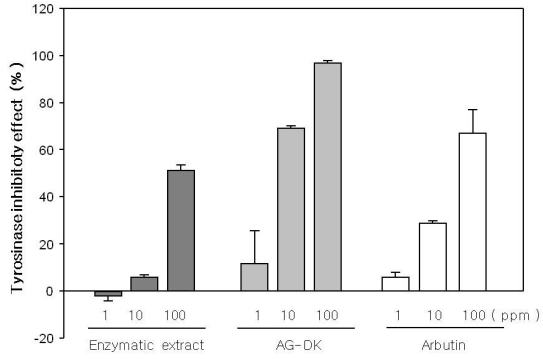

Figure 3. Inhibitory effects of *E. cava* and AG-DK on mushroom tyrosinase activity.

3.3. Tyrosinase Inhibition Activity of *E. cava* and AG-DK

We investigated the effect of the enzymatic extract of *E. cava* and AG-DK on tyrosinase activity using mushroom tyrosinase. As shown in Figure 3, the enzymatic extract of *E. cava* (IC_{50} 97.2 ppm) and AG-DK (IC_{50} 7 ppm) had a strong inhibitory effect on tyrosinase activity (Figure 3). In particular, the inhibitory effects on tyrosinase of AG-DK were much higher than those of arbutin (IC_{50} 59.4 ppm), known as a whitening agent.

3.4. Cytotoxicity test of The Enzymatic Extract of *E. cava* and AG-DK

The enzymatic extract of *E. cava* and AG-DK were treated with RAW 264.7 cells to determine their cytotoxic effect, and cell viability was determined using MTT assay. Figure 4 shows that they were not cytotoxic at

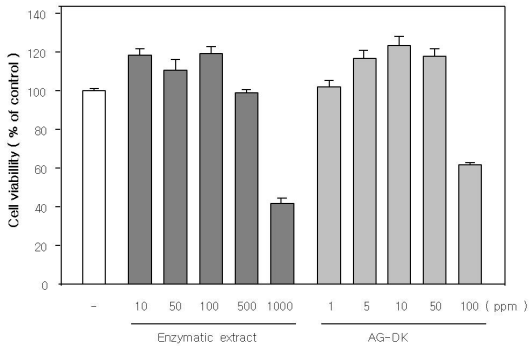

Figure 4. Cytotoxicity test of enzymatic extract of *E. cava* and AG-DK in RAW 264.7 cells.

RAW 264.7 cells in the concentration range (enzymatic extract: to 500 ppm, AG-DK: to 50 ppm).

3.5. Anti-inflammatory Effect of the Enzymatic Extract of *E. cava* and AG-DK

To investigate the anti-inflammatory effect of the enzymatic extract of *E. cava* and AG-DK in RAW 264.7 cells, the inhibition of LPS-induced NO and PGE_2 production were evaluated. NO and PGE_2 production were greatly increased after LPS treatment for 24 h. The *E. cava* enzymatic extract and AG-DK dose-dependently decreased the production of NO and PGE_2 in LPS-stimulated RAW 264.7 cells (Figures 5,6).

4. Conclusion

Many contemporary researchers are interested in finding natural ingredients that are both safe and effective. Enzymes can convert water-insoluble materials into water soluble materials, also this method do not adapt any toxic chemicals. Interestingly, this technique gains high bioactive compound yield and shows enhanced biological activity in comparison with water and organic extract counterparts. In this study, we evaluated various biological activities of the *E. cava* enzymatic extract and its polyphenol extract.

Intracellular and extracellular oxidative stress initiated by reactive oxygen species (ROS) cause skin aging. AGEs are caused by abnormal glycation of proteins, provoke

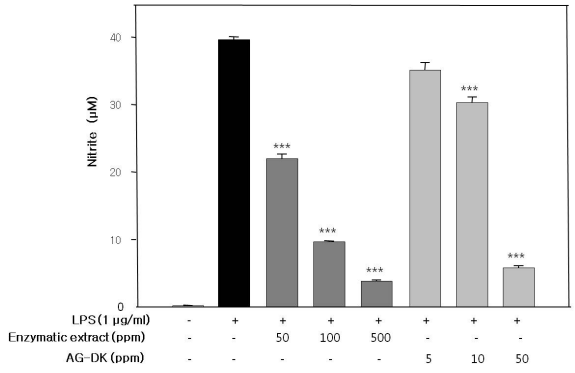

Figure 5. Inhibition of LPS-induced NO production in RAW 264.7 cells by enzymatic extract and AG-DK (***p < 0.001 indicated statistically significant differences from the LPS-treated group.).

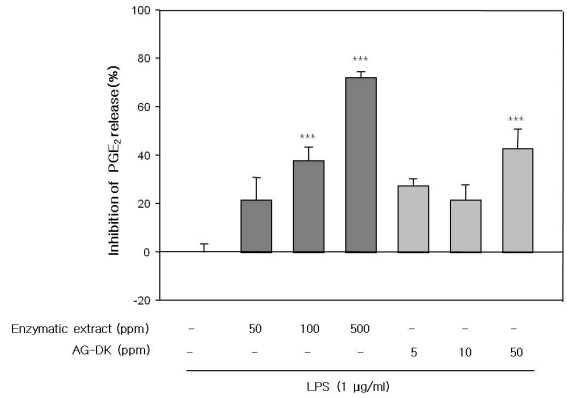

Figure 6. Effects of enzymatic extracts of *E. cava* and AG-DK on LPS-induced PGE_2 production in RAW 264.7 cells (***p < 0.001 indicated statistically significant differences from the LPS-treated group.).

inflammatory responses and accelerate the aging process. We found that the enzymatic extract of *E. cava* and AG-DK showed very high free radical scavenging activity and anti-glycation activity (Figure 1, 2). Thus, the potent anti oxidant and anti-glycation activities of *E. cava* polyphenols might contribute to the elimination of skin risk factors.

Melanogenesis is generated in the melanocytes, located in the basal layer of the epidermis and controlled by tyrosinase [17]. Tyrosinase, which is also referred to as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is considered to be a key enzyme in melanin synthesis [18,19]. The enzymatic extract of *E. cava* and AG-DK exert potent inhibitory effects on mushroom tyrosinase activity (Figure 3). These results indicate that the enzymatic extract of *E. cava* and AG-DK is a good candidate as an inhibitor of melanin formation.

During inflammation, activated macrophages secrete increased amounts of NO, PGE_2 and cytokines, such as interleukin (IL)-1ß, IL-6, and tumor necrosis factor (TNF)-α [20]. The reactive free radical NO, which is synthesized by iNOS, is a major macrophage-derived inflammatory mediator and has also been reported to be involved in the development of inflammatory diseases. PGE_2 is an other import anti-inflammatory mediator and is produced from arachidonicacid metabolites by the catalysis of Cyclooxygenase-2 (COX-2) [21]. Thus, we investigated the anti-inflammatory activities of *E. cava* enzymatic extract and AG-DK on LPS-induced NO and PGE_2 production in murine macrophage RAW 264.7 cells. *E. cava* enzymatic extract and AG-DK dose-dependently decreased the production of NO and PGE_2 in LPS-stimulated RAW 264.7 cells (Figure 5, 6). Based on these results, the *E. cava* enzymatic extract and AG-DK are considered as potential anti-inflammatory ingredients.

These findings suggest that there are potential benefits to applying the *E. cava* enzymatic extract and AG-DK as functional cosmetic skin-care ingredients.

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