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Brown alga *Ecklonia cava* attenuates type 1 diabetes by activating AMPK and Akt signaling pathways

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The antidiabetic therapeutic effect of *Ecklonia cava*, a brown alga, was investigated using streptozotocin-induced type 1 diabetes mellitus rats and C_2C_{12} myoblasts. The methanol extract of *E. cava* (ECM), having a strong radical scavenging activity, significantly reduced plasma glucose level and increased insulin concentration in type 1 diabetes mellitus rats. Moreover, the elevation of plasma ALT in diabetic rats was dramatically restored near to normal range by the treatment of ECM, whereas AST level was not meaningfully altered in any group throughout the experiment. The characteristic indications of diabetes, such as polyphagia and polydipsia, were greatly improved by ECM treatment as well. The mechanism of action of ECM appears to be, at least partially, mediated by the activation of both AMP-activated protein kinase/ACC and PI-3 kinase/Akt signal pathways. Taken together, the present results suggest that *E. cava* has both *in vivo* and *in vitro* antidiabetic effects.

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1. Introduction

Insulin is required for cells to absorb glucose from blood for use as fuel, or for storage. In the patients of diabetes mellitus, this glucose metabolism is altered due to either low levels of insulin secretion (type 1 diabetes) or abnormal resistance to insulin's effects (type 2 diabetes). As a result, the patients of diabetes mellitus have elevated blood glucose level, which is eventually lost in urine, resulting in excessive urine production and dehydration. Consecutively, it causes increased thirst and fluid intake. In addition, diabetes mellitus can cause many complications, including acute (ketoacidosis, etc.) as well as serious long-term (cardiovascular disease, chronic renal failure, retinal damage, nerve damage, etc.) problems. In recent studies, oxidative stress and chronic inflammation have been proposed as a cause of metabolic disorders, including diabetes mellitus (Baynes, 1991; Lee et al., 2004; Park et al., 2006). They have examined the mechanisms behind free radicals, and the possible role of oxidative stress in the pathogenesis of diabetes and diabetic complications on patients and animal models. It has been also suggested that oxidative stress-associated cell signaling is closely linked to its adverse effect on glucose metabolism.

Many natural products have long been used as therapeutics for diabetes in complementary medicine. As an example, *Curcuma longa* has been used for the treatment of diabetes by ayurvedic physicians in India, and polyphenols contained in this natural product are suspected to elucidate some of its pharmacological effect. A study on curcumin (active principle of rhizome of *C. longa*) has shown that tetrahydrocurcumin (THC) inhibits the formation of advanced glycation end products in streptozotocin (STZ)-induced diabetic rat (Pari and Murugan, 2007). The therapeutic potential of other polyphenols has been also demonstrated from the studies of an acute or chronic administration of polyphenols (EGCG from green tea, resveratrol from grape skin) to diabetes mellitus SD rats (Chi et al., 2007; Kao et al., 2000). From these, many scientists have paid attention to the antioxidant property of polyphenols, which may protect cell constituents against oxidative damage and limit the risk of various metabolic disorder diseases (in particular, diabetes mellitus) suffered from oxidative stress.

In general, seaweeds are considered to be a rich source of antioxidants. The potential antioxidant compounds from these seaweeds have been identified as some pigments (fucoxanthin, astaxanthin, carotenoid e.g.) and polyphenols (phenolic acid, flavonoid, tannins e.g.) (Heo et al., 2005). Among these components,

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phlorotannins which is a class of compounds with polymerized phloroglucinol units found in brown algae, especially in *E. cava*, have been known to exhibit high antioxidant activities. From a Korean tradition, brown algae have long been used as a food remedy to promote maternal health after her parturition. Recently, several evidences have demonstrated that *E. cava* crude methanol extract and/or single phlorotannin have various biological activities, including radical scavenging (Athukorala et al., 2006; Kang et al., 2004), antiproliferative (Athukorala et al., 2006; Yuan and Walsh, 2006), matrix metalloproteinase inhibitory (Kim et al., 2006), anti-allergic (Kim et al., 2008), bactericidal and protease inhibitory effects (Ahn et al., 2004). However, its antidiabetic effect remains poorly understood. In this article, we have examined the antidiabetic therapeutic potential of *E. cava* methanol extract using *in vivo* and *in vitro* models.

2. Materials and methods

2.1. Chemicals and reagents

DPPH (2,2-diphenyl-1-picryl-hydrazyl), streptozotocin (STZ), gallic acid, sodium citrate, citric acid, Folin–Ciocalteu's phenol reagent, DMSO (dimethyl sulfoxide), sodium carbonate anhydrous, butylhydroxytoluene (BHT), L-ascorbic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co. (St. Louis, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin were obtained from Gibco-BRL (Grand Island, NY, USA). Antibodies for Phospho-Acetyl-CoA Carboxylase (Ser^{79}), Phospho-AMPKα (Thr^{172}), Phospho-Akt (Ser^{473}), AMPK and Akt were obtained from Cell Signaling Technology (Beverly, MA). All other reagents used were of the purest grade available.

2.2. Plant material and extraction

Eighty percent methanol extracts of several seaweeds (*Ecklonia cava*, *Ecklonia kurome*, *Ishige okamurae* and *Sargassum silliquastrum*) were obtained from Jeju Hi-Tech Industry Development Institute (Jeju Island, Korea). Marine brown alga, *E. cava*, freshly collected during March at the coast of Jeju Island, Korea, was immediately washed with running tap water to remove any contamination of salt, sand and epiphytes. The seaweed samples were briefly rinsed again with fresh distilled water and cut into stem part and leaf part separately. They were dried in the shade with a cool air conditioning, and then stored at −20 °C until use. The frozen samples were lyophilized and homogenized with a grinder before extraction. The methanol extracts were obtained using the methods of Zhang et al. (2006). with minor modifications as described below. An aliquot (10 g) of lyophilized *E. cava* powder was added to 200 ml of 50% methanol with an adjustment to pH 2, and shaken (150 rpm) for 1 h at room temperature. After the extraction, the supernatant was recovered by centrifugation (7000 rpm) at 4 °C for 20 min and the methanol was evaporated using rotary vacuum evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The final aqueous part was lyophilized and kept on −20 °C until use.

2.3. Measurement of total polyphenol content

Total polyphenol content was determined using the method of Zhang et al. (2006) with a minor modification. For polyphenol standard, a stock solution (1 mg/ml) of gallic acid (in distilled water) was prepared and the stock was diluted to give working standards of 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/ml concentrations, respectively. For sample, an aliquot (5 mg) of several seaweeds methanol extract was dissolved in 1 ml of distilled water. To measure total polyphenol content, an aliquot (10 μl) of each sample and standard solutions was mixed with 50 μl of Folin–Ciocalteu's reagent in a 96-well microplate format and incubated for 5 min. The reactant was mixed with 40 μl of 7.5% sodium carbonate solution and incubated in the dark place for 2 h. Absorbance was measured at 750 nm with a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, USA).

2.4. DPPH radical scavenging assay

The antioxidant activity of *E. cava* methanol extract was determined using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method (Borse et al., 2007) with minor modifications. For this, *E. cava* extract (ECM) was prepared as previously described above. A working solution (6×10^{-5} M) of DPPH was also freshly prepared by dissolving it in DMSO and vortex-mixed at the day of experiment. In brief, 900 μl of DPPH solution was mixed with 100 μl of distilled water (Control), or 100 μl of *E. cava* methanol extract (Sample). For the calibration of sample background, 100 μl of *E. cava* extract was mixed with 900 μl of DMSO alone (Sample background) instead of DPPH solution. Butylhydroxytoluene (BHT) and L-ascorbic acid were used as positive controls. An aliquot of Control, Sample solution, or Sample background was transferred to a 96-well microplate and incubated for 1 h in a dark place. The absorbance at 517 nm was measured using a spectrophotometric microplate reader (Bio-Tek Instruments, Inc., Winooski, USA) and DPPH radical scavenging activity of a sample was analyzed using the equation as below.

$$\text{DPPH radical scavenging activity}(\%) = [1(A_1 - (A_2 - A_3))/A_1] \times 100$$

where, A_1 is the absorbance of Control; A_2 is the absorbance of Sample; A_3 is the absorbance of Sample background.

2.5. Animals and diets

Male Sprague–Dawley rats (180–220 g BW, 6 weeks old) were purchased from Central Laboratory Animal Inc. (Korea). The rats were freely fed with a commercial chow diet (Superfeed Co., Korea) and tap water *ad libitum* in a room at 22 ± 2 °C and 60–65% relative humidity with a normal 12 h light–dark cycle. They were acclimatized at least for 1 week before experiment. All the experimental animals were handled according to the guidelines of Gyeongsang National University Guide for the Care and Use of Laboratory Animals.

2.6. Induction of diabetes and treatment of E. cava

The rats were divided into three groups of five animals each as follows for treatments; Control (normal rats treated with physiological saline), diabetes (diabetic rats treated with physiological saline), diabetes-*E. cava* (diabetic rats treated with 300 mg/kg of *E. cava*). For the induction of type 1 diabetes mellitus, rats were administered (i.p.) with streptozotocin (45 mg/kg), freshly prepared in citrate buffer (0.1 M, pH 4.5). Diabetic rats could be identified by the symptoms of polyphagia, polydipsia, and polyuria (18). The status of diabetes mellitus was examined by the analysis of plasma glucose levels, determined at 72 h and then on the day 7 after STZ injection. The rats with plasma glucose higher than 200 mg/dl were used as diabetes mellitus in the present study. For the treatments of ECM and control vehicle (physiological saline), diabetic rats were divided into two groups and administered intraperitoneally with the testing reagents once a day for 3 weeks and they were continuously monitored up to additional one more week. Throughout the experiment, their food intake and water consumption were recorded daily and body weight was monitored weekly.

2.7. Blood biochemical analysis

Once a week, the rats were fasted for 12 h, and then the blood was collected after the brief anesthesia with ethyl ether. For biochemical analysis of blood, the plasma levels of glucose, ALT, and AST were determined colorimetrically using commercial diagnostic kits with IDEXX VetTest Chemistry Analyzer (IDEXX laboratories, Inc., Maine, USA). Plasma insulin was assayed at the end of experiments by using a rat insulin ELISA kit (SHIBAYAGI, Co., Ltd., Gunma, Japan) with a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, USA).

2.8. Histopathological procedure for pancreas

Pancreatic tissues were obtained from the SD rats after ECM or vehicle (physiological saline) treatment for 3 weeks. The tissue fragments were fixed in 10% neutralized formalin solution. After fixing, the tissues were dehydrated and embedded in paraffin. The paraffin blocks were sectioned at 5 μm, and then stained with hematoxylin and eosin (H&E) for observing any histological changes.

2.9. Cell culture

C_2C_{12} (mouse myoblast cell line) were maintained in DMEM supplemented with 10% heat inactivated FBS and antibiotics, at 37 °C in a humidified atmosphere with 5% CO_2. For differentiation into myotubes, cells were reseeded in 6-well plates at a density of 2×10^4 cells/ml. After 48 h (over 80% confluence), the medium was switched to DMEM with 1% (v/v) FBS and was replaced after 2, 4 and 6 days of culture. The treatments of cells with ECM or insulin were initiated as described else where on day 7 when myotube differentiation was complete.

2.10. Western blotting

After the treatments, C_2C_{12} cells were rinsed twice with ice-cold PBS, and then added with 100 μL of lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin). The plate was rocked on ice for 3 min and the cells were scraped using rubber policeman into Eppendorf microcentrifuge tubes. The scraped cells were allowed to lyse for additional 30 min on ice with periodic vortexing. Cell debris was removed by centrifugation (22,000*g*, at 4 °C for 30 min) and the resulting supernatant was collected and determined for its protein concentration using a Bio-Rad protein assay reagent (Bio-Rad, CA, USA). Samples were cooked with SDS sample buffer in boiling water for 5 min, then the proteins (30–40 μg) were separated on 10% SDS–polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Bio-Rad, CA, USA), and

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