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The Protective Effects of Bilberry and Lingonberry Extracts against UV Light-Induced Retinal Photoreceptor Cell Damage in Vitro

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ABSTRACT: Bilberry extract (B-ext) and lingonberry extract (L-ext) are currently used as health supplements. We investigated the protective mechanisms of the B-ext and L-ext against ultraviolet A (UVA)-induced retinal photoreceptor cell damage. Cultured murine photoreceptor (661W) cells were exposed to UVA following treatment with B-ext and L-ext and their main constituents (cyanidin, delphinidin, malvidin, *trans*-resveratrol, and procyanidin). B-ext, L-ext, and constituents improved cell viability and suppressed ROS generation. Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), c-Jun *N*-terminal kinase (JNK), and protein kinase B (Akt) were analyzed by Western blotting. B-ext and cyanidin inhibited phosphorylation of p38 MAPK, and B-ext also inhibited phosphorylation of JNK by UVA. L-ext, *trans*-resveratrol, and procyanidin alleviated the reduction of phosphorylated Akt levels by UVA. Finally, a cotreatment with B-ext and L-ext showed an additive effect on cell viability. Our findings suggest that both B-ext and L-ext endow protective effects against UVA-induced retinal damage.

KEYWORDS: *anthocyanin, resveratrol, procyanidin, retinal photoreceptor, ultraviolet A exposure*

INTRODUCTION

Ultraviolet (UV) radiation, a part of the sunlight spectrum, is a major environmental factor of inducible health hazards that include the etiology of some ocular pathologies such as keratitis,[1,2] pterygium,[3] and cataract.[4,5] UVB has been implicated in the development of periocular skin sunburn and inflammation of the conjunctiva, cornea, and lens, but not of the retina, as UVB is absorbed, while some UVA can reach the retina and lead to damage of inner retina, gradual bleaching of rhodopsin followed by a slow rate of recovery, distortion of mitochondrial structure, and alteration of photoreceptor cell metabolism.[6−8] UVA radiation can also induce DNA breakdown and the production of reactive oxygen species (ROS)[9] that damage the retinal photoreceptor cells. ROS generated by UVA alter mitogen-activated protein kinase (MAPK) signal transduction pathways, such as p38 MAPK and c-Jun *N*-terminal kinase (JNK), and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways, which play crucial roles in the regulation of UVA-induced cellular apoptosis and survival.[10]

Bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.), members of the Ericaceous family, grow in the forests of northern Europe. Bilberry contains 15 different anthocyanins composed of 5 anthocyanidins (delphinidin, cyanidin, malvidin, petunidin, and peonidin) and 3 sugars (glucose, galactose, and arabinose). Bilberry extract (B-ext) possesses potent antioxidant properties,[11] stabilizes collagen fibers, promotes collagen biosynthesis,[12] inhibits platelet aggregation,[13] and improves vascular tone, blood flow, and vasoprotection.[14,15] Furthermore, the bilberry has been reported to improve visual function in an animal model and clinical trial.[16,17] Animal studies have demonstrated that B-ext contributes to the prevention of retinal inflammation[17] and cataracts.[18] Our previous studies have demonstrated that B-ext has a neuroprotective effect against retinal neuronal damage induced by *N*-methyl-D-aspartic acid in mice,[19] and an inhibitory effect against angiogenesis in an oxygen-induced retinopathy model in mice.[20] Additionally, anthocyanins, constituents of bilberry, purple rice, and strawberry, provide protective effects because of their antioxidative capacity to human retinal pigment epithelial cells, murine photoreceptor cells, or human dermal fibroblasts against light-induced damage.[21−23]

Lingonberry is traditionally used for treating frequent urination, sore eyes, toothache, snow blindness, and thrush.[24] Lingonberry extract (L-ext) contains rich phenolic antioxidants (more than 10% *trans*-resveratrol and proanthocyanidin). *trans*-Resveratrol and proanthocyanidin provide protective effects against UV-induced human keratinocyte damage by down-regulating Keap1 expression and activating MAPK.[25,26]

However, to date, the protective effects of bilberry and lingonberry against UV light-induced retinal damage have not been investigated. Therefore, the purpose of our study was to investigate the effects of fruit extracts of both the berries, and to elucidate their protective mechanism against UVA-induced retinal cell damage in cultured retinal photoreceptor cells.

MATERIALS AND METHODS

Materials. B-ext (bilberry ethanol extract containing anthocyanins) and L-ext (lingonberry ethanol extract containing *trans*-resveratrol and proanthocyanidins) were purchased from Beijing Gingko Group Japan

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Co., Ltd. (Tokyo, Japan), who confirmed their constitutions using HPLC. The active constituents of both extracts were analyzed by using high performance liquid chromatography (HPLC) and UV−visible absorption spectroscopy with each standard preparation. B-ext contained 38.4% of anthocyanins, which were 14.1% of delphinidin, 9.1% of cyanidin, and 6.1% of malvidin (Table 1). L-ext contained 10.6% of *trans*-resveratrol and 43.0% of proanthocyanidin. The rest of the composition except the active constituents in both extracts was almost all carbohydrate. Delphinidin, cyanidin, malvidin, and procyanidin B2 were purchased from Extrasynthese (Genay Cedex, France). *trans*-Resveratrol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *N*-Acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A PI3-kinase inhibitor, LY294002, was purchased from Calbiochem (Merck AG, Darmstadt, Germany), and a Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Hoechst 33342, propidium iodide (PI), and 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) were purchased from Invitrogen (Eugene, OR, USA). An antibody against phosphorylated p38 MAPK was purchased from Promega (Madison, WI, USA). Antibodies against total p38 MAPK, phosphorylated JNK, phosphorylated Akt, and total Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against total JNK1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 661W cells were a kind gift from Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA).

Table 1. The Active Constituents of Bilberry and Lingonberry Extracts

extract	constituent	% of extract
bilberry extract	delphinidin	14.1
	cyanidin	9.1
	malvidin	6.1
	total anthocyanin	38.3
lingonberry extract	proanthocyanidin	43.0
	trans-resveratrol	10.6

Cell Culture. The 661W cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged by trypsinization every 2−3 days.

Measurement of Cellular Metabolic Activity Following UVA Light Exposure. The 661W cells (5×10^3 cells/100 μL) were seeded onto a 96-well plate and cultured at 37 °C for 24 h. We first investigated the effects of NAC, bilberry, lingonberry, and their constituents at various concentrations, and we found the effective extract concentrations. In this study, we presented the data on specific extract concentrations of each sample against UVA-induced photoreceptor cell damage. At 70−80% confluence, the medium was replaced by DMEM containing 1% FBS with, for separate samples, 1−30 μg/mL B-ext, L-ext, coapplied B-ext and L-ext at 3−30 μg/mL each, 1−30 μM anthocyanidins, *trans*-resveratrol, procyanidin B2, or 0.1−3 mM NAC, without agent (vehicle group). Treatment with an inhibitor of Akt phosphorylation, LY293002, at a concentration of 3 mM was performed for 1 h before applying the medium containing L-ext. After 1 h of preincubation, the 661W cells were exposed to 4 J/cm^2 UVA light (CL-1000 ultraviolet cross-linker; UVP Inc., Upland, CA, USA) for 20 min, and then the cells were incubated at 37 °C for 24 h. At 24 h after the 20 min of UVA exposure, metabolic activity was measured by using a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8). Briefly, 10 μL of WST-8 reagent was added to each well, followed by incubation for 2 h at 37 °C, after which the absorbance was measured at 492 nm (reference wavelength, 660 nm) by using SkanIt Re for Varioskan Flash 2.4 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell Death Analysis Following UVA Light Exposure. After 24 h the end of UVA exposure, Hoechst 33342 (excitation, 360 nm; emission, 490 nm) and PI were added to the culture medium at final concentrations of 8.1 and 1.5 μM, respectively, followed by incubation for 15 min at 37 °C. Micrographs through fluorescence filters for Hoechst 33342 (U-MWU; Olympus Co., Tokyo, Japan) and PI (U-MWIG; Olympus) were acquired using a charge-coupled device camera (DP30BW; Olympus). Then, the number of dead cells was counted among the total cells.

Measurement of Cellular ROS Production Following UVA Light Exposure. Intracellular ROS production caused by UVA light exposure within the 661W cells was determined using CM-H_2DCFDA. CM-H_2DCFDA is converted to dichlorodihydrofluorescein (DCFH) by an esterase inside the cells. Then, the ROS oxidize a nonfluorescent DCFH to a fluorescent DCFH. After UVA light exposure, CM-H_2DCFDA was immediately added to the culture medium at a final concentration of 10 μM, followed by incubation at 37 °C for 1 h. Fluorescence was then measured using a fluorescence spectrophotometer, SkanIt Re for Varioskan Flash 2.4 (Thermo Fisher Scientific Inc., Waltham, MA, USA), at 488 nm (excitation) and 525 nm (emission). The number of cells was determined by Hoechst 33342 staining and was used to calculate ROS production per cell.[22]

Western Blot Analysis. Following UVA light exposure, the 661W cells were lysed using a cell lysis buffer (RIPA buffer) with phosphatase inhibitor cocktails 2 and 3 (P5726 and P0044; Sigma-Aldrich) and a protease inhibitor (P8340; Sigma-Aldrich). The lysate was centrifuged at 12000*g* for 20 min, and the supernatant was collected for analysis. To determine the protein concentration, a BCA protein assay kit (Thermo Fisher Scientific Inc.) was used with a bovine serum albumin standard. Then, an equal volume of protein sample and sample buffer with 10% 2-mercaptoethanol was electrophoresed using a 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA). For immunoblotting, the following primary antibodies were used: rabbit anti-phospho-p38 MAPK (1:1000; Promega), rabbit anti-p38 MAPK (1:1000; Cell Signaling Technology), rabbit anti-phospho-Akt (1:1000; Cell Signaling Technology), rabbit anti-Akt (1:1000; Cell Signaling Technology), rabbit anti-phospho-JNK (1:1000; Cell Signaling Technology), and rabbit anti-JNK1 (1:1000; Santa Cruz Biotechnology). The secondary antibody was a goat anti-rabbit HRP-conjugated IgG (1:2000). Immunoreactive bands were visualized using a chemiluminescent substrate (ImmunoStarLD; Wako Pure Chem. Inc., Osaka, Japan). Band densities were measured using an imaging analyzer (LAS-4000 mini; Fujifilm, Tokyo, Japan), a gel analysis software (Image Reader LAS-4000; Fujifilm), and a detected band analysis software (Malti Gauge; Fujifilm).

Statistical Analysis. Data are presented as the mean ± SEM. Statistical comparisons were made using one-way analysis of variance followed by Student's *t*-test or Dunnett's multiple-comparison test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Inhibitory Effects of B-Ext and L-Ext on Morphological Alterations, Metabolic Activity Reduction, and Cell Death Following UVA Light Exposure. We investigated the effects of B-ext, L-ext, NAC, a positive control as antioxidant, and their constituents at various concentrations, and we found the effective extract concentrations of each agent. We showed the effects of B-ext, L-ext, and NAC, a positive control antioxidant, on morphological alterations induced by UVA light in the 661W cell cultures. During 24 h of incubation, treatment with 30 μg/mL B-ext, 10 μg/mL L-ext, or 1 mM NAC improved the attenuation of morphological defects induced by UVA light exposure in the 661W cells (Figure 1A). Additionally, a treatment with 10 or 30 μg/mL B-ext, or 10 μg/mL L-ext, but not 30 μg/mL L-ext, significantly inhibited a reduction in the metabolic activity of the 661W cells induced

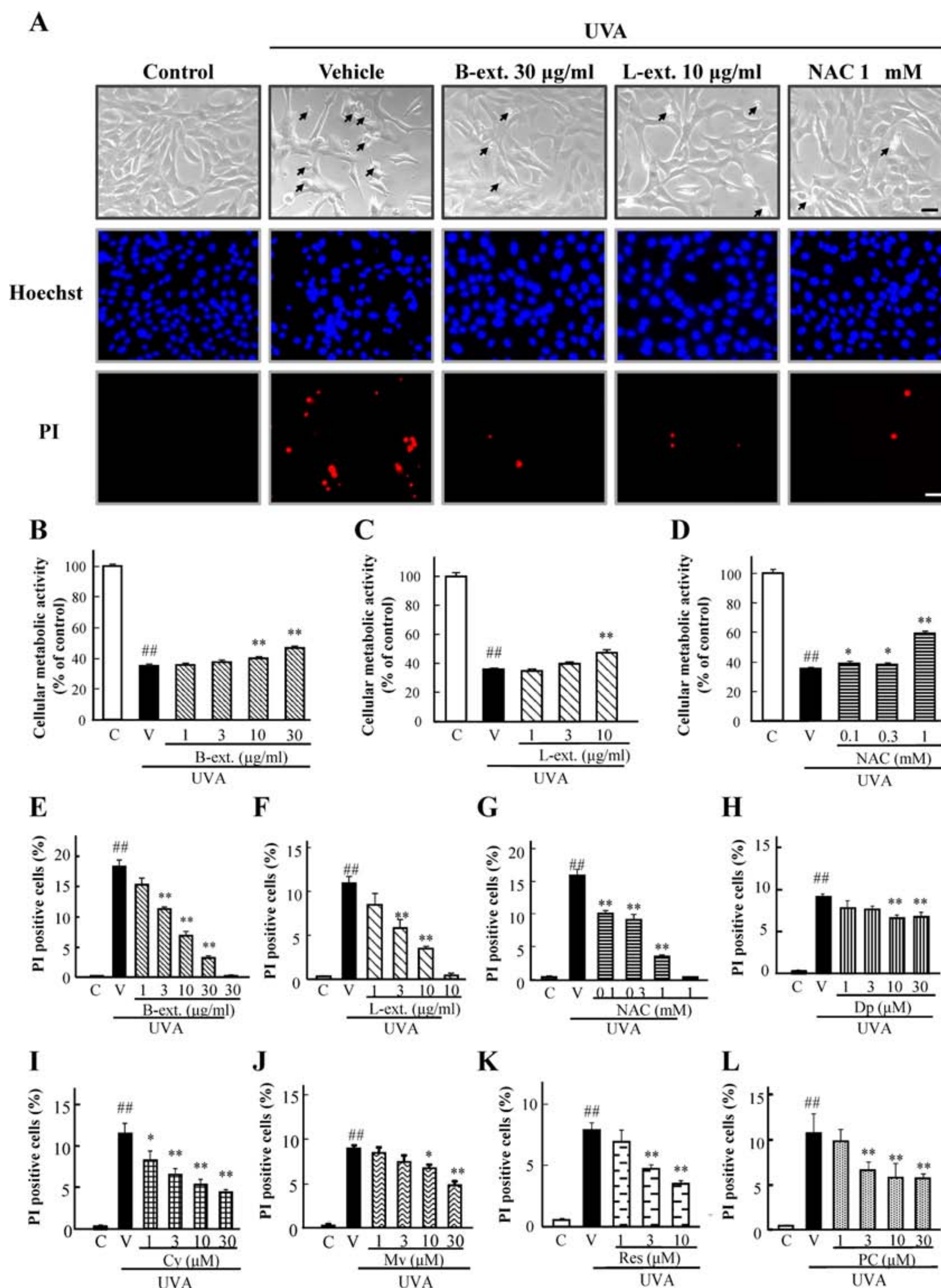

Figure 1. Protective effects of B-ext, L-ext, NAC, and their constituents against UVA-induced 661W cell damage. (A) Cytomorphology of 661W cells and representative fluorescence microscopy of Hoechst 33342 and propidium iodide (PI) staining after 24 h of UVA light exposure. Small arrows indicate apoptotic cells. The scale bar represents 50 μm. Inhibitory effects of B-ext (B), L-ext (C), and NAC (D) on UVA-induced reduction of metabolic activity in 661W cells. Metabolic activity was assessed by incubating the cells in CCK-8 reagent for 2 h at 37 °C, and then photometric data were obtained at 492/660 nm. Inhibitory effects of B-ext (E), L-ext (F), NAC (G), delphinidin (H), cyanidin (I), malvidin (J), resveratrol (K), and procyanidin B2 (L) on UVA-induced retinal cell death of 661W cells. The number of cells exhibiting PI fluorescence was counted, and positive cells are expressed as the percentage of PI-positive to Hoechst 33342-positive cells. Cells were treated with B-ext, L-ext, NAC, delphinidin, cyanidin, malvidin, *trans*-resveratrol, and procyanidin B2 for 1 h, and then exposed to 4 J/cm^2 UVA light. Data are the mean ± SEM (n = 6). C, control; V, vehicle; B-ext, bilberry extract; L-ext, lingonberry extract; NAC, *N*-acetyl-L-cysteine; Dp, delphinidin; Cy, cyanidin; Mv, malvidin; Res, *trans*-resveratrol; PC, procyanidin B2. $^{\#\#}p < 0.01$ vs control (Student's t-test), $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle-treated group (Dunnett's multiple comparison test).

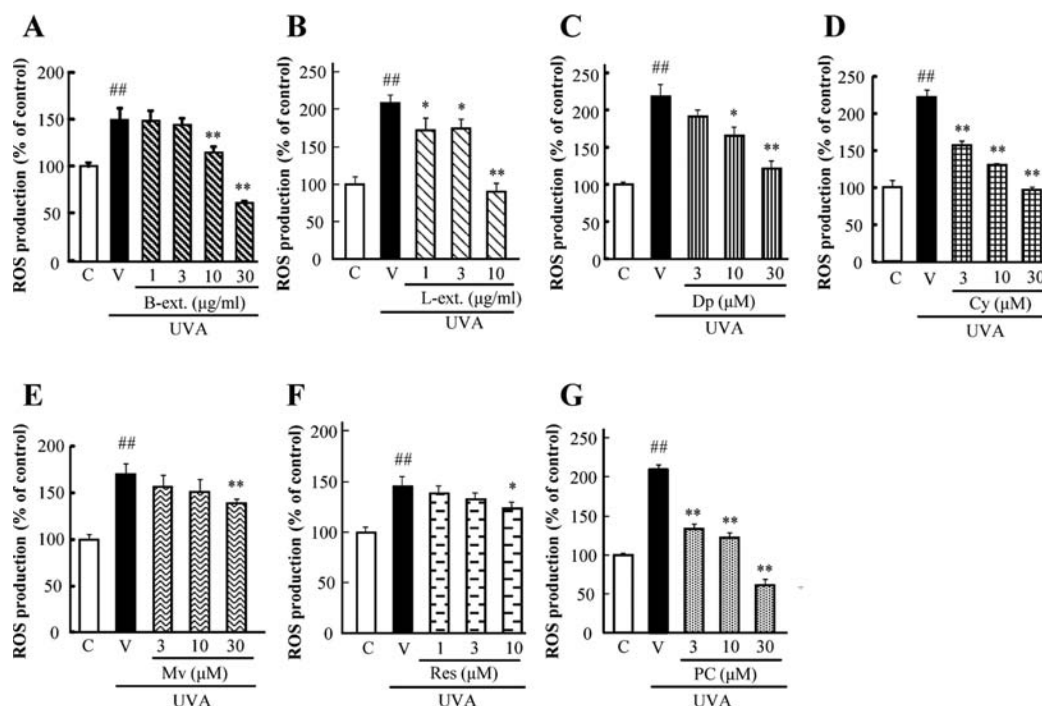

Figure 2. Inhibitory effects of B-ext, L-ext, and their constituents on UVA-induced production of ROS in the 661W cells. The cells were pretreated with B-ext, L-ext, delphinidin, cyanidin, malvidin, *trans*-resveratrol, and procyanidin B2 for 1 h, and then exposed to 4 J/cm^2 UVA light. B-ext (A), L-ext (B), delphinidin (C), cyanidin (D), malvidin (E), *trans*-resveratrol (F), and procyanidin B2 (G) inhibited ROS production in cells induced by UVA light exposure in a concentration-dependent manner. Intracellular ROS levels were determined by measuring the fluorescence of CM-H_2DCFDA (excitation, 488 nm; emission, 525 nm) after UVA exposure for 1 h. Data are the mean ± SEM (n = 5 or 6). C, control; V, vehicle; B-ext, bilberry extract; L-ext, lingonberry extract; Dp, delphinidin; Cy, cyanidin; Mv, malvidin; Res, *trans*-resveratrol; PC, procyanidin B2. $^{\#\#}p < 0.01$ vs control (Student's t-test), $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle-treated group (Dunnett's multiple comparison test).

by UVA light exposure (Figure 1B and Figure 1C). Furthermore, a treatment of 0.1−1 mM NAC also significantly inhibited the reduction of metabolic activity in the 661W cells (Figure 1D). To investigate the protective effects of B-ext, L-ext, and their constituents against UVA-induced death in the 661W cells, we counted the number of UVA-induced dead retinal cells exhibiting PI fluorescence, and expressed the positive cells as the percentage of PI-positive cells to total cell nuclei exhibiting Hoechst 33342 fluorescence. Representative images of Hoechst 33342 and PI staining are shown in Figure 1A. Treatment with 3−30 μg/mL B-ext, 3 or 10 μg/mL L-ext, and 0.1−1 mM NAC significantly inhibited 661W cell death in a concentration-dependent manner (Figure 1E−G). Additionally, treatment with the constituents of B-ext, 10 or 30 μM delphinidin, 1−30 μM cyanidin, and 10 or 30 μM malvidin, and those of L-ext, 3 and 10 μM *trans*-resveratrol, and 3−30 μM procyanidin B2, significantly inhibited 661W cell death in a concentration-dependent manner (Figure 1H−L).

Preventive Effects of B-Ext and L-Ext against UVA-Induced ROS Production in Retinal Photoreceptor Cell Cultures. CM-H_2DCFDA, a cell-permeant indicator of ROS, is nonfluorescent until the removal of its acetal groups by intracellular esterases. Within the cell, esterases cleave CM-H_2DCFDA to release CM-H_2DCFDH, which is converted to a fluorescent product (CM-H_2DCF) upon exposure to ROS. Intercellular ROS production increased because of UVA light exposure, and 10 or 30 μg/mL B-ext, and 1−10 μg/mL L-ext significantly reduced the ROS production in the 661W cells (Figure 2A,B). Additionally, treatment with the constituents of B-ext and L-ext, as indicated earlier, significantly reduced the UVA-induced ROS production in the 661W cells (Figure 2C−G).

Regulation of B-Ext, L-Ext, and Their Constituents on the Phosphorylation of Stress-Response Proteins in Photoreceptor Cells Following UVA Light Exposure. We performed Western blot analysis to investigate the regulatory effect of B-ext, L-ext, and their active constituents (cyanidin, *trans*-resveratrol, and procyanidin B2) on p38 and JNK MAPK stress-response pathways, and the Akt survival-signaling pathway after exposure to UVA light. UVA light exposure increased the phosphorylation of p38 MAPK and JNK, and a treatment with 30 μg/mL B-ext or 10 μM cyanidin, but not that with L-ext, significantly inhibited the phosphorylation of both the proteins in the 661W cells (Figure 3A−C,G,H). Additionally, UVA light exposure decreased the phosphorylation of Akt, and a treatment with 10 μg/mL L-ext, 30 μM procyanidin B2, and 10 μM *trans*-resveratrol, but not that with B-ext, significantly prevented the downregulation of phospho-Akt (Figure 3D−F). Interestingly, treating the 661W cells with *trans*-resveratrol, without UVA light irradiation, enhanced the phosphorylation of Akt. We investigated the relationship between phosphorylation of Akt and 661W cell survival after UVA exposure by using an inhibitor of Akt phosphorylation, LY294002. The protective effect of L-ext against UVA irradiation was reduced because of a pretreatment with 3 mM LY294002 (Figure 4A,B).

Additive Effects of B-Ext and L-Ext on Photoreceptor Cell Survival after UVA Light Exposure. We investigated the additive effects of 30 μg/mL B-ext and 10 μg/mL L-ext on UVA-induced cell damage by measuring the 661W cellular metabolic activity and cell death, because of the extracts'

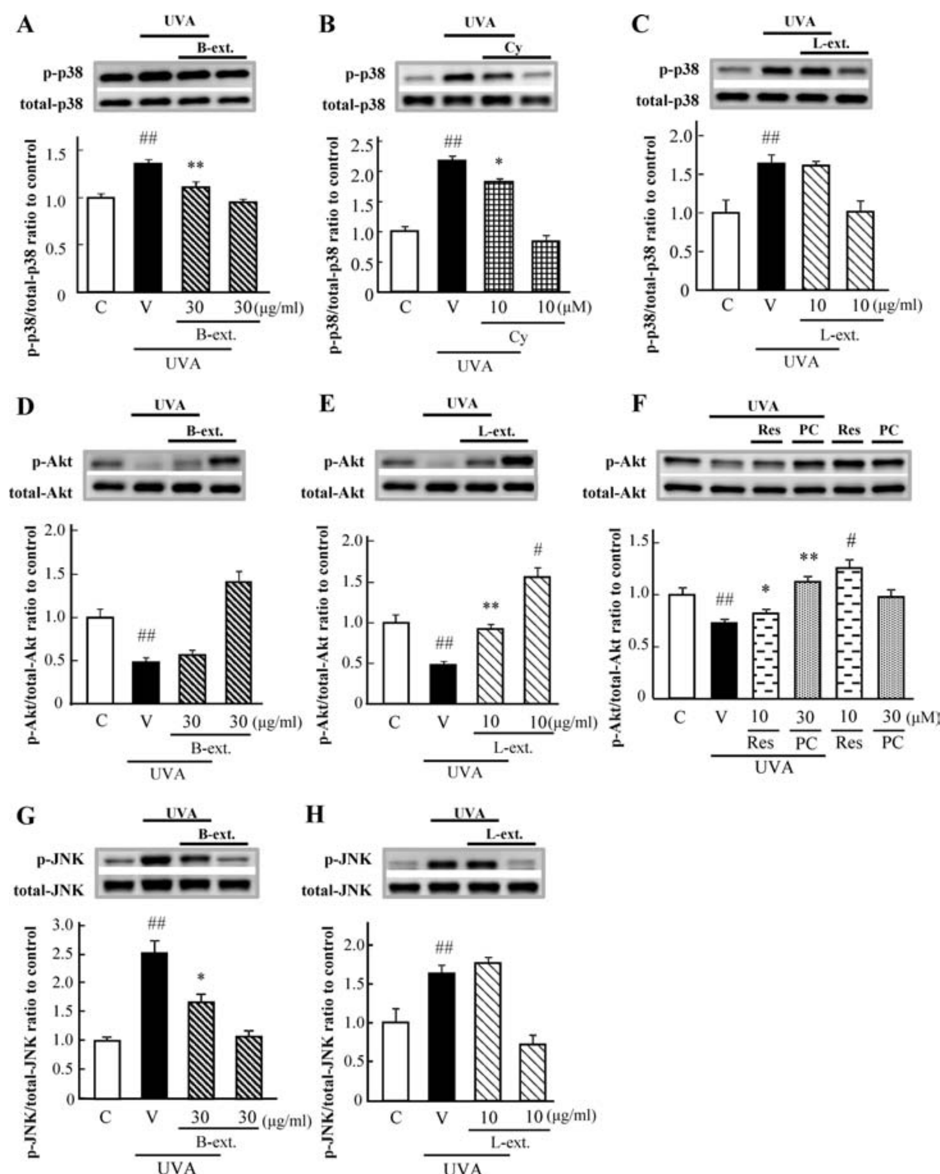

Figure 3. Effects of B-ext, L-ext, and their active constituents, cyanidin, *trans*-resveratrol, and procyanidin B2, on UVA-induced changes of phosphorylated p38 MAPK, Akt, and JNK levels in 661W cells. Cells were pretreated with B-ext, L-ext, cyanidin, *trans*-resveratrol, and/or procyanidin B2 for 1 h, and then exposed to 4 J/cm^2 UVA light. Then, the cells were collected and lysates were analyzed using Western blotting. Data are the mean ± SEM (n = 5 or 6). C, control; V, vehicle; B-ext, bilberry extract; L-ext, lingonberry extract; Cy, cyanidin; Res, *trans*-resveratrol; PC, procyanidin B2. $^{\#\#}p$ < 0.01 vs control (Student's t-test), $^{*}p$ < 0.05, $^{**}p$ < 0.01 vs vehicle-treated group (Dunnett's multiple comparison test).

different effects on the phosphorylation of p38 MAPK and Akt. We found that the protective effects of a combined B-ext and L-ext treatment were more effective against UVA-induced photoreceptor cell damage than a single treatment of either extracts (Figure 5).

■ DISCUSSION

Numerous studies have shown that berry fruits improve visual function and prevent eye diseases such as cataract, diabetic retinopathy, and glaucoma, through the antioxidative activity of polyphenols.[18,20,27,28] In this study, we focused on the preventive effects of bilberry, lingonberry, and their main constituents, including delphinidin, cyanidin, malvidin, *trans*-resveratrol, and procyanidin B2, against retinal injury caused by UVA light exposure in photoreceptor cells.

We first examined the effects of B-ext, L-ext, B-ext containing the 3 main component anthocyanidins (cyanidin, delphinidin, and malvidin), and L-ext containing *trans*-resveratrol and procyanidin B2. In the results, the effective concentrations of B-ext at 10 μg/mL, containing 1.41 μg/mL (4.65 μM) of delphinidin, 0.91 μg/mL (3.17 μM) of cyanidin, and 0.61 μg/mL (1.84 μM) of malvidin, and L-ext at 10 μg/mL, containing 1.06 μg/mL (4.64 μM) of *trans*-resveratrol and 4.30 μg/mL (7.43 μM) of procyanidins, were observed (Figures 1 and 2). Our study results suggested that cyanidin, *trans*-resveratrol, and procyanidins were highly effective components of B-ext and L-ext in inhibiting UVA-induced ROS generation and cell death at concentrations 1−30 μM, 3−10 μM, and 3−30 μM respectively (Figures 1 and 2). Additionally, delphinidin, most abundant in bilberry extract, might possess a protective effect against UVA damage in support of cyanidin. B-ext contains 15 different anthocyanins composed of 5 anthocyanidins (delphinidin, cyanidin, malvidin, petunidin, and peonidin) and 3 sugars (glucose, galactose, and arabinose), however, we could not

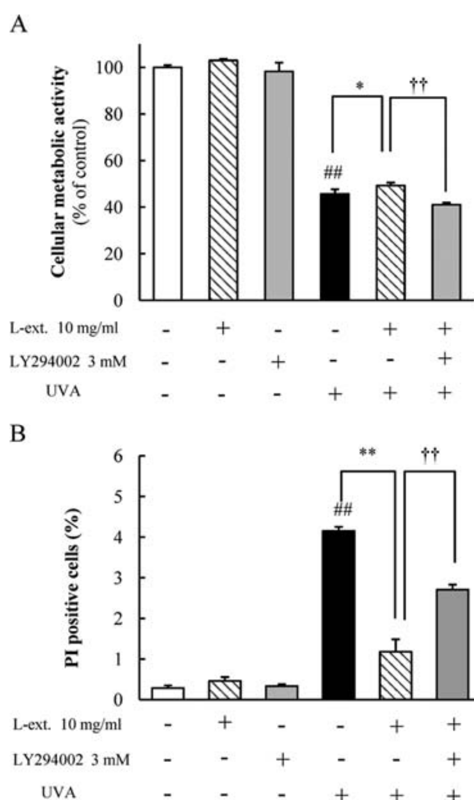

Figure 4. Protective effects of L-ext against UVA-induced 661W cell damage are inhibited by LY294002. The improvement of cellular metabolic activity (A) and prevention of cell death (B) by treatment with 10 μg/mL L-ext were inhibited by 3 mM LY294002 (a PI3K-Akt inhibitor). Cells were pretreated with L-ext and LY294002 for 1 h, and then exposed to 4 J/cm^2 UVA light. Cellular metabolic activity was assessed by incubating the cells in CCK-8 reagent for 2 h at 37 °C, and then photometric data were obtained at 492/660 nm. The number of cells exhibiting PI fluorescence was counted, and positive cells are expressed as the percentage of PI-positive to Hoechst 33342-positive cells. Data are the mean ± SEM (n = 6). C, control; V, vehicle; L-ext, lingonberry extract. $^{\#\#}p < 0.01$ vs nontreatment, $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle-treated group, $^{\dagger\dagger}p < 0.01$ vs 10 μg/mL L-ext -treated group (Student's t-test).

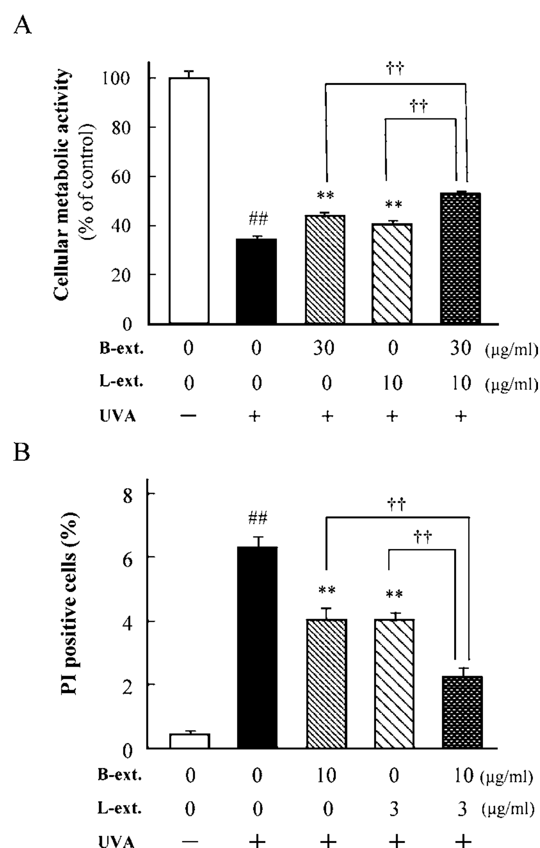

Figure 5. Additive effects of both B-ext and L-ext on UVA-induced 661W cell damage. B-ext and L-ext were coapplied to inhibit the reduction of cellular metabolic activity (A) and cell death (B) induced by UVA light exposure. Cells were pretreated with B-ext, L-ext, and coapplied for 1 h, and then exposed to 4 J/cm^2 UVA light. Cellular metabolic activity was assessed by incubating the cells in the CCK-8 reagent for 2 h at 37 °C, and then photometric data were obtained at 492/660 nm. The number of cells exhibiting PI fluorescence was counted, and positive cells were expressed as the percentage of PI-positive to Hoechst 33342-positive cells. Data are the mean ± SEM (n = 6). B-ext, bilberry extract; L-ext, lingonberry extract. $^{\#\#}p < 0.01$ vs nontreated group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs UVA-treated group, $^{\dagger\dagger}p < 0.01$ vs only B-ext- or L-ext-treated groups (Student's t-test).

purchase all anthocyanins, glycosidic forms, in B-ext. Some anthocyanins were not available for our study. Then, we first investigated the effect of main component anthocyanidins, delphinidin, cyanidin, and malvidin, in bilberry extract against UVA-induced 661W cell damage, and the protective effects of the anthocyanidins were observed in our results (Figures 1 and 2). Subsequently, we used cyanidin-3-glucoside, a glycosidic anthocyanin as a representative in B-ext, and the results were the same as that of cyanidin (data not shown). Thus, in future study, it will be necessary to investigate the protective effect of other glycosidic anthocyanins in B-ext because certain amounts of anthocyanins are absorbed into the bloodstream in the glycosylated form and metabolized in the liver, and then anthocyanin and anthocyanin metabolites shift to the eyes.[29,30]

We have established a relationship between protection against UVA-induced photoreceptor cell damage and the antioxidative activity of B-ext, L-ext, and their constituents. It is already known that UV irradiation induces hydroxyl radical, superoxide anion radical, hydrogen peroxide, and singlet oxygen. First, singlet oxygen is generated from triplet oxygen ($^3O_2$), the ground state of the oxygen molecule, due to absorption UV light energy. Subsequently, singlet oxygen is changed to superoxide anion radical by acquisition of electron. Superoxide anion radical is also generated in cellular mitochondria following the activation of intracellular NADPH oxidase. Superoxide anion radical is metabolized to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is also generated from water by UV irradiation. Finally, hydrogen peroxide is changed to hydroxyl radical by catalytic action of iron or copper ions. Hydroxyl radical bring about lipid peroxidation, protein denaturation, and DNA damage. Furthermore, UV irradiation decreases production of antioxidative enzyme in UV light exposed various cells. Hence, the free radicals are increased by UVA exposure in cells and induce apoptosis in cells. In this study, treatment with NAC, a positive control antioxidant as component of glutathione, alleviated UVA-induced damage of retinal photoreceptor cells (Figure 1), similar to a previous study's report.[31] We previously found that B-ext and its individual constituents, delphinidin, cyanidin, and malvidin, which were the same as anthocyanidin standards purchased from Extrasynthese (Genay Cedex, France), have a free radical scavenging activity against superoxide anion radicals

and/or hydroxyl radicals as shown by the electron-spin resonance method.[32] Additionally, it is known that B-ext, *trans*-resveratrol, and procyanidin B2 improve the antioxidant defense of intracellular glutathione and endogenous superoxide dismutase.[33−35] Thus, as shown in this study, B-ext and L-ext may not only scavenge ROS but also improve the cellular ROS scavenging activity in retinal photoreceptor cells.

p38 MAPK and JNK participate in cellular responses to mitogenic stimuli by oxidative stress and UV light exposure, and the activation of p38 MAPK and JNK induces the apoptotic transcription factor, activator protein-1 (AP-1), by UV light.[36−38] A previous report suggests that activation of p38 MAPK and JNK occurs as result of the generation of intracellular singlet oxygen by UV light.[39] B-ext containing anthocyanins, L-ext containing *trans*-resveratrol, and procyanidin B2 possess an antioxidative capacity against ROS involving singlet oxygen.[40,41] However, this study's results showed an inhibitory effect of only B-ext, but not L-ext, on the activation of p38 MAPK and JNK in the 661W cells. Recently, Kwon et al.[42] and Lim et al.[43] suggested that delphinidin and cyanidin inhibit UV light-induced phosphorylation of MKK4 and MAPK kinase activation of JNK by binding to MKK4 competitively with ATP. Furthermore, UV-stimulated p38 MAPK activation is mediated not only by MKK3 and MKK6 but also by MKK4.[44] Thus, B-ext, including delphinidin and cyanidin, may directly inhibit the UVA light-stimulated activation of p38 MAPK and JNK. UV-induced apoptosis is prevented by a treatment with specific inhibitors of both p38 MAPK and JNK via inhibition of AP-1 activation through the phosphorylation of c-Jun and c-Fos, which is induced by p38 MAPK and JNK.[45,46] Consequently, the inhibitory effects of B-ext and anthocyanidins against UVA-induced cellular damage may contribute to the reduction of ROS production and inhibition of p38 MAPK and JNK phosphorylation.

The PI3K/Akt signaling pathway is a key regulator of cell survival in retinal tissues, including the retinal ganglion cells, inner plexiform layer, inner nuclear layer, and photoreceptor outer segment, as shown by the experiments using an Akt inhibitor.[47] In this study, we observed a decrease in phosphorylated Akt levels immediately after UVA exposure in the 661W cells (Figure 3D−F); L-ext, *trans*-resveratrol, and procyanidin B2, but not B-ext, lessened the attenuation of phosphorylated Akt levels following UVA light exposure (Figure 3D−F). A previous report has suggested that resveratrol prevents cardiomyocyte apoptosis by mediating Akt activation through adenosine A1 and A3 receptors.[48] In this study, the preventive effects of L-ext and its constituents against UVA-induced cellular damage were attenuated by LY294002, a PI3K-Akt inhibitor. Thus, phosphorylation of Akt by L-ext treatment contributed to the former's inhibitory effect against UVA-induced 661W cell death (Figure 4).

Finally, we found that simultaneous treatment with both B-ext and L-ext is more potent than an individual treatment of either against UVA-induced retinal photoreceptor cell damage (Figure 5). Putative mechanisms of action of B-ext and L-ext in UVA-induced photoreceptor cell damage are shown in Figure 6. The additive effect of B-ext and L-ext might be the result of an improvement of Akt reduction and activation of both p38 MAPK and JNK (Figure 5).

Metabolism of anthocyanins, resveratrol, and procyanidins following their oral administration in animals and humans has been reported previously. In previous animal study using pigs after oral administration of blueberry powder, anthocyanins have been identified in the liver, brain, and eyes of pigs.[49] The concentration of anthocyanin in ocular tissues about 700 pg/g of fresh eye tissue weight after intake of water-extracted blueberry powder ranged between 1.3 and 8.5 μmol of anthocyanins/kg of bodyweight/day for 4 weeks. Furthermore, Matsumoto et al. have demonstrated that intraperitoneal administration of blackcurrant anthocyanin at 108 mg/kg body weight leads to maximal concentrations of the compound of 6.72 μg/mL in aqueous humor, 6.89 μg/g in the retina, and 2.30 μg/g in the plasma.[29] However, in previous human study, plasma concentrations of anthocyanins ranged between 0.56 and 4.46 nmol/L after consumption of cranberry juice containing 94.47 mg of anthocyanins in 15 participants.[30] On the other hand, previous in vivo study using murine ocular inflammation in endotoxin-induced uveitis model demonstrated that oral administration of B-ext of 500 mg/kg body weight for 4 days prevented the inflammatory retinal damage, oxidation, rhodopsin decrease, and visual function in mice.[17] Although the plasma concentration of anthocyanins after oral administration may be lower than the effective concentration from in vitro study in our results, anthocyanins may be able to reach ocular tissues and may possess potential efficacy for eye health. In future study, it will be necessary to investigate the protective effect of oral administration of B-ext in animal in vivo study. Several studies have shown the pharmacokinetics and safety of resveratrol and procyanidin in vivo.[50−53] A single oral administration of resveratrol at 0.5, 1.0, 2.5, or 5.0 g to healthy volunteers has shown no toxicity, and the maximal concentrations of resveratrol were 72.6, 117.0, 268.0, and 538.8 ng/mL, respectively (0.32, 0.51, 1.17, and 2.36 μM, respectively), in plasma.[51] These concentrations were approximately the effective doses of 3−10 μM resveratrol used in our study. Holt et al. demonstrated the metabolism of procyanidin B2 in human plasma following a consumption of flavanol-rich cocoa.[52] On average, procyanidin B2 was detected at 5.92

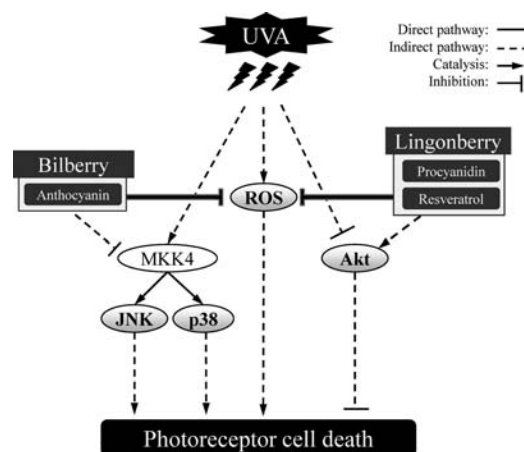

Figure 6. Putative mechanism of B-ext, L-ext, and their constituents against UVA-induced 661W cell damage. UVA irradiation increased production of ROS, phosphorylation of p38 MAPK and JNK, and downregulated the phosphorylation of Akt that induces photoreceptor cell death through the activation of c-Jun, AP-1, and caspase-3. B-ext including anthocyanins reduces ROS production and may inhibit activation of both p38 MAPK and JNK by UVA light exposure via inhibition of MKK4. L-ext including procyanidin and resveratrol reduces ROS production and downregulation of Akt by UVA light exposure. Reactions are represented by direct (solid line) and indirect (dotted line) pathways, catalysis arrows (arrow-headed line), and inhibition arrows (bar-headed line).

μM in the plasma of adult human participants after a single oral administration of 26.4 g of cocoa containing 323 mg of monomeric procyanidins. Therefore, it is possible for procyanidin B2 to reach the effective dose in the eye. However, further research to determine the metabolism in the eye, and to investigate the protective effects against UVA-induced photoreceptor damage in vivo, would be necessary in the case of bilberry anthocyanins, resveratrol, and procyanidins.

In conclusion, we have showed that bilberry and lingonberry protect against UVA-induced retinal photoreceptor cell damage by inhibiting ROS production and regulating phosphorylated p38 MAPK, JNK, and/or Akt levels, which are mediated by anthocyanidin, proanthocyanidin, and *trans*-resveratrol. Because UVA is one of the known causes of various eye diseases, consumption of bilberry and lingonberry extracts, or their coapplication, may prove useful as a prophylactic measure.

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Notes

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■ ABBREVIATIONS USED

B-ext, bilberry extract; CCK-8, cell counting kit-8; CM-H_2DCFDA, 5-(and 6)-chloromethyl-2,7-dichloro-dihydrofluorescein diacetate acetyl ester; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; L-ext, lingonberry extract; PI, propidium iodide; ROS, reactive oxygen species; UVA, ultraviolet A; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt

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