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Antioxidant Activity and Protective Effect of Anthocyanin Oligomers on H_2O_2-Triggered G2/M Arrest in Retinal Cells

Jin-Woo Hwang,[†] Eun-Kyung Kim,[†] Seung-Jae Lee,[†] Yon-Suk Kim,[†] Sang-Ho Moon,[‡] Byong-Tae Jeon,[‡] Si-Heung Sung,[‡] Eun-Tae Kim,[§] and Pyo-Jam Park*[,†]

[†]Department of Biotechnology, [‡]Korean Nokyong Research Center, and [§]Department of Family Medicine, School of Medicine, Konkuk University, Chungju, 380-701, Korea

ABSTRACT: In this study, the free-radical-scavenging properties of anthocyanin oligomers for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, alkyl radical, and hydroxyl radical were evaluated using electron spin resonance (ESR) spectroscopy. The DPPH radical, alkyl radical, and hydroxyl radical scavenging activity of anthocyanin oligomers increased in a dose-dependent manner, with the 50% inhibitory concentration (IC_{50}) value of 13.0, 14.0, and 448.0 μg/mL, respectively. The inhibitory effect of anthocyanin oligomers on lipid peroxidation was examined with ferric thiocyanate (FTC) and thiobarbituric acid (TBA). The inhibitory activity of anthocyanin oligomers was found to be comparable to that of vitamin E. In addition, anthocyanin oligomers enhanced the activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), and glutathione-*S*-transferase (GST, EC 2.5.1.18) in ARPE-19 cells. In addition, anthocyanin oligomers inhibited the H_2O_2-induced G2/M phase arrest in ARPE-19 cells. Taken together, the present results demonstrate that anthocyanin oligomers have high antioxidative activity.

KEYWORDS: *antioxidation, anthocyanin oligomers, ARPE-19, G2/M phase arrest*

INTRODUCTION

Oxidative damage in the human body plays an important causative role in disease initiation and progression. Reactive oxygen species (ROS) are generated and can cause oxidative stress. The importance of ROS and free radicals has attracted increasing attention over the past decade.

A shift in the balance between ROS generation and destruction to overproduction or decreased detoxification has been shown to be associated with many chronic diseases.[1] Damage from free radicals and ROS has been linked to some neurodegenerative disorders and cancers, and oxidation of low-density lipoprotein is a major factor in the promotion of coronary heart disease and atherosclerosis. As a result of these effects, ROS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer, and gastric ulcer.[2] Therefore, the development and utilization of more effective antioxidants are desired.[3] In addition, antioxidants can protect the human body from free radicals and ROS effects and retard the progression of many chronic diseases as well as lipid oxidative rancidity in foods.[4] The types of antioxidants that have been used include antioxidant vitamins (e.g., ascorbic acid, α-tocopherol, β-carotene), inorganic antioxidants (e.g., selenium), and synthetic antioxidants [e.g., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate]. However, the use of these antioxidants in food products is strictly regulated due to industrial efficiency or the potential health hazards caused by such compounds.[5] Therefore, there is a need to identify and characterize more effective natural antioxidants.

Anthocyanin effectively neutralizes free radicals with antioxidants, and antiaging and dementia prevention have been reported. In addition, anthocyanin can promote the reproduction of rhodopsin, which can improve impaired night vision, blurred vision, eye fatigue due to physical and mental fatigue, and various eye diseases caused by a lack of effective rhodopsin. In addition, anthocyanin hinders the creation of arteries, prevents heart disease, reduces the risk of stroke, and has anti-inflammatory and antiseptic effects.

The most active components of fruits, vegetables, and other natural objects are the anthocyanoside oligomers, which occur as small anthocyanidin glycoside polymers that are present as dimers, trimers, tetramers, and pentamers. Anthocyanidin oligomers are hydro- and liposoluble molecules with high bioavailability and are not known to accumulate in the body. Most commercially available bilberry preparations contain only 5−30% anthocyanoside oligomer, where the remaining compounds include inactive nonanthocyanoside impurities such as free sugars, tannin, and organic acids. Using specific fermentation processes, anthocyanoside oligomers can be concentrated to levels higher than 85%.[6]

Myopia causes decreased contrast sensitivity and may be a cause of vague eye discomfort resulting from use of the eyes, which is known as asthenopia. A large adult population study showed that myopia that was fully corrected with spectacles was associated with lower mean contrast sensitivity in the higher spatial frequencies when compared with the emmetropic condition.[7] Moreover, visual acuity and contrast sensitivity under reduced light conditions, namely, mesopic visual function, was lower in myopia subjects who were corrected by spectacle and photorefractive keratectomy.[8]

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Therefore, the goal of the present study was to evaluate the antioxidative effects of anthocyanin oligomers on human retinal pigment epithelium (RPE) cells (designated ARPE-19).

MATERIALS AND METHODS

Materials. Propidium iodide (PI), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and (4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were purchased from Sigma Chemical Co. (St. Louis, MO). RNase A, pBR 322 DNA, and Tween-20 were purchased from Novagen Inc. (Darmstadt, Germany), Takara Biomedicals (Tokyo, Japan), and USB Co. (Cleveland, OH). Dulbecco's modified Eagle's medium (DMEM):nutrient mixture F12 (1:1 mixture), trypsin−DTA (ethylenediaminetetraacetic acid), penicillin/streptomycin, and certified fetal bovine serum were supplied by Invitrogen Co. (Carlsbad, CA). Triton X-100 and MW electrophoresis calibration kits were purchased from Amersham-Pharmacia (Piscataway, NJ). Anthocyanin oligomers from products manufactured by Kitolife (Pyeong-Taek, Korea) were used. These products were manufactured using specific fermentation processes.[6]

Synthesis and Analysis of Anthocyanin Oligomer. Anthocyanin oligomers was fermented using the method described by Lee et al.,[6] which involves using the anthocyanin monomer and *Aspergilus niger*. The mold was then removed by centrifugation at 0.012 rcf, and the samples were lyophilized in a freeze drier for 3 days. The molecular weight of the anthocyanin oligomers was measured using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, UltrafleXtreme MALDI time-of-flight (TOF) mass spectrometer, Bruker Daltonics, MA) equipment of the Korea Basic Science Institute. Also, the fermentation results were compared to the results obtained for the anthocyanin monomer

Electron Spin Resonance (ESR) Measurement. *DPPH Radical Scavenging Activity.* DPPH radical scavenging activity was measured using the method described by Lee et al.[9] A 30 μL anthocyanin oligomers solution (or ethanol itself as control) was added to 30 μL of DPPH (60 μM) in an ethanol solution. After being mixed vigorously for 10 s, the solution was transferred to a 100 μL quartz capillary tube, and the scavenging activity of anthocyanin oligomers for the DPPH radical was measured using a JESFA ESR spectrometer. The spin adduct was measured on an ESR spectrometer exactly 2 min later. The experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; and sweep time, 30 s. The DPPH radical scavenging activity was calculated using the following equation in which H and H_0 are the relative peak heights of the radical signals with and without the sample, respectively:

$$\text{radical scavenging activity} = \left(\frac{1 - H}{H_0}\right) \times 100\% \quad (1)$$

Alkyl Radical Scavenging Activity. Alkyl radicals were generated as described by Kim et al.[10] Briefly, 20 μL of 40 mM AAPH was mixed with 20 μL of phosphate buffered-saline (PBS), 20 μL of 40 mM 4-POBN, and 20 μL of anthocyanin oligomers solution. The mixture was vortexed and incubated at 37 °C for 30 min. Subsequently, the reaction mixture was transferred to a sealed capillary tube, and the spin adduct was recorded under the following controlled spectrometric conditions: modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9441 MHz; magnetic field, 336.5 ± 5 mT; and sweep time, 30 s. The peroxyl radical scavenging ability was also calculated using eq 1.

Hydroxyl Radical Scavenging Activity. Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber−Weiss reaction, and the generated hydroxyl radicals rapidly reacted with nitrone spin trap DMPO.[10] The resultant DMPO−OH adducts were detected using an ESR spectrometer. The anthocyanin oligomers solution (20 μL) was mixed with DMPO (0.3 M, 20 μL), $FeSO_4$ (10 mM, 20 μL), and H_2O_2 (10 mM, 20 μL) in a phosphate buffer solution (pH 7.4) and then transferred into a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 200; and sweep time, 4 min. The hydroxyl radical scavenging ability was also calculated using eq 1.

Lipid Peroxidation Inhibition Assay Using the Ferric Thiocyanate (FTC) Method. A screw-cap vial (φ = 38 mm × 75 mm) containing a mixture of 4 mg of sample in 4 mL of 99.5% ethanol, 4.1 mL of 2.51% linoleic acid in 99.5% ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0), and 3.9 mL of water (final concentration, 0.02%, w/v) was placed in an oven at 40 °C in the dark.[11] A 9.7 mL portion of 75% (v/v) ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 2.0 × 10−2 M ferrous chloride in 3.5% hydrochloric acid were added to 0.1 mL of this mixture in a test tube (φ = 1.5 mm × 14.5 cm). Three minutes after the addition of ferrous chloride, the absorbance was measured at 500 nm. This step was repeated every 24 h until the control reached its maximum absorbance value.

Lipid Peroxidation Inhibition Assay Using the Thiobarbituric Acid (TBA) Method. The samples prepared for the FTC method were used for this assay. One milliliter of 20% aqueous trichloroacetic acid and 2 mL of 0.67% aqueous thiobarbituric acid were added to 2 mL of the sample solution in a 10 mL centrifuge tube.[12] The mixture was placed in a boiling water bath for 10 min. After being cooled, the mixture was centrifuged at 0.8 rcf for 30 min. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was measured on the basis of the absorbance at the final day of the FTC assay.

Cell Culture. Human adult RPE cells (ARPE-19, ATCC #CRL-2302) were maintained in a 1:1 mixture of DMEM and F-12 nutrient mixture supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2, and 90% relative humidity. Upon reaching 90% confluency, the cells were trypsinized and seeded into the appropriate tissue culture vessels for each experiment.

Cytotoxicity Assay in Vitro. To determine the effect of purified peptide on the viability of normal human retina cells, a colorimetric MTT assay was performed. Normal human retina cells, ARPE-19, were seeded at 0.5 × 10^4 cell/well in 96-well microliter plates in DMEM/F12 medium containing 10% FBS. After 24 h of incubation in a humidified 5% (v/v) CO_2/air environment at 37 °C, 20 μL of MTT dye solution was added to each well. After 4 h of incubation, 200 μL of the solubilization/stop solution was added to dissolve the formazan crystals, and the mixture was incubated at 37 °C overnight. The absorbance was read using a microplate reader (Tecan Trading AG, Männedorf, Switzerland) at 540 nm.

Determination of Antioxidative Enzyme Activity. *SOD (EC 1.15.1.1) Activity.* The ARPE-19 cells (0.5 × 10^4) were plated, allowed to attach overnight, and exposed to H_2O_2 and various concentrations of the anthocyanin oligomer for 1 day at 37 °C. The cells were collected, washed with PBS, and pelleted by centrifugation at 15.7 rcf for 5 min. Cell pellets were incubated in lysis buffer consisting of 30 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM Na_3VO_4, 25 mM NaF, 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail for 60 min on ice. After insoluble factions were removed by centrifugation at 15.7 rcf (4 °C) for 30 min, the supernatant was collected, and the protein concentration was determined using a BCA protein assay kit. The supernatant fraction was used to determine SOD activity, which was measured using kits from Fluka according to the manufacturer's instructions.

CAT (EC 1.11.1.6) Assay. CAT activity was measured on the basis of the rate of H_2O_2 decomposition.[13] This method is based on H_2O_2 degradation through the action of CAT contained in the examined samples. In this procedure, 50 mM phosphate buffer (pH 7.0) was used and 30 mM H_2O_2 was used as the substrate. CAT activity was expressed as μmol H_2O_2/min/mg protein.

GPx (EC 1.11.1.9) Activity Assay. The activity of GPx was determined following the oxidation of nicotinamide adenine

dinucleotide phosphate (NADPH) using *tert*-butyl hydroperoxide as a substrate.[14] This reaction proceeded through the action of GPx contained in the samples using *tert*-butyl hydroperoxide (3 mM) as substrate in 0.5 M phosphate buffer, pH 7.0, at 37 °C. The activity of GPx was expressed as nmol NADPH/min/mg protein.

GST (EC 2.5.1.18) Assay. The activity of GST toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined as described by the method of Habig et al.[15] This method is based on the reaction of CDNB with the SH group of GSH catalyzed by GST contained in the samples. The reaction proceeded in the presence of 1 mM GSH in phosphate buffer (pH 6.5) at 37 °C. GST activity was expressed as nmol GSH/min/mg protein. All chemicals were products manufactured by Sigma.

The GST Assay Kit (Sigma) utilizes CDNB, which is suitable for the broadest range of GST isozymes. There is an increase in the absorbance at 340 nm after conjugation of the thiol group of glutathione to the CDNB substrate.

Flow Cytometer Analysis. For sub-G1 and cell cycle analysis, ARPE-19 cells were suspended in ethanol with 0.5% Tween-20 and left for 24 h at 4 °C. The cells were harvested by centrifugation, resuspended in 1 mL of PBS with 0.05 mg/mL propidium iodide and 10 μL/mL RNase A, and incubated at 37 °C for 30 min. Apoptotic cell death was assessed by measuring the hypodiploid DNA content of the cells using a flow cytometer. Cells belonging to the sub-G1 population were considered apoptotic cells, and the percentage of cells in each phase of the cell cycle was determined.

Statistical Analysis. The data are presented as mean ± SD. The paired *t* test was used for comparisons between H_2O_2-treated and the anthocyanin-oligomer-treated group. All analyses were performed using an SPSS system (SPSS institute, Chicago, NC).

RESULTS AND DISCUSSION

Anthocyanin oligomers, including many dimer, trimer, and tetramer oligomers, were produced by biochemical and biological engineering. The produced anthocyanin oligomers were analyzed by MALDI-TOF/MS. In this analysis, the molecular weights of the dimer (602), trimer (890), and tetramer (1178) anthocyanin oligomers were determined. In addition, the molecular weight of the anthocyanin monomers was shown to be 331 Da (Figure 1A). Thus, this approach was economically suited for the biosynthesis of anthocyanin oligomers (Figure 1B).

The free-radical-scavenging activity of the anthocyanin oligomers is shown in Figure 2A. DPPH is a stable free radical and has been widely used to evaluate the free-radical-scavenging activity of natural antioxidants. In this study, the DPPH radical scavenging activities were 90.30% at 62.5 μg/mL (data not recorded here), and the IC_{50} value of the anthocyanin oligomers was 13.0 μg/mL.

In addition, the alkyl radical spin adduct of 4-POBN/free radicals was generated from AAPH at 37 °C after 30 min, and a decrease in the ESR signals was observed at increasing anthocyanin oligomers concentrations. The alkyl radical scavenging activity was 93.11% at 500 μg/mL (Figure 2C), and the IC_{50} value of the anthocyanin oligomers was 14.0 μg/mL.

The hydroxyl radical generated in the Fe^{2+}/H_2O_2 system was trapped by DMPO, which formed the spin adduct detected by ESR spectroscopy, and the typical 1:2:2:1 ESR signal of the DMPO−OH adduct was observed. The height of the third peak in the spectrum represents the relative amounts of DMPO−OH adduct. The addition of the anthocyanin oligomers resulted in a decrease in the peak corresponding to the DMPO−OH adduct. Hydroxyl radical scavenging activities of 85.95% at 2.0 mg/mL (Figure 2E) were observed, and the IC_{50} value of anthocyanin oligomers was determined to be 0.448 mg/mL. These results clearly demonstrate that the anthocyanin

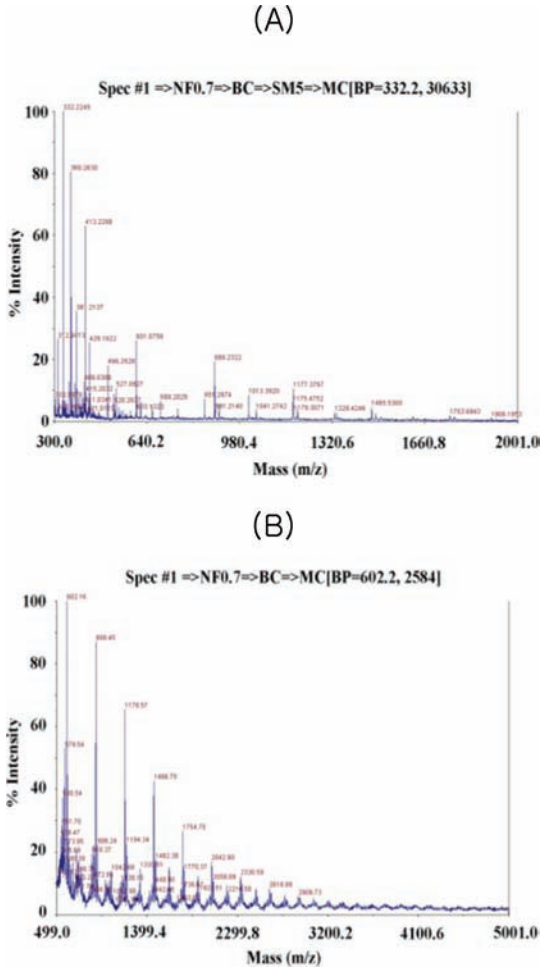

Figure 1. Biosynthesis of anthocyanin oligomers from anthocyanin monomers. The anthocyanin oligomer was synthesized by fermentation using *A. niger* at 25 °C for 3 day. Before biosynthesis (A) and after biosynthesis (B).

oligomers possesses antioxidative activity and can effectively scavenge DPPH and alkyl and hydroxyl radicals.

The DPPH radical scavenging activity of the anthocyanin oligomers was similar to the activity of ascorbic acid. In addition, this study demonstrated the effectiveness of anthocyanin oligomers for the inhibition of lipid peroxidation. Also, the anthocyanin oligomers prepared in this study were more active than anthocy anin oligomers extracted from litchi.[16]

In other studies, free-radical-scavenging meaurement by ESR showed higher effects after isolation and purification.[10,17] Various molecular weight composition was used in this study of anthocyanin oligomers. However, isolated and purified anthocyanin oligomers with a single molecular weight show a higher free-radical-scavenging activity.

The antioxidant activity of anthocyanin oligomers was determined using FTC and TBA methods and compared with that of vitamin E. The effects of anthocyanin oligomers and vitamin E on peroxidation of linoleic acid emulsions are shown in Figure 3A. When the individual activity of samples was measured using the FTC method, the anthocyanin oligomers and vitamin E were shown to have similar absorbance values. The absorbance value of the control increased on day 2 and reached maximum levels on day 5 and finally dropped on day 6. Figure 3 shows the total

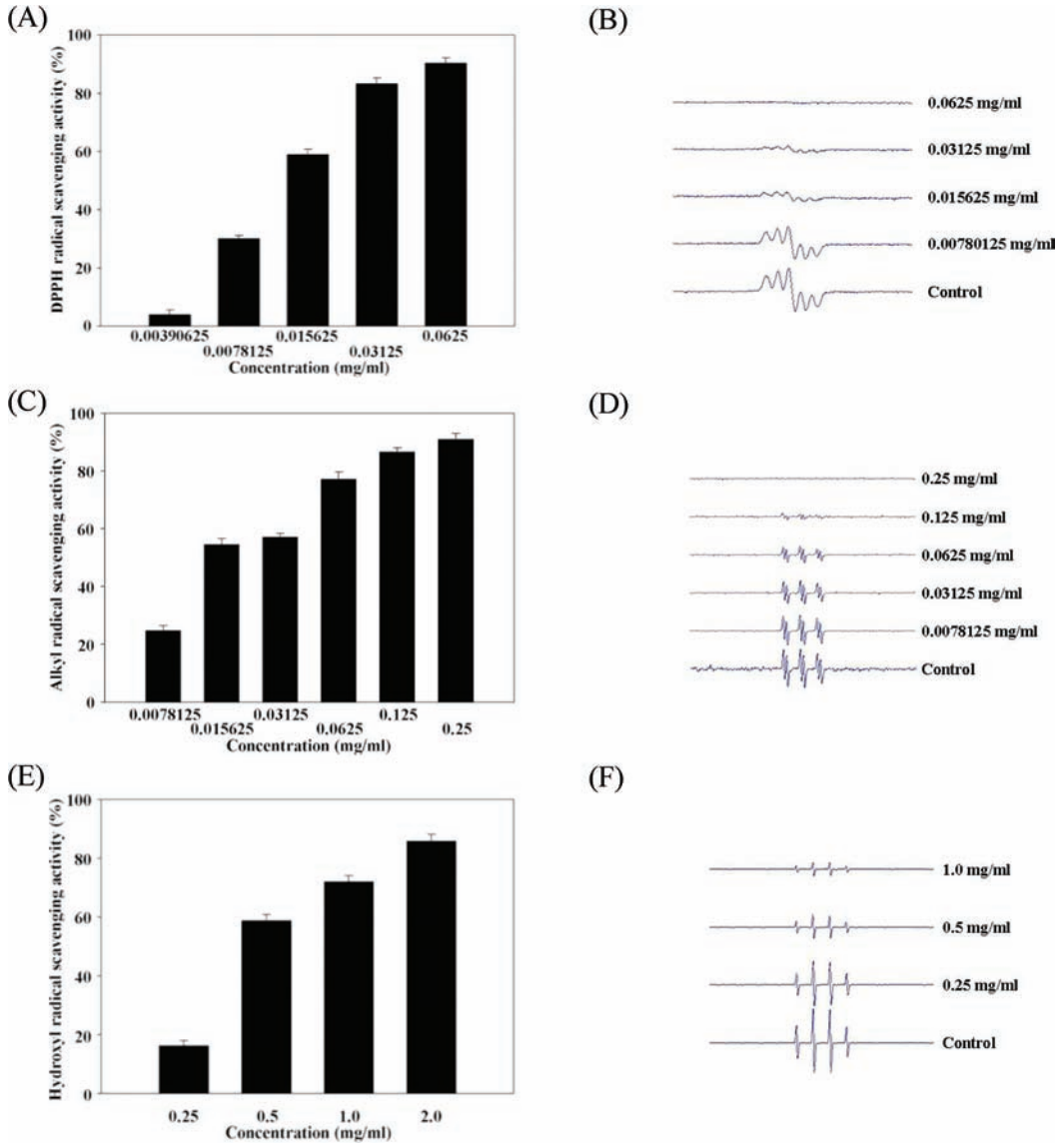

Figure 2. Free-radical-scavenging activity of the anthocyanin oligomers: DPPH radical (A), DPPH radical spectra (B), alkyl radical (C), alkyl radical spectra (D), hydroxyl radical (E), and hydroxyl radical spectra (F).

antioxidant activity of anthocyanin oligomers as measured using the FTC and TBA methods. When the FTC method was used, the anthocyanin oligomers were shown to have higher activity (91%) than vitamin E (88%). However, when the TBA method was used, vitamin E had higher antioxidant activity (95%) than the anthocyanin oligomers (87%). No significant differences were observed between the total antioxidant activity of anthocyanin oligomers and vitamin E for both methods (Figure 3).

Antioxidants are known to alleviate oxidative stress, which is believed to be one of the major causes for the accumulation of mutations in the genome. Peroxide is gradually decomposed to lower molecular compounds during the oxidation process, which were measured using the FTC and TBA methods. The amount of peroxide at the primary stage of linoleic acid peroxidation was measured using the FTC method, whereas the TBA method measures the secondary stage. Total antioxidant activity of anthocyanin oligomers was determined using the FTC and TBA methods. Total antioxidant activity measured using the FTC method was higher than the activity measured using the TBA method. This may indicate that the amount of peroxide in the initial state of lipid peroxidation was greater than the amount of peroxide in the secondary stage. Furthermore, secondary products such as malondialdehyde are not stable for long periods of time and will turn into alcohol and acid, which cannot be detected by spectrophotometer.[18,19]

The viability and proliferation activity of retinal cells, ARPE-19, after incubation with different concentrations of the anthocyanin oligomers and H_2O_2 were measured. The proliferation activity of the cells was measured using the colorimetric MTT assay. In these experiments, ARPE-19 cells were seeded at a density of 0.5×10^4 cells/mL and incubated with the anthocyanin oligomers for 18 h. Toxicity analysis revealed a slow increase in mitochondrial dehydrogenase activity with an increase in H_2O_2 concentration. On the other hand, ARPE-19 showed 60% viability against 0.5 mM of H_2O_2 (data not showed). This result is in agreement with a report by Wang et al.[20]

The addition of the anthocyanin oligomer increased ARPE-19 proliferation in a noncytotoxic manner. This observation

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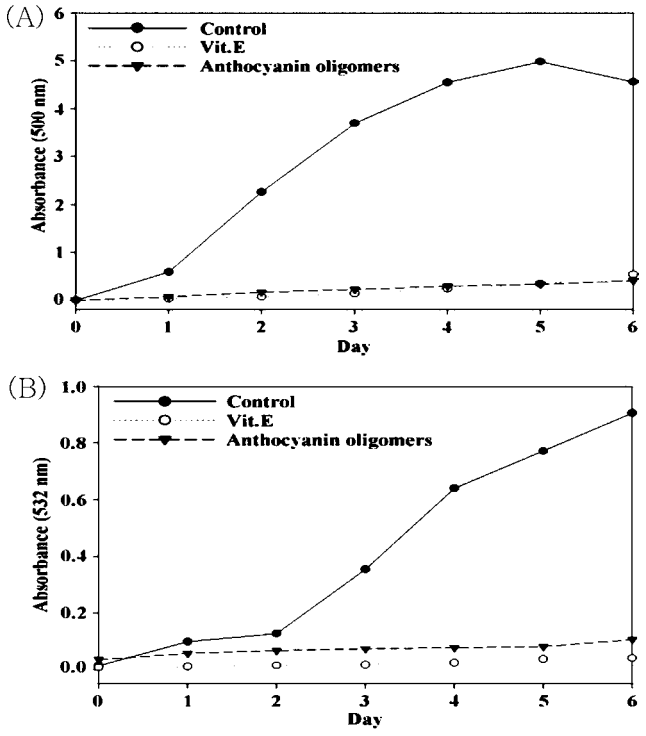

Figure 3. Inhibitory effect of the anthocyanin oligomer on lipid peroxidation using FTC (A) and TBA (B) methods.

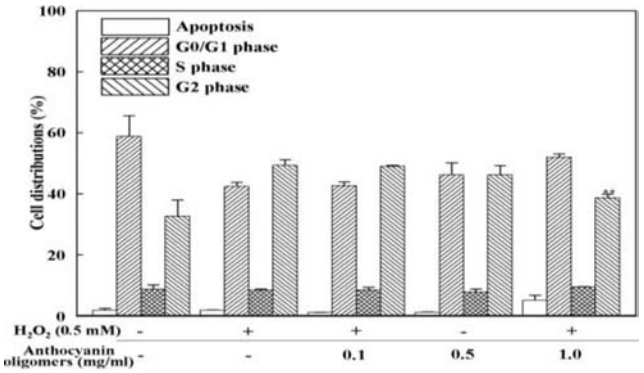

Figure 4. Cell death and cell cycle of ARPE-19 after anthocyanin oligomers treatment prior to H_2O_2 treatment. The cells were treated with 0.1, 0.5, and 1.0 mg/mL of the anthocyanin oligomers prior to the addition of 0.5 mM H_2O_2 for 24 h. Means ± SD of triplicate experiments. **Values ($p < 0.01$) are significantly different as analyzed by paired t test compared to the oxidative damage with sample-treated group.

demonstrated that the anthocyanin oligomer promoted the proliferation of ARPE-19 in a dose-dependent manner.

Also, due to strong reactivity with biomolecules, ·OH is capable of doing more damage to biological systems than any other ROS.[21] Therefore, the protective effects H_2O_2 were evaluated for anthocyanin oligomers, which have the highest free-radical-scavenging activity. The retinal cell protective effect of the anthocyanin oligomers was determined using cell cycle analysis with a flow cytometer. The cells were treated with various concentrations of the anthocyanin oligomers prior to treatment with 0.5 mM H_2O_2 for 24 h.

In the oxidative stress group, G2/M phase arrest was observed. However, in the anthocyanin-oligomers-treated group, G2/M phase arrest was reduced. These results indicate that the anthocyanin oligomers protects retinal cells against H_2O_2-induced oxidative damage by inhibiting G2/M phase arrest in a dose-dependent manner (Figure 4).

Damage to growing cells causes a temporary pause in G1/S or G2/M phase until the damage is repaired. When damage is severe, cells may either undergo apoptosis or enter a dormant G0 state. Regulation of cell cycle progression is controlled by many factors, including cyclin accumulation and degradation; phosphorylations of Cdks, cyclins, and other proteins; regulation of cyclin/Cdk dimerization; and the binding of a number of Cdk inhibitory proteins. Movement of cells from G2 → M is regulated by cyclin A and cyclin B/Cdc2. Cyclin B/Cdc2 kinase activity peaks in late G2 and remains high until its degradation. This kinase has been identified as the principal component of the mitosis promoting factor (MPF). In mammalian cells, three cyclin B isoforms have been characterized: cyclin B1, cyclin B2, and cyclin B3. Cyclin B1 plays an important role in maintaining the G2/M transition and its progression.[22]

SOD, which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. The effects of H_2O_2 and various concentrations of the anthocyanin oligomer on SOD enzyme activity were determined. Treatment with H_2O_2 and various concentrations of anthocyanin oligomer for 1 day resulted in a modest, but statistically significant, increase in SOD activity (Figure 5A).

CAT is an antioxidative enzyme ubiquitously present in mammalian and nonmammalian aerobic cells that contain a cytochrome system. CAT was initially isolated from the liver and later from blood, bacterial, and plant sources. The enzyme contains four ferrihemoprotein groups per molecule. The enzyme has a molecular mass of 240 kDa. CAT activity varies greatly between different tissues. The activity is highest in the liver and kidney and lowest in connective tissues. In eukaryotic cells, the enzyme is concentrated in subcellular organelles called peroxisomes (microbodies).[23]

CAT catalyzes the decomposition of H_2O_2 to water and oxygen. H_2O_2 is formed in the eukaryotic cell as a byproduct of various oxidase and superoxide dismutase reactions. H_2O_2 is highly deleterious to the cell, and the accumulation of H_2O_2 results in oxidation of cellular targets such as DNA, proteins, and lipids, which can lead to mutagenesis and cell death. Removal of H_2O_2 from the cell by CAT protects against oxidative damage to the cell. The role of CAT in oxidative stress-related diseases has been widely studied.[24] CAT activity was the same in the control and H_2O_2- and anthocyanin-oligomer-treated groups (Figure 5B).

In addition GPx provides a mechanism for detoxification of peroxides in living cells.[25] This reaction plays a crucial role in protecting cells from damage by free radicals that are formed by peroxide decomposition. Lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid peroxidation. GPx enzymes reduce peroxides to alcohols using glutathione and prevent the formation of free radicals. GPx activity was shown to have similar effects in the H_2O_2- and anthocyanin-oligomer-treated groups (Figure 5C).

GST are a group of enzymes that are important in the detoxication of many different xenobiotics in mammals. These enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics and

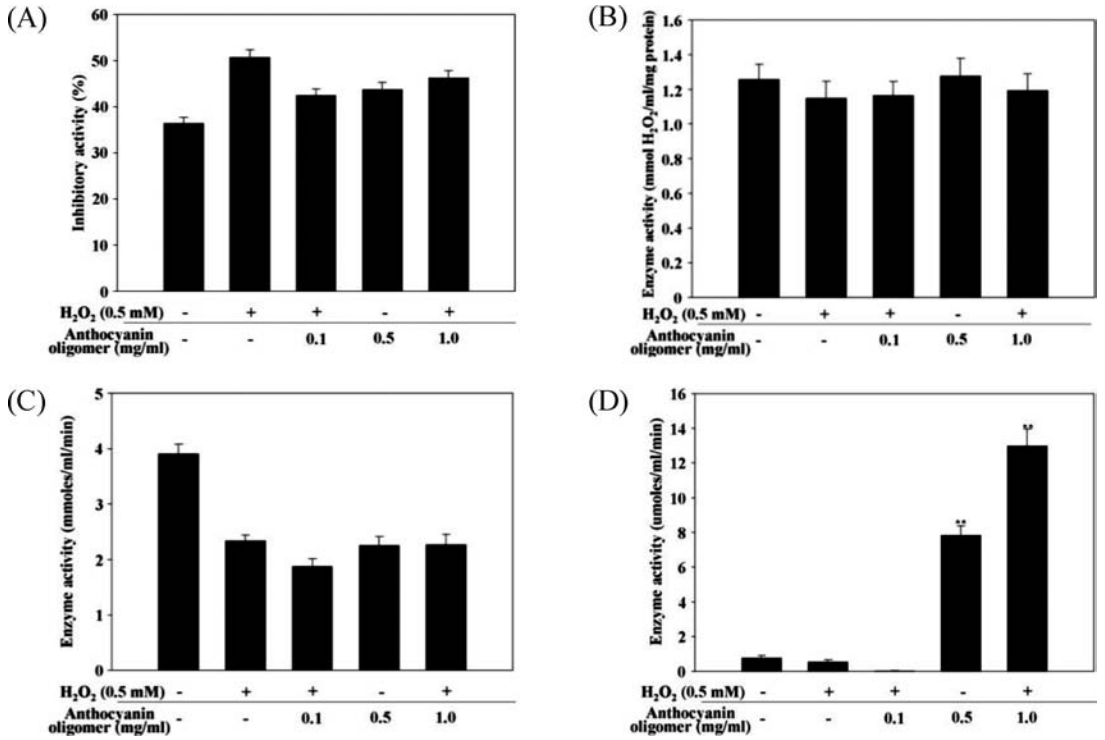

Figure 5. Effect of anthocyanin oligomers on SOD (A), CAT (B), GPx (C), and GST (D) activity in retinal cells. Values are given as the mean ± SD. **Values ($p < 0.01$) are significantly different as analyzed by paired t test compared to oxidative damage with the sample-treated group.

thereby defend cells against the mutagenic, carcinogenic, and toxic effects of the compounds. GST activity has been observed in plants,[26] insects,[27] yeast,[28] bacteria,[29] and in most mammalian tissues, especially in the liver, which plays a key role in detoxification. There are several classes of GST isozymes, and these classes differ in their specificity toward xenobiotic or endogenous substrates. GST activity was significantly increased in the anthocyanin-oligomer-treated group at 0.5 and 1.0 mg/mL (Figure 5D).

In conclusion, in this study, anthocyanin oligomer showed antioxidative activity and inhibited G2/M phase arrest. Its free-radical-scavenging properties were investigated by ESR spectroscopy, and inhibition of lipid oxidation was measured using the FTC and TBA methods. Second, its possible protective effects on retinal cell damage against oxidative stress were demonstrated by cell cycle and antioxidative enzyme activity. On the basis of the combined results of this study, the following conclusions can be made:

Anthocyanin oligomers scavenged free radicals such as DPPH and alkyl and hydroxyl radicals in a dose-dependent manner. The IC_{50} values were 13.0, 14.0, and 448.0 μg/mL, respectively. In the FTC method, anthocyanin oligomer concentrations of 1.0 mg/mL showed similar activity as vitamin E. In addition, anthocyanin oligomer concentrations of 1.0 mg/mL showed inhibitory activity in the TBA method. SOD, CAT, GPx, and GST activities were significantly higher in the 1.0 mg/mL anthocyanin-oligomer-treated group than the H_2O_2-treated group. G2/M phase arrest was higher in the H_2O_2-treated group than the anthocyanin-oligomer-treated group.

Thus, further studies are being conducted to evaluate G2/M phase arrest-related factors using the westhern blot method.

AUTHOR INFORMATION

Corresponding Author

*Tel: +82-43-840-3588. Fax: +82-43-852-3616. E-mail: parkpj@kku.ac.kr.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Je, J. Y.; Park, P. J.; Kim, S. K. Free radical scavenging properties of heterochitooligo-saccharides using an ESR spectroscopy. *Food Chem. Toxicol.* **2004**, *42*, 381−387.

(2) Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **1993**, *20*, 21−29.

(3) Oktay, M.; Glin, I.; Kfreviolu, I. Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *LWT-Food Sci. Technol.* **2003**, *36*, 263−271.

(4) Glin, I.; Bykokurolu, M. E.; Kfreviolu, I. Metal chelating and hydrogen peroxide scavenging effects of melatonin. *J. Pineal Res.* **2003**, *34*, 278−281.

(5) Cashman, J. R. Monoamine oxidase and flavin-containing monooxygenases, In *Biotransformation*; Guengerich, F. P., Ed.; Elsevier: New York, 1997; pp 69−96.

(6) Lee, J. H.; Lee, H. K.; Kim, C. Y.; Hong, Y. J.; Choe, C. M.; You, T. W.; Seong, G. J. Purified high-dose anthocyanoside oligomer administration improves nocturnal vision and clinical symptoms in myopia subjects. *Br. J. Nutr.* **2005**, *93*, 895−899.

(7) Ogden, T. E.Visual acuity and contrast sensitivity. In *Retina*, 2nd ed.; Mosby: St. Louis, MO, 1994; p 149.

(8) Owsley, C.; Sloane, M. E. Contrast sensitivity, acuity, and the perception of "real-world" targets. *Br. J. Ophthalmol.* **1987**, *71*, 791−796.

(9) Lee, S. J.; Kim, E. K.; Hwang, J. W.; Kim, C. G.; Choi, D. K.; Lim, B. O.; Moon, S. H.; Jeon, B. T.; Park, P. J. Neuroprotective effect of

Hericium erinaceum against oxidative stress on PC-12 cells. *J. Korean Soc. Appl. Biol. Chem.* **2010**, *54*, 112−117.
(10) Kim, E. K.; Lee, S. J.; Jeon, B. T.; Moon, S. H.; Kim, B. K.; Park, T. K.; Han, J. S.; Park, P. J. Purification and characterisation of antioxidative peptides from enzymatic hydrolysates of venison protein. *Food Chem.* **2009**, *114*, 1365−1370.
(11) Saha, K.; Lajis, N. H.; Israf, D. A.; Hamzah, A. S.; Khozirah, S.; Khamis, S.; Syahida, A. Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *J. Ethnopharmacol.* **2004**, *92*, 263−267.
(12) Mackeen, M. M.; Ali, A. M.; Lajis, N. H.; Kawazu, K.; Hassan, Z.; Amran, M.; Habsah, M.; Mooi, L. Y.; Mohamed, S. M. Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex. T. Anders. *J. Ethnopharmacol.* **2000**, *72*, 395−402.
(13) Clairborne, A. In *Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Ed.; CRC Press Inc.: Boca Raton, FL, 1984.
(14) Tamura, M.; Oschino, N.; Chance, B. Some characteristics of hydrogen and alkyl-hydroperoxides metabolizing systems in cardiac tissue. *J. Biochem.* **1982**, *92*, 1019−1031.
(15) Habig, W. H.; Pubst, M. J.; Jakoby, W. B. Glutathione S-transferase. *J. Biol. Chem.* **1974**, *249*, 7130−7139.
(16) Xuewu, D.; Yueming, J.; Xinguo, S.; Zhaoqi, Z.; John, S. Antioxidant properties of anthocyanins extracted from litchi (*Litchi chinenesis* Sonn.) fruit pericarp tissues in relation to their role in the pericarp browning. *Food Chem.* **2007**, *101*, 1365−1371.
(17) Lee, S. J.; Kim, E. K.; Hwang, J. W.; Oh, H. J; Cheong, S. H.; Moon, S. H.; Jeon, B. T.; Lee, S. M.; Park, P. J. Purification and characterisation of an antioxidative peptide from enzymatic hydrolysates of duck processing by-products. *Food Chem.* **2010**, *123*, 216−220.
(18) Ottolenghi, A. Interaction of ascorbic acid and mitochondria lipids. *Arch. Biochem. Biophys.* **1959**, *79*, 355.
(19) Hakimŏglu, F.; Kızıl, G.; Kanay, Z.; Kızıl, M.; Isı, H. The effect of ethanol extract of *Hypericum lysimachioides* on lipid profile in hypercholesterolemic rabbits and its *in vitro* antioxidant activity. *Atherosclerosis* **2007**, *192*, 113−122.
(20) Wang, Z. Y.; Shen, L. j.; Tu, L. L.; Hu, D. N.; Liu, G. Y.; Zhou, Z. L.; Lin, Y.; Chen, L. H.; Qu, J. Erythropoietin protects retinal pigment epithelial cells from oxidative damage. *Free Radical Biol. Med.* **2009**, *46* (8), 1032−1041.
(21) Halliwell, B. Antioxidant defence mechanisms: From the beginning to the end (of the beginning). *Free Radical Res.* **1999**, *31*, 261−272.
(22) Zhang, Z.; Huang, C.; Li, J.; Leonard, S. S.; Lanciotti, R.; Butterworth, L.; Shi, X. Vanadate-induced cell growth regulation and the role of reactive oxygen species. *Arch. Biochem. Biophys.* **2001**, *392*, 311−320.
(23) Zamocky, M.; Koller, F. Understanding the structure and function of catalases: Clues from molecular evolution and in vitro mutagenesis. *Prog. Biophys. Mol. Biol.* **1999**, *72*, 19−66.
(24) Tome, M. E.; Baker, A. F.; Powis, G.; Payne, C. M.; Briehl, M. M. Catalase-overexpressing thymocytes are resistant to glucocorticoid-induced apoptosis and exhibit increased net tumor growth. *Cancer Res.* **2001**, *61*, 2766−2733.
(25) Ursini, F.; Maiorino, M.; Brigelius, F. R.; Aumann, K. D.; Roveri, A.; Schomburg, D.; Flohe, L. Diversity of glutathione peroxidases. *Methods Enzymol.* **1995**, *252*, 38−52.
(26) Mozer, T. J.; Tiemeier, D. C.; Jaworski, E. G. Purification and characterization of corn glutathione S-transferase. *Biochemistry* **1983**, *22* (5), 1068−1072.
(27) Toung, Y. P.; Hsieh, T. S.; Tu, C. P. *Drosophila* glutathione S-transferase 1−1 shares a region of sequence homology with the maize glutathione-S-transferase III. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 31−35.
(28) Tamaki, H.; Yamamoto, K.; Kumagai, H. Expression of two glutathione S-transferase genes in the yeast *Issatchenkia orientalis* is induced by *o*-dinitrobenzene during cell growth arrest. *J. Bacteriol.* **1999**, *181*, 2958−2962.
(29) Piccolomini, R.; Ilio, C. D.; Aceto, A.; Allocati, N.; Faraonea, A.; Cellini, L.; Ravagnan, G.; Federici, G. Glutathione transferase in bacteria: Subunit composition and antigenic characterization. *J. Gen. Microbiol.* **1989**, *135*, 3119−3125.