Journal of Food and Nutrition Research, 2014, Vol. 2, No. 6, 277-280
Available online at http://pubs.sciepub.com/jfnr/2/6/2

DOI:10.12691/jfnr-2-6-2

Protective Effects of Berry Extracts on Hydrogen Peroxide-induced Rat Brain Neuronal Cell Damage *In Vitro*

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Received May 13, 2014; Revised June 03, 2014; Accepted June 09, 2014

Abstract The purpose of this study was to evaluate the antioxidative activities and protective effects of boysenberry, black currant, and blueberry extracts on neuronal cells. We found that berry extracts had different levels of antioxidant efficacy in various assays, including cell viability assays using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). In addition, reactive oxygen species (ROS) were measured using 2',7'–dichlorofluorescein diacetate (DCF-DA) and flow cytometry analysis. Fruit extracts possessed strong antioxidant activities as measured using the malondialdehyde (MDA) assays. These extracts effectively inhibited lipid peroxidation. Black currant fruit extract significantly reduced intracellular ROS accumulation resulting from hydrogen peroxide treatment in PC12 cells. Black currant fruit more effectively inhibited lactate dehydrogenase (LDH) release into the medium than other tested fruit extracts did. The present results indicate that fruit extracts have the potential to protect neurons from hydrogen peroxide-induced cell damage and should be considered as a prospective functional food and therapeutic product.

Keywords: *antioxidant, hydrogen peroxide, neuro-protection, boysenberry, black currant, blueberry*

Cite This Article: Chang-Ho Jeong, Chi-Woen Jang, Dong-Chan Kum, Koo Yul Lee, Seung Yuan Lee, Sun Jin Hur, and Seung-Jae Lee, "Protective Effects of Berry Extracts on Hydrogen Peroxide-induced Rat Brain Neuronal Cell Damage *In Vitro*." *Journal of Food and Nutrition Research*, vol. 2, no. 6 (2014): 277-280. doi: 10.12691/jfnr-2-6-2.

1. Introduction

Reactive oxygen species (ROS) include free radicals, such as hydroxyl radicals, superoxide radicals, and singlet oxygen. Various radicals are known to be major causes of some chronic and degenerative diseases, including malaria, heart disease, inflammation, cancer, human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS), and neurological disorders such as Alzheimer's, and Parkinson's disease [1]. Synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, and tertiary butylhydroquinone are used to reduce ROS formation in natural products [1,2]. However, the use of synthetic antioxidants is under increasing scrutiny because of potential health risks associated with such compounds. Although synthetic antioxidants are effective and cheap compared to natural antioxidants, their application is restricted because of potential risks related to health [2]. Therefore, there is new interest in finding natural and safe antioxidant agents from natural sources.

Berries contain micronutrients and macronutrients, including fibers, minerals, and vitamins. Various health benefits and disease prevention conferred by berry extracts have been attributed to large amounts of bioactive phytochemicals, including phenolic acids, anthocyanins, flavonols, and ellagitannins [4,5]. Many of the biological effects of phenolics are related to free radical scavenging and inhibition of cancer cell growth. Plants typically produce anthocyanins as a protective mechanism against environmental stress factors, including UV, extreme temperatures, and drought [6]. Anthocyanins effectively neutralize free radicals. Antioxidant, anti-aging, and dementia prevention effects of anthocyanins have been reported [7,8]. Anthocyanins inhibit angiogenesis, prevent heart disease, reduce the risk of stroke, and have anti-inflammatory and antiseptic effects [9].

Here we observed that berry extracts protect neuronal cells from hydrogen peroxide-induced damage. These results demonstrate that inhibition of apoptosis-inducing toxic substances in neuronal cells with berry extracts may be a useful approach for disease-specific therapy.

2. Materials and Methods

2.1. Materials

Boysenberry, black currant, and blueberry (*Rubus ursinus* × *Rubus idaeus*, *Ribes nigrum L.*, and *Vaccinium spp.*) native to America and New Zealand were imported from Wooyang Frozen Food Co., Ltd (Chungnam, Korea). Folin & Ciocalteu's phenol reagent, 2',7'-dichlorofluorescein diacetate (DCF-DA), hydrogen peroxide solution, 3-[4,5-dimethy-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay kit, and LDH release assay kit were obtained from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA).

2.2. Determination of Total Phenolic Compound Concentration

The phenolic compound concentration of extracts from boysenberry, black currant, and blueberry were determined using a protocol similar to that of the Folin-ciocalteu assay [10]. Briefly, 0.1 mL of extracts from boysenberry, black currant, and blueberry were mixed with 50 µL of 50% Folin-Ciocalteau reagent and 150 µL of 20% sodium carbonate (Na_2CO_3). After 30 min at room temperature, the absorbance of the solution was measured at 750 nm using a spectrophotometer (SECOMAM, Ales, France). The concentration of polyphenols was calculated using a standard curve generated using gallic acid. The results were expressed as mg of gallic acid equivalents (mg GAE/g fruit juice).

2.3. Cell Culture

PC-12 (KCLB 21721) cells, the standard model for neuronal function studies, were obtained from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen Corporation (Carlsbad, CA, USA). PC-12 cells were cultured and maintained in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. All the treatments were performed at 30% confluence.

2.4. Lipid Peroxidation Assay

PC-12 cells (2.0×10^5 cells) were first incubated with 31, 62, 125, and 250 µg/mL concentrations of extracts from boysenberry, black currant, and blueberry for 1 h, and then incubated with 1.0 mM hydrogen peroxide for 24 h. We mixed 200 µL of each supernatant with 400 µL of trichloroacetic acid solution and then boiled it at 95°C for 30 min. The absorbance was measured at 532 nm. MDA levels were determined using a colorimetric method with malondialdehyde tetrabutyl-ammonium as the standard. Results are given as µM concentration [11].

2.5. Oxidative Stress Assay

Oxidative stress was measured using the 2',7'–dichlorofluorescein diacetate (DCF-DA) assay. PC-12 cells (1.0×10^4 cells/well) were treated at 37°C for 48 h with a range of fruit extract concentrations. After incubation, the cells were washed three times and then incubated with 200 µM hydrogen peroxide. We added 10 µM DCF-DA to each well, following incubation for 45 min at 37°C. After adding hydrogen peroxide at a concentration of 200 µM, the fluorescence of each sample was monitored for up to 60 min at excitation and emission wavelengths of 485 nm and 538 nm, respectively [7].

2.6. MTT Assay

Cytotoxicity was determined using the MTT assay. In brief, 1.5×10^4 PC-12 cells were seeded in 96-well microtiter plates in complete medium (Dulbecco's Modified Eagle's medium with 10% fetal bovine serum). After incubation for 24 h in a humidified 5% (vol/vol) CO^2/air environment at 37°C, cells were treated with various concentrations (0, 25, 50, 100, and 200 µg/mL) of berry extract. Cells were then treated with 200 µM hydrogen peroxide for 3 h. The culture medium was aspirated and 200 µL of MTT dye solution (150 µL) was added to each well. After incubation for 4 h, the medium was aspirated and the crystals were dissolved with dimethyl sulfoxide. The absorbance at 540 nm was measured using a microplate reader [12].

2.7. Neuro-Protection from Oxidative Stress

Release of LDH (indicating loss of plasma membrane integrity [13]) was measured using an LDH detection kit according to the manufacturer's instructions (Sigma, MO, USA). PC-12 cells were pelleted by centrifugation (250 × *g*) for 5 min at RT, 100 µL of the supernatants were transferred into new wells, and LDH was determined using the *in vitro* toxicology assay kit.

2.8. Statistical Analysis

Statistical analyses were performed 3 times for all the experiments. The data are expressed as the mean ± one standard error of mean (SEM). Statistical analyses were assessed by Student's *t*-test for paired data. Graph Pad Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA) was used. Significant differences ($p < 0.05$) between the mean values of the triplicate samples were determined for various assays.

3. Results

The total polyphenols contents in berries were determined using regression equations from calibration curves and expressed in gallic acid equivalents (GAE). The polyphenol content of boysenberry, black currant, and blueberry extracts were 392, 344, and 164 mg GAE per 100 g respectively (data not shown). In addition, berry extracts significantly reduced MDA levels in PC-12 cells compared to those in the control group (Figure 1A). Among the three kinds of berries tested, the highest lipid peroxidation inhibition was observed with black currant extract, which inhibited activity by 65% at a concentration of 200 µg/mL. Black currant had a similar inhibitory effect on lipid peroxidation to that of vitamin C, which was used as a positive control. In addition, intracellular ROS level was measured using an oxidation-sensitive, fluorescent probe, DCF-DA. As shown in Figure 1B, hydrogen peroxide-treated cells displayed increased DCF

fluorescence compared to those in the control group. However, the generation of ROS was inhibited by extracts from boysenberry, black currant, and blueberry fruit. These results are indicative that accumulated ROS is an important mediator of hydrogen peroxide-induced apoptosis. PC-12 cells were treated with various concentrations of extracts from boysenberry, black currant, and blueberry fruit, with or without hydrogen peroxide (200 µg/mL) to determine effects on cytotoxicity. Cytotoxicity was assessed using the MTT assay in triplicate. Extracts from boysenberry, black currant, and blueberry fruit did not induce significant cytotoxicity, and vitamin C treatment alone did not affect cytotoxicity (Figure 2A). Boysenberry, black currant, and blueberry extracts protected PC-12 cells from hydrogen peroxide-induced cell death in a dose-dependent manner. These fruit extracts also significantly inhibited hydrogen peroxide-induced LDH release (Figure 2B).

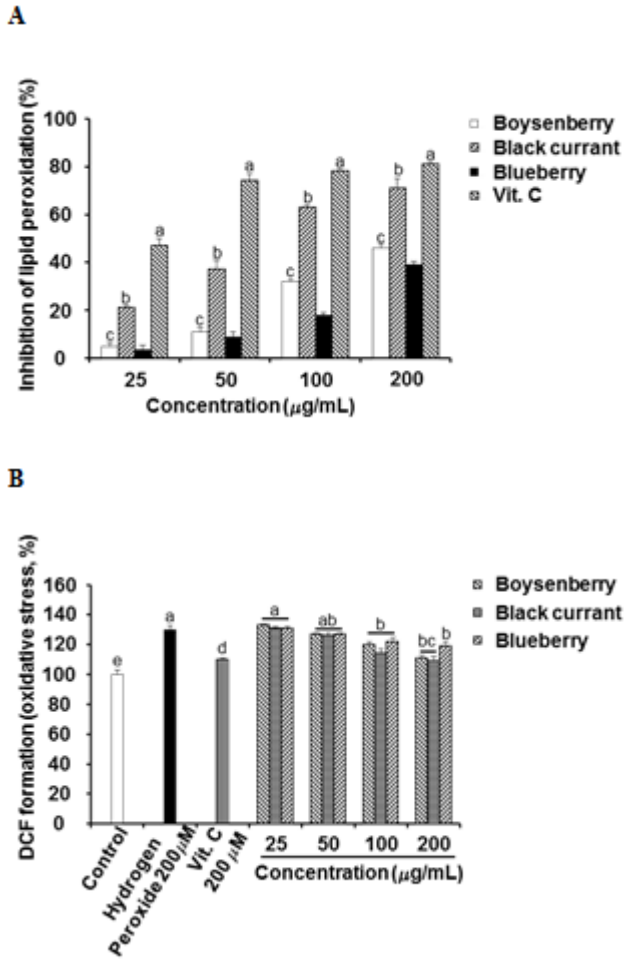

Figure 1. Antioxidant activity of boysenberry, black currant, and blueberry extracts. Malondialdehyde assay for both ferric ion- and ascorbic acid-induced lipid peroxidation (A). Effects of boysenberry, black currant, and blueberry extracts on ROS production, determined in the presence and absence of hydrogen peroxide in PC-12 cells (B). [a-d] Significantly different at the 0.05 level by Duncan's multiple range test

4. Discussion

The observed protective effects of the berry extracts against hydrogen peroxide-induced damage in rat neuronal cells is most likely due to the antioxidant properties of its constituents, mainly polyphenols [4]. Polyphenols are secondary metabolites present in vegetal tissues, flowers, and fruits. Polyphenols include a wide variety of molecules containing at least one aromatic ring with one or more hydroxyl groups. Many polyphenols are known to exist in plants. They are important in the human diet as antioxidants. The potential protective role of polyphenols in ingested fruits against oxidative damage diseases, such as neuronal disease, heart disease, liver disease, and cancer has been a recent research focus [7].

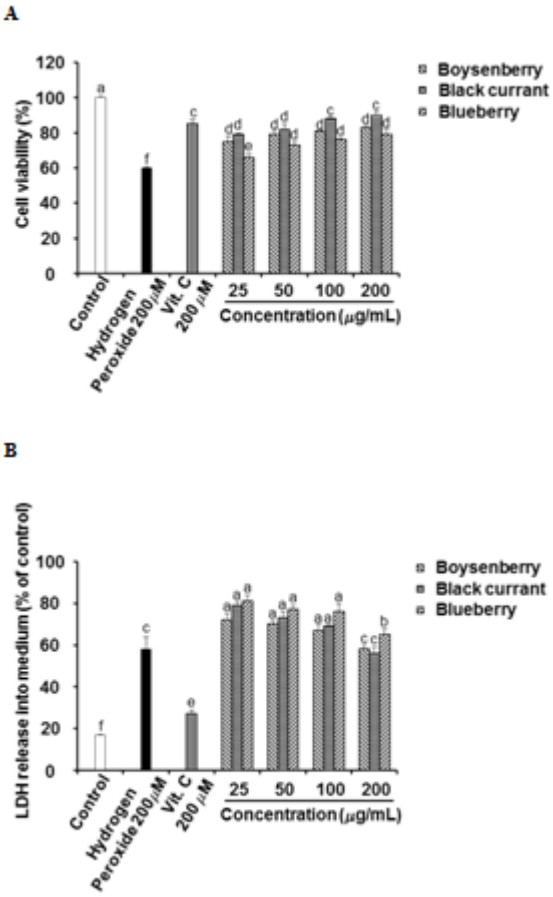

Figure 2. Neuro-protective effect of boysenberry, black currant, and blueberry extracts. Hydrogen peroxide-induced cytotoxicity (A) and membrane damage (B) in PC-12 cells. [a-f] Significantly different at the 0.05 level by Duncan's multiple range test

Of the many biomolecules that can undergo fatal oxidative damage in the presence of ROS, membrane lipids are especially sensitive to oxidation by this physiological process [14]. For this reason, we used brain homogenates for the investigation of oxidative stress-induced lipid peroxidation [15]. ROS are by-products of normal cellular metabolism of molecular oxygen. Previous studies indicate that neurodegenerative disorders, such as Alzheimer's, Huntington's, and Parkinson's Diseases can be caused by ROS [1,3]. Hydroxyl radicals are capable of more damage to biological systems than any other ROS because of strong reactivity with biomolecules [16].

Increased plasma LDH activity may be due to apoptosis or necrosis leading to leakage of the enzyme into the blood stream [17,18]. Thus, berry extracts may impart protection against hydrogen peroxide-induced oxidative injury, which may result in organ damage.

In another study, berry extract was shown to have strong antioxidant and anticancer activities [19,20,21,22]. Here we confirm these reported *in vitro* effects using a

different cell-based system and the extracts of three kinds of berries containing phenolic compounds. Berry extracts significantly inhibited hydrogen peroxide-induced oxidative damage. *Corema album* berry and miracle berry had a similar total phenolic content (121.4 mg GAE/100 g FW and 1448.3 mg GAE/100 g fresh weight (FW), respectively) as in other reported berries in this study, such as blackberry (435.0 mg GAE/100 g FW) (Du et al., 2014). In addition, our results are in accordance with previous reports that raspberry, bilberry, lingon berry, and black currant inhibited lipid oxidation [23].

The phytochemical milieu of berry extracts is rich in bioactive polyphenolic compounds that may play an important role in human health and nutrition.

5. Conclusions

Here we demonstrate that extracts of boysenberry, black currant, and blueberry fruit are neuro-protective *in vitro*. These extracts were analyzed for total polyphenol content. Subsequently, these extracts were found to have strong antioxidant, lipid peroxidation inhibitory, and membrane integrity maintenance effects on PC-12 cells. However, there is currently no evidence that polyphenols would significantly improve the antioxidant status of an organism after berry consumption. Therefore, it is important to investigate the inhibition of oxidative damage by various berries in *in vivo* systems.

Acknowledgments

This study was supported by agenda project (Grant No. PJ009376012014) of the Rural Development Administration, Republic of Korea.

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