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Sea Buckthorn (*Hippophae rhamnoides* L.) Leaf Extracts Protect Neuronal PC-12 Cells from Oxidative Stress

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The present study was carried out to investigate the antioxidative and neuroprotective effects of sea buckthorn (*Hippophae rhamnoides* L.) leaves (SBL) harvested at different times. Reversed-phase high-performance liquid chromatography analysis revealed five major phenolic compounds: ellagic acid, gallic acid, isorhamnetin, kaempferol, and quercetin. SBL harvested in August had the highest total phenolic and flavonoid contents and antioxidant capacity. Treatment of neuronal PC-12 cells with the ethyl acetate fraction of SBL harvested in August increased their viability and membrane integrity and reduced intracellular oxidative stress in a dose-dependent manner. The relative populations of both early and late apoptotic PC-12 cells were decreased by treatment with the SBL ethyl acetate fraction, based on flow cytometry analysis using annexin V-FITC/PI staining. These findings suggest that SBL can serve as a good source of antioxidants and medicinal agents that attenuate oxidative stress.

Keywords: Antioxidant capacity, apoptosis, flow cytometry, high-performance liquid chromatography, total phenolics, total flavonoids

Introduction

Hippophae rhamnoides ssp. *turkestanica* L. (family Elaeagnaceae), commonly known as sea buckthorn, is a thorny, nitrogen-fixing, deciduous shrub widely distributed throughout the temperate zones of Asia and Europe, and throughout subtropical zones, especially at high altitude [1, 2]. Sea buckthorn blooms in April, and its fruits are usually collected through August to October. Fruit ripening occurs approximately 100 days after pollination. Sea buckthorn is used extensively in oriental traditional medicines to treat cough, skin diseases, gastric ulcers, asthma, and lung disorders [2]. All parts of this plant are considered to be rich source of a large number of bioactive substances [2]. Sea buckthorn contains bioactive compounds, including vitamin C, minerals, monosaccharides, organic acids, carotenoids, vitamin E, tannins, and flavonoids such as quercetin, myricetin, and kaempferol [2, 3].

An imbalance between the generation of free radicals and antioxidant defenses to detoxify them leads to oxidative stress in the body. To prevent cellular damage from oxidative stress, enhancement of the endogenous oxidative defense system can be achieved via dietary or pharmacological consumption of antioxidants [4]. Natural antioxidants have been reported to prevent oxidation of proteins and peroxidation of lipids from oxidative stress induced by reactive oxygen species (ROS) generation [5]. Many studies have reported that antioxidants such as polyphenols possess anti-inflammatory, anti-neurodegenerative, anti-mutagenic, anti-carcinogenic, and anti-diabetic effects [6, 7]. Fruits, leaves, and seeds of sea buckthorn have been reported to have a variety of biological and therapeutic properties, including anti-atherogenic [8], anti-inflammatory [9], anti-ulcerogenic [10], hepatoprotective [11], tissue

regenerative [12], and immunomodulatory [13, 14] properties. An extract of sea buckthorn leaves (SBL) exhibited potent antioxidant activity [15, 16]. Alcoholic SBL extract inhibited the cytotoxic effect of the oxidative stress caused by hypoxic exposure in C-6 glioma cells [17].

Oxidative stress is associated with the pathogenesis of neurodegenerative diseases such as Alzheimer's diseases, in particular excitotoxicity and apoptosis, the two main causes of neuronal cell death. Many studies have shown increased oxidation of proteins, DNA, and lipids by ROS in the brains of Alzheimer's disease patients [18, 19]. Furthermore, exogenous hydrogen peroxide can elevate oxidative stress beyond the protective capacity of endogenous antioxidant defenses, and induce apoptosis and necrosis in cultured cortical neurons [20, 21]. Therefore, use of effective antioxidants might be a potential strategy to suppress oxidative stress in the body.

Like green tea leaves, SBL can be utilized in consumer products such as cosmetic ingredients, food additives, and beverages. The time at which SBL are harvested should be taken into account to obtain SBL with higher amounts of antioxidants, because the maturity of leaves is related to changes in antioxidant profiles. Antioxidative phenolic profiles of SBL harvested at different times have not yet been determined. Limited information is available on the protective effects of bioactive compounds in SBL on neuronal cells. Therefore, our aims in the present study were to evaluate the total phenolic and flavonoid contents as well as antioxidant capacity of SBL harvested at different times and to quantitatively identify the major phenolics of SBL. Furthermore, neuronal PC-12 cells were used to investigate the neuroprotective effects of SBL against hydrogen peroxide (H_2O_2)-induced oxidative stress.

Materials and Methods

Sample

Leaves of sea buckthorn were collected from Ganghwa Island, Republic of Korea in June, July, and August 2010 and then dried in the shade. The dried leaves were powdered in an electric grinder. The ground leaves were sealed and stored in the refrigerator at 4°C.

Reagents

Folin-Ciocalteu's phenol reagent, gallic acid, ABTS, ascorbic acid, DPPH, aluminum chloride, catechin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), H_2O_2, an in vitro lactate dehydrogenase toxicology assay kit, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), ellagic acid, quercetin, kaempferol, and isorhamnetin were purchased from Sigma-Aldrich Co., LLC (USA). 2,2′-Azobis(2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Pure Chemical Industries, Ltd. (Japan). Dulbecco's phosphate-buffered saline (DPBS), Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), and penicillin/streptomycin were purchased from Welgene Inc. (Korea). The BD annexin V:FITC apoptosis detection kit I was purchased from BD Biosciences (USA). All other reagents used were of analytical grade.

Extraction and Fractionation

Phenolics from SBL were extracted in 80% (v/v) aqueous methanol. SBL were homogenized using a Polytron homogenizer (PT 10/35; Kinematica, Switzerland) at 15,000 rpm for 2 min in 100 ml of 80% (v/v) aqueous methanol. The homogenized mixture was further sonicated for 20 min with continual N_2 purging. The mixture was filtered through Whatman #2 filter paper (Whatman International Limited, UK) using a chilled Büchner funnel and rinsing with 50 ml of absolute methanol. The remaining residue in the funnel was re-extracted using the procedure described above. The filtrates were combined and evaporated to dryness under reduced pressure using a rotary evaporator (Eyela, Japan) in a water bath at 37°C. Extraction was performed in three independent replications.

The solid obtained after evaporation was dissolved in 1 L of distilled water, which was subjected to liquid–liquid extraction with the same volume of each of the following four organic solvents in sequence: *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The SBL fractions were evaporated to dryness and kept at −20°C until analysis.

Determination of Total Phenolic Content

The total phenolic content of SBL extracts was measured with a colorimetric assay using Folin-Ciocalteu's phenol reagent [22]. Briefly, 0.2 ml of appropriately diluted extract was mixed with 2.6 ml of deionized water, and then 0.2 ml of Folin-Ciocalteu's phenol reagent was added to the mixture. At 6 min after the addition of the reagents, 2 ml of 7% (w/v) Na_2CO_3 solution was added. The absorbance of the mixture was measured at 750 nm at 90 min after incubation. The total phenolic content in SBL extracts was expressed as g gallic acid equivalents (GAE)/100 g dry weight (DW). Three replicate extractions were analyzed.

Determination of Total Flavonoid Content

Levels of total flavonoids in SBL extracts were measured using a colorimetric assay [22]. Five hundred microliters of the extract was mixed with 3.2 ml of deionized water, and 150 µl of 5% (w/v) $NaNO_2$ was added to the mixture. At 5 min, 150 µl of 10% (w/v) $AlCl_3$ was added. At 6 min, 1 ml of 1 M NaOH was added. The absorbance of the mixture was measured immediately at 510 nm. The total flavonoid content of SBL extracts was expressed as g catechin equivalents (CE)/100 g DW. Triplicate extracts were analyzed.

Determination of Antioxidant Capacity Using ABTS Radicals

The antioxidant capacity of SBL extracts was measured using a

solution containing ABTS radicals [23]. This solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. The reaction between ABTS radicals and appropriately diluted samples was performed at 37°C for 10 min. The antioxidant capacity of SBL extracts was expressed as g vitamin C equivalents (VCE)/100 g DW.

Determination of Antioxidant Capacity Using DPPH Radicals

The antioxidant capacity of SBL extracts was also determined using DPPH radical chromogens [23]. The absorbance of DPPH radicals in 80% (v/v) aqueous methanol was set at 0.650 ± 0.020 at 517 nm. DPPH radicals and appropriately diluted samples were reacted at 23°C for 30 min. The antioxidant capacity of SBL extracts was expressed as g VCE/100 g DW.

Quantification of Phenolics Using HPLC

SBL ethyl acetate fractions were refluxed under 1 N HCl in a water bath at 90°C for 30 min. Phenolics in the acid-hydrolyzed SBL ethyl acetate fractions were analyzed using an HPLC system (Agilent 1100 series; Agilent Technologies, Inc., USA) equipped with autosampler, vacuum degasser, quaternary pump, and photodiode array detector. Chromatographic separation was performed using a C18 reversed-phase analytical column (Zorbax Eclipse XDB-C18, 4.6 × 250 mm, 5 μm; Agilent Technologies, Inc.). The injection volume was 20 μl. The flow rate was kept at 1.0 ml/min. The detector was set at 280, 320, and 370 nm. The solvent gradient compositions of binary mobile phases (solvent A, 0.1% (v/v) formic acid in distilled water; solvent B, 0.1% (v/v) formic acid in acetonitrile) were as follows: 92% A/8% B at 0 min, 89% A/11% B at 5 min, 85% A/15% B at 15 min, 80% A/20% B at 35 min, 60% A/40% B at 55 min, 40% A/60% B at 85 min, 92% A/8% B at 85.1 min, and 92% A/8% B at 100 min. Phenolic compounds of SBL ethyl acetate fractions were tentatively identified by comparing UV-visible spectra, retention times, and spike-input of authentic standards.

Cell Culture

PC-12 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C.

Protective Effects of the SBL Ethyl Acetate Fraction on PC-12 Cells

The cytotoxicity and cell viability of the SBL ethyl acetate fraction were determined using an MTT reduction assay. PC-12 cells at a density of 2×10^4 cells per well in 96-well plates were incubated for 3 h. After removal of the medium, the PC-12 cells were treated with serum-free medium containing various concentrations of SBL ethyl acetate fraction. After 24 h, the PC-12 cells were treated with 150 μM H_2O_2 for 1 h, and then with 0.5 mg/ml MTT for 3 h. The resulting formazan product was dissolved by the addition of DMSO. The amount of MTT formazan was determined by measuring the absorbance using a microplate reader (Infinite M200, Tecan Austria GmbH, Austria) at 570 and 630 nm. The cytotoxicity and viability were expressed as the percentage (%) of viable cells relative to control cells (100%) that were not treated with stress and test samples.

Measurement of Lactate Dehydrogenase (LDH) Release

PC-12 cells at a density of 2×10^4 cells per well in 96-well plates were incubated for 3 h. After removal of the medium, the PC-12 cells were treated with serum-free medium containing various concentrations of SBL ethyl acetate fraction. After 24 h, the PC-12 cells were treated with 150 μM H_2O_2 for 1 h. An in vitro toxicology assay kit was used for the LDH assay. Damage of the plasma membrane was evaluated by measuring the amount of the intracellular enzyme LDH released into the medium. The absorbance was measured using test and reference wavelengths of 490 and 690 nm, respectively.

Measurement of Intracellular Oxidative Stress

Levels of intracellular oxidative stress were evaluated using the fluorescent probes DCFH-DA and DHE. PC-12 cells at 2×10^4 cells per well in 96-well plates with 100 μl of RPMI-1640 medium containing FBS were incubated for 24 h. The cells were treated with various concentrations of the SBL ethyl acetate fractions for 24 h. The cells were incubated with 50 μM of DCFH-DA or 5 μM of DHE in HBSS for 30 min and treated with 100 μM H_2O_2 in HBSS for 1 h. The fluorescence was measured using a microplate reader (Infinite M200; Tecan Austria GmbH). DCFH-DA was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, and DHE was measured at an excitation wavelength of 480 nm and an emission wavelength of 610 nm. The ROS production level was expressed as the percentage (%) decrease in fluorescence intensity of the control (not exposed to stress and test samples).

Analysis of Apoptotic Cells Using Flow Cytometry

Apoptotic PC-12 cells were analyzed using a flow cytometry system (BD FACS Aria 2; BD Corporation, USA). After treatment of cells with the SBL ethyl acetate fraction for 24 h, apoptosis of the PC-12 cells was induced by adding H_2O_2 for 24 h. The PC-12 cells were washed with cold PBS and then resuspended in binding buffer and stained with annexin V/propidium iodide (PI) staining solution for 15 min. Apoptotic cells were quantitated using a flow cytometry system with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. FL1 represents annexin V fluorescence, whereas FL2 shows PI fluorescence. At least 10,000 events were recorded. Apoptotic cells were expressed as a percentage of the total cell number.

Statistical Analysis

All experiments were performed in triplicates. Statistical analyses were performed using SAS software (ver. 8.2; SAS Institute, Inc., USA). One-way analysis of variance was performed to evaluate differences between average values. Significant differences were verified by Duncan's multiple-range test at the $p < 0.05$ confidence level.

Results and Discussion

Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid contents of SBL extracts are shown in Table 1. The content (g GAE/100 g DW) of total phenolics in leaves varied according to harvest season as follows: August (11.67) > July (9.93) > June (8.62). SBL harvested in August had a significantly ($p < 0.05$) higher total phenolic content than the other SBL harvests tested in this study. As SBL increased in maturity from June to August, so did the total phenolic content. In contrast to our findings, a previous study reported that the total phenolic content of sea buckthorn berry decreased as the harvesting time was delayed [24]. It has also been reported that the content of total phenolics decreases with increasing maturation in acerola fruit [25]. Similar to our findings in this study, the subcritical water extract of SBL had a total phenolic content of 7.6−9.4 g GAE/100 g DW [16]. However, aqueous and hydroalcoholic extracts of SBL had total phenolic contents of approximately 4.05 and 5.63 g GAE/100 g DW, respectively [15], which were lower than those found in the current study.

Contents (g CE/100 g DW) of total flavonoids decreased in the following order: August (2.18) > July (1.70) > June (1.60). SBL harvested in August had a significantly ($p < 0.05$) higher total flavonoid content than the other SBL harvests tested (Table 1). A previous study reported that the subcritical water extract of SBL had a total flavonoid content of 4.7−6.6 g rutin equivalents (RE)/100 g DW [16]. It has been reported that total flavonoid contents of SBL aqueous and hydroalcoholic extracts were approximately 1.49 and 2.08 g RE/100 g DW, respectively [15].

Antioxidant Capacity

Two different methods were used to measure the antioxidant capacity (expressed as VCE) of SBL. ABTS radical scavenging assays of SBL harvested at different times (June to August) revealed that SBL harvested in August had the highest antioxidant capacity at 32.30 g VCE/100 g DW, whereas SBL harvested in June had the lowest at 22.70 g VCE/100 g DW (Table 1). The antioxidant capacity measured using ABTS radicals increased as the harvest period was delayed from June to August. The antioxidant capacity of SBL tested in this study differed significantly ($p < 0.05$) among harvest times. A previous study reported that the antioxidant activity of SBL was 10.9 and 14.3 g Trolox equivalents (TE)/100 g DW for aqueous and hydroalcoholic extracts, respectively, using the ABTS assay [15].

Similar to results of the ABTS assay, SBL harvested in August had the highest antioxidant capacity at 20.60 g VCE/100 g DW according to the DPPH assay. The antioxidant capacity measured in the DPPH assays was significantly different ($p < 0.05$) among SBL harvested at different times. SBL harvested in August had the highest total phenolic and total flavonoid contents as well as antioxidant capacity among the SBL tested in this study. Therefore, the subsequent experiments were performed using SBL harvested in August. It was previously reported that the aqueous and hydroalcoholic extracts of SBL had 12.0 and 16.7 g TE/100 g DW, respectively [15], using the DPPH assay. A previous study also reported that the subcritical water extract of SBL had an antioxidant capacity of 19.5−35.3 g RE/100 g DW measured using the DPPH assay [16].

Quantification of Phenolics in the SBL Ethyl Acetate Fraction Using HPLC

The concentrations of five compounds (kaempferol, quercetin, isorhamnetin, ellagic acid, and gallic acid) in SBL harvested at different times are shown in Table 2. SBL harvested in August had the highest amounts of all five phenolics compared with SBL harvested in June and July. The concentrations of five compounds quantified using HPLC were found to increase as the harvesting time was delayed from June to August. The sum (mg/100 g DW) of

Table 1. Total phenolic and flavonoid contents as well as antioxidant capacity of sea buckthorn leaves harvested at different times.

Harvest time	Total phenolics (g GAE[1]/100 g DW[2])	Total flavonoids (g CE[3]/100 g DW)	Antioxidant capacity (g VCE[4]/100 g DW)	
			ABTS	DPPH
June	8.62 ± 0.41[5b6]	1.60 ± 0.14[b]	22.70 ± 2.56[b]	15.55 ± 0.57[c]
July	9.93 ± 0.78[b]	1.70 ± 0.23[b]	25.75 ± 2.11[b]	17.67 ± 0.96[b]
August	11.67 ± 0.83[a]	2.18 ± 0.10[a]	32.30 ± 2.62[a]	20.60 ± 0.98[a]

[1-4]GAE, DW, CE, and VCE stand for gallic acid equivalents, dry weight, catechin equivalents, and vitamin C equivalents, respectively.

[5]Data are presented as the mean ± standard deviation ($n = 3$).

[6]Means with different superscripts in the same column are significantly different by Duncan's multiple-range test ($p < 0.05$).

Table 2. Concentrations (mg/100 g of dry weight) of phenolic compounds of sea buckthorn leaves harvested at different times.

Harvest time	Phenolic compound					
	Ellagic acid	Gallic acid	Isorhamnetin	Kaempferol	Quercetin	Sum
June	494.1 ± 2.3[1c2]	330.7 ± 2.7[b]	29.2 ± 1.9[c]	109.8 ± 8.2[b]	28.9 ± 1.3[b]	992.7 ± 10.2[c]
July	532.7 ± 1.5[b]	299.4 ± 0.7[c]	35.1 ± 2.1[b]	129.8 ± 10.2[a]	19.6 ± 0.9[c]	1,016.6 ± 13.0[b]
August	672.2 ± 0.9[a]	372.5 ± 0.9[a]	61.7 ± 1.5[a]	141.9 ± 3.1[a]	53.6 ± 2.3[a]	1,301.9 ± 6.1[a]

[1]Data are presented as the mean ± standard deviation (n = 3).

[2]Means with different superscripts in the same column are significantly different by Duncan's multiple-range test ($p < 0.05$).

the amounts of the five phenolic compounds (kaempferol, quercetin, isorhamnetin, ellagic acid, and gallic acid) in SBL decreased as follows: August (1,301.9) > July (1,016.6) > June (992.7). The contents of quercetin and isorhamnetin in SBL harvested in August were approximately two times higher than those from leaves harvested in June (Table 2). SBL from the hilly regions of the North-West Himalayas were found to have flavonol content (isorhamnetin, kaempferol, and two quercetin glycosides) of approximately 156.9 and 141.5 mg/100 g DW for aqueous and hydroalcoholic extracts, respectively [15]. Another study reported that the sum of flavonols (isorhamnetin, kaempferol, and quercetin-3-glucoside) found in SBL extracted with subcritical water ranged from 21.5 to 58.2 mg/100 g DW [16].

The contents of physiologically active substances such as kaempferol, quercetin, isorhamnetin, and vitamins A, C, and E in berries and leaves of sea buckthorn are known to be affected by maturity, harvest season, cultivar, cultivation environment, and location [24, 26–29]. Similar to our HPLC results, SBL has been reported to contain various flavonoids, including quercetin, kaempferol, and isorhamnetin [26, 30]. The HPLC results showing the highest amounts of all five phenolics in SBL harvested in August were consistent with those observed for total phenolic and flavonoid contents, and antioxidant capacity in this study. These results suggest that harvest time should be taken into account when products containing SBL, such as teas, are manufactured. This strategy will lead to the development of good quality processed products containing more biologically active ingredients.

Neuroprotective Effects of the SBL Ethyl Acetate Fraction Harvested in August

The cytotoxicity of the SBL ethyl acetate fraction harvested in August on PC-12 cells was evaluated using the MTT assay. The ethyl acetate fraction was not considered cytotoxic when cell viability was greater than 80%. The SBL ethyl acetate fraction had no cytotoxicity up to 20 μg/ml (data not shown).

Neuroprotective effects of the SBL ethyl acetate fraction against H_2O_2-induced oxidative stress in PC-12 cells are shown in Fig. 1. When PC-12 cells were treated with H_2O_2 for 1 h, PC-12 cell viability decreased to 34.8% compared with control cells (100%) that were not treated with H_2O_2. Pretreatment of cells with the SBL ethyl acetate fraction at concentrations of 5, 10, and 20 μg/ml for 24 h before exposure to H_2O_2 (150 μM) increased the viability of PC-12 cells in a dose-dependent manner (Fig. 1). The SBL ethyl acetate fraction increased cell viability due in part to blocking mitochondrial dysfunction against oxidative stress (Fig. 1). This is consistent with the results of a previous study that reported restoration of the mitochondrial transmembrane potential in C-6 glioma cells pretreated with SBL [17].

Effects of the SBL Ethyl Acetate Fraction Harvested in August on the Viability of PC-12 Cells

LDH is released into the external environment when

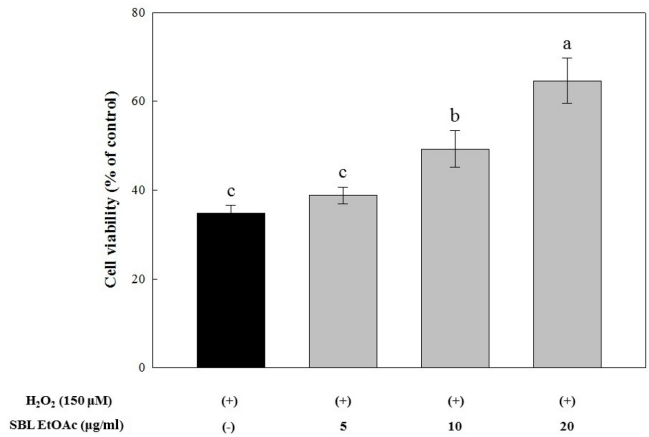

Fig. 1. Protective effects of the ethyl acetate fraction (EtOAc) of sea buckthorn leaves (SBL) harvested in August on the viability of PC-12 cells exposed to oxidative stress, based on the MTT reduction assay.

The data are displayed with mean ± standard deviation (bars) of three replicates. Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

cellular membranes are damaged by oxidative stress. Pretreatment of PC-12 cells with the SBL ethyl acetate fraction maintained the membrane integrity against H_2O_2-induced oxidative damage in a dose-dependent manner (Fig. 2). When PC-12 cells were exposed to H_2O_2 for 1 h, the LDH release level increased up to approximately 187.8% compared with the control group (100%). At an SBL ethyl acetate fraction concentration of 20 μg/ml, LDH release from PC-12 cells decreased up to 114.1% compared with the stress control (187.8%). These results suggest that phenolic compounds in SBL have the potential to protect neuronal cells from plasma membrane damage induced by oxidative stress, which might help treat neurodegenerative diseases such as Alzheimer's disease.

Effects of the SBL Ethyl Acetate Fraction Harvested in August on Intracellular Oxidative Stress in PC-12 Cells

Intracellular oxidative stress levels in PC-12 cells measured using the DCFH-DA and DHE assays are shown in Fig. 3. Exposure of neuronal PC-12 cells to oxidative stress resulted in an about 2-fold increase in ROS production in the DCFH-DA assay (Fig. 3A). Pretreatment of neuronal PC-12 cells with the SBL ethyl acetate fraction inhibited the intracellular oxidative stress production induced by 150 μM H_2O_2 in a dose-dependent manner (Fig. 3A). Oxidized DHE in cells generates a red fluorescent signal. H_2O_2-induced oxidative stress in PC-12 cells increased DHE staining by 143% compared with that in the control (Fig. 3B). The production of oxidized DHE by PC-12 cells decreased according to the concentration of the ethyl acetate fraction in a dose-dependent manner (Fig. 3B). At an SBL ethyl acetate fraction concentration of 20 μg/ml, the intracellular oxidative stress decreased to the control level (100%) in both the DCFH-DA and DHE assays (Fig. 3).

Consistent with the previous experimental findings, the SBL ethyl acetate fraction reduced intracellular oxidative stress production in a dose-dependent manner. The compounds kaempferol, quercetin, ellagic acid, and gallic acid, which are found in SBL, have been reported to have higher antioxidant capacity than ascorbic acid [31]. Isorhamnetin isolated from sea buckthorn marc showed

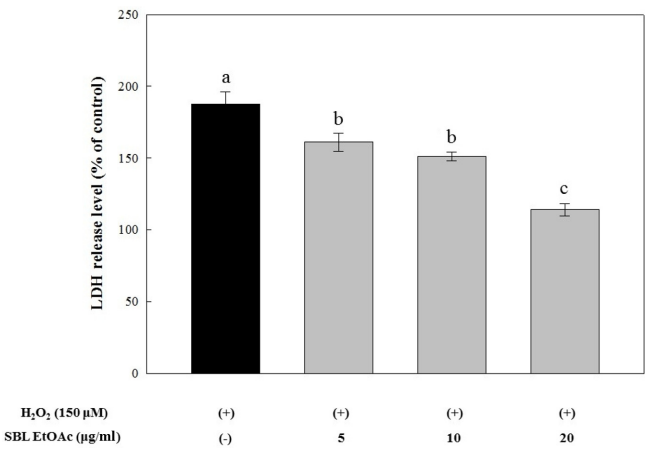

Fig. 2. Protective effects of the ethyl acetate fraction (EtOAc) of sea buckthorn leaves (SBL) harvested in August on the viability of PC-12 cells exposed to oxidative stress, based on the lactate dehydrogenase (LDH) release assay.

The data are displayed with mean ± standard deviation (bars) of three replicates. Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

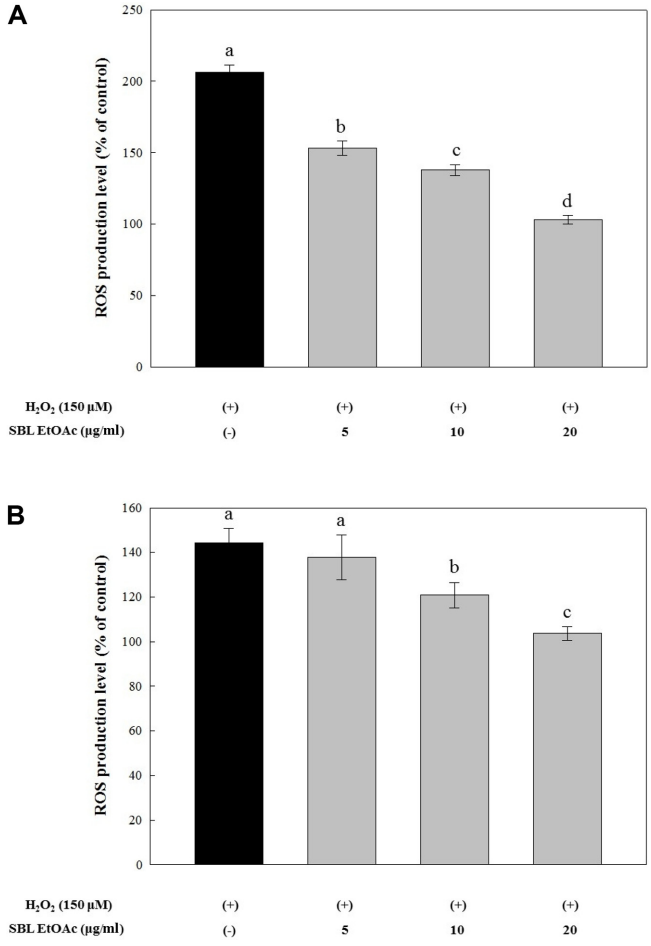

Fig. 3. Effects of the ethyl acetate fraction (EtOAc) of sea buckthorn leaves (SBL) harvested in August on intracellular oxidative stress in PC-12 cells exposed to oxidative stress, based on the DCFH-DA (**A**) and DHE (**B**) assays.

The data are displayed with mean ± standard deviation (bars) of three replicates. Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

DPPH radical-scavenging and iron-chelating activities and was suggested as an alternate antioxidant to synthetic antioxidants [32]. Flavonoids such as quercetin and kaempferol have been shown to reduce oxidative stress in cells, partly due to quenching generated ROS [33, 34]. The decreased intracellular oxidative stress that we observed in PC-12 cells might be associated with the antioxidant capacity of phenolic compounds such as ellagic acid, gallic acid, isorhamnetin, kaempferol, and quercetin found in SBL.

Effects of the SBL Ethyl Acetate Fraction Harvested in August on Apoptosis of PC-12 Cells

Quantitative measurement of apoptotic cells by flow cytometry was performed using the annexin V-FITC/PI

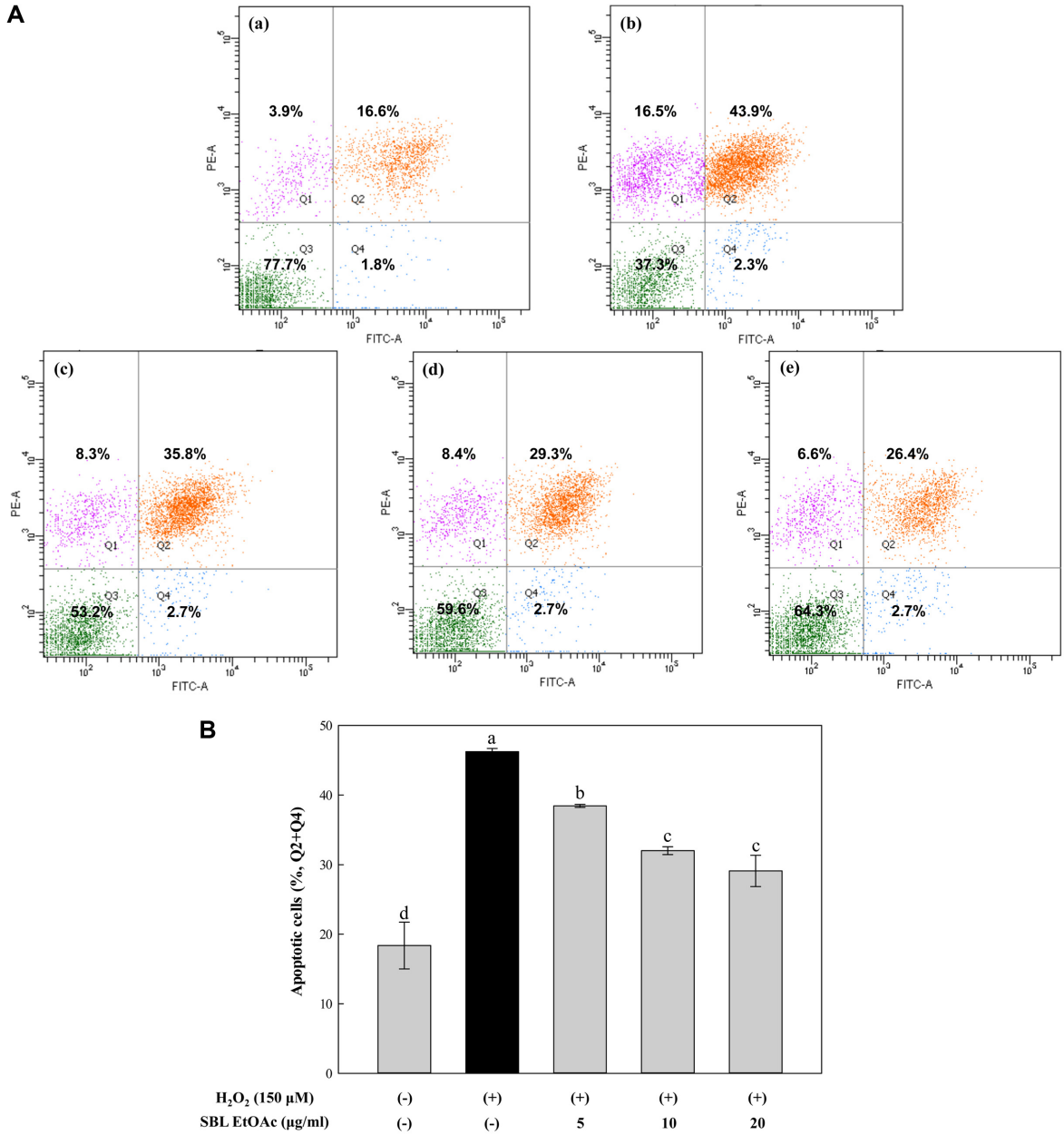

Fig. 4. Flow cytometric analysis of annexin V-FITC/propidium iodide double staining in PC-12 cells treated with the ethyl acetate fraction (EtOAc) of sea buckthorn leaves (SBL) harvested in August to assess apoptotic cells.

(**A**) Dot plots for flow cytometric analysis of apoptotic cells; (a) control, (b) H_2O_2 150 μM, (c) H_2O_2 150 μM + SBL EtOAc 5 μg/ml, (d) H_2O_2 150 μM + SBL EtOAc 10 μg/ml, and (e) H_2O_2 150 μM + SBL EtOAc 20 μg/ml. (**B**) Bar graph quantifying the percentage of apoptotic PC-12 cells. Values shown are the mean of three independent experiments.

detection kit (Fig. 4). Apoptotic cells stain with annexin V-FITC, whereas necrotic cells stain with PI. Annexin V-positive cells were assigned to the upper-right and lower-right quadrants, named Q2 and Q4, respectively. PC-12 cells in Q2 (annexin V-FITC positive/PI positive) corresponded to late-stage apoptotic cells, whereas those in Q4 (annexin V-FITC positive/PI negative) corresponded to early-stage apoptotic cells. Living cells without signs of apoptosis in the lower-left quadrant (Q3) were negative for both annexin V-FITC and PI staining.

Treatment of PC-12 cells with H_2O_2 resulted in a decrease in viable cells from 77.7% to 37.3% of double-negative cells (Fig. 4A). The population of early apoptotic cells following treatment with the SBL ethyl acetate fraction was not significantly different between the control and treatment groups (Fig. 4A), indicating that this fraction had no influence on the population of early apoptotic cells. Treatments with the SBL ethyl acetate fraction, however, decreased the relative proportions of late apoptotic cells from 43.9% to 26.4% (Fig. 4A). When PC-12 cells were treated with 150 μM of H_2O_2 for 24 h, the apoptotic cell ratio more than doubled to 46.2% compared with the H_2O_2-untreated control (18.4%; Fig. 4B). Pre-treatments of cells with the SBL ethyl acetate fraction at concentrations of 5, 10, and 20 μg/ml led to a 38.5%, 32.0%, and 29.1% decrease in apoptotic cells, respectively (Fig. 4B).

Quercetin is known to have neuroprotective effects via SIRT1 activation, which can protect PC-12 cells from apoptosis [35]. Kaempferol has been reported to prevent normal lung and liver cells from H_2O_2-induced apoptosis through caspase-3 activation [36]. Ellagic acid ameliorated the activation of caspase-3 under the nephrotoxicity induced by oxidative stress, partly due to its antioxidant and anti-apoptotic activities [37]. Gallic acid protected PC-12 cells against amyloid-β-induced apoptosis in a dose-dependent manner [38]. Gallic acid, however, was reported to induce neuronal cell death, related to apoptosis induced by H_2O_2 [39]. In this study, the significant decrease in the apoptotic neuronal cells indicated that the SBL ethyl acetate fraction effectively enhanced the viability and decreased the apoptosis of PC-12 cells in a dose-dependent manner (Fig. 4). These results suggest that the preventive effects of the SBL ethyl acetate fraction on PC-12 viability are associated with inhibition of apoptotic cell death.

In conclusion, pre-treatment of PC-12 cells with the SBL ethyl acetate fraction increased their viability and membrane integrity, partly due to a decrease in intracellular oxidative stress. The relative proportion of total apoptotic PC-12 cells was decreased by treatment with the SBL ethyl acetate fraction. The total phenolic and flavonoid contents as well as antioxidant capacity of SBL increased significantly ($p < 0.05$) as the harvest was delayed. Therefore, the optimal harvest time of SBL should be considered when manufacturing processed products, such as tea. SBL might serve as a good source of antioxidants that can potentially counteract the oxidative stress-induced mitochondrial dysfunction and apoptosis of neuronal cells.

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