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Sweet and Sour Cherry Phenolics and Their Protective Effects on Neuronal Cells

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The identification of phenolics from various cultivars of fresh sweet and sour cherries and their protective effects on neuronal cells were comparatively evaluated in this study. Phenolics in cherries of four sweet and four sour cultivars were extracted and analyzed for total phenolics, total anthocyanins, and their antineurodegenerative activities. Total phenolics in sweet and sour cherries per 100 g ranged from 92.1 to 146.8 and from 146.1 to 312.4 mg gallic acid equivalents, respectively. Total anthocyanins of sweet and sour cherries ranged from 30.2 to 76.6 and from 49.1 to 109.2 mg cyanidin 3-glucoside equivalents, respectively. High-performance liquid chromatography (HPLC) analysis revealed that anthocyanins such as cyanidin and peonidin derivatives were prevalent phenolics. Hydroxycinnamic acids consisted of neochlorogenic acid, chlorogenic acid, and *p*-coumaric acid derivatives. Glycosides of quercetin, kaempferol, and isorhamnetin were also found. Generally, sour cherries had higher concentrations of total phenolics than sweet cherries, due to a higher concentration of anthocyanins and hydroxycinnamic acids. A positive linear correlation ($r^2 = 0.985$) was revealed between the total anthocyanins measured by summation of individual peaks from HPLC analysis and the total anthocyanins measured by the pH differential method, indicating that there was in a close agreement with two quantifying methods for measuring anthocyanin contents. Cherry phenolics protected neuronal cells (PC 12) from cell-damaging oxidative stress in a dose-dependent manner mainly due to anthocyanins. Overall results showed that cherries are rich in phenolics, especially in anthocyanins, with a strong antineurodegenerative activity and that they can serve as a good source of biofunctional phytochemicals in our diet.

KEYWORDS: Anthocyanins; oxidative stress; PC 12 cells; reversed-phase HPLC; sweet and sour cherries; total phenolics

INTRODUCTION

Humans routinely consume a wide range of phenolics from fruits, vegetables, and cereals. Phenolics are a diverse group of aromatic compounds with at least one hydroxyl group, which include derivatives such as glycosides. Phenolics are important in determining the sensory quality of foods such as color, taste, and flavor. For instance, phenolics are involved in enzymatic browning reactions, which commonly take place in fresh and processed products of various fruits and vegetables (*1*, *2*) and are known to interact with proteins in foods (*3*). The composition and concentration of phenolics are significantly influenced by the stage of maturity, cultivars, cultural practices, geographic origin, growing season, climatic conditions, postharvest storage conditions, and food-processing procedures (*4*−*6*).

Many dietary phenolics are known to provide health-improving benefits due to their various biological activities. These possible health beneficial effects included antioxidant, antiallergic, anticarcinogenic, antimicrobial, antimutagenic, and antiinflammatory properties (*7*−*11*). Also, some phenolics protected neuronal cells from the oxidative stress induced by reactive oxygen species (ROS) or amyloid β protein, which may be related to the pathogenesis of Alzheimer's disease (*12*−*14*). Phenolics may have long-term human health benefits and may reduce the risk of some chronic diseases such as cancer and heart disease (*15*). A dietary intake of phenolics was epidemiologically found to have an inverse relationship with the risk of

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dementia, of which the most well-known type is Alzheimer's disease (*16*).

Cherries are known to have anthocyanins as the major phenolics (*17*). Cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside have been identified in sweet cherries (*18*−*20*). Sour cherries are known to contain various anthocyanins such as cyanidin 3-sophoroside, cyanidin 3-glucosylrutinoside, cyanidin 3-glucoside, and cyanidin 3-rutinoside (*19*, *21*). It was also reported that sour cherries had cyanidin 3-arabinosylrutinoside, pelargonidin 3-glucoside, and peonidin 3-rutinoside (*21*, *22*). Among hydroxycinnamates, sweet and sour cherries had neochlorogenic acid and *p*-coumaroylquinic acid as the predominant compounds (*4*, *19*, *20*). Flavonols and flavan-3-ols were also found in sweet and sour cherries, which included catechin, epicatechin, quercetin 3-glucoside, quercetin 3-rutinoside, and kaempferol 3-rutinoside (*4*, *19*, *20*).

Cherries are still under-researched and less utilized among fruits. Sweet cherries (*Prunus avium* L.) are mainly consumed as fresh fruit, whereas sour cherries (*Prunus cerasus* L.) are mostly used in processed products such as freezing, canning, and juices (*23*). Only a few studies have performed the comparative analysis for the quantification and identification of phenolics between sweet and sour cherries (*19*, *23*). Furthermore, there is little information available on the possible health benefits of dietary phenolics in fresh cherries on animal cells exposed to cell-damaging oxidative stress. The objectives in this study were to comparatively analyze dietary functional phenolics of fresh sweet and sour cherries and to investigate the in vitro effect of cherry phenolics on protection of neuronal PC 12 cells, derived from a transplantable rat pheochromocytoma, under cell-damaging oxidative stress.

MATERIALS AND METHODS

Cherry. Sweet and sour cherries were picked at commercial maturity during the 2004 harvest season at the New York State Agricultural Experiment Station orchards in Geneva, New York. Four sweet cherry cultivars were cv. Hartland, cv. Hedelfingen, cv. Regina, and cv. Black Gold. Four sour cherry cultivars included cv. Danube, cv. Balaton, cv. Schattenmorelle, and cv. Sumadinka. All eight cherry cultivars have relatively dark skins. Two sweet cherries (cv. Regina and cv. Black Gold) have relatively less-pigmented flesh, whereas all of the others have pretty dark flesh. Immediately upon arrival in the laboratory after harvest, fresh cherry samples were stored at 2−5 °C in a cold room.

Chemicals. Folin−Ciocalteu's phenol reagent, chlorogenic acid, quercetin, quercetin 3-galactoside, quercetin 3-rutinoside, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylformide, *p*-coumaric acid, sodium dodecyl sulfate, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Cyanidin, cyanidin 3-glucoside, cyanidin 3-rutinoside, isorhamnetin, isorhamnetin 3-rutinoside, kaempferol 3-rutinoside, peonidin 3-glucoside, and quercetin 3-glucoside were obtained from Extrasynthese (Genay, France). Cyanidin 3-glucosylrutinoside and peonidin 3-rutinoside were purchased from Polyphenols Laboratories (Sandnes, Norway). Ham's F12K medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). All other chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade.

Extraction. Phenolics were extracted from various sweet and sour cultivars of cherries using homogenization and sonication (*24*). Cherry seeds were removed by hand with care. Fresh cherries, 50 g, and 100 mL of absolute methanol were mixed in 250 mL round-bottomed flask under N_2 purging. The sample was homogenized in an ice bath using a Polytron homogenizer (PT 10/35, Kinematica, Kriens-Luzern, Switzerland) set at the level of 7 for 2 min. The homogenized sample was further sonicated for 20 min with continual N_2 purging. Whatman no. 2 filter paper (Whatman International Limited, Kent, England) was used to filter the sample using a chilled Büchner funnel with 50 mL of absolute methanol. For the re-extraction of the residue, 1 min of homogenization was done using 80% aqueous methanol and the same conditions were used for sonication and filtration steps. The two filtrates were transferred to a 1 L round-bottomed flask with an additional 50 mL of 80% aqueous methanol, which was evaporated using a rotary evaporator at 40 °C water bath until the volume of extract was reduced to 20−40 mL. The remaining phenolic extract was first dissolved in 50 mL of absolute methanol and then diluted to a 100 mL final volume using distilled deionized water (DDW). The phenolic extract was centrifuged at refrigerated temperature at 12000*g* for 20 min and then stored under nitrogen gas at −18 °C before analysis. Phenolic extraction for sweet and sour cherries was done in three replications.

Total Phenolics. Total phenolics of fresh sweet and sour cherries were measured by the spectrophotometric analysis (*25*). Briefly, an aliquot (0.2 mL) of appropriately diluted extracts was mixed with 2.6 mL of DDW. A reagent blank using 2.8 mL of DDW was prepared. At zero min, Folin−Ciocalteu's phenol reagent, 0.2 mL, was added to the mixture and then shaken. Two mL of 7% Na_2CO_3 solution was added after 6 min. After 90 min at 23 °C, the absorbance was read against the prepared blank at 750 nm. Total phenolics in various cherry cultivars were expressed as mg gallic acid equivalents (GAE)/100 g of fresh cherry. Each extract was analyzed in three replications.

Total Anthocyanins. The quantification of total anthocyanins of fresh cherries was evaluated by the pH differential method (*26*). Phenolic extracts of cherries in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) were measured simultaneously at 510 and 700 nm after 15 min of incubation at 23 °C. The content of total anthocyanins was expressed in mg cyanidin 3-glucoside equivalents (CGE)/100 g of fresh cherry. A molar absorptivity of 26900 L/mol cm was used for cyanidin 3-glucoside (molecular weight of 449.2 g/mol).

Fractionation of Phenolics Using C18 Sep-Pak Cartridge. For the easier separation of phenolics during HPLC analysis, a simple fractionation of cherry phenolic extracts was performed using preconditioned C18 Sep-Pak cartridges to separate anthocyanins from nonanthocyanin phenolics (*24*). Two C18 Sep-Pak cartridges were connected and preconditioned by sequentially eluting with 10 mL of ethyl acetate, 10 mL of absolute methanol, and 10 mL of 0.01 N aqueous HCl through the cartridges. A phenolic extract, 200 μL, was loaded onto the cartridges, which were washed with 6 mL of 0.01 N aqueous HCl to remove sugars, acids, and other water-soluble compounds. To dry cartridges, a current of nitrogen gas was allowed to pass through the connected C18 Sep-Pak cartridges for 10 min. Cartridges were rinsed with 20 mL of ethyl acetate to elute phenolic compounds other than anthocyanins, from which eluent was collected in a 50 mL test tube. The adsorbed anthocyanins were eluted from the cartridges with 10 mL of methanol with 0.1% (v/v) HCl, which also was collected into another 50 mL test tube. The solvents of the nonanthocyanin fraction and anthocyanin fraction were removed using a rotary evaporator under reduced pressure at 40 °C. Nonanthocyanin fractions and anthocyanin fractions were dissolved in 50% aqueous methanol and DDW, respectively, which was stored at −4 °C under nitrogen gas to prevent oxidation until HPLC analysis.

Acid Hydrolysis and Alkaline Digestion. Acid hydrolysis was performed after heating the manually collected sample of single peak containing 1.1 M HCl at 90 °C for 60 min, which resulted in the identification of aglycone of unknown anthocyanin and also confirmed the aglycones of kaempferol 3-rutionoside and isorhamnetin 3-rutinoside via HPLC analysis (*5*). Alkaline digestion of the sample having individual unknown hydroxycinnamic acid manually collected was done to detach the acyl substituent on phenolic acid (*27*). Digestion began to add 2 mL of 10% (w/v) KOH solution to 1 mL of sample of manually collected HPLC peak. The mixture was allowed to react at room temperature for 8 min and then was neutralized with 12 N HCl with care. The resulting samples hydrolyzed by acid and digested by alkaline were filtered with 0.45 μm PTFE and PVDF before its HPLC loading, respectively.

HPLC Analysis of Phenolics. HPLC analysis of cherry phenolics was performed using a reversed-phase HPLC system (Hewlett-Packard

Table 1. Total Phenolics and Total Anthocyanins in Sweet and Sour Cherries

cultivars	total phenolics[a]	total anthocyanins[b]
sweet Hartland	146.8 ± 19.3 b[c]	76.6 ± 12.2 c
sweet Hedelfingen	96.1 ± 20.1 a	40.3 ± 7.3 ab
sweet Regina	104.3 ± 6.6 a	40.7 ± 2.2 a
sweet Black Gold	92.1 ± 12.3 a	30.2 ± 8.7 a
sour Danube	161.7 ± 1.3 b	65.5 ± 3.0 c
sour Balaton	146.1 ± 20.8 b	49.1 ± 0.8 b
sour Schattenmorelle	295.5 ± 3.9 c	72.4 ± 5.8 c
sour Sumadinka	312.4 ± 8.5 d	109.2 ± 6.2 d

[a] Total phenolics are expressed as mg GAE/100 g of fresh cherry. [b] Total anthocyanins are expressed as mg CGE/100 g of fresh cherry. [c] Data are reported as means ± standard deviations with three replications. Means in a column followed by different letters are significantly different using analysis of variance at the level of $p < 0.05$.

model 1100; Palo Alto, CA) with a C18 reversed-phase Symmetry Analytical column (5 μm × 250 mm × 4.6 mm; Waters Corp., Milford, MA), a 20 μL sample loop, a photodiode array detector, a quaternary pump, and a vacuum degasser. Linear solvent gradient of binary mobile phases (solvent A, 0.1% H_3PO_4 in HPLC grade water; solvent B, 0.1% H_3PO_4 in HPLC grade acetonitrile) during HPLC analysis was applied as follows (total 60 min): 92% A/8% B at 0 min, 89% A/11% B at 4 min, 87% A/13% B at 25 min, 80% A/20% B at 27.5 min, 40% A/60% B at 50 min, 92% A/8% B at 55 min, and 92% A/8% B at 60 min (*5*). The flow rate was at 1.0 mL/min. The detector was set at 520 nm for anthocyanins, at 370 nm for flavonols, and 320 nm for hydroxycinnamic acids. UV−visible spectra of phenolics were simultaneously recorded from 200 to 600 nm during sample running. Cherry phenolics were tentatively identified with a spiked input of their authentic standards, in addition to comparisons of their UV−visible spectra and retention times.

For quantification of phenolics by HPLC analysis, the standard curves of individual phenolics that relate various concentrations of authentic standard solutions to the areas of their corresponding peaks were obtained. The concentration of each phenolic compound in various cherry cultivars was determined from the standard curves.

Cell Culture. The PC 12 cell line (ATCC, Manassas, VA) was derived from a transplantable rat pheochromocytoma. PC 12 cells were cultured in Ham's F12K medium containing 15% horse serum, 2.5% fetal bovine serum, 50 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 5% CO_2.

Determination of Cell Viability. PC 12 cells were plated at a density of 10^4 cells/well on 96 well plates in 100 μL of Ham's F12K. Cell viability was evaluated using two different in vitro assays such as MTT reduction and trypan blue exclusion assays. The cell viability was determined by the conventional MTT reduction assay (*28*). The cells were incubated with 0.25 mg MTT/mL (final concentration) for 2 h at 37 °C, and the reaction was stopped by adding solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a microplate reader (Bio-Rad, Hercules, CA) at 570 and 630 nm for test and reference wavelengths, respectively. The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Cells with damaged membranes appeared blue due to their accumulation of dye. The dye of 0.4% trypan blue was added to PC 12 cells. After 5 min, cells were loaded into a hematocytometer and counted for the dye uptake. The number of viable cells was calculated as a percentage of the total cell population.

RESULTS AND DISCUSSION

Total Phenolics. Total phenolics of four sweet cherry cultivars and four sour cherry cultivars are shown in **Table 1**. The total phenolics of sweet cherry cultivars were in a range from 92.1 to 146.8 mg GAE/100 g with an average of 109.8 mg. Sour cherry cultivars had the total phenolics of 146.1 to 312.4 mg with an average of 228.9 mg. Generally, sour cherries contained a higher level of total phenolics than sweet cherries. Sour cv. Sumadinka was found to have the highest total phenolics among the eight cherry cultivars tested, whereas sweet cv. Black Gold had the lowest. Sour cv. Sumadinka had about 3.4 times higher total phenolics than sweet cv. Black Gold. Total phenolics in sweet cherry grown in Spain was reported to be at about 99.9 mg (*17*), and sweet cherries in Portugal showed the total phenolics at 92.7−264 mg (*4*), which was similar to those of four sweet cherry cultivars in this study. Total phenolics of four different sour cherry cultivars harvested in 2003 from the same NY orchard was reported to be in a range of 254.1−398.5 mg (*5*).

Total Anthocyanins. Table 1 shows total anthocyanins of sweet cherries and sour cherries, evaluated by the colorimetric analysis. Total anthocyanins of sweet cherries were between 30.2 (cv. Black Gold) and 76.6 (cv. Hartland) mg CGE/100 g, whereas total anthocyanins of sour cherries were between 49.1 (cv. Balaton) and 109.2 (cv. Sumadinka) mg. The average content of total anthocyanins of sour cherries was about 1.6 times higher than that of sweet cherries. As observed on total phenolics, a wide range of the concentrations of total anthocyanins was revealed among different cherry cultivars tested. Sour cv. Sumadinka with the highest total anthocyanins among cherry cultivars contained about 3.6 times higher total anthocyanins than sweet cv. Black Gold. Total anthocyanins in various sweet cherry cultivars have been reported to be 28.2−62.1 mg (*18*). Four sour cherry cultivars harvested in 2003 from the same NY orchard were reported to have total anthocyanins of 45.0−67.1 mg (*5*).

To compare total phenolics and total anthocyanins of sweet and sour cherries in this study with previous reports for the same or different cultivars, total phenolics and total anthocyanins of sweet and sour cherries by other researchers are presented in **Table 2**.

HPLC Analysis of Phenolics. Phenolics of sweet and sour cherries were fractionated into anthocyanin fractions and nonanthocyanin fractions for the HPLC analysis. Anthocyanins were found to be the principal subclass of flavonoids among phenolics individually identified in fresh cherries (**Table 3**). All sweet cherries had neither cyanidin derivative nor cyanidin 3-glucosylrutinoside. Previously, sweet cherries were also reported to contain no cyanidin 3-glucosylrutinoside (*19*). All eight cultivars of cherries commonly contained cyanidin 3-glucoside and cyanidin 3-rutinoside.

Sour cherries had cyanidin 3-glucosylrutinoside as the major anthocyanin, whereas sweet cherries had cyanidin 3-rutinoside as the major anthocyanin (*19*, *20*, *22*, *29*). Cyanidin 3-rutinoside composed 77.3−86.6% of total anthocyanins in sweet cherries. Cyanidin 3-glucosylrutinoside consisted of 76.8−86.9% of total anthocyanins in sour cherries. Anthocyanins have been reported to be the dominant phenolics in sweet cherries, where the glycosides of cyanidin were the major compounds and the glycosides of peonidin and pelargonidin were the minor compounds (*20*, *23*). Four sweet cherries contained anthocyanins at 31.0−92.6 mg/100 g. Anthocyanins of sweet cherry cultivars (Burlat, Saco, Summit, and Van) quantified by HPLC were in the range of 19.4−95.7 mg/100 g (*4*), which are in good accordance with the results in this study. As observed in total anthocyanins measured by colorimetric analysis, sour cherries were generally found to contain a higher amount of anthocyanins evaluated by HPLC analysis than sweet cherries.

A positive linear relationship ($r^2 = 0.985$) was shown between total anthocyanins determined by the colorimetric method and the sum of individual anthocyanins analyzed by HPLC in eight

Table 2. Total Phenolics and Total Anthocyanins in Various Sweet and Sour Cherries by Other Workers

cultivars	total phenolics[a]	total anthocyanins[b]	literature cited
	sweet		
4-70	99.9	63.3	*17*
2C-61-22		231.9*	*20*
13N-7-70		1.6*	*20*
13S-10-40		297.5*	*20*
13S-39-51		40.7*	*20*
Bing	97.4	28.1	*18*
	185–194	26.1–63.7	*19*, *23*
		224.7*	*20*
Burlat	119–141	44.7–95.7*	*4*
Lambert	117.2	28.2	*18*
		198.4*	*20*
Napoleon	144.0	30.9	*18*
Petrovka	197.0	63.4	*18*
Rainer	75	0.5	*23*
Royal Ann	229	0.5–0.63	*19*, *23*
Saco	171–264	26.5–61.9*	*4*
Sam		227.0*	*20*
Stella		154.2*	*20*
Stella Compact	121.0	35.2	*18*
Summit	92.7–159	21.4–36.6*	*4*
		82.0*	*20*
Sylvia		245.2*	*20*
Van	94–144	19.4–47.1*	*4*
		150.8*	*20*
	sour		
I 21 (33)		15.9*	*21*
II 7 (30)		14.1*	*21*
II 9 (11)		35.5*	*21*
Balaton	254.1	45.0	*5*
		128.1*	*5*
		23.6	*29*
English Morello		11.5*	*21*
Karneol	366.0	56.8	*5*
Kroeker	398.5	67.1	*5*
Montmorency	407	8.7	*23*
		2.2*	*21*
		7.5*	*29*
Northstar	335.2	66.8	*5*

[a] Total phenolics are expressed as mg GAE/100 g of fresh cherry. [b] Total anthocyanins evaluated by the colorimetric analysis are expressed as mg CGE/100 g of fresh cherry, whereas the data evaluated by HPLC analysis and followed by an asterisk * are as mg/100 g of fresh cherry.

cherry cultivars (data not shown). Total anthocyanins evaluated by the colorimetric analysis were almost the same levels with the sum of individual anthocyanins by HPLC analysis. Therefore, it is believed that total anthocyanins measured by colorimetric assay can be comparable to the sum of individual anthocyanins quantified using HPLC.

The flavonol glycosides in cherries included quercetin 3-rutinoside, quercetin 3-glucoside, kaempferol 3-rutinoside, and isorhamnetin 3-rutinoside (**Table 4**). Sour cv. Danube had no kaempferol 3-rutinoside. Quercetin 3-rutinoside was a major flavonol in cherry cultivars except sour cv. Balaton and cv. Sumadinka, of which the dominant flavonol was isorhamnetin 3-rutinoside. The presence of isorhamnetin 3-rutinoside in sweet and sour cherries has not yet been reported. To identify aglycone for the peak containing isorhamnetin 3-rutinoside, its HPLC peak was repeatedly collected by multiple injections and then hydrolyzed in acidic conditions. The resulting hydrolyzate was analyzed again by HPLC. On the basis of its UV−visible spectrum, its retention time, and spiked input of authentic isorhamnetin, the peak was tentatively identified as the glycoside of isorhamnetin.

Sweet and sour cherries commonly contained neochlorogenic acid, *p*-coumaric acid derivative 1, and chlorogenic acid, while *p*-coumaric acid derivative 2 was not found in all sweet cherries and sour cv. Schattenmorelle (**Table 5**). The *p*-coumaric acid derivative 1 could be tentatively designated as *p*-coumaroylquinic acid based on the data previously reported in the literature (*4*, *19*, *20*). Neochlorogenic acid was found to be the major phenolic compound in sweet and sour cherries, the amount of which was relatively higher in sour cultivars than sweet cultivars. Among hydroxycinnamic acids, chlorogenic acid derivatives composed 45.6−91.2% for sweet cherries and 75.5−92.5% for sour cherries. Gao and Mazza (*20*) previously reported that neochlorogenic acid and *p*-coumaroylquinic acid were the major hydroxycinnamic acids in various sweet cherry cultivars. Chaovanalikit and Wrolstad (*19*) showed that the level of hydroxycinnamic acids of cherries ranged from 30 to 87.0 mg chlorogenic acid equivalents (CAE)/100 g, which is higher than that of the cherries studied here (12.3−40.1 mg CAE/100 g).

Viability of PC 12 Cells. The effects of cherry phenolics on the viability of neuronal PC 12 cells exposed to the oxidative stress were evaluated by two different in vitro methods such as MTT and trypan blue assays (**Table 6**). Oxidative stress to the brain has been implicated in the development of neurodegenerative diseases such as Alzheimer's disease (*30*). Oxidative stress leads to the accumulation of ROS such as H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$, even if ROS can be generated as byproducts of normal cellular processes. Because H_2O_2 can easily pass through the cellular membrane and form hydroxyl radicals in the presence of metals, it is an important risk factor to oxidative stress in neurodegeneration (*31*). It was previously reported that H_2O_2 treatment caused ~5 times intracellular accumulation of oxidative stress in PC 12 cells as compared with that of the control (*32*).

Table 3. Individual Anthocyanins (mg/100 g) in Sweet and Sour Cherries

cultivars	cyanidin derivative[a]	cyanidin 3-glucosyl-rutinoside	cyanidin 3-glucoside	cyanidin 3-rutinoside	peonidin 3-rutinoside	sum[b]
sweet Hartland			12.06 ± 3.82[c]	74.74 ± 20.28	5.84 ± 1.60	77.44 ± 20.82
sweet Hedelfingen			5.97 ± 1.00	34.43 ± 9.99	0.80 ± 0.48	35.98 ± 9.41
sweet Regina			3.70 ± 0.95	40.37 ± 1.75	2.69 ± 0.76	39.54 ± 2.00
sweet Black Gold			2.26 ± 0.27	26.82 ± 9.51	1.90 ± 0.53	25.28 ± 8.2
sour Danube	10.71 ± 3.18	111.39 ± 18.33	2.24 ± 0.38	20.67 ± 3.19		63.69 ± 3.26
sour Balaton	3.07 ± 0.32	88.95 ± 6.91	1.43 ± 0.11	15.45 ± 1.45	1.76 ± 0.55	45.57 ± 3.41
sour Schattenmorelle	4.81 ± 0.62	146.15 ± 18.85	1.81 ± 0.31	21.97 ± 2.94		68.76 ± 8.15
sour Sumadinka	11.93 ± 1.56	227.66 ± 55.69	2.79 ± 0.70	19.72 ± 3.61		98.64 ± 13.09

[a] The concentration of cyanidin derivative is expressed as CGE. The aglycone of cyanidin derivative with an unidentified moiety substitution was identified via acid hydrolysis and then followed by HPLC analysis on the basis of its UV–visible spectrum, its retention time, and spiked input of cyanidin aglycone standard. [b] The sum of individual anthocyanins detected by HPLC analysis is presented as CGE. In brief, the area of individual peaks of anthocyanins found in cherries was converted into CGEs by using the standard curve of authentic cyanidin 3-glucoside. The corresponding CGEs of each anthocyanin were summed. [c] The data are presented with means ± standard deviations ($n = 3$).

Table 4. Flavonols (mg/100 g) in Sweet and Sour Cherries

cultivars	quercetin 3-rutinoside	quercetin 3-glucoside	kaempferol 3-rutinoside	isorhamnetin 3-rutinoside
sweet Hartland	4.69 ± 0.99[a]	0.79 ± 0.22	0.36 ± 0.03	0.13 ± 0.01
sweet Hedelfingen	1.99 ± 0.74	0.30 ± 0.05	0.41 ± 0.21	0.09 ± 0.03
sweet Regina	2.85 ± 0.37	0.22 ± 0.02	1.39 ± 0.21	0.12 ± 0.02
sweet Black Gold	1.14 ± 0.22	0.16 ± 0.003	0.30 ± 0.08	0.08 ± 0.02
sour Danube	2.52 ± 0.12	0.32 ± 0.04		0.33 ± 0.02
sour Balaton	0.97 ± 0.30	0.21 ± 0.05	0.30 ± 0.08	1.12 ± 0.15
sour Schattenmorelle	4.36 ± 0.10	0.44 ± 0.01	1.29 ± 0.06	2.65 ± 0.04
sour Sumadinka	1.40 ± 0.54	0.22 ± 0.03	0.37 ± 0.07	1.57 ± 0.48

[a] The data are presented with means ± standard deviations ($n = 3$).

Table 5. Hydroxycinnamic Acids (mg/100 g) in Sweet and Sour Cherries

cultivars	neochlorogenic acid[a]	*p*-coumaric acid derivative[b] 1	chlorogenic acid	*p*-coumaric acid derivative 2
sweet Hartland	8.83 ± 0.34[c]	6.84 ± 0.60	0.65 ± 0.18	
sweet Hedelfingen	10.18 ± 2.78	1.00 ± 0.18	0.21 ± 0.13	
sweet Regina	6.71 ± 0.18	3.80 ± 0.36	0.50 ± 0.04	
sweet Black Gold	2.90 ± 0.54	3.61 ± 0.88	0.13 ± 0.02	
sour Danube	18.06 ± 2.68	0.89 ± 0.07	0.58 ± 0.16	0.63 ± 0.13
sour Balaton	21.88 ± 4.04	1.85 ± 0.34	1.35 ± 0.02	2.42 ± 0.45
sour Schattenmorelle	6.74 ± 0.14	4.06 ± 0.26	5.77 ± 0.25	
sour Sumadinka	27.79 ± 6.98	2.70 ± 0.68	0.93 ± 0.38	2.21 ± 0.84

[a] The level of neochlorogenic acid is expressed as CAE. Because of no commercial availability of authentic neochlorogenic acid standard, it was identified with the comparison of a previously reported pattern of its HPLC separation and of its UV–visible spectrum with chlorogenic acid standard (*39*). [b] The level of each *p*-coumaric acid derivative is expressed as *p*-coumaric acid equivalents. The individual HPLC peak of each *p*-coumaric acid derivative was repeatedly collected by multiple injections and then digested in alkaline conditions. The digested sample was analyzed again by HPLC. On the basis of its UV–visible spectrum, its retention time, and spiked input of authentic *p*-coumaric acid, each peak was identified as *p*-coumaric acid derivative. [c] The data are presented with means ± standard deviations ($n = 3$).

Table 6. Effects of Cherry Phenolics on Neuronal PC 12 Cell Viability (%) Exposed on H_2O_2-Induced Oxidative Stress[a]

	cell viability (%)[b]	
cultivars	MTT reduction	trypan blue exclusion
sweet Hartland	63.8 ± 1.5[c]	59.7 ± 5.8
sweet Hedelfingen	43.3 ± 7.5*[d]	49.8 ± 3.1
sweet Regina	50.1 ± 2.7	50.7 ± 2.6
sweet Black Gold	32.5 ± 5.1*	43.1 ± 3.0*
sour Danube	67.1 ± 4.2	62.1 ± 1.4
sour Balaton	44.1 ± 5.9*	45.4 ± 2.7*
sour Schattenmorelle	57.7 ± 1.4	54.9 ± 2.9
sour Sumadinka	86.9 ± 2.2	73.6 ± 3.9

[a] Neuronal PC 12 cells were preincubated for 10 min with cherry phenolic extracts and then were treated with H_2O_2 (400 μM) for 2 h. [b] The control group received treatment with neither H_2O_2 nor cherry phenolic extracts. The viability of PC 12 cells, treated 400 μM H_2O_2 only, was 40.5% for MTT reduction assay and 42.4% for trypan blue exclusion assay as compared to control (100%). [c] Results are represented as means ± standard deviation ($n = 3$). [d] A significant effect ($p < 0.05$) was observed on H_2O_2-induced oxidative stress in PC 12 cells except the results followed by an asterisk *.

The MTT assay is a simple colorimetric assay to measure cell viability, which is based on the ability of viable cells only to reduce the pale yellow MTT to a dark blue formazan. The amount of the formazan product created is proportional to the number of viable cells. With exposure to cell-damaging oxidative stress, neuronal cells only treated with H_2O_2 showed ~40% cell viability by MTT assay as compared with the control without H_2O_2 insults and cherry phenolics. The different levels of phenolics from sweet and sour cherries revealed the dose-dependent effects on the viability of neuronal PC 12 cells (**Table 6**). Sour cv. Sumadinka increased ~2.1 times higher cell viability as compared to cells with H_2O_2 insults only. Generally, PC 12 cell viability increased due to the treatment of phenolic extracts of cherries. Previously, PC 12 cells pretreated with apple phenolics before H_2O_2 exposure showed significant reduction of H_2O_2-induced oxidative damage (*32*).

Oxidative stress of H_2O_2 is known to lead to the lipid peroxidation of cellular membrane and ultimately to the cell death. The trypan blue exclusion assay was used to know if there was the cytoprotection of cherry phenolics on membrane damage caused by H_2O_2 treatment. Viable cells maintained the membrane integrity and allowed trypan blue dye not to pass through the membrane. Cherry phenolics protected the cellular membrane from oxidative stress as compared to cells only pretreated with H_2O_2 (**Table 6**). About 58% of PC 12 cells only with H_2O_2 treatment had dye accumulation inside cells, indicating that H_2O_2-induced oxidative stress apparently caused the severe damage on plasma membrane. On the other hand, the oxidative damage in neuronal cells was reduced after their exposure to cherry phenolic extracts, where the significant cytoprotection from oxidative stress was revealed by various cherry cultivars except sweet cv. Black Gold and sour cv. Balaton. The above results from two different assays for cell viability demonstrated that cherry protected neuronal PC 12 cells from cell-damaging oxidative H_2O_2 toxicity in vitro.

To evaluate how anthocyanins play more an important role in in vitro cytoprotection, linear regression of the first order was performed between anthocyanins and cell viability on the basis of percentage (%) of control (**Figure 1**). Eight cherry cultivars used for two cell viability assays contained the final anthocyanin concentrations of ~5.2−18.7 mg CGE/L in wells. Cherry anthocyanins protected the neuronal cells from oxidative damage in a dose-dependent manner. The higher the amount of anthocyanins is, the more increased viability of neuronal cells. The cell viability measured by MTT and trypan blue assays showed higher correlation (r^2) of 0.867 and 0.684 to anthocyanins, respectively (**Figure 1**). This highly positive correlation

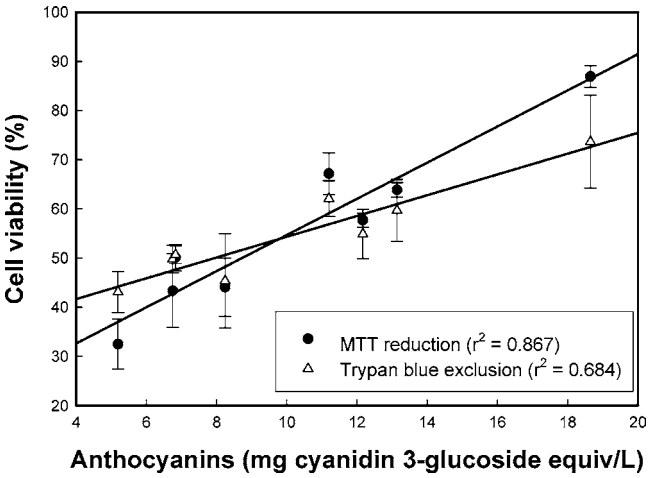

Figure 1. Relationship between anthocyanins in eight cherry cultivars and PC 12 cell viability evaluated by two in vitro biological assays. The concentrations of anthocyanins used for each cell viability assay were the final concentrations in the microplate wells. Each point is represented as mean ± standard deviation ($n = 3$).

of anthocyanins to PC 12 cell viability may suggest that cherry anthocyanins protected effectively neuronal cells from cell-damaging oxidative stress.

Flavonoids have been known to reduce oxidative stress in biological systems due to their antioxidant capacities (*12*−*14*, *33*). Anthocyanins, one of major groups in flavonoids, play an important biological role as potent antioxidants, the properties of which include the chelation of metal ions, inhibition of lipid oxidation, and scavenging of free radicals (*34*−*36*). It was previously reported that phenolics from various plant sources including apples protected PC 12 cells from oxidative stress (*14*, *32*, *37*, *38*). These previous researches have been related to nonanthocyanin flavonoids such as flavan-3-ols (*38*), flavonols (*32*, *33*), flavones (*14*), flavanones (*13*), and isoflavones (*37*). Unlike various plant sources described above, cherries possess a relatively higher amount of anthocyanins, which comprised >70% of total phenolics. Therefore, it is believed that anthocyanins from cherries played a more important role in antineurodegenerative activity than the other phenolics.

In conclusion, various cultivars of fresh cherries showed different contents of total phenolics and total anthocyanins. Anthocyanins were identified as the dominant phenolics in sweet and sour cherries, the aglycones of which were cyanidin and peonidin. Flavonols were the glycosides of quercetin, kaempferol, and isorhamnetin. Hydroxycinnamic acids consisted of the derivatives of chlorogenic acid and *p*-coumaric acid. The HPLC analysis of cherries revealed that phenolic composition and the level of individual phenolics varied in a wide range among different cultivars. There was a close agreement between two analytical methods; total anthocyanins measured by the pH differential method and total anthocyanins measured by summation of individual peaks from HPLC analysis. Generally, sour cherries had higher total phenolics than sweet cherries, which can be attributed to higher concentrations of anthocyanins and hydroxycinnamic acids in sour cherries. Cherry phenolics showed the protective effect on PC 12 cells against cell-damaging oxidative stress in a dose-dependent manner mainly due to anthocyanins. Overall results indicated that cherries are rich in phenolics, especially in anthocyanins, with a strong antineurodegenerative activity, and thus, they can serve as a good source of biofunctional phytochemicals in our diet, providing health-promoting effects in humans.

ABBREVIATIONS USED

CAE, chlorogenic acid equivalents; CGE, cyanidin 3-glucoside equivalents; DDW, distilled deionized water; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species.

LITERATURE CITED

(1) Nicolas, J. J.; Richard-Forget, F. C.; Goupy, P. M.; Amiot, M.-J.; Aubert, S. Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109−157.

(2) Lee, C. Y.; Kagan, V.; Jaworski, A. W.; Brown, S. K. Enzymatic browning in relation to phenolic compounds and polyphenoloxidase activity among various peach cultivars. *J. Agric. Food Chem.* **1990**, *38*, 99−101.

(3) Siebert, K. J. Effects of protein-polyphenol interaction on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353−362.

(4) Gonçalves, B.; Landbo, A.-K.; Knudsen, D.; Silva, A. P.; Moutinho-Pereira, J.; Rosa, E.; Meyer, A. S. Effect of ripeness and postharvest storage on the phenolic profiles of cherries (*Prunus avium* L.). *J. Agric. Food Chem.* **2004**, *52*, 523−530.

(5) Kim, D.-O.; Padilla-Zakour, O. I. Jam processing effect on phenolics and antioxidant capacity in anthocyanin-rich fruits: cherry, plum, and raspberry. *J. Food Sci.* **2004**, *69*, S395−S400.

(6) Kim, D.-O.; Kim, Y. J.; Heo, H. J.; Freer, J.; Padilla-Zakour, O. I.; Lee, C. Y. Phenolics and antioxidant capacity in selected New York State plums. *N. Y. Fruit Q.* **2004**, *12* (4), 9−12.

(7) Murakami, A.; Nakamura, Y.; Ohto, Y.; Yano, M.; Koshiba, T.; Koshimizu, K.; Tokuda, H.; Nishino, H.; Ohigashi, H. Suppressive effects of citrus fruits on free radical generation and nobiletin, an antiinflammatory polymethoxyflavonoid. *Biofactors* **2000**, *12*, 187−192.

(8) Benavente-García, O.; Castillo, J.; Marin, F. R.; Ortuño, A.; Río, J. A. D. Uses and properties of Citrus flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505−4515.

(9) Do, J.-R.; Kang, S.-N.; Kim, K.-J.; Jo, J.-H.; Lee, S.-W. Antimicrobial and antioxidant activities and phenolic contents in the water extract of medicinal plants. *Food Sci. Biotechnol.* **2004**, *13*, 640−645.

(10) Wang, H.; Provan, G. J.; Helliwell, K. Tea flavonoids; their functions, utilisation and analysis. *Trends Food Sci. Technol.* **2000**, *11*, 152−160.

(11) Yoshimura, Y.; Nakazawa, H.; Yamaguchi, F. Evaluation of the NO scavenging activity of procyanidin in grape seed by use of the TMA-PTIO/NOC 7 ESR system. *J. Agric. Food Chem.* **2003**, *51*, 6409−6412.

(12) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433−446.

(13) Heo, H. J.; Kim, D.-O.; Shin, S. C.; Kim, M. J.; Kim, B. G.; Shin, D.-H. Effect of antioxidant flavanone, naringenin, from *Citrus junos* on neuroprotection. *J. Agric. Food Chem.* **2004**, *52*, 1520−1525.

(14) Heo, H. J.; Kim, D.-O.; Choi, S. J.; Shin, D. H.; Lee, C. Y. Potent inhibitory effect of flavonoids in *Scutellaria baicalensis* on amyloid β protein-induced neurotoxicity. *J. Agric. Food Chem.* **2004**, *52*, 4128−4132.

(15) Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables—The millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36*, 703−725.

(16) Commenges, D.; Scotet, V.; Renaud, S.; Jacqmin-Gadda, H.; Barberger-Gateau, P.; Dartigues, J.-F. Intake of flavonoids and risk of dementia. *Eur. J. Epidemiol.* **2000**, *16*, 357−363.

(17) Serrano, M.; Guillén, F.; Martínez-Romero, D.; Castillo, S.; Valero, D. Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. *J. Agric. Food Chem.* **2005**, *53*, 2741−2745.

(18) Mozetič, B.; Trebše, P.; Hribar, J. Determination and quantitation of anthocyanins and hydroxycinnamic acids in different cultivars of sweet cherries (*Prunus avium* L.) from Nova Gorica Region (Slovenia). *Food Technol. Biotechnol.* **2002**, *40*, 207−212.

(19) Chaovanalikit, A.; Wrolstad, R. E. Anthocyanins and polyphenolic composition of fresh and processed cherries. *J. Food Sci.* **2004**, *69*, FST73−FST83.

(20) Gao, L.; Mazza, G. Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries. *J. Agric. Food Chem.* **1995**, *43*, 343−346.

(21) Chandra, A.; Nair, M. G.; Iezzoni, A. Evaluation and characterization of the anthocyanin pigments in tart cherries (*Prunus cerasus* L.). *J. Agric. Food Chem.* **1992**, *40*, 967−969.

(22) Chandra, A.; Rana, J.; Li, Y. Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *J. Agric. Food Chem.* **2001**, *49*, 3515−3521.

(23) Chaovanalikit, A.; Wrolstad, R. E. Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. *J. Food Sci.* **2004**, *69*, FST67−FST72.

(24) Kim, D.-O.; Lee, C. Y. Extraction and isolation of polyphenolics. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons: New York, 2002; pp I1.2.1−I1.2.12.

(25) Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(26) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV−visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons: New York, 2001; pp F1.2.1−F1.2.13.

(27) Durst, R. W.; Wrolstad, R. E. Separation and characterization of anthocyanins by HPLC. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons: New York, 2001; pp Units F1.3.1−F1.3.13.

(28) Heo, H.-J.; Cho, H.-Y.; Hong, B.; Kim, H.-K.; Kim, E.-K.; Kim, B.-G.; Shin, D.-H. Protective effect of 4′,5-dihydroxy-3′,6,7-trimethoxyflavone from *Artemisia asiatica* against Aβ-induced oxidative stress in PC12 cells. *Amyloid* **2001**, *8*, 194−201.

(29) Wang, H.; Nair, M. G.; Iezzoni, A. F.; Strasburg, G. M.; Booren, A. M.; Gray, J. I. Quantification and characterization of anthocyanins in Balaton tart cherries. *J. Agric. Food Chem.* **1997**, *45*, 2556−2560.

(30) Smith, M. A.; Rottkamp, C. A.; Nunomura, A.; Raina, A. K.; Perry, G. Oxidative stress in Alzheimer's disease. *Biochim. Biophys. Acta* **2000**, *1502*, 139−144.

(31) Varadarajan, S.; Yatin, S.; Aksenova, M.; Butterfield, D. A. Alzheimer's amyloid β-peptide-associated free radical oxidative stress and neurotoxicity. *J. Struct. Biol.* **2000**, *130*, 184−208.

(32) Heo, H. J.; Kim, D.-O.; Choi, S. J.; Shin, D. H.; Lee, C. Y. Apple phenolics protect in vitro oxidative stress-induced neuronal cell death. *J. Food Sci.* **2004**, *69*, S357−S360.

(33) Heo, H. J.; Lee, C. Y. Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. *J. Agric. Food Chem.* **2004**, *52*, 7514−7517.

(34) Kim, D.-O.; Lee, C. Y. Comprehensive study of vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 253−273.

(35) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304−309.

(36) Chun, O. K.; Kim, D.-O.; Lee, C. Y. Superoxide radical scavenging activity of the major polyphenols in fresh plums. *J. Agric. Food Chem.* **2003**, *51*, 8067−8072.

(37) Heo, H. J.; Kim, M. J.; Suh, Y. M.; Choi, S. J.; Mun, N. S.; Kim, H. K.; Kim, E. K.; Shin, D. H. Protective effects of daidzein on oxidative stress-induced neurotoxicity and scopolamine-mediated cognitive defect. *J. Food Sci.* **2005**, *70*, S91−S94.

(38) Heo, H. J.; Lee, C. Y. Epicatechin and catechin in cocoa inhibit amyloid β protein induced apoptosis. *J. Agric. Food Chem.* **2005**, *53*, 1445−1448.

(39) Nakatani, N.; Kayano, S.; Kikuzaki, H.; Sumino, K.; Katagiri, K.; Mitani, T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). *J. Agric. Food Chem.* **2000**, *48*, 5512−5516.

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