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Phenolic Compounds from Blueberries Can Inhibit Colon Cancer Cell Proliferation and Induce Apoptosis

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Research has shown that diets rich in phenolic compounds may be associated with lower risks of several chronic diseases including cancer. This study systematically evaluated the bioactivities of phenolic compounds in rabbiteye blueberries and assessed their potential antiproliferation and apoptosis induction effects using two colon cancer cell lines, HT-29 and Caco-2. Polyphenols in three blueberry cultivars, Briteblue, Tifblue, and Powderblue, were extracted and freeze-dried. The extracts were further separated into phenolic acids, tannins, flavonols, and anthocyanins using an HLB cartridge and LH20 column. Some individual phenolic acids and flavonoids were identified by HPLC with >90% purity in anthocyanin fractions. The dried extracts and fractions were added to the cell culture medium to test for antiproliferation activities and induction of apoptosis. Flavonol and tannin fractions resulted in 50% inhibition of cell proliferation at concentrations of 70−100 and 50−100 μg/mL in HT-29 and Caco-2 cells, respectively. The phenolic acid fraction showed relatively lower bioactivities with 50% inhibition at ∼1000 μg/mL. The greatest antiproliferation effect among all four fractions was from the anthocyanin fractions. Both HT-29 and Caco-2 cell growth was significantly inhibited by >50% by the anthocyanin fractions at concentrations of 15−50 μg/mL. Anthocyanin fractions also resulted in 2−7 times increases in DNA fragmentation, indicating the induction of apoptosis. The effective dosage levels are close to the reported range of anthocyanin concentrations in rat plasma. These findings suggest that blueberry intake may reduce colon cancer risk.

KEYWORDS: Anthocyanins; antiproliferation; apoptosis; blueberries; cancer; polyphenols

INTRODUCTION

Recent evidence from epidemiological studies shows that diets rich in fruits and vegetables are associated with a lower risk of chronic diseases including cancer (*1*−*3*). Phenolic acids, flavonoids, and anthocyanins may contribute to this effect (*4*). These compounds are present in all fruits and vegetables (*5*). Polyphenols function as antioxidants, scavenging free radicals (*6*), inhibiting or activating enzymes, or functioning as metal chelators (*7*, *8*), thus preventing damage to lipids, proteins, and nucleic acids. In addition to their antioxidant properties, polyphenols have been reported to decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis, and exhibit phytoestrogenic activity (*9*−*11*). All of these functions may contribute to cancer prevention.

Much information is available on the reported inhibitory effects of specific plant phenolic compounds and extracts on mutagenesis and carcinogenesis and the role of antioxidant activity in these effects (*12*). Some scientists believe that diets supplemented with a single or few compounds do not have the same health benefits as a diet rich in fruits and vegetables. Isolated pure compounds may either lose their bioactivity or may not behave the same way as the compound in whole foods (*13*). Zhou et al. (*14*) evaluated the effects of soy phytochemical concentrate (SPC) and tea on prostate cancer. They found that the combination of SPC and black tea synergistically inhibited prostate tumorigenicity, final tumor weight, and metastases to lymph nodes in vivo. Dietary SPC or tea treatment alone did not reduce the incidence of tumor metastasis to lymph nodes. It has been suggested that the combination of phytochemicals in fruits and vegetables is critical to their powerful antioxidant and anticancer activity (*15*, *16*). Thus, studies of specific crops should be a good way to provide valuable information on their health benefits.

Blueberries (belong to the Vacciniaceae) can be classified as highbush, lowbush, and rabbiteye. They have been considered to be one of the fruits with the highest antioxidant potentials (*17*−*19*). Some studies have evaluated the bioactivities of blueberry. Bomser et al. (*20*) found that the ethyl acetate extracts

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and further hexane/chloroform subfraction of lowbush blueberry (*V. angustifolium* Ait) significantly induced quinone reductase (QR) activity, an enzyme involved in phase II xenobiotic detoxification. In the same study, the crude extracts significantly inhibited the induction of ornithine decarboxylase (ODC). Smith et al. (*21*) also evaluated the bioactivity of wild blueberry (*V. angustifolium* Ait). They found that the crude 70% acetone extract (flavonoid-rich) exhibited clear, significant induction of QR activity. In an evaluation of the bioactivity of lowbush blueberry (*Vaccinium angustifolium*) and highbush blueberry (*Vaccinium corymbosum*), Katsube et al. (*22*) found that the ethanol extract significantly inhibited proliferation of HL60 human promyelocytic leukemia cells and HCT116 human colon carcinoma cells in the range from 2 to 6 mg/mL.

Studies with a systematic evaluation of anticancer activity (antiproliferation and induction of apoptosis) of phenolic compounds in blueberry are very limited. In addition, studies have shown that the contents of phenolic compounds in blueberries vary widely. Our previous study evaluated the phenolic contents of different cultivars of blueberry, including rabbiteye (*Vaccinium ashei* Reade) and southern highbush (*Vaccinium corymbosum* L. hybrids) blueberries, and found significant differences in polyphenol contents among different cultivars (*17*). This makes the direct evaluation of the bioactivities of different varieties critical. The total anthocyanin contents in rabbiteye have been reported to be ∼100 mg/100 g of fresh weight (FW), and the flavonoids ranged from 33 to 387 mg/100 g of FW (*17*). Our objectives in this study were to systematically evaluate the bioactivities of phenolic compounds in rabbiteye blueberries, to assess the potential antiproliferative and apoptosis induction effects on colon cancer cells, and to identify the possible active components.

MATERIALS AND METHODS

Chemicals and Reagents. Pure standards of gallic acid, *p*-hydroxybenzoic acid, (+)-catechin, caffeic acid, (−)-epicatechin, *p*-coumaric acid, ferulic acid, ellagic acid, quercetin, and kaempferol were purchased from Sigma (St. Louis, MO). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards are delphinidin 3-*O*-β-glucopyranoside (Dp-Glc), cyanidin 3-*O*-β-galactopyranoside (Cy-Gal), cyanidin 3-*O*-β-glucopyranoside (Cy-Glc), petunidin 3-*O*-β-glucopyranoside (Pt-Glc), peonidin 3-*O*-β-galactopyranoside (Pn-Gal), peonidin 3-*O*-β-glucopyranoside (Pn-Glc), malvidin 3-*O*-β-glucopyranoside (Mv-Glc), and peonidin 3-*O*-α-arabinopyranoside (Pn-Ara). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Milwaukee, WI). Acetonitrile, methanol, orthophosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), formic acid, and water (HPLC grade) were purchased from Fisher Scientific (Norcross, GA). MTT Cell Proliferation Assay kits were purchased from ATCC (Manassas, VA). Cell Death Detection ELISA kits (Boehringer Mannheim, Roche) were purchased from Roche (Indianapolis, IN).

Sample Collection. Blueberries were collected from the field in 2004. The blueberry cultivars collected were Briteblue (Chula, GA), Tifblue (Alma, GA), and Powderblue (Chula, GA). All cultivars were grown with irrigation or under conditions of adequate rainfall. Samples were frozen and stored at −40 °C until use.

Extraction and Fractionation. Polyphenolic fractions were obtained using a modified procedure reported by Youdim et al. (*23*) and Oszmianski et al. (*24*). Briefly, 100 g of berries was homogenized in 300 mL of acetone/methanol/water/formic acid (40:40:20:0.1, v/v/v/v). Crude extracts were freeze-dried using a UNITOP 600L freeze-dryer (Virtis, Gardiner, NY) and resolubilized in water, applied to an activated Oasis HLB cartridge (Waters Corp., Milford, MA), and washed with water, 15% methanol in water, and finally methanol acidified with formic acid. The 15% methanol fraction eluted the phenolic acids, and the acidified methanol eluted the anthocyanins and other components of interest. The fraction containing the anthocyanins was dried again and resolubilized in 50% methanol in water and applied to a Sephadex LH20 column (Amersham Biosciences AB, Uppsala, Sweden). The column was then washed with 70% methanol acidified with 10% formic acid to elute anthocyanins and flavonols. The LH20 column was then washed with 70% acetone to elute the tannins or procyanidins. After freeze-drying the anthocyanins and flavonol fraction, the fraction was resolubilized in 5% formic acid in water and applied to the second Oasis HLB cartridge. The cartridge was washed with 5% formic acid, followed by ethyl acetate, and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols, and the acidified methanol eluted the anthocyanins. All fractions were freeze-dried and resolubilized for the cell proliferation assay. Extraction and fractionation were repeated five times, and the fractions were pooled together to obtain sufficient amount for the bioassay.

Total Polyphenols Measurement. Total phenolics were measured according to the Folin−Ciocalteu reagent method (*25*). The extract/fractions were dissolved in 80% methanol, and 20 μL of sample solution was introduced in a test tube, 1.0 mL of Folin−Ciocalteu reagent and 0.8 mL of sodium carbonate (7.5%) were added, and the contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a Shimadzu 300 UV−vis spectrophotometer (Shimadzu UV-1601, Norcross, GA). The total phenolic content was expressed as gallic acid equivalents (GAE), using a standard curve generated with 50, 100, 200, 300, and 400 mg/L of gallic acid.

Hydrolysis. For the phenolic acids and flavonoids, fractions were dissolved in methanol containing 1.2 N HCl (40 mL of methonal + 10 mL of 6 N HCl). The samples were vortexed for 1 min and then placed in a water bath at 80 °C while shaking at 200 rpm for 2 h for acid hydrolysis of phenolic glycosides to aglycons. For the anthocyanin hydrolysis, fractions were dissolved in 50% methanol solution containing 2 N HCl (50 mL of methanol + 33 mL of water + 17 mL of 37% HCl). Samples were placed in a water bath at 90 °C with shaking at 200 rpm for 1 h for acid hydrolysis of anthocyanins to anthocyanidins. The hydrolyzed sample was cooled in dark and filtered through a 0.2 μm syringe nylon filter. A 20 μL aliquot of filtered sample was injected into the HPLC for analysis.

HPLC Analysis. HPLC was performed with a Hewlett-Packard (Avondale, PA), model 1100 liquid chromatograph with quaternary pumps and a diode array UV−visible detector coupled to an HP ChemStation. For the analysis of phenolic acids and flavonoids, a procedure previously reported by our laboratory was used (*17*): column, Beckman Ultrasphere C18 ODS 4.6 × 250 mm; column temperature, 40 °C; mobile phase (A) water/methanol (70:30 v/v) with 1% formic acid, (B) ethanol, and (C) 1% formic acid in water; flow rates, 1.3 mL/min from 0 to 5 min and 1.0 mL/min from 5.01 to 75 min; gradients, at 0 min 100% solvent C, at 5 min 100% solvent C, at 5.01 min 50% solvent C, at 10 min solvent B, at 10.01 min 5% solvent B and 45% solvent C, at 20 min 15% solvent B, at 25 min 15% solvent B, at 60 min 50% solvent B, at 60.01 min 100% solvent B, at 65 min 100% solvent B, at 65.01 min 50% solvent C, at 75 min 50% solvent C. Phenolic compounds were detected at wavelengths of 260, 280, 320, and 360 nm.

For the anthocyanin and anthocyanidin analysis, the mobile phase was solvent A, orthophosphoric acid/methanol/water (5:10:85, v/v/v), and solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradient for the separation was 100% solvent A at 0 min, 90% solvent A and 10% solvent B at 5 min, and 50% solvent A and 50% solvent B at 25 min, with a 5 min postrun with HPLC grade water. Anthocyanin and anthocyanidin were detected at 520 nm.

Cell Cultures. Two cancer cell lines were purchased from ATCC (Manassas, VA): (1) HT-29 human colon, colorectal adenocarcinoma cultured in ATCC medium (McCoy's 5a medium with 1.5 mM L-glutamine, 90%; fetal bovine serum, 10%); (2) Caco-2, human colon, colorectal adenocarcinoma cultured in ATCC medium (minimum essential medium (Eagle) with 2 mM l-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate, 80%; fetal bovine serum, 20%). Cancer cells were cultured in the specific medium in an incubator

Table 1. Total Polyphenolic Content (Weight Percent) of Different Extracts/Fractions from Blueberries[a]

cultivar	crude extract	phenolic acids	flavonols	tannins
Briteblue	6.0 ± 0.5	14.9 ± 1.1	76.6 ± 1.5	87.9 ± 0.9
Tifblue	6.4 ± 0.3	15.1 ± 0.9	86.5 ± 0.6	85.4 ± 2.5
Powderblue	4.9 ± 0.2	12.4 ± 1.2	75.3 ± 1.1	86.9 ± 3.3

[a] Total polyphenols are expressed as gallic acid equivalents (GAE), and the total polyphenolic contents percentage was calculated on the basis of GAE/fraction weight × 100%. Values are averages of triplicate analyses ± standard error.

with 5% CO_2 under 37 °C. The medium was changed two to three times per week.

Cell Antiproliferation Assay. After digestion with trypsin−EDTA, uniform amounts (∼1 × 10^4 in the case of HT-29 and ∼5000 in Caco-2) of cells in growth media were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation at 37 °C in 5% CO_2, the growth medium was replaced with 100 μL of medium containing different concentrations of fruit extracts/fractions. Eight concentrations of extracts/fractions in 2× dilution factor were applied. Depending on different fractions, the highest concentrations in crude extract and phenolic acid fractions were a few milligrams per milliliter and the highest concentrations in the rest of the fractions were around a few hundred micrograms per milliliter. The subsequent concentrations were $^1/_2$, $^1/_4$, ..., $^1/_{256}$ of the highest. Control cultures received everything but the fruit extracts/fractions, and blank wells contained 100 μL of growth medium and extract/fraction without cells. Except for anthocyanin fractions that were water soluble, dimethyl sufoxide (DMSO) was added initially to the extracts/fractions to help dissolve the sample. The final DMSO content was 0.25%. Therefore, the control for these treatments also contained 0.25% DMSO. None of the extracts/fractions added changed the pH (≈7.2) of the culture medium (data not shown). After 48 h of incubation, cell proliferation was determined using the ATCC MTT Cell Proliferation Assay at 570−655 nm with a Bio-Rad model 680 microplate reader (Hercules, CA). To better explain the antiproliferation results, the inhibition of cell proliferation was calculated on the basis of the following formula:

$$\text{inhibition percentage} = (\text{cell no. in control} - \text{cell no. in treatment})/(\text{cell no. in control} - \text{original cell no. before addition of extract or carrier}) \times 100\%$$

IC_{50} is the extract concentration under which a 50% inhibition of cell proliferation occurred.

Detection of Apoptosis. An early event in apoptosis is DNA fragmentation and release and activation of an endogenous endonuclease. The endonuclease cleaves double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosome is tightly complexed with the core histones and is therefore protected from cleavage. DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA using a cell death detection ELISA kit (Boehringer Mannheim, Roche). Briefly, the cytosolic fraction (13000*g* supernatant) of cancer cells (∼1 × 10^5 of HT-29 or 5 × 10^4 of Caco-2) after treatment with different concentrations of anthocyanin extract was used as antigen source in a sandwich ELISA with a primary antihistone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. The DNA fragmentation was measured at 405−490 nm using a Bio-Rad model 680 microplate reader.

Statistical Analysis. The bioactivities of different fractions in three cultivars were statistically compared. Statistical analysis was conducted by General Linear Model (GLM) followed by Duncan's multiple-range test at $\alpha = 0.05$ (*26*).

RESULTS

Extraction and Fractionation. The total polyphenolic contents (weight percent) of different fractions are shown in **Table 1**. Total polyphenols are expressed as gallic acid equivalents (GAE), and the total polyphenolic contents percentage was calculated as GAE/fraction weight × 100%. The crude extracts had the lowest total polyphenolic contents representing ∼5%. Flavonols and the condensed tannin/procyanidin fractions had high purity with ∼80% of total polyphenolic contents. In all three cultivars except for Tifblue, procyanidin fractions had higher total polyphenolic contents than flavonol fractions. The phenolic acid fraction had relatively low (12−15%) total polyphenolic contents compared to other fractions.

Some of the individual phenolic acids and flavonoids were identified by comparing their retention times and characteristic spectra with standards. The contents of individual phenolic acids and flavonoids in different fractions of blueberries are shown in **Table 2**. In the phenolic acid fractions, no ferulic acids were detected in Briteblue and no coumaric acids were detected in Powderblue. The profile of phenolic acids contents varied among different cultivars. The major phenolic acids detected in Briteblue fraction were caffeic and *p*-coumaric acids, whereas the major compound identified in Tifblue fraction was gallic acid (3.2% of total weight or 21% of the total polyphenolic content of fraction). However, some amount of catechin was found in the phenolic acid fractions of all three cultivars.

Although the flavonol fractions had high total polyphenolic contents, only a small amount of individual compounds were identified. Major flavonols found in the fractions were quercetin and kaempferol. The contents of quercetin ranged from 4.4% in Tifblue to 8.5% in Powderblue.

The highest purity of isolated fractions was observed in the anthocyanin fractions. Major individual anthocyanins in the anthocyanin fractions are presented in **Table 3**. **Figure 1** shows the analytical HPLC chromatogram of blueberry anthocyanin fractions. Ten major peaks and three small peaks were observed in all three cultivars (**Figure 1 B−D**). Seven different anthocyanins were identified on the basis of the retention time and characteristic spectra. The total weights of the identified anthocyanins were added to obtain the percentage of total identified anthocyanins in the total weight of whole fraction. More than 86% of whole fractions were identified in Tifblue and Powderblue, and ∼75% were identified in Briteblue. Because some of the peaks (**Figure 1**) could not be identified, acid hydrolysis was conducted to evaluate the anthocyanidin profile. Five standards (Dp-Glc, Cy-Gal, Pt-Glc, Pn-Gal, and Mv-Glc) were hydrolyzed in a water bath and run in HPLC to yield the characteristic retention times and spectra. **Figure 2** shows the peaks of the five anthocyanidins. The anthocyanidin profiles of three fractions were obtained through acid hydrolysis (**Figure 2 B−D**). **Table 4** shows the individual anthocyanidins in the anthocyanin fractions after acid hydrolysis. The amount of different anthocyanidins is expressed on the basis of the specific weight of anthocyanins, Dp-Glc, Cy-Gal, Pt-Glc, Pn-Gal, and Mv-Glc, because most of the anthocyanins in blueberry are monoglycosides. The weight percentages were then calculated to assess the purity of different anthocyanin fractions. The highest purity was obtained in Tifblue and Powderblue, with ∼98% anthocyanidin monoglycosides. About 89% of anthocyanidin monoglycosides were observed in Briteblue fraction. In general, five different anthocyanidins (Dp, Cy, Pt, Pn, and Mv) were identified and no other peak was found in any of the three cultivars (**Figure 2**).

Cell Antiproliferation. The inhibition of HT-29 and Caco-2 cancer cell proliferation by crude extracts of blueberries is shown in **Figure 3**. In both cell lines, the IC_{50} in the crude extract ranged from 1000 to 3000 μg/mL. The Briteblue crude extract showed the highest antiproliferation activity among the three

Table 2. Individual Phenolic Acids and Flavonoids (Weight Percent) in Different Fractions of Blueberries[a]

cultivar	phenolic acids				flavonoids			
	gallic acid	caffeic acid	*p*-coumaric acid	ferulic acid	catechin	epicatechin	quercetin	kaempferol
				Phenolic Acid Fractions				
Briteblue	0.4 ± 0.0 (2.4 ± 0.1)	3.2 ± 0.0 (21.7 ± 0.1)	2.5 ± 0.0 (17.0 ± 0.1)	ND	2.3 ± 0.1 (15.1 ± 0.5)	ND	ND	ND
Tifblue	3.2 ± 0.0 (21.4 ± 0.2)	1.1 ± 0.0 (7.5 ± 0.1)	0.3 ± 0.0 (1.7 ± 0.1)	0.4 ± 0.0 (2.3 ± 0.1)	1.9 ± 0.1 (12.4 ± 0.4)	ND	ND	ND
Powderblue	0.8 ± 0.0 (6.5 ± 0.1)	1.3 ± 0.0 (10.3 ± 0.2)	ND	0.4 ± 0.0 (3.2 ± 0.1)	2.8 ± 0.1 (22.5 ± 0.4)	ND	ND	ND
				Flavonol Fractions				
Briteblue	0.7 ± 0.1 (1.0 ± 0.1)	0.7 ± 0.1 (0.9 ± 0.1)	0.6 ± 0.1 (0.7 ± 0.1)	ND	ND	ND	5.7 ± 0.3 (7.5 ± 0.4)	2.2 ± 0.2 (2.9 ± 0.3)
Tifblue	0.6 ± 0.1 (0.7 ± 0.1)	0.3 ± 0.0 (0.3 ± 0.0)	ND	0.3 ± 0.0 (0.3 ± 0.0)	ND	1.4 ± 0.1 (1.7 ± 0.1)	4.4 ± 0.2 (5.1 ± 0.3)	3.4 ± 0.2 (3.9 ± 0.2)
Powderblue	0.8 ± 0.1 (1.1 ± 0.1)	0.9 ± 0.1 (1.2 ± 0.1)	ND	0.6 ± 0.0 (0.8 ± 0.1)	ND	ND	8.5 ± 0.3 (11.3 ± 0.3)	5.9 ± 0.3 (7.8 ± 0.4)

[a] Data are percentage by weight of individual compounds in the whole weight of specific fraction. Data in parentheses are percentage of individual compounds in the total polyphenols of specific fraction. Values are averages of triplicate analyses ± standard error. ND, not detected.

Table 3. Major Individual Anthocyanins (Weight Percent) in the Anthocyanin Fractions[a]

cultivar	Dp-Glc	Cy-Gal	Cy-Glc	Pt-Glc	Pn-Gal	Pn-Glc	Mv-Glc	total anthocyanins
Briteblue	15.6 ± 0.3	5.7 ± 0.3	4.7 ± 0.1	21.5 ± 0.5	4.6 ± 0.0	14.2 ± 0.3	8.5 ± 0.3	74.7 ± 1.6
Tifblue	7.6 ± 0.2	14.9 ± 0.4	5.9 ± 0.0	20.4 ± 0.3	8.6 ± 0.1	20.5 ± 0.5	8.1 ± 0.1	86.0 ± 1.5
Powderblue	12.1 ± 0.1	16.4 ± 0.3	8.1 ± 0.1	25.2 ± 0.4	3.5 ± 0.3	14.7 ± 0.3	7.2 ± 0.2	87.2 ± 1.7

[a] Values are averages of triplicate analyses ± standard error. Abbreviations: Dp-Glc, delphinidin 3-*O*-β-glucopyranoside; Cy-Gal, cyanidin 3-*O*-β-galactopyranoside; Cy-Glc, cyanidin 3-*O*-β-glucopyranoside; Pt-Glc, petunidin 3-*O*-β-glucopyranoside; Pn-Gal, peonidin 3-*O*-β-galactopyranoside; Pn-Glc, peonidin 3-*O*-β-glucopyranoside; Mv-Glc, malvidin 3-*O*-β-glucopyranoside.

cultivars in both HT-29 and Caco-2 cells. The bioactivities of different blueberry cultivars varied with different cancer cell lines. The highest IC_{50} in HT-29 was observed in Powderblue, whereas in Caco-2, the highest IC_{50} was found in Tifblue among all three cultivars.

All four fractions, phenolic acids, flavonols, tannins or procyanidins, and anthocyanins, showed significantly higher bioactivities, probably because of their higher purity, compared to the crude extracts. The inhibition of HT-29 cancer cell proliferation by different fractions is shown in **Figure 4**. The phenolic acid fraction showed the lowest antiproliferation activities among the four fractions with an IC_{50} of ~1000 μg/mL (**Figure 4A**). The anthocyanin fraction showed the highest antiproliferation activity among the four fractions (**Figure 4D**). For Briteblue anthocyanins, the IC_{50} was ~25 μg/mL. This was significantly lower when compared with Tifblue and Powderblue, although the anthocyanin content of the Briteblue fraction was lower than that of the other two cultivars (**Table 4**). The IC_{50} values for Tifblue and Powderblue were ~50 μg/mL. Intermediate bioactivities were observed in tannin and flavonol fractions. The IC_{50} for the tannin fractions and flavonol fractions ranged from 70 to 100 μg/mL. In the tannin fractions, Briteblue showed the highest bioactivity among the three cultivars, although their total polyphenolic contents were very close (**Table 1**). No significant difference was observed among the flavonol fractions from different cultivars.

A similar trend in the antiproliferation activities of the four fractions was found in Caco-2 cells (**Figure 5**). Again, the phenolic acid fraction showed the lowest antiproliferation activities among the four fractions, whereas the anthocyanin fraction showed the highest antiproliferation activity. The lowest IC_{50} (highest bioactivity) was observed at ~15 μg/mL anthocyanin fraction in Caco-2 cells. Significant inhibition effects were observed with as low as 1 μg/mL anthocyanins. No significant difference was found in the anthocyanin fractions of the three cultivars, unlike in the HT-29 cells, for which Briteblue anthocyanin showed the highest bioactivities. In the flavonol fractions, Briteblue showed the highest antiproliferation activities with an IC_{50} at ~50 μg/mL. In the procyanidin fraction, all three cultivars showed similar bioactivities with IC_{50} values of ~100 μg/mL (**Figure 5C**).

It is important to note that a statistically significant increase in HT-29 cell proliferation was observed with 4.7 μg/mL of the tannin fraction from the Briteblue cultivar (**Figure 4C**). Increases were also observed at similar dosage levels with tannin fractions from Tifblue and Powderblue cultivars, although a statistical analysis showed no significant effects.

Apoptosis. DNA fragmentation in HT-29 and Caco-2 cells after treatment with anthocyanin fractions is shown in **Figure 6**. From **Figure 6A**, we can see that anthocyanins resulted in 3−7 times increases in DNA fragmentation compared with no treatment control. Because DNA fragmentation is the primary physiological characteristic of apoptosis, our results suggest that anthocyanin can induce programmed cell death in HT-29 colon cancer cells. The highest DNA fragmentation level was observed at ~40 μg/mL, representing a 7 times increase compared to the control. Further increases of anthocyanin did not result in increased level of DNA fragmentation, although very few live cells existed at those high dosage levels (**Figure 4D**). Similar results were obtained in Caco-2 cell lines when treated with the Tifblue anthocyanin fraction (**Figure 6B**). Anthocyanin treatment resulted in a 2−4 times increase in DNA fragmentation. The highest DNA fragmentation level was observed with ~80 μg/mL anthocyanin fraction. A further increase in anthocyanin level slowed DNA fragmentation. As a control reagent, KOH resulted in a significant decrease in DNA fragmentation compared with no treatment.

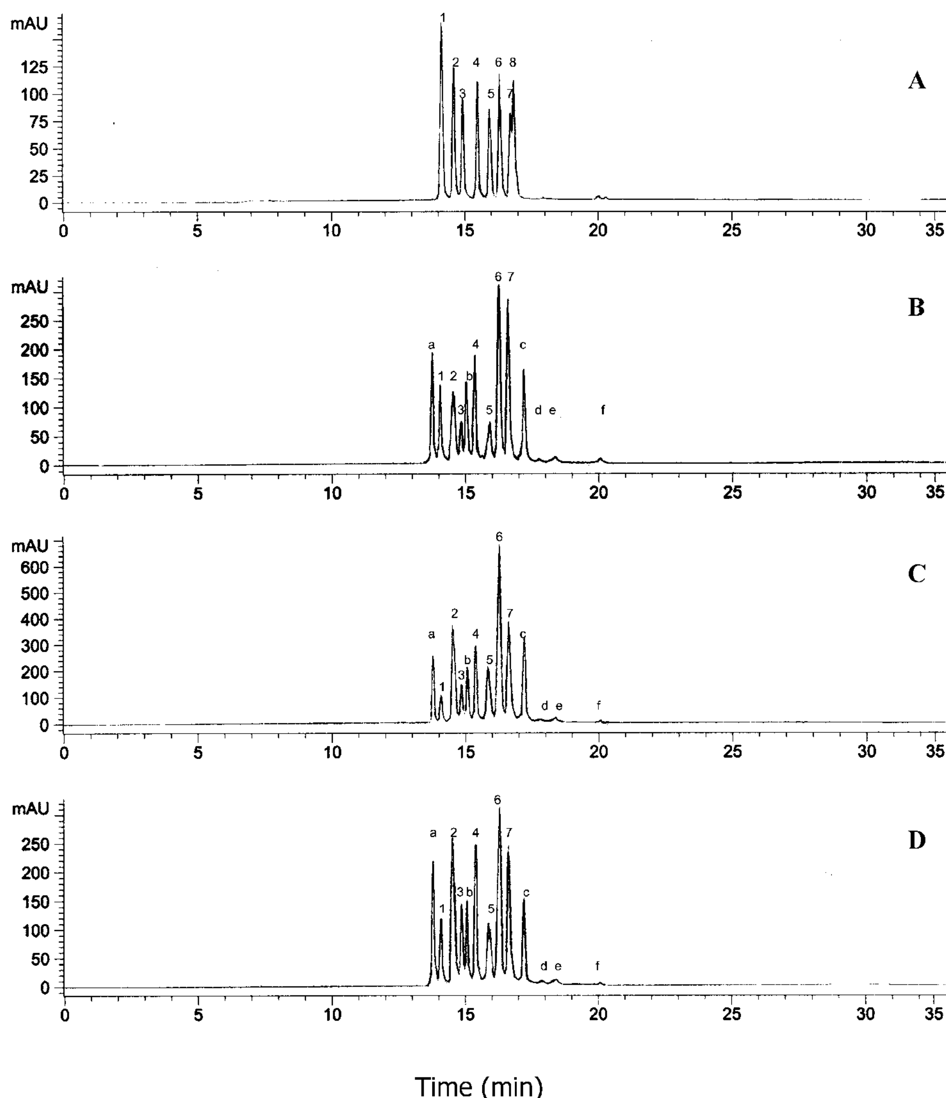

Figure 1. Chromatogram of analytical HPLC of blueberry anthocyanin fractions: (**A**) anthocyanin standards; (**B**) Briteblue fraction; (**C**) Tifblue fraction; (**D**) Powderblue fraction. Peaks: (1) Dp-Glc; (2) Cy-Gal; (3) Cy-Glc; (4) Pt-Glc; (5) Pn-Gal; (6) Pn-Glc; (7) Mv-Glc; (8) Pn-Ara; (a–f) unidentified.

DISCUSSION

The phenolic contents of blueberry have been reported by others (*27*−*29*). In general, the phenolic acid profile of the phenolic acid fraction in this paper is consistent with previous analytical measurements (*17*, *30*). Caffeic acid, as reported here, was not at detectable levels in Briteblue and Tifblue blueberries in our previous studies (*17*). This could be due to seasonal and environmental differences. The Powderblue cultivar was not evaluated in our previous study. Catechin was found in the phenolic acid fraction, probably because catechin has a polarity similar to that of phenolic acids. This similarity can be observed from their close retention times. The two major flavonols (kaempferol and quercetin) in the flavonol fractions in the current study were in good agreement with the reports of Kader et al. (*30*).

The profile of anthocyanins in the current study is in good agreement with previous results (*30*−*32*). Five anthocyanidins (Dp, Cy, Pt, Pn, and Mv) were reported in Tifblue and Powderblue cultivars by Ballington et al. (*31*). This is a perfect match with the anthocyanidin profile of the anthocyanin fractions in the current study. Although three peaks could not be identified because we did not have the standards, all seven major anthocyanins identified matched with previous results. Our hydrolyzed products (aglycons) were in good concurrence with individual anthocyanin measurements except in the Briteblue cultivar, for which ∼50% Pn was lost. More malvidin was found after hydrolysis. This indicates that some unidentified peaks were malvidin monoglycosides. From the studies of Ballington et al. (*31*), the other two could be Mv-gal and Mv-ara.

The loss of phenolic compounds can occur during the fractionation process. In addition, certain phenolic compounds that were not detectable can become detectable because of the concentration effects during the fractionation and freeze-drying process.

In the healthy state, cell renewal/proliferation is balanced by cell death/apoptosis. During tumor development a shift toward proliferation may alter the balance. Deregulated cell proliferation and suppression of apoptosis provides a minimal "platform" for further neoplastic progression. Scientists have stated that targeting of these critical events should have potent and specific therapeutic consequences (*33*). In other words, even minute effects on apoptosis induction and cell antiproliferation may help maintain balance, thereby decreasing the chance of cancer progression.

Our antiproliferation and apoptosis induction results are in good agreement with several previous studies. In an evaluation of the bioactivities of bilberry, Katsube et al. (*22*) stated that it

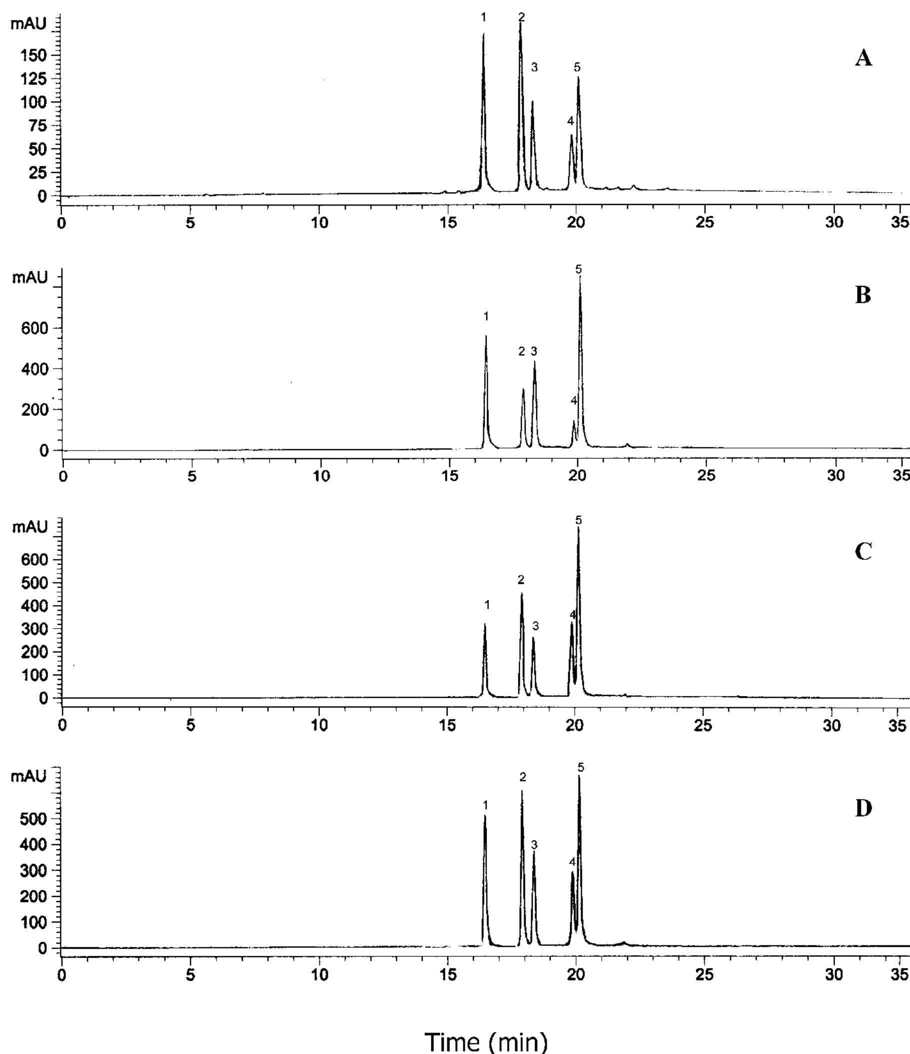

Figure 2. Chromatogram of analytical HPLC of blueberry anthocyanin fractions after acid hydrolysis: (**A**) anthocyanidin standards; (**B**) Briteblue fraction; (**C**) Tifblue fraction; (**D**) Powderblue fraction. Peaks: (1) delphinidin; (2) cyanidin; (3) petunidin; (4) peonidin; (5) malvidin.

Table 4. Major Individual Anthocyanidins (Weight Percent) in the Anthocyanin Fraction after Acid Hydrolysis[a]

cultivar	delphinidin	cyanidin	petunidin	peonidin	malvidin	total anthocyanidins
Briteblue	18.6 ± 0.9	10.0 ± 0.4	30.3 ± 0.5	9.1 ± 1.3	21.4 ± 0.6	89.3 ± 3.2
Tifblue	12.6 ± 0.8	17.5 ± 1.1	22.1 ± 0.8	25.2 ± 1.2	20.0 ± 0.7	97.4 ± 4.1
Powderblue	20.0 ± 0.3	21.2 ± 0.6	26.2 ± 0.7	15.5 ± 0.9	16.8 ± 1.1	98.7 ± 3.3

[a] Values are averages of triplicate analyses ± standard error. The amount of different anthocyanidins is expressed on the basis of the specific weight of anthocyanins.

was the anthocyanins in the bilberry extract that inhibited the growth of HL-60 human leukemia cells through apoptosis. They also found that the inhibitory effects of malvidin and delphinidin were greater than those of the flavonols. In the current study, we cannot state that the anticancer activities of flavonols were lower than those of anthocyanins in rabbiteye blueberry because the purity of the flavonol fractions was much lower than that of the anthocyanin fractions. Instead, we can suggest that anthocyanin was one of the major components that inhibited colon cancer cell proliferation and induced apoptosis. For a comparison of effective dosage levels, in a study by Katsube et al. (*22*), 2−6 mg/mL of an ethanol extract of selected berries (including blueberry) resulted in a significant inhibition of HL-60 and HCT116 cell growth. Anthocyanin fractions obtained by semipreparative HPLC in the same study resulted in higher levels of cancer cell inhibition and apoptosis at 300 μg/mL. This is in good agreement with our findings for HT-29 and Caco-2 cells in the current study. The IC_{50} of crude blueberry extracts ranged from 1000 to 3000 μg/mL, and the IC_{50} of anthocyanin fractions ranged from 15 to 50 μg/mL. The lower effective dosage level in the current study could be because we used different cancer cell lines. In another study using chokeberry, anthocyanin-rich extracts (AREs) inhibited HT-29 cell growth by ∼50% after 48 h of exposure at 25 μg/mL (*34*). This is very close to the IC_{50} level in anthocyanin fractions we observed in the current study. In the study by Zhao et al. (*34*) the AREs contained 737 mg of total polyphenolics and 102 mg of monomeric anthocyanin per gram of extract. The purity of anthocyanin fractions in the current studies was ∼90%.

In the current study, 70−100 μg/mL of procyanidin fractions inhibited HT-29 and Caco-2 cell growth by ∼50%. These effective dosage levels were close to those reported for lowbush (wild) blueberries (*35*). In lowbush blueberry (*V. angustifolium* Ait.), proanthocyanidin-rich fractions exhibited significant an-

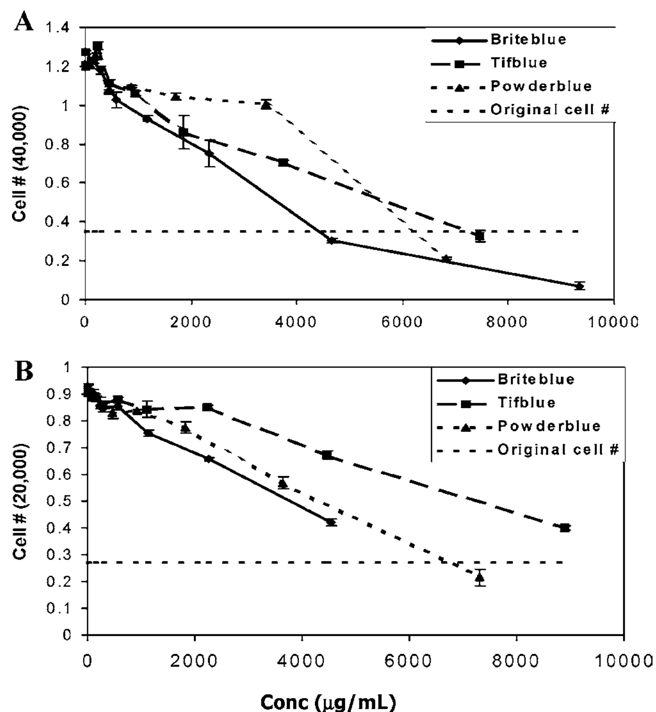

Figure 3. Inhibition of HT-29 (**A**) and Caco-2 (**B**) cancer cell proliferation by crude extracts of blueberries (mean ± SD, $n = 8$). The x-axis gives the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h of incubation and before treatments were applied.

tiproliferation activities in human prostate (LNCaP) and murine liver (Hepa 1c1c7) cancer cell lines (*35*). The evaluated concentration of proanthocyanidin-rich fractions was 20 μg/mL. The authors stated that there was a significant positive correlation between proanthocyanidin content of different fractions and antiproliferation activity.

The cancer prevention effects of Cy-glc were evaluated in human promyelocytic leukemia cell line HL-60 and Jurkat T-leukemia cells (*36*). The IC_{50} was 174.9 μg/mL Cy-glc in Jurkat cells, with a higher effective concentration in HL-60 cells after 24/30 h of exposure. Although the calculation formula was slightly different from that used in the current study, we can still find some interesting comparisons. Fimognari et al. (*36*) observed that in Jurkat cells, 12.5 μg/mL Cy-glc was sufficient to induce significant characteristics of apoptosis. Necrotic cells were observed at higher concentration. This finding is in good agreement with our DNA fragmentation results. Low DNA fragmentation was observed at very high dosage levels, and the highest DNA fragmentation levels were observed around or lower than the IC_{50}. This suggests that anthocyanins can induce apoptosis at a low/appropriate level, but promote necrosis at high dosages. This phenomenon has also been found with other compounds, such as the antitumor drug fostriecin. Fostriecin is a good apoptosis-inducing agent, yet it can cause necrosis in human promyelocytic HL-60 and lymphocytic MOLT-4 leukemic cells after it reaches certain dosage levels (*37*). Identification of the effective dosage levels of anthocyanins can provide valuable information for further clinical studies and cancer prevention.

The slight increase in HT-29 cell proliferation at low concentrations of the tannin fraction is interesting. A similar phenomenon was observed in Jurkat and HL-60 cells after treatment with Cy-glc (*36*), although the authors did not specify if the increase was statistically significant. The underlying mechanisms are unclear and need further study. This phenomenon further clarifies the importance of the identification of the effective dosage levels associated with different phenolic compounds and other phytochemicals.

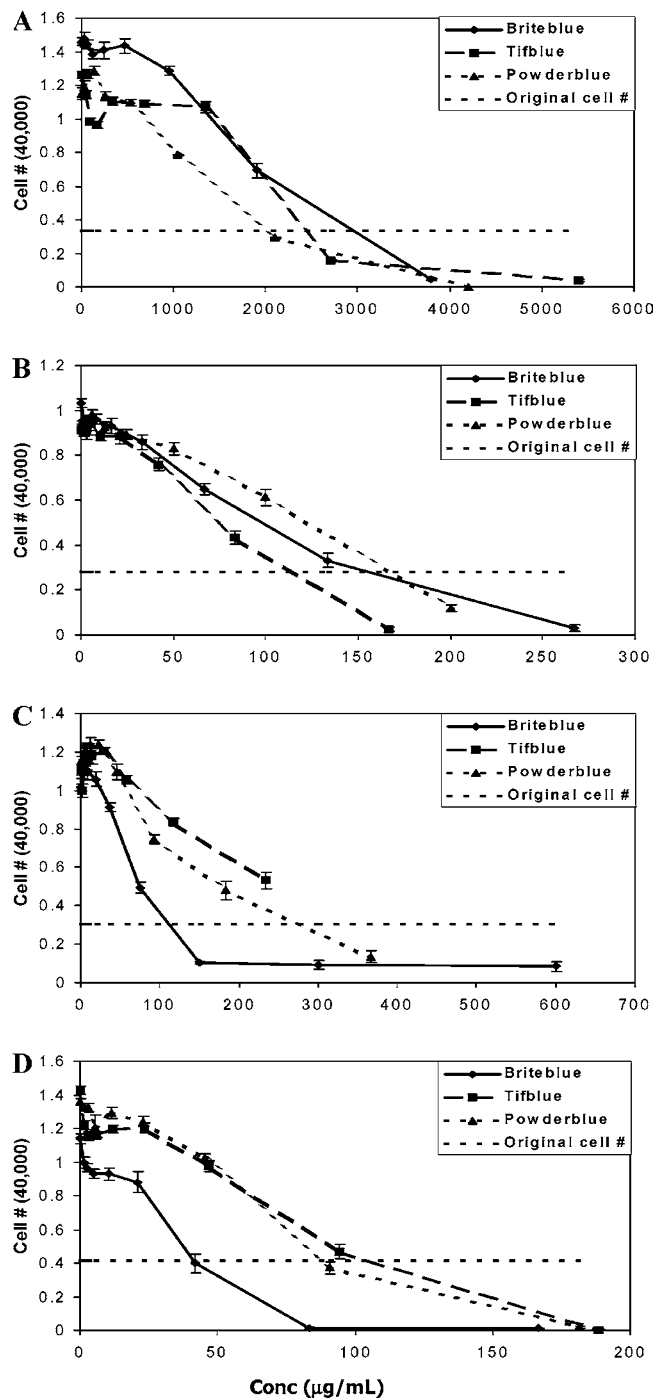

Figure 4. Inhibition of HT-29 cancer cell proliferation by different fractions of blueberries (mean ± SD, $n = 8$): (**A**) phenolic acid fraction; (**B**) flavonol fraction; (**C**) tannin fraction; (**D**) anthocyanin fraction. The x-axis gives the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h of incubation and before treatments were applied.

Apoptosis is one of the most important mechanisms for antitumor activity (*38*). It is a complicated process involving many factors, and researchers are continuing to clarify its role in cancer control. It has been reported that the apoptosis induction effects of Cy-glc in Jurkat T cells can be explained by a modulation of p53 and bax protein expression (*36*). The

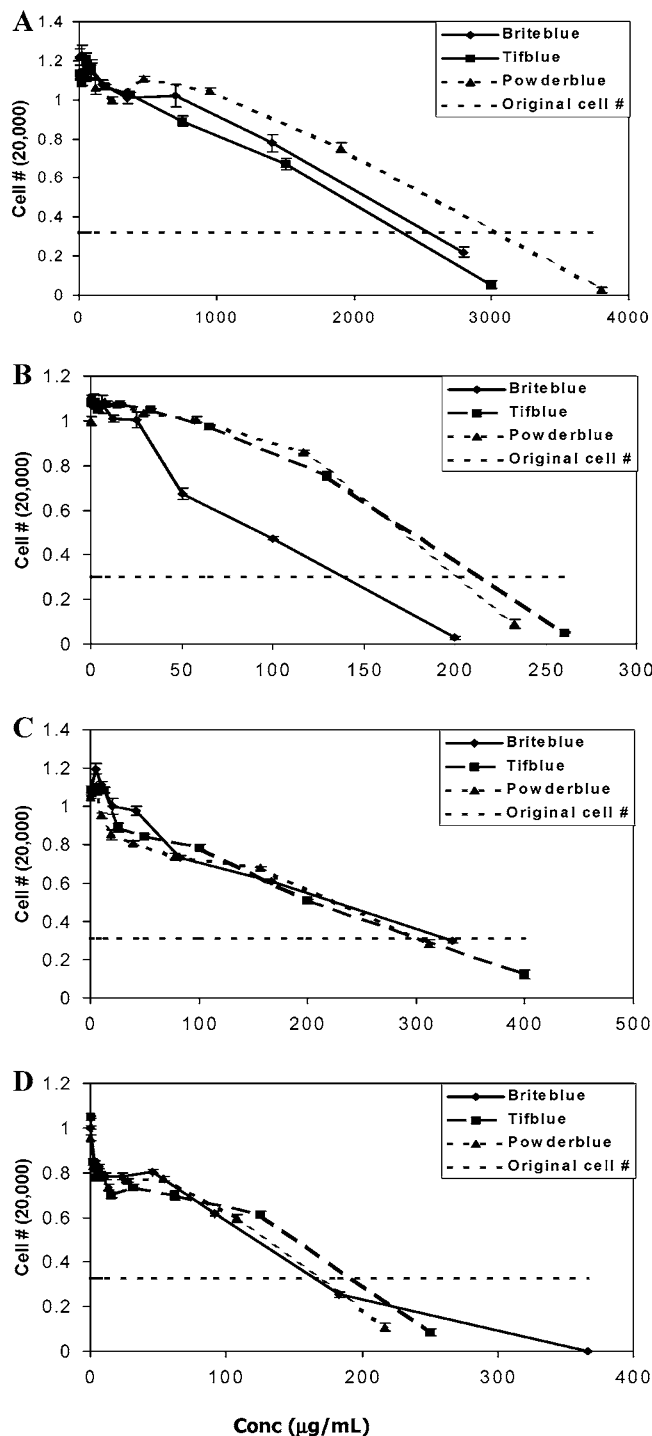

Figure 5. Inhibition of Caco-2 cancer cell proliferation by different fractions of blueberries (mean ± SD, $n = 8$): (**A**) phenolic acid fraction; (**B**) flavonol fraction; (**C**) tannin fraction; (**D**) anthocyanin fraction. The *x*-axis gives the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h of incubation and before treatments were applied.

mechanisms involved in apoptosis induction by blueberry extracts need further investigation. Studies are ongoing in our laboratory to further clarify the possible mechanisms.

To correctly relate the in vitro study results to human disease outcomes, where exposure to polyphenols is chronic and at relatively low concentrations, information about bioavailability and metabolism is required (*39*). Studies have shown that anthocyanin concentration can reach a few micrograms per milliliter in rat plasma. Miyazawa et al. (*40*) reported 1.563 μg/mL Cy-glc in plasma after rats were fed fruit anthocyanins. The effective antiproliferation concentrations of anthocyanin fractions in the current study are close to this level; for instance, the IC_{50} was ~15 μg/mL in Caco-2 cells. In addition, a significant antiproliferation effect (10−20% inhibition) was observed at a level of ~1 μg/mL in Caco-2 and HT-29 colon cancer cell lines. This is indeed within the reported absorption level. The reported concentration of anthocyanins in human plasma is 0.005−0.05 μg/mL (assuming an average molecular mass of 465 g/mol for unit conversion) (*41*). Scientists have pointed out that the bioavailability of anthocyanins could have been underestimated, because some important metabolites might have been ignored or the methods used might need to be optimized for the analysis of anthocyanin metabolites (*41*). In addition, higher levels of phenolic compounds have been detected in animal intestine after oral supplementation (*42*, *43*). Phenolic compounds could inhibit colon cancer development in human intestines before they are absorbed and are detected in the plasma.

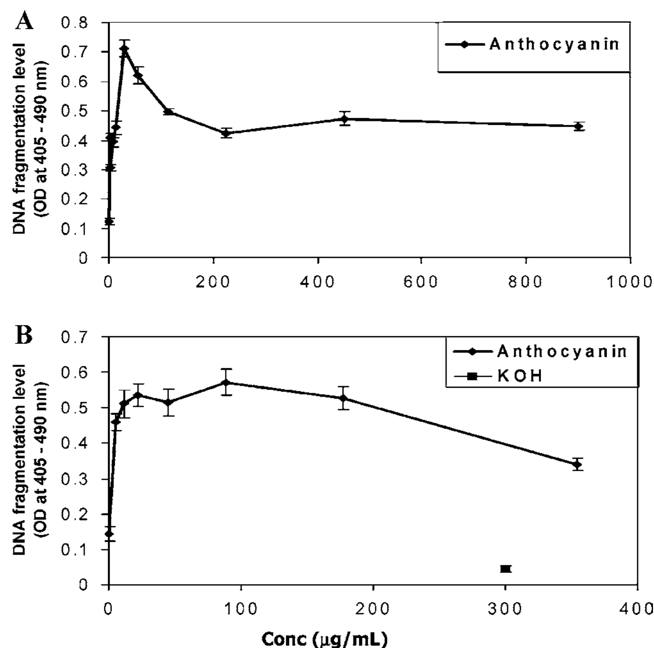

Figure 6. DNA fragmentation in HT-29 and Caco-2 cells associated with anthocyanin fractions (mean ± SD, $n = 3$): (**A**) anthocyanin fractions of Briteblue in HT-29 cells; (**B**) anthocyanin fractions of Tifblue in Caco-2 cells. The *x*-axis gives the concentration (μg/mL) of extracts in the culture medium. 1 mol/L of KOH was used as a control reagent to induce necrosis.

Without doubt, in vivo animal studies and human intervention studies will provide more solid evidence in this area. Phenolic compounds may act differently under in vivo conditions as a result of metabolism or by altering growth factors that inhibit or promote tumor growth, by affecting the activity of detoxification enzymes, or by changing the immune response of the host. Animal studies are currently in progress in our laboratory for further clarification of the bioactivities of phenolic compounds in blueberries.

In conclusion, our study found that phenolic compounds in rabbiteye blueberry could inhibit colon cancer cell proliferation and induce cancer cell apoptosis. Further studies are required to clarify the mechanisms and to evaluate the bioavailability and metabolism of phenolic compounds in blueberries before it can be determined that blueberry intake can reduce colon cancer risk.

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