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Structure−Function Relationships of Anthocyanins from Various Anthocyanin-Rich Extracts on the Inhibition of Colon Cancer Cell Growth

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Anthocyanins are potent antioxidants and may be chemoprotective. However, the structure−function relationships are not well understood. The objectives of this study were to compare the chemoprotective properties of anthocyanin-rich extracts (AREs) with variable anthocyanin profiles to understand the relationship between anthocyanin chemical structure and chemoprotective activity, measured as inhibition of colon cancer cell proliferation. Additionally, the chemoprotective interaction of anthocyanins and other phenolics was investigated. AREs with different anthocyanin profiles from purple corn, chokeberry, bilberry, purple carrot, grape, radish, and elderberry were tested for growth inhibition (GI_{50}) using a human colorectal adenocarcinoma (HT29) cell line. All AREs suppressed HT29 cell growth to various degrees as follows: purple corn ($GI_{50} \sim 14$ μg of cy-3-glu equiv/mL) > chokeberry and bilberry > purple carrot and grape > radish and elderberry ($GI_{50} > 100$ μg of cy-3-glu equiv/mL). Anthocyanins played a major role in AREs' chemoprotection and exerted an additive interaction with the other phenolics present. Statistical analyses suggested that anthocyanin chemical structure affected chemoprotection, with nonacylated monoglycosylated anthocyanins having greater inhibitory effect on HT-29 cell proliferation, whereas anthocyanins with pelargonidin, triglycoside, and/or acylation with cinnamic acid exerted the least effect. These findings should be considered for crop selection and the development of anthocyanin-rich functional foods.

KEYWORDS: Anthocyanins; colon cancer; HT29; cell proliferation; anthocyanin-rich extracts

INTRODUCTION

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive red, orange, purple, and blue colors of most fruits and vegetables. Interest in anthocyanins as natural colorants and value-added ingredients has increased due to their color characteristics and potential health benefits. Anthocyanins are the most abundant dietary flavonoids. Anthocyanin consumption has been estimated to be as high as ∼200 mg/day/person (*1*), although a more recent study (*2*) reported anthocyanin consumption at about 12.5 mg/day/person in the United States, compared to the average daily intake of other flavonoids (23 mg/person) (*3*). Many fruits and vegetables are rich in anthocyanins, including berries, purple carrot, purple corn, red radish, red cabbage, and others. Their anthocyanin profiles may vary greatly according to the commodity, with differences in the type of aglycone, type and number of glycosylations, and presence of acylating groups. Structures of anthocyanidins (anthocyanin aglycones) commonly found in fruits and vegetables are shown in **Figure 1**.

Colon cancer is the third most common cancer and the third leading cause of cancer death for both men and women in the United States (*4*). Epidemiological studies (*5*) show a protective effect of fruits and vegetables against chronic diseases, including cancer, and anthocyanins may contribute to those protective effects. Anthocyanin-rich foods and anthocyanin pigments have been suggested as potential agents to reduce the risk of colon cancer by inhibiting proliferation of human colon cancer cells in vitro (*6*−*10*). Anthocyanin-rich extracts (AREs) from berries including blueberries, black currant, black chokeberries, lingonberries, cherries, and raspberries suppressed the proliferation of HT29 cells in a dose-dependent manner (*6*). AREs from grape, bilberry, and chokeberry suppressed HT29 cell proliferation by 50% at concentrations of 25−75 μg/mL (equivalents as cyanidin 3-glucoside), with chokeberry being the most potent

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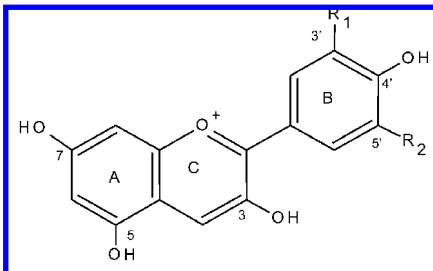

Anthocyanidins	Substitutes		λ_{max} (nm) visible spectra	Molecular Weight
	R_1	R_2		
Pelargonidin	H	H	494 (orange)	271
Cyanidin	OH	H	506 (orange-red)	287
Delphinidin	OH	OH	508 (red)	303
Peonidin	OCH_3	H	506 (orange-red)	301
Petunidin	OCH_3	OH	508 (red)	317
Malvidin	OCH_3	OCH_3	510 (bluish-red)	331

Figure 1. Structures of common anthocyanidins in fruits and vegetables.

inhibitor; however, the same concentrations had little effect on the growth of nontransformed colon epithelial cells (NCM460) (*7*).

Anthocyanin-rich fractions from bilberries (*8*), blueberries (*9*), and red wine (*10*), rather than other fractions, were found to effectively inhibit the growth of many human colon cancer cell lines, including CaCo-2 (*9*), HCT116 (*8*), HCT15 (*10*), HL60 (*8*), and HT29 (*9*). Different in vivo studies have shown that anthocyanin-rich foods and anthocyanin pigments from food were potent inhibitors of carcinogenesis in the colon (*11*−*13*).

Anthocyanin structure−function relationships are not well understood and are difficult to assess from the literature as methods to evaluate growth inhibitory activity of anthocyanin-rich sources have varied in sample preparation methods and screening assays (*6*−*10*). Thus, the aim of the present research was to understand the relationship between anthocyanin chemical structures and their corresponding biological activity. For this purpose, we evaluated the colon cancer cell chemoprotective activity of several commercial AREs with different and distinctive anthocyanin profiles that are being used by the food industry as natural colorants or value-added food ingredients. Synergistic or antagonistic effects between anthocyanins and other phenolics were also evaluated.

MATERIALS AND METHODS

Anthocyanin Sources. Seven AREs with different pigment profiles were used (**Table 1**). These were chokeberry (*Aronia meloncarpa* E.), elderberry (*Sambucus nigra* L.), bilberry (*Vaccinium myrtillus* L.), grape (*Vitis vinifera* L.), purple carrot (*Daucus dacota* L.), purple corn (*Zea mays* L.), and red radish (*Raphanus sativus* L.). All extracts were commercially available and were kindly donated by the suppliers (**Table 1**).

HT29 Cell Line. The HT29 cell line derived from a colorectal adenocarcinoma (HTB 38; American Type Culture Collection, Manassas, VA) was grown in McCoy's 5A medium (Fisher Scientific, Fair Lawn, NJ), which was supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) at 37 °C and 5% CO_2 atmosphere.

Reagents and Solvents. All reagents and solvents for HPLC analyses, sample fractionation, and the Sulforhodamine B (SRB) assay were purchased from Fisher Scientific. Folin−Ciocalteu phenol reagent and standard of gallic acid (crystalline gallic acid, 98% purity) were purchased from Sigma (St. Louis, MO).

Methodology Optimization. The SRB assay is a colorimetric method used to determine cell proliferation by measuring the cellular protein content. The detailed methodology for the SRB assay is described later. For reliable measurements, the absorbance readings should be within the linear range, which is affected by cell seeding density. To determine the appropriate seeding density, the concentration of cell seeding in the 24-well plate had to be determined to satisfy the linear regression with the final reading after 72 h of incubation. Different levels of cell number (0, 1×10^3, 5×10^3, 1×10^4, 5×10^4, and 1×10^5 cells/mL) were plated and assayed with SRB after 72 h of growth. Treatments were seeded with four replicates per plate. A line regression was obtained to determine the optimal cell seeding concentration.

The ARE preparation procedure was also optimized to minimize the effects of residual acids used during sample preparation on cell proliferation using chokeberry extracts as a test sample. Chokeberry ARE was semipurified using a solution acidified either by 0.01% hydrochloride acid (HCl) or by 1% acetic acid. Solvents and acids were removed via rotary evaporation or rotary evaporation plus lyophilization.

Semipurification of Anthocyanin Extracts. Commercial AREs were semipurified using the method described by Giusti and Wrolstad (*14*), modified for use of the extracts on cell cultures. Briefly, about 0.5 g of each commercial ARE was dissolved in 25 mL of deionized water, sonicated for 15 min in an FS28H Ultrasonic Cleaner from Fisher Scientific, and filtered through a Whatman no. 4. The sample was semipurified by passing 3−5 mL aliquots through the C18 Sep-Pak solid cartridge (5 g) (Waters Corp., Milford, MA). Anthocyanins and other phenolics were bound to the C18 cartridge, whereas sugars and other polar compounds were removed with 30 mL of 1% acetic acid-acidified water, followed by 10 mL of hexane to remove 1 volume of water dead column. Anthocyanins and other phenolics were recovered from the cartridge with 30 mL of methanol containing 1% acetic acid and 5 mL of deionized water. The methanol was removed by rotary evaporation at 40 °C in a Büchi Rotavapor (Brinkmann Instruments, Inc., Westbury, NY), and the residue was taken up to about 10 mL with deionized water in a volumetric flask and then frozen at −70 °C. Samples were then lyophilized in a Labconco freeze-dry system (Labconco Corp., Kansas City, MO). Dried samples were stored in a freezer until treatments were performed.

Fractionation of Anthocyanins and Other Phenolics. Anthocyanin-rich extracts were separated into an anthocyanin fraction (ACN) and another phenolics fraction (OPF) by using solid phase extraction. About 0.5 g of each commercial ARE was dissolved in 25 mL of deionized water, sonicated for 15 min, and filtered with a Whatman no. 1 filter paper. The filtrate (∼5 mL) was passed through a C18 Sep-Pak solid cartridge (5 g, Waters Corp.) previously activated with methanol followed by acidified water (*14*). Anthocyanins and other phenolics were bound to the C18 cartridge, whereas sugars and other polar compounds were removed with 30 mL of 0.1% HCl-acidified water, followed by 10 mL of hexane. The non-anthocyanin phenolics (OPF) were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin fraction was eluted with 30 mL of methanol containing 1% acetic acid followed by 5 mL of deionized water. The OPF was mixed with 5 mL of deionized water before rotary evaporation of the solvents and to keep the procedure similar to the one followed for ACN fraction. Organic solvents were removed by rotary evaporation at 40 °C, and each residue was taken to 10 mL with deionized water, then frozen and lyophilized. Dried samples were stored until used for biological tests. For that, dried ACN and OPF were redissolved in equal amounts of deionized distilled water. In addition, a reconstituted ARE was prepared by mixing equal volumes of ACN and OPF to determine if the fractionation procedure had altered the composition or chemoprotection of the ARE components.

Cell Growth Inhibition. Human colorectal adenocarcinoma HT29 cells were plated at 1.3×10^4 cells/well in 24-well plates (Falcon) using McCoy's 5A medium containing 10% fetal bovine serum. Cells were allowed to grow for 24 h to achieve log phase growth at the time of treatment (time 0). Semipurified AREs, ACN, and OPF were tested on HT29 cells for their inhibitory effect at concentrations ranging from 0 to 200 μg/mL as cyanidin-3-glucoside equivalents in growth media. The ratio of ACN and OPF was kept the same as in the AREs. Therefore, the OPF was quantified as ACN equivalents at the same volume. HT29 cell growth was determined by using the SRB assay

Table 1. Major Anthocyanin Profiles in Seven Commercial Anthocyanin-Rich Sources

source	aglycone[a]	glycosylation	acylation	ref	supplier[b]
chokeberry (*Aronia meloncarpa* E.)	Cy	C3: monoglycoside	none	*29*	1
elderberry (*Sambucus nigra* L.)	Cy	C3: mono-, diglycoside C3 and C5: di-, triglycoside	none	*29*	1
bilberry (*Vaccinium myrtillus* L.)	Dp, Cy, Pt, Pn, Mv	C3: monoglycoside	none	*34*	1
purple corn (*Zea mays* L.)	Cy, Pt, Pn	C3: monoglycoside	one aliphatic acid	*35*	2
purple carrot (*Daucus carota* L.)	Cy	C3: di-, triglycoside	one cinnamic acid	*36*	3
radish (*Raphanus sativus* L.)	Pg	C3 and C5: triglycoside	more than one cinnamic acid	*37*	4
grape (*Vitis inifera* L.)	Dp, Cy, Pt, Pn, Mv	C3: monoglycoside	one cinnamic acid	*21*	5

[a] Abbreviations: Cy, cyanidin; Dp, delphinidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; Mv, malvidin. [b] 1, Artemis International, Inc. (Fort Wayne, IN); 2, Globenatural International S.A. (Chorrillos-Lima, Peru); 3, Overseal Foods Ltd. (Derbyshire, U.K.); 4, RFI Ingredients (Blauvelt, NY); 5, Polyphenolics, Inc. (Madera, CA).

after an additional 48 h of incubation. Every treatment was plated in four replications, whereas the experiments were repeated three to four times. The percentage growth inhibition was calculated as

$$\%\ \text{growth inhibition} = 100 - (T_{trt} - T_0) \times 100/(T_{ctr} - T_0) \quad (1)$$

T_{trt} is the absorbance of sample with treatment of anthocyanins at 565 nm, T_0 is the absorbance of sample after the first 24 h incubation (time 0) prior to the treatment of anthocyanins, and T_{ctr} is the absorbance of sample without treatment of anthocyanins after the total incubation.

The growth inhibition of *i*% (GI*i*) indicated that the anthocyanins or others caused an *i*% reduction in the net protein increase in control cells. GI*i* values were calculated from every regression equation.

The interaction of anthocyanins with other phenolics was further studied by using the combination index (CI) reported by Chou and Talalay (*15*). The combination index values were calculated on the drug effect equation to compare the concentrations of individual anthocyanin fraction and other phenolics fraction with their relative concentration when they were applied together to inhibit the same amount of HT29 cell growth. The equation used was the following:

$$CI_i = \frac{[ACN_0]}{[ACN_1]} + \frac{[OPF_0]}{OPF_1} \quad (2)$$

$[ACN_1]$ and $[OPF_1]$ are the concentrations of ACN and OPF to inhibit certain amounts (*i*%) of cell growth when they are tested separately on the cells. $[ACN_0]$ and $[OPF_0]$ are the concentrations of ACN and OPF to inhibit certain amounts (*i*%) of cell growth when they are tested together.

Sulforhodamine B Assay. The detailed methodology for the SRB assay was described by Skehan and co-workers (*16*). Briefly, cells were fixed by addition of 250 μL of 50% trichloroacetic acid (TCA) at 4 °C for 1 h. TCA and media were removed, and wells were washed with water five times and dried at room temperature. SRB (0.4% in acetic water) of 500 μL was added to each well to stain cells at room temperature for 20 min. Wells were washed using 1% acetic acid five times and dried at room temperature. The incorporated dye was then solubilized with 1 mL of 10 mM Tris for 5 min at room temperature on a shaker. The absorbance at wavelength of 565 nm was measured using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Monomeric Anthocyanins. The total monomeric anthocyanin content was measured by the pH differential method (*17*). An HP UV−visible spectrophotometer (Agilent Technologies, Inc., Palo Alto, CA) was used to read absorbance at the maximum visible wavelength of absorption of each extract (ranging from 500 to 525 nm) and at 700 nm. Monomeric anthocyanins were calculated as equivalents of cyanidin-3-glucoside, using the extinction coefficient of 26900 L cm^{-1} mg^{-1} and a molecular mass of 449.2 g/L (*17*) or, in the case of radish, which contains only pelargonidin derivatives, as equivalents of pelargonidin-3-glucoside, using the extinction coefficient of 31600 L cm^{-1} mg^{-1} and a molecular mass of 433.2 g/L (*14*). Disposable cuvettes of 1 cm path length were used.

Total Phenolics. Total phenolics were measured using the modified microscale protocol for Folin−Ciocalteu colorimetry (*18*). Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve. Instead of using cuvettes, 24-well plates were read in a Multi-Detection Microplate Reader. Briefly, samples and a concentration series of gallic acid calibration standards (0−500 mg/L) were put in 2 mL vials. Deionized water was added to 1.6 mL, followed by 100 μL of Folin−Ciocalteu reagent. Vials were mixed well by inverting and standing at room temperature for 5 min. Then 300 μL of 20% sodium carbonate solution was added and thoroughly mixed. The final volume was 2 mL. Samples were incubated in 40.0 ± 0.1 °C for 20 min and cooled to room temperature immediately in ice. Each sample (1 mL) was transferred to a 24-well plate, and absorbance was measured at 765 nm.

HPLC Analysis. A high-performance liquid chromatograph (HPLC) system on a 2695 Separation Module (Waters) equipped with an autosampler, a 996 photodiode array detector, and Empower software was used to monitor the phenolic composition of extracts used.

A reversed-phase 5 μm Symmetry C18 column (4.6 × 150 mm, Waters Corp., Milford, MA) fitted with a 4.6 × 22 mm Symmetry 2 micro guard column (Waters Corp.) was used. The solvents used were (A) 1% formic acid in water and (B) 100% acetonitrile. Solvents and samples were filtered though 0.45 μm poly(tetrafluoroethylene) membrane filters (Pall Life Sciences, Ann Arbor, MI) and 0.45 μm polypropylene filters (Whatman Inc., Clifton, NJ), respectively. Separation was achieved by using a linear gradient from 0 to 25% B in 20 min. An injection volume of 50 μL with a 1 mL/min flow rate was used. Spectral information over the wavelength range of 270−700 nm was recorded.

Statistical Analysis. Regression analysis was used to model the growth inhibition of cells with the different ARE treatments. The Tukey HSD test was used to evaluate mean differences among GI_{50} values or growth inhibition (%) when at the same concentration in a one-way ANOVA model. The Student *t* test was used to determine differences in the mean combination index (CI) values compared with a null hypothesized CI = 1 ($p < 0.05$). The effects of anthocyanin chemical structure on the properties of the growth inhibition in colon cancer cells were evaluated by classifying anthocyanins according to the type of aglycone (six typical aglycones), number of glycosidic substitutions (one, two, or three), and acylation (no acylation versus aliphatic or cinnamic acid substitutions). The multiple linear regression analysis in a general linear univariate model was conducted to determine if the presence of acylating groups, type of aglycone, and number of sugar substitutions on the anthocyanin molecule could affect their chemoprotective properties using a stepwise procedure to remove variables that were not significantly related to anthocyanin growth inhibitory activity.

All analyses were performed using SPSS (14.0) software. For all statistical calculations, $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Methodology Development. The SRB assay was applied throughout this study to determine cell growth. This method was chosen over the commonly used MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay because it avoids the interference of residual anthocyanins in the final reading. In the SRB assay, the interference of the pigments in the media is eliminated by fixing the cells in situ and removal of all residual media and anthocyanins by washing with acidified

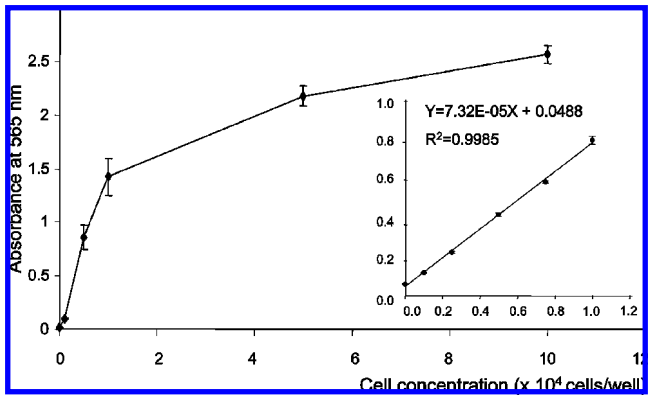

Figure 2. Effect of seeding concentration of HT29 cells in 24-well plate on final reading via SRB assay after 72 h of incubation. Values are represented as mean ± standard error ($n = 4$).

water. SRB is a colorimetric cytotoxicity assay and indirectly determines cell proliferation by measuring the amount of protein that binds to the dyes and determining their absorbance. It is important that the final optical density is within the linear range to retain methodological sensitivity. The final cell density is affected by seeding density, cell size, growth rate, and incubation duration. Therefore, we determined the HT29 seeding density that would satisfy the optical density linearity after 72 h of incubation. When the seeding density was $>5 \times 10^4$ cells/well (absorbance ~ 1.5 units), the final optical density deviated from the linear range (**Figure 2**). The seeding density in the 24-well plate was then determined in a linear range that was obtained with cells seeded at concentrations of up to 1×10^4 cells/well (**Figure 2**). The seeding concentration of 1.3×10^4 HT29 cells/well showed an absorbance of ~1 at 565 nm after 72 h of incubation, which was calculated on the regression curve and equation ($R^2 = 0.9985$) shown in **Figure 2**. Therefore, the seeding concentration of 1.3×10^4 HT29 cells/well was used for this study.

Anthocyanins exhibit improved stability under acidic conditions. For that reason, solvents were acidified to stabilize anthocyanins during semipurification and fractionation. However, residual acids in the extracts could affect cell growth and result in an overestimation of growth inhibition obtained with the extracts (*19*). To minimize these effects, different acids were evaluated as well as the different drying procedures used to remove solvents and acids. Chokeberry ARE was semipurified using solution acidified either by 0.01% hydrochloride acid (HCl) or by 1% acetic acid. The solvents and acids were removed via rotary evaporation or rotary evaporation plus lyophilization. The sample prepared using 0.01% HCl-acidifed solvents dried using a rotary evaporator showed significantly higher inhibition than the two samples prepared with 1% acetic acids ($p < 0.05$) regardless of the drying process (**Figure 3**). The sample prepared with 0.01% HCl-acidifed solvents and dried with a combination of rotary evaporation plus lyophilization also showed higher inhibition than samples prepared with 1% acetic acid-acidifed solvents, although not significantly different ($p > 0.05$) in **Figure 3**. These results suggest that rotary evaporation followed by lyophilization was more efficient in removing acids from the samples than rotary evaporation alone and that the HCl residue was more toxic to the cells than the acetic acid residue. For that reason, samples for our biological tests were prepared using 1% acetic acid acidification of the solvents followed by drying with a rotary evaporator followed by lyophilization.

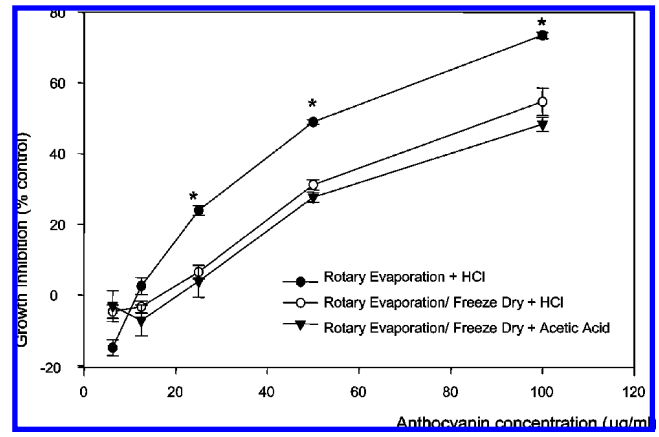

Figure 3. Effect of sample preparation on the growth of HT29 cells: rotary evaporation + HCl, sample was prepared by 0.01% HCl-acidified solvent, which was removed by rotary evaporator; rotary evaporation/freeze-dry + HCl, sample was prepared by 0.01% HCl-acidified solvent, which was removed by rotary evaporation plus lyophilization; rotary evaporation/freeze-dry + acetic acid, sample was prepared by 1% acetic acid-acidified solvent, which was removed by rotary evaporation plus lyophilization. Values are represented at mean ± standard error ($n = 4$).

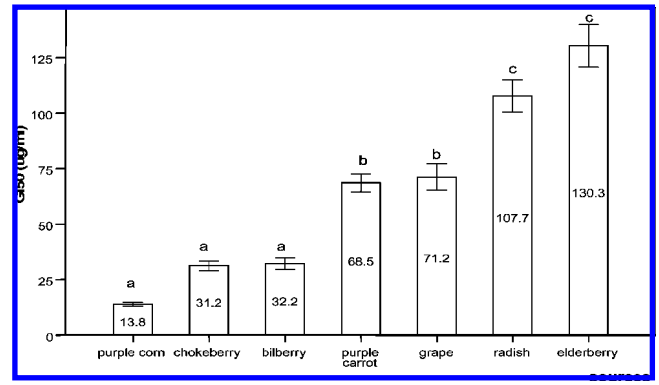

Figure 4. GI_{50} of anthocyanin-rich extracts (based on the monomeric anthocyanins) from seven natural sources on the growth inhibition of HT29 cell line. Groups a−c were divided into homogeneous subsets of GI_{50} means of anthocyanin-rich extract from different sources. Anthocyanin-rich extracts were significantly different among groups, whereas anthocyanin-rich extracts within the same group were not significantly different at the 0.05 level by least significant difference test (Tukey HSD). Values are represented as equivalents of cyanidin 3-glucoside (μg/mL) and pg 3-glucoside (μg/mL) for radish.

Growth Inhibition of AREs on HT29 Cell Line. All AREs exhibited a dose-dependent inhibitory effect on the growth of HT29. The concentration to inhibit 50% cell proliferation (GI_{50} values) was calculated for all different AREs and statistically divided into three homogeneous groups by Tukey HSD test at the level of $p < 0.05$ (**Figure 4**). Lower GI_{50} values indicate more potent growth inhibitory activity of a given extract. The AREs from purple corn, containing mostly cyanidin-3-glucoside derivatives, showed the greatest growth inhibition with GI_{50} values of 13.8. In the same group were chokeberry and bilberry with GI_{50} values of 31.2 and 32.2 μg/mL, respectively. The second group included AREs from purple carrot and grape (GI_{50} = 68.5 and 71.2 μg/mL, respectively). Radish and elderberry (GI_{50} = 107.7 and 130.3 μg/mL, respectively) showed relatively lower effectiveness to suppress the HT29 proliferation as compared to the other groups. These results on chokeberry, bilberry, and grape are in agreement with our previous study. Zhao et al. reported that chokeberry was most effective, then

bilberry, followed by grape at concentrations of 25−75 μg/mL (equivalents as cyanidin 3-glucoside) to inhibit 50% HT29 cell proliferation (*7*).

The GI_{50} numbers obtained on the colorectal cell model were compared with reported literature on anthocyanin concentration in the colon contents, as a point of reference of the concentrations that might be achieved in the proximity of the colon tissue. A study on pigs reported that the ACN remaining from the whole GI tract 4 h after black raspberry feeding (~50 mg of ACN/kg of body weight) was 41.7 ± 4.9%, whereas it was about 7% in the colon segment (*20*). Anthocyanin concentration in rat feces after dietary supplementation of AREs (4% of the diet) has been reported as 700−2000 μg/g of wet weight (*21*). The GI_{50} values obtained in the present study were much lower than the concentrations of anthocyanins reported in the colon, suggesting that it may be possible through the consumption of anthocyanin-rich foods to achieve concentrations of anthocyanins in the GI tract that are large enough to exert chemoprotection in vivo. Recent in vivo studies have shown that anthocyanin-rich extracts or foods exert chemoprotection in oral (*22*), esophageal (*23, 24*), and colon (*11*−*13*) cancers. These data indicate that the consumption of anthocyanins would be effective in the prevention of gastrointestinal cancers in humans. However, few in vivo studies have reported the effective chemoprotection of anthocyanins on cancers that need delivery of phytochemicals through the blood stream, such as lung cancer. Carlton et al., for example, found that dietary lyophilized strawberries failed to inhibit lung tumorigenesis in mice (*25*). Low bioavailability of anthocyanins might explain this because different studies have found low anthocyanin levels in human serum (69.3−245.8 μg as cyanidin 3-glucoside equiv/L) measured 2 h after the consumption of chokeberry anthocyanin extracts containing 1.3 g of anthocyanins (*26*) or in human plasma (7.5−97.8 μg as cyanidin 3-glucoside equiv/L) measured 1 h after the consumption of 330 mL of black currant juice (*27*). The referenced anthocyanin concentration in the blood was much lower than the GI_{50} values obtained with all AREs in our experiment. This suggests that anthocyanins may not be present in the plasma in concentrations high enough to inhibit cell proliferation on tissues that require delivery of phytochemicals through the bloodstream.

Growth Inhibition of ACN and OPF fractions on HT29 Cell Line. The role of anthocyanins in the chemoprotection of ARE was tested by isolating chokeberry anthocyanins from other phenolics and then tested separately on HT29 cells. The purity of the anthocyanin fraction was determined to be 93.1 ± 0.3% calculated from the percent area of anthocyanins to total area of compounds with absorption in the 270−700 nm range (mostly other phenolics), as shown in the maximum plot (**Figure 5**). The max plot shows all peaks at their maximum intensity of absorption in the 270−700 nm range. Anthocyanin peaks showed a maximum absorbance at 520 nm, and the non-anthocyanin peaks in the maximum plot chromatograph were relatively very small.

Chokeberry ARE, ACN, and OPF all showed dose-dependent inhibition of HT29 cell proliferation (**Figure 6**). Cytotoxicity was evident when the anthocyanin concentration of ARE and ACN was 75 μg/mL or higher, as the growth inhibition exceeded 100% of control cells. The ACN fraction of the ARE contributed the majority of the chemoprotective effects of chokeberry AREs. The ACN at concentrations of 75 μg/mL or higher resulted in a growth-inhibitory effect almost to the same degree as did the ARE containing the same amount of anthocyanins. The concentration of OPF, which was equivalent to the amount isolated

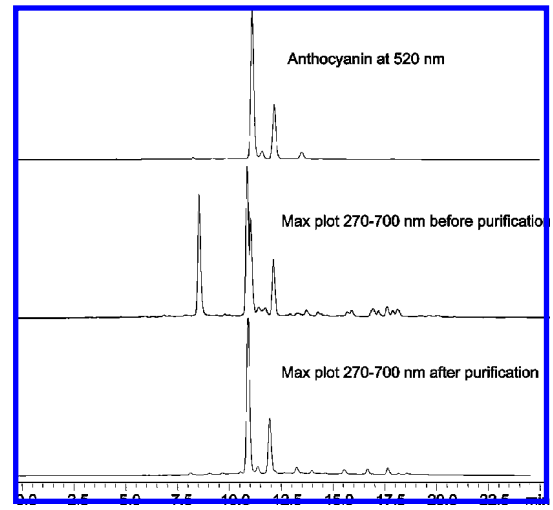

Figure 5. Purity of chokeberry anthocyanins determined by HPLC chromatograph.

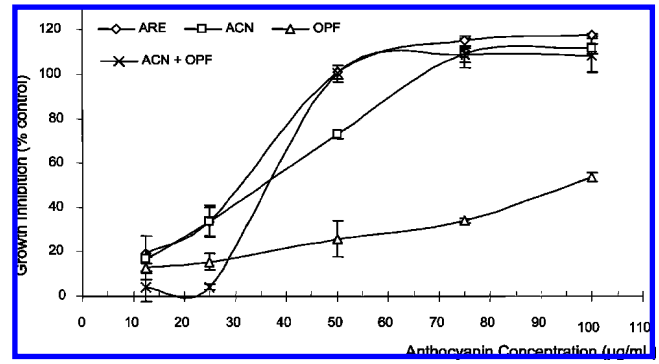

Figure 6. Growth inhibition of anthocyanin-rich extracts from chokeberry on HT29 cell line: ARE, anthocyanin-rich extract; ACN + OPF, reconstitution of anthocyanin fraction and other phenolic fraction; ACN, anthocyanin fraction; OPF, other phenolic fraction. Values are represented as mean absorbance ± standard error ($n = 4$).

from the ARE containing 75 μg/mL ACN, however, resulted in relatively low inhibition of HT29 cell growth (GI ~ 25%). Thus, the OPF fraction contributed to the growth inhibition of ARE only at lower concentrations and to a much lesser extent than the ACN fraction.

AREs were reconstituted by mixing the two different fractions (anthocyanins plus other phenolics) according to their original ratios in chokeberry ARE and tested on the HT29 cell line system. The growth inhibition of HT29 cells was very similar to the original ARE's (**Figure 6**), suggesting that our methodology separated anthocyanins successfully from other phenolics without significantly changing their biological activities.

These findings are in agreement with those of others (*8*), suggesting that anthocyanins are the primary antiproliferative components present in anthocyanin-rich commodities and/or extracts. Anthocyanin fractions from bilberry suppressed the growth of HL60 colon cancer cells to a greater extent than other fractions (*8*). In another study, an anthocyanin-rich fraction (purity about 75−87%, w/w) had the greatest antiproliferative effect on HT29 when compared to phenolic acids, tannins, and flavonol fractions from blueberries (*9*). An anthocyanin fraction from red wine also had higher growth inhibition of HCT15 human colon cancer cell line than other flavonoid fractions (*10*). Thus, evidence is mounting that anthocyanins may play a major role in the chemoprotective action of anthocyanin-rich foods or commodities.

Table 2. Combination Index (CI) Values[a] of the Interaction between Anthocyanin Fraction and Other Phenolics

source	combination index		
	GI_{25}	GI_{50}	GI_{100}
anthocyanins + other phenolics combination effect	1.222 (0.266) additive	1.144 (0.279) additive	1.221 (0.097) additive

[a] The combination index values were calculated on the multiple drug effect equation. A CI of <1, =1, and >1 indicates synergistic, additive, and antagonistic effects, respectively. Each value represents the mean of three independent experiments carried out in four replicates. Student's *t* tests were computed to evaluate if significant differences in the mean CI values compared with a null hypothesized CI of 1 at the level of 0.05. Values are represented as means (standard error), $n = 4$.

Chemoprotective Interaction of Anthocyanins and Other Phenols. The interaction of anthocyanins with other phenolics was further studied on the HT29 cell system by using the combination index (CI) reported by Chou and Talalay (*15*). A CI of <1, 1, or >1 indicates synergistic, additive, and antagonistic effects, respectively.

An additive or synergistic interaction of anthocyanins, proanthocyanidins, and flavonols from cranberries has been suggested for the growth inhibition of HT29 and HCT116 cells (*28*). The combination indices obtained with our fractions (CI = 1.2/1.4/1.2 at levels of GI_{25}, GI_{50}, and GI_{100}) were not significantly different from the null hypothesized CI = 1 at the level of 0.05 (**Table 2**), suggesting that the interaction between chokeberry anthocyanins and other phenolics is additive. Anthocyanins and other phenolics in chokeberry might exert chemoprotective effects by a similar mechanism, resulting in an additive effect on the growth inhibition of HT29 cells.

Effect of Anthocyanin Chemical Structure on Chemoprotective Activity. Anthocyanin profiles of the seven materials evaluated in this study are listed in **Table 1**. Anthocyanins in purple corn are monoglucosylated cyanidin, peonidin, and pelargonidin, with cyanidin-3-glucoside being the most abundant (**Table 3**). Purple corn ARE demonstrated the highest growth inhibitory activity among the extracts evaluated in this study, suggesting that cyanidin glucoside anthocyanins may be effective chemoprotective compounds. Chokeberry contains mainly cyanidin derivatives monoglycosylated with galactose, arabinose, or xylose, whereas elderberry has cyanidin derivatives that are mono-, di-, or triglycosylated (3-glucoside, 3-sambubioside, 3-samubioside-5-glucoside, and 3,5-diglucoside) (*29*). Chokeberry and elderberry both have the cyanidin as the aglycone and are nonacylated anthocyanins. The differences between them are the type, position, and number of sugar moieties attached to the cyanidin aglycone. Purple carrot is another source of cyanidin derivatives with acylated or nonacylated complex sugar substitution patterns: cyanidin-3-xylosyl-galactoside, 3-xylosyl-glucosyl-galactoside, and cyanidin-3-xylosyl-glucosyl-galactoside acylated with one cinnamic acid (*30*). The GI_{50} of purple carrot ARE was between those of chokeberry and elderberry. The differences in biological activity observed among these sources of ARE containing the same type of aglycone point toward the importance of the substitution on the aglycone molecule as a modulator of bioactivity. Our results suggest that the 3,5-glycosylation pattern on anthocyanidins might indicate lower biological activities as compared to glycosylation on position 3 only.

Anthocyanins in grapes are a mixture of five different aglycones with a glucose moiety and are acylated or nonacylated with one cinnamic acid. Both the purple carrot and grape have the cinnamic acid acylation, and their inhibitory effects were not significantly different from each other. However, radish ARE containing mostly pelargonidin-3-sophoroside-5-glucoside acylated with one or two cinnamic acids showed a relatively low inhibitory effect as compared to other acylated anthocyanin sources such as carrot and grape. These differences might be due to the type of aglycone or glycosylation pattern. Among the sources assayed in this experiment, the two extracts with the lowest growth inhibition for HT29 cells, radish and elderberry, were the only ones that contained anthocyanins with glycosylation at 3,5-diglycosides. This further supports the hypothesis that a 3,5-glycosylation pattern on anthocyanidins lowers biological activity.

Statistical Analyses of the Relationship between Bioactivity and Pigment Profiles. To better understand the anthocyanin structure−function relationships, the anthocyanin compositional data for the different AREs used in this study were collected, and the percent of each individual anthocyanin (as determined by analytical HPLC) was used to determine the relative proportions of the different types of aglycone, glycosylation, and acylation in each extract (**Table 3**). The relationship between these chemical characteristics and biological activity, determined as the GI_{50}, was then analyzed statistically using multiple regression analyses. There were six variables for the type of aglycone, including cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. Three variables were associated with the type of glycosylation: mono-, di-, and triglycoside. Anthocyanins were also classified into three acylation categories: nonacylated anthocyanins, anthocyanins acylated with aliphatic acid, and anthocyanins acylated with cinnamic acid. Multiple linear regression analysis determined that the GI_{50} was dependent on the chemical structure of the pigments, and all three characteristics of the molecule (aglycone, glycosylation, and acylation) affected the GI_{50}. The following equation ($R^2 = 0.977$, $p < 0.001$) relating GI_{50} and chemical structure was generated:

Table 3. Structural Contribution of Anthocyanins in Different Anthocyanin-Rich Extracts and Their GI_{50} on the Growth of HT29 Cell Line[a]

source	GI_{50}(μg/mL)	aglycone (%)						glycosylation (%)			acylation (%)		
		Cy	Pg	Pn	Dp	Pt	Mv	Mono	Di	Tri	NA	Ali	Cinn
chokeberry	31.2	100	0	0	0	0	0	100	0	0	100	0	0
elderberry	130.3	100	0	0	0	0	0	45.54	44.67	9.8	100	0	0
bilberry	32.2	23.42	0	8.1	26.97	16.06	25.45	100	0	0	100	0	0
purple corn	13.8	73.37	6.53	20.09	0	0	0	99.99	0	0	93.62	6.37	0
purple carrot	68.5	100	0	0	0	0	0	0	46.1	53.9	58.03	0	41.97
radish	107.7	0	100	0	0	0	0	0	0	100	0	0	100
grape	71.2	3.03	0	28.61	6.47	15.03	44.32	21.25	76.21	0	85.29	0	12.17

[a] Abbreviations: Cy, cyanidin; Dp, delphinidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; Mv, malvidin; Mono, monoglycoside; Di, diglycoside; Tri, triglycoside; NA, none; Ali, aliphatic acid; Cinn, cinnamic acid.

$$\log GI_{50} = -4.473 - 0.07NA + 0.046CA + 0.014Pg - 0.01MG + 0.005TG,\ R^2 = 0.977 \quad (3)$$

NA is the percentage of nonacylated anthocyanins, CA is the percentage of anthocyanins acylated with cinnamic acids, Pg is the percentage of anthocyanins with pelargonidin as aglycone, MG is the percentage of anthocyanins glycosylated with monoglycoside, and TG is the percentage of anthocyanins glycosylated with triglycoside.

Pelargonidin ($R = 0.537$, $p = 0.006$), triglycoside ($R = 0.609$, $p = 0.001$), and acylation with cinnamic acid ($R = 0.591$, $p = 0.002$) were significantly correlated to the base-10 logarithm of GI_{50} values, suggesting that anthocyanin with pelargonidin, triglycoside, or acylation with cinnamic acid would have the least growth inhibitory effect on HT29 cells. Monoglycoside ($R = -0.724$, $p < 0.0001$) and nonacylation ($R = -0.568$, $p = 0.003$) were negatively correlated with the log GI_{50} values, indicating that anthocyanins with monoglycoside might be more potent inhibitors of the proliferation of HT29 cancer cells. In other studies, anthocyanin biological activity was shown to be affected by their chemical structures (*10, 31*−*33*). Effects of anthocyanin structures on their biological activities are dependent on experimental models (*31*−*33*). In our experimental data, pelargonidin derivatives showed relatively low inhibition on HT29 cell in vitro. Zhang et al. found that malvidin exerted the greatest inhibition among cyanidin, delphinidin, pelargonidin, and petunidin, closely followed by pelargonidin at 200 μg/mL on AGS (stomach), HCT-116 (colon), NCI-H460 (lung), MCF-7 (breast), and SF-268 (central nervous system) cancer cell growth by MTT assay (*33*). In another study, cyanidin 3-sophoroside-5-glucoside or cyanidin 3-sophoroside-5-glucoside acylated with cinnamic acids showed a greater antimutagenicity activity than did peonidin-type anthocyanins with the same structure patterns by using *Salmonella typhimurium* TA 98 (*32*). Yoshimoto et al. also found that deacylation of the cyanidin-type pigment increased antimutagenicity, whereas the antimugenicity of peonidin-type pigment markedly decreased after deacylation (*32*). In our study, anthocyanins without acylation were more potent inhibitors of HT29 cell proliferation than acylated anthocyanins. The glycosidic pattern also affected the anthocyanin's biological activities. Koide et al. found that an extract containing mostly cyanidin- glucoside and cyanidin rhamnoside was more effective in suppressing the HCT15 cell growth in vitro than the extract rich in cyanidin rhamnoside (*31*), suggesting that cyanidin-3-glucoside resulted in greater cell growth inhibition than the corresponding cyanidin-3-rhamnoside. In our study, anthocyanin triglycosides such as 3-diglycoside-5-glycoside might have decreased anthocyanin antiproliferative action on HT29 cells.

Regression analysis of structural characteristics of anthocyanins and GI_{50} values found a significant relationship between the chemoprotective activities of anthocyanins and multiple characteristics of anthocyanins, the type of aglycones, sugars, and acylated acids, and the position and degree of glycosylation and acylation.

The chemical structures of anthocyanins do have a significant impact on their biological activity, and data suggest that nonacylated monoglycosylated anthocyanins are more potent inhibitors of colon cancer cell growth proliferation, whereas pelargonidin aglycone and triglycosylation seemed to exert lower inhibitory effect on colon cancer cell proliferation. More research is being carried out to better understand the partial contribution of the different portions of the molecule and their relationships.

ABBREVIATIONS USED

ARE, anthocyanin-rich extract; ACN, anthocyanin fraction; AP, reconstitution of anthocyanin fraction and other phenolic fraction; CI, combination index; OPF, other phenolics fraction; HPLC, high-performance liquid chromatograph; $GI_{25}/GI_{50}/GI_{100}$, the concentration inhibiting 25%/50%/100% cell growth; GIT, gastrointestinal tract.

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