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Effects of Commercial Anthocyanin-Rich Extracts on Colonic Cancer and Nontumorigenic Colonic Cell Growth

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Commercially prepared grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.), and chokeberry (*Aronia meloncarpa* E.) anthocyanin-rich extracts (AREs) were investigated for their potential chemopreventive activity against colon cancer. The growth of colon-cancer-derived HT-29 and nontumorigenic colonic NCM460 cells exposed to semipurified AREs (10−75 μg of monomeric anthocyanin/mL) was monitored for up to 72 h using a sulforhodamine B assay. All extracts inhibited the growth of HT-29 cells, with chokeberry ARE being the most potent inhibitor. HT-29 cell growth was inhibited ~50% after 48 h of exposure to 25 μg/mL chokeberry ARE. Most importantly, the growth of NCM460 cells was not inhibited at lower concentrations of all three AREs, illustrating greater growth inhibition of colon cancer, as compared to nontumorigenic colon cells. Extracts were semipurified and characterized by high-pressure liquid chromatography, spectrophotometry, and colorimetry. Grape anthocyanins were the glucosylated derivatives of five different anthocyanidin molecules, with or without *p*-coumaric acid acylation. Bilberry contained five different anthocyanidins glycosylated with galactose, glucose, and arabinose. Chokeberry anthocyanins were cyanidin derivatives, monoglycosylated mostly with galactose and arabinose. The varying compositions and degrees of growth inhibition suggest that the anthocyanin chemical structure may play an important role in the growth inhibitory activity of commercially available AREs.

KEYWORDS: Anthocyanins; colon cancer; HT-29 colon cancer cells; NCM460 colon cells; cell growth; chokeberry; bilberry; grape

INTRODUCTION

Anthocyanins, natural pigments present in fruits and vegetables, have shown considerable potential in the food industry as safe and effective food colorants (*1*, *2*). Recently, interest in anthocyanin-rich foods and extracts has intensified because of their possible health benefits. Anthocyanins are potent antioxidants (*3*−*6*). Anthocyanin fractions extracted from different sources, including flower petals (*7*), grape rinds and red rice (*8*), red soybeans and red beans (*9*), *Vaccinium* species (*10*), purple corn (*11*), and different cherry and berry extracts (*12*−*14*), have demonstrated anticancer activity.

In vitro, anthocyanin fractions more effectively inhibited growth of human intestinal carcinoma HCT-15 cells than did flavonoids (*7*, *9*, *15*). HCT116 colon cancer cells were inhibited by anthocyanin-containing berry extracts including cowberry, strawberry, blueberry, and bilberry extracts (*13*). Similarly, tart cherry anthocyanins and their aglycon cyanidin were shown to inhibit the growth of human colon cancer cell lines HT-29 and HCT116 (*14*). In vivo, the tart cherry extract inhibited the intestinal tumor development in Apc(min) mice (*14*), suggesting that anthocyanins as well as the aglycons may reduce the risk of intestinal cancer. Freeze-dried black raspberries (*12*) and purple corn (*11*) have been shown to inhibit azoxymethane-induced colon tumors in rats.

However, the relationship of anthocyanin structure to anticarcinogenic activity has not been well established. Koide and co-workers (*8*, *9*) reported that different sources of anthocyanins and the presence of glycosylations might affect the inhibition of HCT-15 cancer cell growth. Bioabsorption studies have demonstrated that the structure of anthocyanins greatly affects uptake (*16*, *17*). We (unpublished data), and others (*18*), have observed that the method of preparation of anthocyanin extracts greatly affects the biological activity. Therefore, we investigated the chemopreventive activity of anthocyanin extracts commercially prepared for the food industry as natural colorants.

The first objective of the present study was to compare the effects of commercially available anthocyanin-rich extracts (AREs) with different anthocyanin profiles on the growth of colon cancer cells. The AREs from grape, bilberry, and chokeberry were selected on the basis of their anthocyanin high-pressure liquid chromatography (HPLC) profiles provided by the commercial companies. Grapes contain acylated glucoside derivatives of a variety of anthocyanidins, whereas bilberry

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anthocyanins are all nonacylated. Chokeberries have a simple pigment profile, with all of the anthocyanins being cyanidin derivatives.

The second objective was to investigate whether these AREs also affect the growth of normal colon epithelial cells to determine whether a differential response in growth inhibition may be observed as has been reported in oral epithelial cells exposed to various chemopreventive agents (*19*, *20*). To begin to elucidate active components, we characterized extracts for anthocyanin composition and total phenolics and assessed the effect of cell incubation on the color of the culture medium. We report significant growth inhibition of colon cancer, but not normal colon cells, by all AREs. Chokeberry extract was the most potent inhibitor at early time points, and with longer exposure bilberry ARE also significantly inhibited colon cancer cell growth. To our knowledge, this is the first report of differential inhibition of growth of colon cancer compared to normal colon cells by AREs. These results suggest that commercial AREs may be effective chemopreventive agents.

MATERIALS AND METHODS

Anthocyanin Sources. Commercially available colored extracts with different anthocyanin profiles were used for this study. The grape (*Vitis vinifera)* extract was supplied by Polyphenolics, Inc. (Madera, CA). The bilberry (*Vaccinium myrtillus* L.) and chokeberry (*Aronia meloncarpa* E.) extracts were supplied by Artemis International, Inc. (Fort Wayne, IN).

Reagents. All HPLC reagents were purchased from Fisher Scientific (Fair Lawn, NJ). Standards and reagents for phenolic acid analyses were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. An HT-29 cell line derived from a colorectal adenocarcinoma (HTB 38; American Type Culture Collection, Manassas, VA) was grown in McCoy's 5A medium (Cambrex Bio Science, Walkersville, MD). The nontransformed, nontumorigenic, immortalized colon NCM460 cell line (InCell Corp., San Antonio, TX) derived from human normal colon mucosa (*21*, *22*) was grown in M3 Base culture medium (InCell). All media were supplemented with 10% fetal bovine serum (FBS; Cambrex Bio Science). Both cell lines were incubated for up to 72 h at 37 °C in a 5% CO_2 atmosphere.

Cell Growth Assays. HT-29 and NCM460 cells were plated at 5×10^4 cells/well in 24-well plates with the appropriate medium. Cells were grown for 24 h to attain log phase growth at the time of ARE addition (time 0). To determine the time and concentration effect of the three AREs on the growth of colon cells, the HT-29 and NCM460 cells were exposed to different concentrations of the extracts on the basis of their monomeric anthocyanin content. Cells were incubated in the presence of ARE for 24, 48, and 72 h at concentrations of 25, 50, and 75 μg of monomeric anthocyanin/mL of growth medium for grape and bilberry AREs, and 10, 25, and 50 μg of monomeric anthocyanin/mL for chokeberry ARE. These dose ranges were based on results obtained in preliminary experiments (data not shown). There were four replications for each treatment concentration at each time point. Two controls were used: cells grown in medium and cells grown in medium with the acidified water vehicle (0.01% HCl) added at the greatest volume used in the ARE treatment. To avoid a possible effect of well position on cell growth, treatments were randomly assigned within the 24 wells. Cell growth was assessed using a sulforhodamine B in vitro toxicology assay kit (Sigma) as described previously (*23*). Briefly, this colorimetric method measures the total cellular protein, as an absorbance at 490 nm, which reflects the changes in cell number. The absorbance at 490 nm was measured spectrophotometrically using a microplate reader, Elx800 (Bio-Tek Instruments, Inc., Winooski, VT). The percentage of growth inhibition was calculated as

$$(OD_{\text{cells exposed to vehicle HCl}} - OD_{\text{cells exposed to AREs}})/OD_{\text{cells exposed to vehicle HCl}} \times 100$$

Semipurification of Anthocyanin-Rich Extracts. Anthocyanins were semipurified by solid-phase extraction using a C18 cartridge (Waters Corp., Milford, MA) as described by Giusti and Wrolstad (*2*). A 1 g portion of each anthocyanin extract was dissolved in 10 mL of deionized water and passed through the C18 cartridge. Anthocyanins and other phenolics were bound to the C18 cartridge, while sugars and other polar compounds were removed with 0.01%-HCl-acidified water. Anthocyanins and other phenolics were recovered with ethanol containing 0.01% HCl. The alcohol was removed by rotary evaporation at 40 °C, and each solute was redissolved in 10 mL of 0.01%-HCl-deionized water.

Monomeric Anthocyanin and Polymeric Color Measurement. The monomeric anthocyanin content and polymeric color measurements were determined by a pH-differential method and a bisulfite bleaching method, respectively (*24*). A Shimadzu 1601 UV spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for spectral measurements at 420, 520, and 700 nm. The content of monomeric anthocyanin was calculated as cyanidin-3-glucoside, using an extinction coefficient of 26900 L cm^{-1} mol^{-1} and a molar mass of 449.2 g mol^{-1} (*25*).

HPLC Analyses of Anthocyanins. A Waters Delta 600 HPLC instrument equipped with a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and Millenium 32 software (Waters Corp.) was used for the analyses. The mobile phase was composed of the following solvents: (A) 1% phosphoric acid, 10% acetic acid, 5% acetonitrile, 84% water, (B) 100% acetonitrile. Solvents and samples were filtered though 0.45 cm poly(tetrafluoroethylene) membrane filters (Pall Life Sciences, Ann Arbor, MI) and 0.45 μm polypropylene filters (Whatman Inc., Clifton, NJ), respectively.

Anthocyanins were separated on a Symmetry C18 5 μm, 4.6 × 150 mm column fitted with a 22 × 4.6 mm Symmetry 2 micro guard column (Waters). Separation was achieved by using a linear gradient from 0 to 30% solvent B in 35 min. An injection volume of 50 μL with a 1 mL/min flow rate was used. Spectral information over the wavelength range of 260−600 nm was collected throughout the separation time.

Total Phenolics Measurement. Total phenolics were measured using a modification of the Folin−Ciocalteu method for total phenols (*26*). The absorbance of the samples and standards was measured at 755 nm. Total phenols were calculated as gallic acid equivalents based on a gallic acid standard curve.

Color Measurement. Changes in the color of the medium during cell growth were assessed by comparing the color of McCoy's 5A medium, with or without 50 μg of chokeberry anthocyanin/mL of medium, and the color after 24 h of incubation with or without 4×10^6 HT-29 cells. Chokeberry ARE was selected because it was the most potent inhibitor of cancer cell growth within 24 h of exposure. Samples of the medium were measured using an XE ColorQuest colorimeter (Hunter Labs Inc., Reston, VA) and using the transmittance mode, illuminant C, and a 2° observer angle. Hunter CIELCh (lightness, L^*), chroma (C^*), hue (h), and haze values were obtained.

Statistical Analysis. Data on the growth of cells at different ARE treatments were analyzed using the PROC mixed program for SAS Version 8.1 (SAS Institute Inc., Cary, NC). The fixed portion of the model included the effects of the combinations of cell lines, treatments, and different time points. The residual was defined as random. Model residuals examined for the assumptions of the analysis of variance were satisfactory. Contrasts were used to test for differences in the response across time for different treatments within and between cell lines. Pairwise comparisons of means were based on t statistic probabilities, and values of $p < 0.05$ were considered significant.

RESULTS

Inhibition of Colonic Cancer and Nontumorigenic Colonic Cell Growth. As seen in **Figure 1A**, grape ARE significantly inhibited HT-29 cell growth in a time- and dose-dependent manner. After 24 h of exposure to 75 μg of monomeric anthocyanin/mL of grape ARE, HT-29 cell growth was inhibited by 12% ($p < 0.05$). With increased time of exposure to 48 and 72 h, the percentage of growth inhibition of HT-29 cells compared to the control by 75 μg of monomeric anthocyanin/mL increased to 35% and 55%, respectively. A similar time-

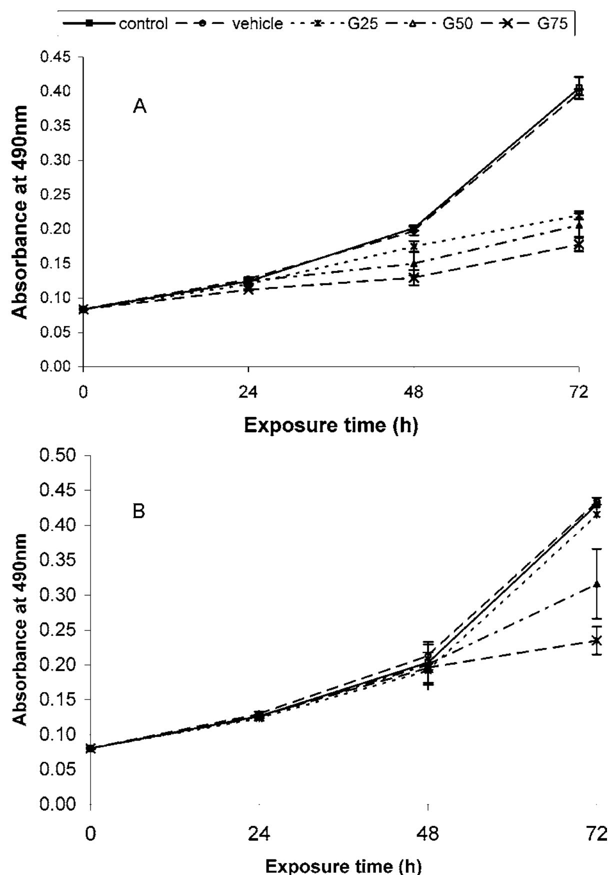

Figure 1. Effect of grape ARE on growth of (**A**) HT-29 colon cancer cells and (**B**) normal NCM 460 colon cells. Control cells were grown in medium only. HCL represents cells grown in medium containing the same volume of 0.01% (v/v) HCL as used for extracts. G25, G50, and G75 represents cells that were treated with grape ARE at concentrations of 25, 50, and 75 μg of monomeric anthocyanin/mL of medium, respectively. Values are represented as mean absorbance ± standard error.

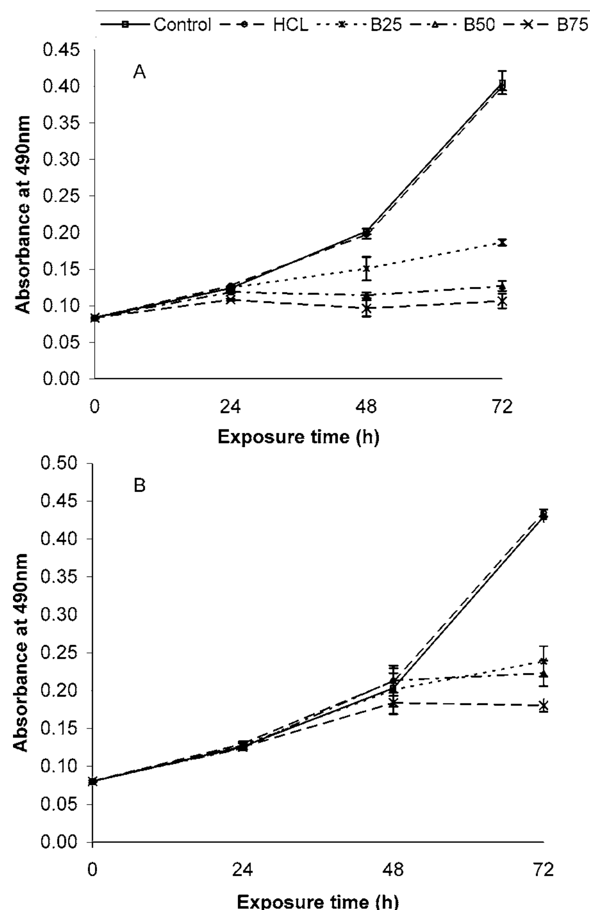

Figure 2. Effect of bilberry ARE on growth of (**A**) HT-29 colon cancer cells and (**B**) normal NCM 460 colon cells. Control and HCL were the same as in **Figure 1**. B25, B50, and B75 represent treatment with bilberry ARE at concentrations of 25, 50, and 75 μg of monomeric anthocyanin/mL of medium, respectively. Values are represented as mean absorbance ± standard error.

dependent increase in cell growth inhibition was observed at concentrations of 25 and 50 μg of monomeric anthocyanin/mL of grape ARE (**Figure 1A**). Trypan blue exclusion determined that cells remained viable (data not shown). In contrast, growth of NCM460 cells was not significantly ($p > 0.05$) inhibited at any time point by 25 or 50 μg of monomeric anthocyanin/mL of grape ARE (**Figure 1B**). Significant inhibition of NMC460 cells occurred only after 72 h of incubation with the highest concentration of grape ARE when the percentage of inhibition reached 46%.

Similar results were obtained with bilberry ARE (**Figure 2**). Although the HT-29 growth inhibition at 25 μg of monomeric anthocyanin/mL of bilberry ARE was not significant ($p = 0.40$) at 24 h of exposure, after 48 and 72 h exposures significant growth inhibition of 24% and 53% ($p = 0.02$), respectively, was observed. Higher concentrations of bilberry ARE at 50 and 75 μg of monomeric anthocyanin/mL significantly ($p < 0.01$) inhibited the growth of the HT-29 cells in a time-dependent manner (**Figure 2A**). At 75 μg of monomeric anthocyanin/mL, the HT-29 cell growth was inhibited by 15% at 24 h, 51% at 48 h, and 73% by the end of 72 h of exposure. Comparatively, NCM460 cell growth was not significantly inhibited ($p > 0.05$) after 24 or 48 h at any concentration, but was inhibited by all concentrations after 72 h of incubation (**Figure 2B**).

At all concentrations and time points, chokeberry ARE inhibited HT-29 cell growth to a greater degree ($p = 0.01$) than either grape or bilberry ARE (**Figure 3**) when extracts were added on the basis of monomeric anthocyanin content. After 24 h of exposure, growth inhibition of HT-29 cells was observed with as low as 10 μg of monomeric anthocyanin/mL of chokeberry ARE ($p < 0.01$), and after 72 h of exposure, growth of cells was inhibited by 45% ($p < 0.01$) as compared to the controls. A similar pattern was observed at concentrations of 25 and 50 μg of monomeric anthocyanin/mL of chokeberry ARE (**Figure 3A**) with growth inhibition reaching 52% and 70%, after 48 and 72 h, respectively. NMC460 cell growth was significantly inhibited only after 48 h at the highest concentration and after 72 h by all concentrations tested (**Figure 3B**).

Composition of Anthocyanin-Rich Extracts. The anthocyanin profiles of grape, bilberry, and chokeberry ARE were analyzed by HPLC monitored at 520 nm (**Figure 4**). Grape ARE contained glucoside derivatives of five different anthocyanidins (peaks 3−7) and their acylated counterparts (seen as late-eluting peak 12 in **Figure 4A**). The pigments in the bilberry ARE were all nonacylated and had five different aglycons glycosylated with galactose (peaks 1, 3, and 9), glucose (peaks 2, 4, 6, and 8), and arabinose (peaks 4, 5, and 10) (**Figure 4B**). The chokeberry ARE had a simple pigment profile, with two major anthocyanins that were monoglycosylated with different sugar substitutions: galactose (69%, peak 1) and arabinose (23%, peak 3). Both of the anthocyanins were cyanidin derivatives (**Figure 4C**).

Chokeberry ARE had a higher content of total phenolics (737 mg/g of extract) compared to either grape ARE (589 mg/g) or bilberry ARE (322 mg/g). The monomeric anthocyanin content

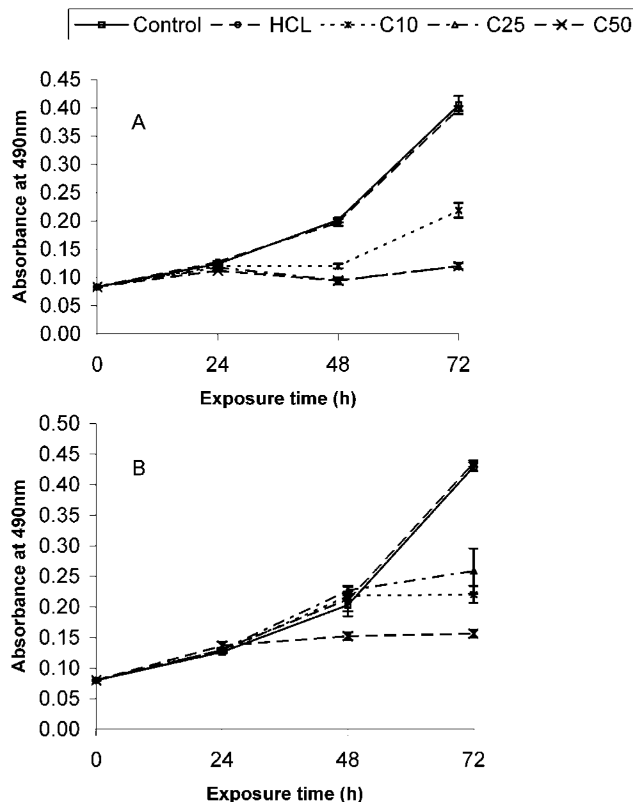

Figure 3. Effect of chokeberry ARE on growth of (**A**) HT-29 colon cancer cells and (**B**) normal NCM 460 colon cells. Control and HCL were the same as in **Figure 1**. C10, C25, and C50 represent treatment with chokeberry ARE at concentrations of 10, 25, and 50 μg of monomeric anthocyanin/mL of medium, respectively. Values are represented as mean absorbance ± standard error.

was highest in grape ARE (210 mg/g extract), and the contents in bilberry ARE and chokeberry ARE were similar to each other, 92 and 102 mg/g of extract, respectively. Therefore, the percentage of total phenolics that were monomeric anthocyanins was calculated (w/w) to be highest in grape (35.6%), followed by bilberry (28.7%) and chokeberry (13.8%). The polymeric color percentage, which represents the percentage of color that is being contributed by polymerized anthocyanins, was highest in the chokeberry ARE (20%) followed by bilberry ARE (14.2%) and grape ARE (10.7%).

Growth Inhibition Based on Phenolic Contents. Because the total phenolic levels in grape, bilberry, and chokeberry ARE were different at the same monomeric anthocyanin concentration, we also compared growth inhibition of HT-29 cells at a concentration of 200 μg/mL total phenolics (**Figure 5**). Grape ARE demonstrated a 12% growth inhibition within 24 h, which was a small but significant increase compared to bilberry ARE (7%) and chokeberry ARE (7%). But with increased exposure time to 48 and 72 h, the HT-29 cells were more effectively inhibited by bilberry and chokeberry ARE.

Effect of Cells on ARE Color. During the cell culture experiments, we observed a change in the color of the anthocyanin-containing medium following incubation with the cells. To determine whether this was potentially due to uptake or metabolism of the extract by the cells, or due simply to incubation conditions, we incubated the chokeberry ARE in the medium with and without HT-29 cells and measured color changes quantitatively using the CIELCh system. As expected, there were significant changes in L^*, C^*, and h in the cell medium immediately following addition of ARE (**Table 1**). L^*

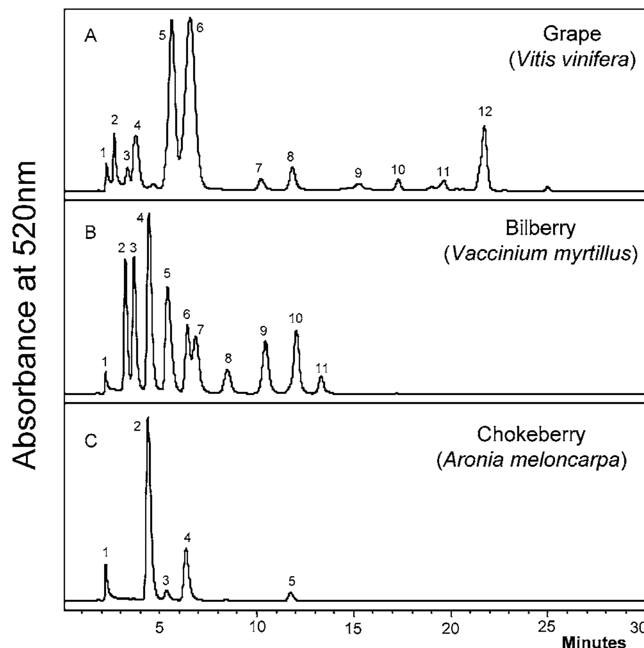

Figure 4. HPLC profiles of semipurified (**A**) grape, (**B**) bilberry, and (**C**) chokeberry ARE. (**A**) Grape: peaks 3, delphinidin-3-glucoside (8); 4, cyanidin-3-glucoside (27); 5, petunidin-3-glucoside (41); 6, peonidin-3-glucoside (2); 7, malvidin-3-glucoside (3); 11, acylated petunidin-3-glucoside with *p*-coumaric acid (9); other peaks unknown. (**B**) Bilberry: peaks 1, delphinidin-3-galactoside (11); 2, delphinidin-3-glucoside (13); 3, cyanidin-3-galactoside/delphinidin-3-arabinoside (20); 4, cyanidin-3-glucoside/petunidin-3-galactoside (15); 5, cyanidin-3-arabinoside (8); 6, petunidin-3-glucoside (9); 7, peonidin-3-galactoside/petunidin-3-arabinoside (4); 8, peonidin-3-glucoside (8); 9, malvidin-3-galactoside (9); 10, malvidin-3-arabinoside (2). (**C**) Chokeberry: peaks 1, cyanidin-3-galactoside (69); 2, cyanidin-3-glucoside (4); 3, cyanidin-3-arabinoside (23); 4, cyanidin-3-xyloside (4). The peak number is that from the figure. The number in parentheses is the percent area of the peak.

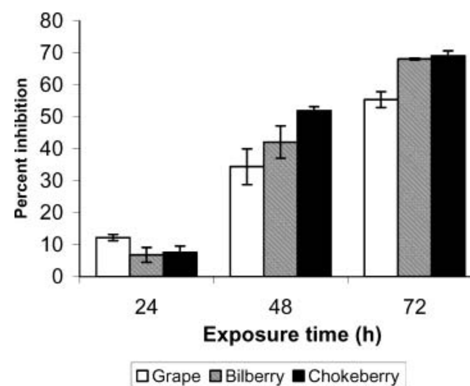

Figure 5. Percentage inhibition of HT-29 colon cancer cells after 24, 48, and 72 h of exposure to 200 μg of total phenolics/mL of medium of grape, bilberry, and chokeberry ARE. Values are represented as mean absorbance ± standard error.

values represent lightness ranging from 0 (black) to 100 (white). Addition of ARE resulted in a decrease in lightness from 76 to 22. Addition of cells did not change the lightness in either the control or ARE medium, although the lightness increased in the ARE medium after 24 of incubation without cells. Chroma is the measurement of saturation, and higher values represent higher saturation of color, which explains the increase in chroma upon addition of ARE to the medium. The chroma of the medium containing 50 μg/mL of monomeric chokeberry ARE

Table 1. Colorimetric Measurements of Growth Medium in the Presence of Chokeberry Anthocyanin-Rich Extract and HT-29 Cells after 24 h of Exposure

	time (h)	no. of cells ($\times 10^6$)	L^*	C^*	h (deg)	haze	pH
McCoy's 5A medium	0	0	76.49	24.85	16.64	6.49	7.50
	24	0	75.57	26.27	8.61	5.8	7.54
	24	4	78.91	20.93	20.11	22.62	7.52
McCoy's 5A medium + ARE	0	0	22.23	41.71	23.1	6.04	7.47
	24	0	53.89	43.95	46.94	15.94	7.41
	24	4	54.84	24.93	36.95	40.35	7.41

decreased from 41.7 to 24.9 after 24 h of incubation with 4×10^6 cells, indicating that the cells may have taken up anthocyanins and other colored phenolics/compounds from the medium. There was little change in the chroma of the ARE medium incubated without cells (**Table 1**). The hue angle between 0° and 90° is representative of red (0°), orange, and yellow (90°) colors. The addition of ARE to the medium resulted in little change in hue from 16 to 23, remaining in the red angle range. For medium without ARE, the hue of the medium remained in the red range during incubation regardless of whether cells were present. The ARE medium hue changed toward orange (h^* of 47) after 24 h of incubation without cells, with less of a change occurring following incubation with cells (**Table 1**).

Medium samples were clear of haze at time zero. After 24 h of incubation, medium with no anthocyanins showed an increase in haze only in the presence of cells. This might be due to the release of cell metabolites. The increase in haze in the presence of cells in the ARE medium after 24 h of incubation was much more pronounced. Haze increased from ~6 to 15 in the absence of cells, and up to 40 when cells were present in the medium.

The pH of the control medium was at a neutral level (~7.5) after 24 h of incubation with or without cells. With addition of ARE, the pH decreased slightly to 7.47. After 24 h of incubation with or without cells, the pH of the ARE medium decreased to 7.41. In general, the pH levels of the medium were still neutral throughout the experiment with or without ARE.

DISCUSSION

AREs Inhibit Cancer but Not Normal Colon Cell Growth. Although there are numerous reports of inhibition of cancer cell growth in vitro by various anthocyanin-containing extracts or purified anthocyanin fractions (*8*−*10*, *13*, *14*, *27*, *28*), to our knowledge, we are the first to compare the effects of these compounds on nontransformed colon cells versus cancerous colon cell growth. Clearly, it is of interest to find compounds or mixtures of compounds that will inhibit cancer cell growth while having little to no cytotoxic effect on normal cells. The NCM460 cells are nontumorigenic derived from normal colon mucosa that has not been infected or transfected with any genetic information (*21*). The cells are positive only to colonic epithelial cell antigens, and some of the cells are positive for mucin synthesis.

In previous studies, different sources of anthocyanins have exhibited varying degrees of inhibition of colon cancer cells (*8*, *9*). Kamei and co-workers (*7*) demonstrated that suppression of growth of malignant intestinal carcinoma HCT-15 cells by anthocyanins was dose-dependent. Kang and co-workers (*14*) demonstrated that anthocyanins from tart cherries inhibited cell growth of colon cell line HT-29 and HCT116 by 50% at concentrations of 585 and 260 μM, respectively, with no cytotoxic effect. In our study, the increase in percentage inhibition with time was mainly due to growth of control cells over time as there was little or no change in the growth of cells exposed to the AREs. The concentrations that were used did not have any cytotoxic effect on the colon cancer cells; rather the effect of the AREs was more cytostatic. Our laboratory has also previously reported cytostatic inhibition of cell proliferation by chokeberry ARE (*29*), as we determined that more than 90% of the cells were alive at the end of 72 h of exposure to the extract. Kamei and colleagues (*7*) demonstrated that anthocyanins, such as delphinidin, cyanidin, and pelargonidin, significantly inhibited HCT-15 intestinal cancer cell growth as compared with other flavonoids.

Time of Exposure. Some interesting observations can be made by comparison of the effect of the three AREs on both cell lines at three time points. As discussed above, at the lowest concentrations, the chokeberry ARE inhibited the HT-29 cells at the earliest time points. At these early time points (24 and 48 h) and low concentrations (10 and 25 μg/mL of medium), the NCM460 cell growth was not affected. On the basis of these results, the chokeberry ARE appears to be the most effective chemopreventive ARE among the three that were tested. However, by 72 h, even the lowest concentration of chokeberry ARE significantly inhibited the growth of NCM460 cells. The bilberry ARE and grape ARE at the low concentrations inhibited growth of HT-29 cells but had little effect on the growth of NCM460 cells. By 72 h of exposure all three AREs affect the growth of the nontransformed colon cells. The time point that best predicts chemopreventive activity in vivo is not known, but is currently under investigation in our laboratory.

Chokeberry ARE Has the Greatest Inhibitory Effect on HT-29. The greater inhibition of HT-29 cell growth within 24 h by chokeberry ARE as compared to grape or bilberry AREs may be related to the differences in the anthocyanin profiles of these extracts, or to the presence of other phenolics. In chokeberry ARE, cyanidin-3-galactoside and cyanidin-3-arabinoside were the two predominant anthocyanins, comprising 70% and 25% of monomeric anthocyanin, respectively. Bilberry extracts contained more cyanidin-3-galactoside than grape extracts though the major anthocyanins are derivatives of delphinidin and malvidin. Though the antioxidant activity of anthocyanins was reported to be associated with their structures (*3*), the relationship between the purified anthocyanins and chemopreventive activity remains to be clearly elucidated. Meriers and co-workers (*27*) found that cyanidin-3-galactoside was a more efficient inhibitor of the growth of the human vulva carcinoma cell A431 than malvidin, the typical anthocyanin in red grapes. Similarly, the aglycon cyanidin has been reported to be a potent inhibitor of colon cancer cell growth (*14*) and an inhibitor of cyclooxygenase enzyme activity (*5*).

Monomeric and Polymeric Anthocyanin Content. Anthocyanin pigments are responsible for the red, purple, and blue colors of many fruits and vegetables. Anthocyanins play a critical role in the color quality; however, anthocyanins are relatively unstable and often undergo degradation during processing and storage. Monomeric anthocyanins condense to form polymeric complexes; therefore, polymeric color is used as an indicator of anthocyanin conversion. Measurement of total anthocyanin content along with indices for the degradation of these pigments is useful in assessing color quality (*24*). Among the three sources of anthocyanin used in the present study, grape ARE had a lower polymeric color, indicating monomeric anthocyanins from grape may be more stable than those from bilberry and chokeberry.

Total Phenolics in Anthocyanin-Rich Extracts. Phenolic compounds comprise one of the largest groups of plant metabolites, and have antioxidative, anti-inflammatory, and anticarcinogenic activities (*30*). The inhibitory effect on tumor promotion has been demonstrated for numerous compounds, including curcumin, caffeic acid, ferulic acid, and chlorogenic acid (*30*). The AREs used in the present study contained other phenolics that may contribute to the growth inhibitory activity of the extracts; therefore, inhibition of HT-29 cell growth was also compared on the basis of the concentration of total phenolics in the extracts. The results obtained were similar to those when extracts were added on the basis of anthocyanin content, suggesting that neither total phenolics nor total anthocyanins are predictive of colon cancer cell growth. This is in agreement with the observations of Liu and co-workers (*31*), who reported that, although total phenolics and flavonoids in different varieties of raspberries were correlated with antioxidant activity, there was no relationship between total phenolics and inhibition of liver cancer cell growth. Whether differences in anthocyanin or other phenolic composition or an unidentified component is responsible is yet to be determined.

Medium Color Change after Cell Incubation. During preliminary experiments with cells incubated with high concentrations (100−200 μg/mL) of monomeric anthocyanin, we observed that cells developed a brown coloration, suggesting that the cells might have taken up anthocyanin pigments. The results of the present study indicated that HT-29 cells affected the color characteristics of AREs in McCoy's 5A medium. The changes may be explained by pigment absorption by the cells or by the metabolism of the pigments during the incubation period. Youdim and co-workers (*32*) demonstrated the ability of endothelial cells to incorporate anthocyanins from elderberry into the membrane and cytosol, resulting in significant protective effects against oxidative damage.

Although anthocyanins are natural colorants and exist widely in many fruits, flowers, and other plant materials, they are not stable pigments. Anthocyanins undergo reversible structural transformations and dramatic changes in color with changes in pH. At a pH of 3 or below, the color of anthocyanins ranges from orange to blue-red, depending on the chemical structure, and exists predominantly as a flavylium cation (*33*). This is the stable form of anthocyanin. As the pH is raised, hydration and proton-transfer reactions can occur, generating a number of chemical structures: the first reaction produces a colorless carbinol pseudobase that can undergo ring opening to a chalcone pseudobase; the latter reactions give rise to quinonoidal bases, with formation of purple quinonoid anions after further deprotonation. These anions exist as the orange to purple flavilium cation at pH 1, colorless carbinol pseudobase or chalcone at pH 4.5, and blue quinoidal base at pH 7 (*30*). With increased temperature, anthocyanin degradation increases too. In the present study, McCoy's 5A medium was at pH 7.4, a pH at which anthocyanins are an unstable blue quinoidal base. Seeram and co-workers (*34*) studied the degradation products of anthocyanin from tart cherries under the same experimental conditions as used in the present study. In the absence of cells, anthocyanins degraded to three benzoic acid derivatives, protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid in McCoy's 5A medium after 72 h of incubation. However, these compounds were not detected in McCoy's 5A medium containing 10% FBS. All media used in the present study contained 10% FBS, and colon cell growth was inhibited although the pH and temperature were not optimum for the stability of anthocyanins. This suggests that the active forms may be anthocyanin degradation products.

In conclusion, the present study demonstrates that commercial AREs prepared from grapes, bilberries, and chokeberries inhibit HT-29 colon cancer cell growth at lower concentration and at earlier time points than nontumorigenic NCM colon cells. We have recently also observed that growth of other colon cancer cell lines is also inhibited by chokeberry ARE in a concentration-dependent manner (unpublished data). At this time, reasons for the different susceptibilities of colon cancer versus nontumorigenic cell lines are unknown, but may involve differences in gene expression between NCM cells and tumor cell lines (*35*), or possibly differences in growth medium composition. Chokeberry ARE, containing monoglycosylated cyanidin derivatives, inhibited colon cancer cell growth to a greater extent than grape and bilberry AREs when inhibition was compared at similar concentrations of monomeric anthocyanin. Chemical structure may play an important role in the inhibitory activity. Anthocyanin-rich products would have added value based on the potential biological activity of their components, making them more attractive alternatives to the use of artificial food colors by the food industry.

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LITERATURE CITED

(1) Strack, D. W., V. The anthocyanins. In *The Flavonoids: Advances in research since 1986*; Harborne, J. B., Ed.; Chapman & Hall: New York, 1994.

(2) Giusti, M. M.; Wrolstad, R. E. Characterization of red radish anthocyanins. *J. Food Sci.* **1996**, *61*, 322−326.

(3) Wang, H.; Nair M. G.; Strasburg, G. M.; Chang, Y.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294−296.

(4) Pool-Zobel, B. L.; Bub, A.; Schroder, N.; Rechkemmer, G. Anthocyanins are potent antioxidants in model systems but do not reduce endogenous oxidative DNA damage in human colon cells. *Eur. J. Nutr.* **1999**, *38*, 227−34.

(5) Seeram, N. P.; Momin, R. A.; Nair, M. G.; Bourquin, L. D. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362−9.

(6) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7.

(7) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, *13*, 590−4.

(8) Koide, T.; Kamei, H.; Hashimoto, Y.; Kojima, T.; Hasegawa, M. Antitumor effect of hydrolyzed anthocyanin from grape rinds and red rice. *Cancer Biother. Radiopharm.* **1996**, *11*, 273−7.

(9) Koide, T.; Hashimoto, Y.; Kamei, H.; Kojima, T.; Hasegawa, M.; Terabe, K. Antitumor effect of anthocyanin fractions extracted from red soybeans and red beans in vitro and in vivo. *Cancer Biother. Radiopharm.* **1997**, *12*, 277−280.

(10) Bomser, J.; Madhavi, D. L.; Singletary, K.; Smith, M. A. In vitro anticancer activity of fruit extracts from *Vaccinium* species. *Planta Med.* **1996**, *62*, 212−6.

(11) Hagiwara, A.; Miyashita, K.; Nakanishi, T.; Sano, M.; Tamano, S.; Kadota, T.; Koda, T.; Nakamura, M.; Imaida, K.; Ito, N.;

Shirai, T. Pronounced inhibition by a natural anthocyanin, purple corn color, of 2- amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-associated colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine. *Cancer Lett.* **2001**, *171*, 17−25.

(12) Harris, G. K.; Gupta, A.; Nines, R. G.; Kresty, L. A.; Habib, S. G.; Frankel, W. L.; LaPerle, K.; Gallaher, D. D.; Schwartz, S. J.; Stoner, G. D. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr. Cancer* **2001**, *40*, 125−33.

(13) Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, *51*, 68−75.

(14) Kang, S. Y.; Seeram, N. P.; Nair, M. G.; Bourquin, L. D. Tart cherry anthocyanins inhibit tumor development in Apc(*Min*) mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13−9.

(15) Kamei, H.; Hashimoto, Y.; Koide, T.; Kojima, T.; Hasegawa, M. Anti-tumor effect of methanol extracts from red and white wines. *Cancer Biother. Radiopharm.* **1998**, *13*, 447−52.

(16) Wu, X.; Cao, G.; Prior, R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* **2002**, *132*, 1865−71.

(17) McGhie, P. E.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539−4548.

(18) Smith, M. A. L.; Marley, K. A.; Seigler, D.; Singletary, K.; Meline, B. Bioactive properties of wild blueberry fruits. *J. Food Sci.* **2000**, *65*, 352−356.

(19) Ding, H.; Han, C.; Gibson-D'Ambrosio, R.; Steele, V. E.; D'Ambrosio, S. M. Piroxicam selectively inhibits the growth of premalignant and malignant human oral cell lines by limiting their progression through the S phase and reducing the levels of cyclins and AP-1. *Int. J. Cancer* **2003**, *107*, 830−6.

(20) D'Ambrosio, S. M.; Gibson-D'Ambrosio, R.; Milo, G. E.; Casto, B.; Kelloff, G. J.; Steele, V. E. Differential response of normal, premalignant and malignant human oral epithelial cells to growth inhibition by chemopreventive agents. *Anticancer Res.* **2000**, *20*, 2273−80.

(21) Moyer, M. P.; Manzano, L. A.; Merriman, R. L.; Stauffer, J. S.; Tanzer, L. R. NCM460, a normal human colon mucosal epithelial cell line. *In Vitro Cell Dev. Biol. Anim.* **1996***, 32*, 315−317

(22) Zhao, D.; Keates, A. C.; Kuhnt-Moore, S.; Moyer, M. P.; Kelly, C. P.; Pothoulakis, C. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. *J. Biol. Chem.* **2001**, *276*, 44464−44471.

(23) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−12.

(24) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV−Visible spectroscopy. In *Current procotols in food analytical chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons: New York, 2001; pp F1.2.1−F1.2.13.

(25) Giusti, M. M.; Rodriguez-Saona, L. E.; Wrolstad, R.E Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **1999**, *47*, 4631−4637.

(26) Singleton, V. L. *Modern methods of plant analysis*; Springer-Verlag: Berlin, Heiedelberg, 1988.

(27) Meiers, S.; Kemeny, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958−62.

(28) Xue, H.; Aziz, R. M.; Sun, N.; Cassady, J. M.; Kamendulis, L. M.; Xu, Y.; Stoner, G. D.; Klaunig, J. E. Inhibition of cellular transformation by berry extracts. *Carcinogenesis* **2001**, *22*, 351−6.

(29) Malik, M.; Zhao, C.; Schoene, N.; Guisti, M. M.; Moyer, M. P.; Magnuson, B. A. Anthocyanin-rich extract from Aronia melanocarpa E induces a cell cycle block in colon cancer but not normal colonic cells. *Nutr. Cancer* **2003**, *46*, 186−196.

(30) Yang, C. S.; Landau, J. M.; Huang, M.; Newmark, H. L. Inhibition of cancinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.

(31) Liu, M.; Li, X. Q.; Weber, C.; Lee, C. Y.; Brown, J.; Liu, R. H. Antioxidant and antiproliferative activities of raspberries. *J. Agric. Food Chem.* **2002**, *50*, 2926−30.

(32) Youdim, K. A.; Martin, A.; Joseph, J. A. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radical Biol. Med.* **2000**, *29*, 51−60.

(33) Wrolstad, R. E.; Durst, R. W.; Giusti, M. M.; Rodriguez-Saona, L. E. Analysis of anthocyanins in nutraceuticals. In *Quality Management of Nutraceuticals*; Ho, C.-T., Zheng, Q. Y., Eds.; American Chemical Society: Washington, DC, 2002; pp 42−62.

(34) Seeram, N. P.; Bourquin, L. D.; Nair, M. G. Degradation products of cyanidin glycosides from tart cherries and their bioactivities. *J. Agric. Food Chem.* **2001**, *49*, 4924−9.

(35) Nimmrich, I.; Erdmann, S.; Melchers, U.; Finke, U.; Hentsch, S.; Moyer, M. P.; Hoffman, I., Muller, O. Seven genes that are differentially transcribed in colorectal tumor cell lines. *Cancer Lett*. **2000**, *160*, 37−43.

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