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Study of Anticancer Activities of Muscadine Grape Phenolics in Vitro

WEIGUANG YI,† JOAN FISCHER,‡ AND CASIMIR C. AKOH*,†

Department of Food Science and Technology, The University of Georgia, Athens, Georgia 30602-7610, and Department of Foods and Nutrition, The University of Georgia, Athens, Georgia 30602-3622

Muscadine grapes have unique aroma and flavor characteristics. Although a few studies reported high polyphenols content of muscadine grapes, little research has been conducted to evaluate the phenolic compounds bioactivities in any muscadine grape cultivar. The objective of this study was to evaluate the effect of phenolic compounds in muscadine grapes on cancer cell viability and apoptosis. Four cultivars of muscadine (Carlos, Ison, Noble, and Supreme) were assessed in this study. Phenolic compounds were extracted from muscadine skins and further separated into phenolic acids, tannins, flavonols, and anthocyanins using HLB cartridge and LH20 column. Some individual phenolic acids and flavonoids were identified by HPLC. Anthocyanin fractions were more than 90% pure. The effect of different fractions on the viability and apoptosis of two colon cancer cell lines (HT-29 and Caco-2) was evaluated. A 50% inhibition of cancer cell population growth for the two cell lines was observed at concentrations of 1−7 mg/mL for crude extracts. The phenolic acid fractions showed a 50% inhibition at the level of 0.5−3 mg/mL. The greatest inhibitory activity was found in the anthocyanin fraction, with a 50% inhibition at concentrations of ∼200 μg/mL in HT-29 and 100−300 μg/mL in Caco-2. Anthocyanin fractions also resulted in 2−4 times increase in DNA fragmentation, indicating the induction of apoptosis. These findings suggest that polyphenols from muscadine grapes may have anticancer properties.

KEYWORDS: Anthocyanins; apoptosis; cell viability; colon cancer; muscadine grapes; phenolic acids

INTRODUCTION

The genus *Vitis* belongs to the botanical family *Vitaceae* (vine family). It is divided into two subgenera, *Euvitis* and *Muscadinia*, based on various morphological criteria and somatic chromosome number, with the former having 38 and the latter having 40. *Euvitis* are bunch grapes that bear berries in a cluster and have a concentrated harvest period during which the entire cluster, or bunch, is harvested as a uniformly ripe, intact unit. In contrast, muscadine cultivars are harvested as individual berries that ripen over an extended harvest period. Most muscadine grape cultivars are hybrids derived from the principal muscadine species *Vitis rotundifolia* Michx. Muscadine grapes have been shown to give profitable yields of fruit with unique aroma and flavor characteristics.

The phytochemical profiles of muscadine have been documented by a few studies. Muscadine grapes are good sources of ellagic acid, resveratrol, quercetin, and other flavonols (*1*, *2*). Muscadine is distinguishable from most other grapes variety in that it has high content of ellagic acid and anthocyanin 3,5-diglucosides (*3*, *4*). Currently, many researchers are focusing on the health benefit of dietary polyphenols, because of their potential antioxidative, antiinflammatory, and anticarcinogenic activities (*5*−*7*). In addition, many efforts have been spent studying the bioavailability of polyphenols in humans. Many polyphenols are absorbed and have been detected in animal and human plasma, including phenolic acids, flavanones, quercetin, proanthocyanidins, and anthocyanins (*8*). Unfortunately, very few studies have been done to evaluate the potential health benefits of muscadine grapes (*9*, *10*). Any information on the potential anticancer benefits of muscadine grapes will be highly valuable.

Colorectal cancer is the second leading cause of cancer death in North America and Europe and the fourth most common form of cancer worldwide (*11*). Many researchers are focusing on the health benefits of phytochemicals in relation to colon cancer. Higher levels of phenolic compounds have been detected in animal intestine than in other tissues after oral supplementation (*12*, *13*). Phenolic compounds could inhibit colon cancer development in human intestines before they are absorbed and are detected in the plasma. The objectives in the current study were to systematically evaluate the potential anticancer activities of polyphenols in different cultivars of muscadine grapes using colon cancer cell lines and to identify the active compounds.

* To whom correspondence should be addressed. Telephone: (706) 542-1067. Fax: (706) 542-1050. E-mail: cakoh@uga.edu.
† Department of Food Science and Technology.
‡ Department of Foods and Nutrition.

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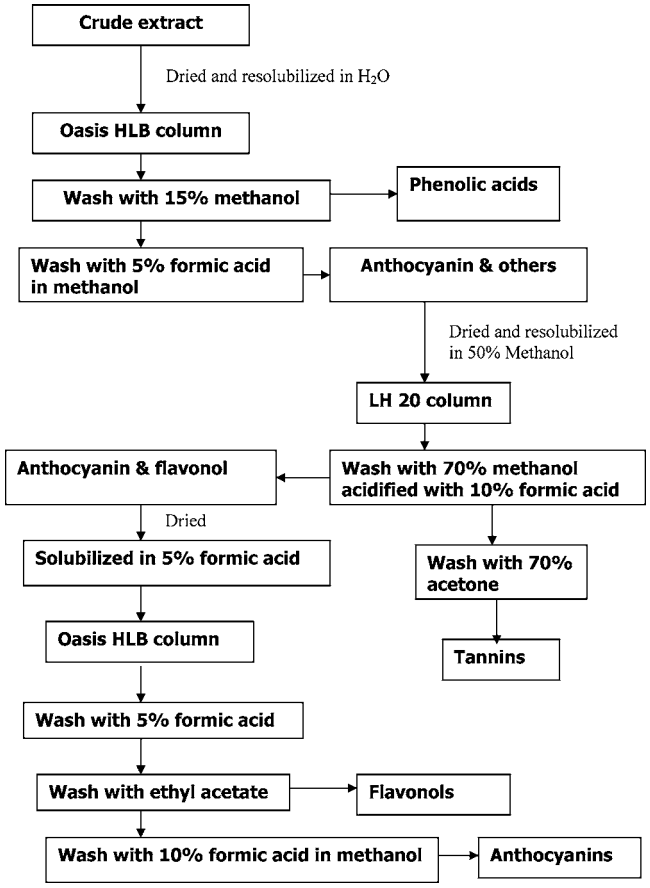

Figure 1. Procedures of fractionation of different phenolic compounds.

MATERIALS AND METHODS

Chemicals and Reagents. Pure standards of gallic acid, ellagic acid, myricetin, quercetin, kaempferol, and *trans*-resveratrol 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). Folin-Ciocalteu reagent, dimethyl sufoxide (DMSO), and vanillin were purchased from Sigma (St. Louis, MO).

Acetonitrile, methanol, *O*-phosphoric 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. Muscadine grapes were collected randomly from Paulk Vineyards (Wray, GA) at the time of optimum harvest maturity. Four cultivars, which include one bronze (Carlos) and three purple (Ison, Noble, and Supreme), were harvested in 2004. Samples were frozen and stored at −40 °C until use.

Extraction and Fractionation. Skins of muscadine grapes were removed, and 50 g of skins was homogenized in 500 mL of acetone/methanol/water/formic acid (40:40:20:0.1, v/v/v/v). Phenolic fractions were obtained using a modified procedure reported by Youdim et al. (*14*) and Oszmianski et al. (*15*). **Figure 1** shows the schematics of the fractionation procedure. The crude extract was freeze-dried using a UNITOP 600L freeze-dryer (Virtis, Gardiner, NY). The dried extract was resolubilized in water, and applied to an activated Oasis HLB cartridge (Waters Corp., Milford, MA) that was washed with water, 15% methanol in water, and finally with methanol acidified with 5% formic acid. The 15% methanol fraction eluted the phenolic acids, and the acidified methanol eluted the anthocyanins and other flavonoids. 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 proanthocyanidins. After freeze-drying the anthocyanin 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 in medium (or DMSO when necessary) for the cell viability and apoptosis assay. Extraction and fractionation were repeated five times and the fractions were pooled to obtain a sufficient amount for the bioassay.

Total Polyphenols Measurement. The extract/fractions were dissolved in 80% methanol. Total polyphenols were measured according to the Folin−Ciocalteu reagent method (*16*). Twenty microliters of sample solution was introduced into 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 for complete reaction and removal of air bubbles. Absorption at 765 nm was measured in a Shimadzu 300 UV−vis spectrophotometer (Shimadzu UV-1601, Norcross, GA). The total polyphenols 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.

Proanthocyanidin Measurement. Measurement of proanthocyanidins was based on the vanillin assay (*17*, *18*). Briefly, tannin fractions of different muscadine cultivars were dissolved in methanol, and 250 μL of sample solutions was added to 1.75 mL of vanillin reagent (0.5 g of vanillin in 50 mL of 70% sulfuric acid). The mixtures were incubated at 20 °C for 15 min. Absorption at 765 nm was measured in a Shimadzu 300 UV−vis spectrophotometer. Blanks (tannin fractions without vanillin reagent) were also measured and subtracted from the sample−reagent reading. The proanthocyanidin quantification was expressed as catechin equivalents (CAE), using a standard curve generated with 250, 500, 1000, and 1500 mg/L of catechin.

Hydrolysis. Acid hydrolysis was conducted to convert phenolic glycosides into aglycons. In the phenolic acid fractions and flavonol fractions, samples were dissolved in methanol containing 1.2 N HCl (40 mL of methanol + 10 mL of 6 N HCl). The sample solutions were placed in a water bath at 80 °C while shaking at 200 rpm for 2 h. For the anthocyanin hydrolysis, anthocyanin 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 samples were cooled in ice bath in the 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. For the analysis of phenolic acids and flavonoids, a procedure previously reported by our laboratory was used (*1*). A Beckman Ultrasphere C18 ODS 4.6 × 250 mm column was used with column temperature at 40 °C. The mobile phases were solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and solvent C, water. The gradients were as follows: at 0 min, 100% solvent A; at 5 min, 90% solvent A and 10% solvent B; at 25 min, 30% solvent A and 70% solvent B; and at 30 min, 30% solvent A and 70% solvent B, with 5 min postrun with 100% solvent C. The flow rate was 1 mL/min. Phenolic compounds were detected at wavelengths of 260, 313, and 360 nm.

For the anthocyanin and anthocyanidin analysis, the same column was used. The mobile phases were solvent A, *O*-phosphoric acid/methanol/water (5:10:85, v/v/v), and solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradients were as follows: 100% solvent A at 0 min, 90% solvent A and 10% solvent B at 5 min, 50% solvent A and 50% solvent B at 25 min, with 5 min postrun with HPLC-grade water. Anthocyanin and anthocyanidin were detected at 520 nm.

Cell Cultures. Cancer cell lines were purchased from ATCC (Manassas, VA). Two cancer cells were used: (1) HT-29 human colon, colorectal adenocarcinoma cultured in ATCC McCoy's 5a medium with 1.5 mM L-glutamine−fetal bovine serum (90%:10%); and (2) Caco-2 human colon, colorectal adenocarcinoma cultured in ATCC 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−fetal bovine serum (80%:20%). Cancer cells were grown in an incubator with 5% CO_2 at 37 °C. Medium was changed two or three times per week.

Cell Viability Assay. Uniform amounts of cells in growth media ($\sim 1 \times 10^4$ in the case of HT-29 and ~5000 in Caco-2) were inoculated into each well of a 96-well, flat-bottom plate. The growth medium was replaced with 100 μL of media containing different concentrations of muscadine extracts/fractions after 24 h of incubation at 37 °C in 5% CO_2. 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, DMSO was added initially to help dissolve the phenolic acid, flavonol, and tannin fractions. The final DMSO content was 0.25%. None of the extracts/fractions added changed the pH (about 7.2) of the culture medium (data not shown). After 48 h of incubation, viable cells were determined by ATCC MTT assay at 570−655 nm using a Bio-Rad Model 680 Microplate Reader (Hercules, CA). Briefly, a mitochondrial enzyme in living cells, succinate dehydrogenase, reduced the yellow tetrazolium salt (MTT) to insoluble purple formazan crystals. Therefore, the amount of formazan produced was proportional to the number of viable cells (*19*). Inhibition of cell population growth was calculated using the following formula:

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

IC_{50} is the extract concentration under which a 50% inhibition of cell population growth occurred. IC_{50} was calculated by interpolation from dose−response curves. With anthocyanin fractions, IC_{25} (the extract concentration under which a 25% inhibition occurred) was also calculated.

Detection of Apoptosis. DNA fragmentation was measured by quantification of cytosolic oligonucleosome-bound DNA using a cell death detection ELISA kit (Boehringer Mannheim, Roche). Briefly, $\sim 1 \times 10^5$ HT-29 or 5×10^4 Caco-2 cancer cells after being treated with different concentrations of anthocyanin extract were lysed and the lysate was centrifuged at 13 000*g* for 15 min. The supernatant (cytosolic fraction) was used as antigen source in a sandwich ELISA with a primary anti-histone antibody coated to the microtiter plate. After incubation at 20 °C for 90 min, the conjugate solution (a secondary anti-DNA antibody coupled to peroxidase) was added. After incubation at 20 °C for another 90 min, the substrate (ABTS) solution was added. 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 four cultivars were statistically compared. Statistical analysis was conducted by the General Linear Model (GLM) followed by Duncan's multiple-range test at $\alpha = 0.05$ (SAS 8.2, SAS Inst., Inc., 1999).

RESULTS

Total Polyphenols Measurement and Proanthocyanidin Estimation. The phenolic compounds extracted from the skins of muscadine grapes were separated into phenolic acids, flavonols, tannin/proanthocyanidins, and anthocyanins. The total polyphenol contents/proanthocyanidin estimation of different extracts/fractions from muscadine grapes are shown in **Table 1**. The total polyphenolic content percentage was calculated on the basis of GAE/(fraction weight) × 100% in order to quantify the purity of different extracts/fractions. As expected, the crude extract had lower purity (less than 10% of total polyphenolic content) than the other fractions. High total polyphenolic contents were observed in flavonol and tannin fractions. The total polyphenolic contents of flavonol fractions ranged from 76.3% in Carlos to 86.1% in Supreme. Tannin fractions contained from 85.1% (Carlos) to 93.2% (Noble) total polyphenols. Phenolic acid fractions had relatively lower purity than the other fractions with total polyphenols ranging from 14.3% to 27.4%.

Table 1. Total Polyphenolic Content/Proanthocyanidin Estimation (Weight Percent) of Different Extracts/Fractions from Muscadine Grapes

cultivars	crude extract	phenolic acids	flavonols	tannins
Carlos	8.9 ± 0.2[a]	24.9 ± 0.4	76.3 ± 2.2	85.1 ± 4.4 (64.6 ± 2.2)[b]
Ison	8.2 ± 0.3	14.3 ± 0.3	86.0 ± 1.4	86.1 ± 4.0 (67.8 ± 1.5)
Noble	7.9 ± 0.3	25.8 ± 0.3	82.4 ± 2.1	93.2 ± 2.5 (74.7 ± 2.4)
Supreme	9.3 ± 0.2	27.4 ± 0.7	86.1 ± 1.5	90.5 ± 3.3 (81.6 ± 2.9)

[a] Values represent total phenolic content percentages. Total polyphenols were expressed as gallic acid equivalents (GAE), and the total phenolic content percentage was calculated on the basis of GAE/(fraction weight) × 100%. Results are averages of triplicate analyses ± standard error. [b] Values without parenthesis represent total phenolic content percentages. Values in parentheses are proanthocyanidin content percentages. The proanthocyanidin estimations were expressed as catechin equivalents (CAE), and the proanthocyanidin content percentage was calculated on the basis of CAE/(fraction weight) × 100%. Results are averages of triplicate analyses ± standard error.

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

cultivars	ellagic acid	resveratrol	myricetin	quercetin	kaempferol
			Phenolic Acid Fraction		
Carlos	4.2 ± 0.1 (16.7 ± 0.5)	0.4 ± 0.0 (1.5 ± 0.1)	ND[b]	0.4 ± 0.0 (1.6 ± 0.1)	ND
Ison	1.8 ± 0.1 (12.8 ± 0.8)	0.4 ± 0.0 (3.0 ± 0.0)	0.3 ± 0.0 (2.4 ± 0.1)	1.1 ± 0.0 (7.7 ± 0.1)	ND
Noble	4.3 ± 0.3 (16.6 ± 1.2)	0.3 ± 0.0 (1.1 ± 0.0)	0.9 ± 0.0 (3.4 ± 0.1)	1.1 ± 0.0 (4.5 ± 0.1)	ND
Supreme	3.4 ± 0.1 (12.6 ± 0.4)	0.2 ± 0.0 (0.7 ± 0.0)	ND	1.2 ± 0.0 (4.3 ± 0.1)	ND
			Flavonol Fraction		
Carlos	5.9 ± 0.2 (7.7 ± 0.2)	7.1 ± 0.1 (9.3 ± 0.1)	3.6 ± 0.1 (4.7 ± 0.1)	5.0 ± 0.1 (6.6 ± 0.1)	1.3 ± 0.0 (1.7 ± 0.1)
Ison	6.6 ± 0.5 (7.6 ± 0.6)	2.4 ± 0.1 (2.8 ± 0.1)	2.6 ± 0.1 (3.1 ± 0.1)	2.9 ± 0.1 (3.4 ± 0.2)	1.7 ± 0.1 (2.0 ± 0.1)
Noble	8.1 ± 0.6 (9.8 ± 0.7)	4.0 ± 0.1 (4.9 ± 0.1)	4.1 ± 0.1 (5.0 ± 0.2)	2.7 ± 0.2 (3.3 ± 0.2)	1.7 ± 0.1 (2.1 ± 0.1)
Supreme	4.1 ± 0.4 (4.8 ± 0.5)	2.5 ± 0.1 (2.9 ± 0.1)	3.0 ± 0.3 (3.5 ± 0.3)	3.5 ± 0.2 (4.1 ± 0.2)	0.8 ± 0.0 (0.9 ± 0.1)

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

The vanillin assay can specifically measure the content of proanthocyanidins instead of all phenols (*20*). The proanthocyanidin content percentages of tannin fractions are shown in parentheses in **Table 1**. The proanthocyanidin content percentage was calculated as CAE/(fraction weight) × 100%. Tannin fractions contained from 64.6% (Carlos) to 81.6% (Supreme) of proanthocyanidin.

Individual Phenolic Compounds Assessment. Table 2 shows the individual phenolic acids and flavonoids in phenolic acid fractions and flavonol fractions. The identification of individual phenolic compounds was based on the retention times and characteristic spectra of standards. The major identified compound in the phenolic acid fraction was ellagic acid.

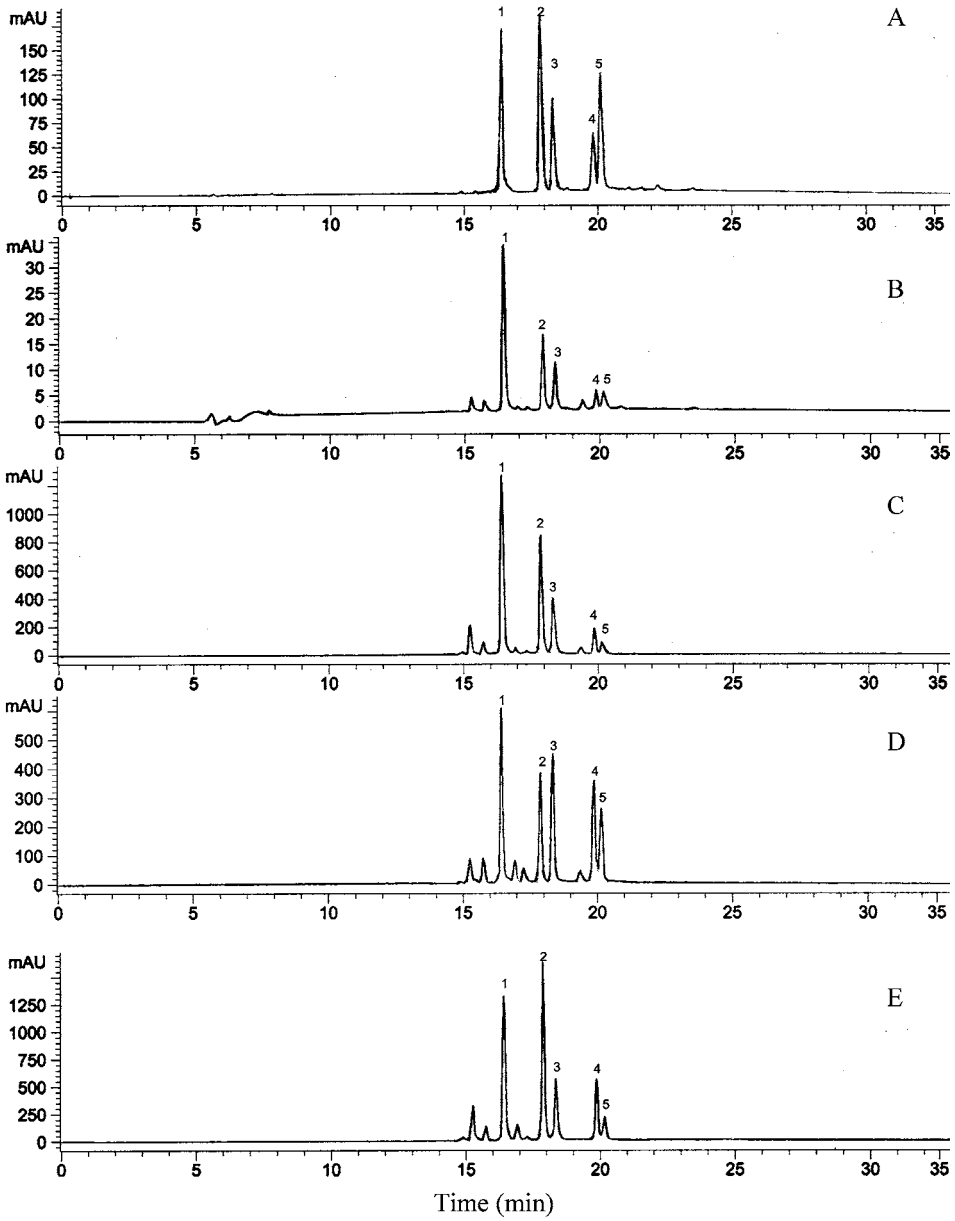

Figure 2. Chromatogram of analytical HPLC of muscadine anthocyanin fractions after acid hydrolysis: (**A**) anthocyanidin standards, (**B**) Carlos fraction, (**C**) Ison fraction, (**D**) Noble fraction, (**E**) Supreme fraction. Peaks: (1) delphinidin, (2) cyanidin, (3) petunidin, (4) peonidin, and (5) malvidin.

Fractions from Carlos, Noble, and Supreme contained about 4% ellagic acid on a dry weight percentage. That represents about 15% of the total polyphenol content. Ison had relatively lower contents of ellagic acid (1.8%). No kaempferol was detected in this fraction of the four cultivars, but low levels of resveratrol and quercetin were found. Myricetin was detected in Ison and Noble, but not in Carlos and Supreme.

All five compounds (ellagic acid, resveratrol, myricetin, quercetin, and kaempferol) were found in flavonol fractions. The levels of myricetin ranged from 2.6% (Ison) to 4.1% (Noble), and quercetin ranged from 2.7% (Noble) to 5.0% (Carlos) of fraction dry weight. Relatively lower levels of kaempferol were detected in the range of 0.8−1.7%. The resveratrol content in flavonol fractions ranged from 2.4% in Ison to 7.1% in Carlos. A moderate amount of ellagic acid remained in flavonol fractions and ranged from 4.1% to 8.1%.

Among the four different fractions, the anthocyanin fraction had the highest purity with more than 90% of the components (except for Carlos) identified. Anthocyanin fractions were first injected into the HPLC without hydrolysis. Five peaks were obtained in every cultivar. None of these five peaks matched the retention time and characteristic spectra of eight anthocyanin standards (monoglycosides). The anthocyanidin profiles were obtained after acid hydrolysis (**Figure 2B−E**). Five anthocyanin standards (Dp-Glc, Cy-Gal, Pt-Glc, Pn-Gal, and Mv-Glc) were hydrolyzed to yield the characteristic retention times and spectra of anthocyanidins. Five dominant anthocyanidins were identified in the four cultivars. It has been reported that the predominant anthocyanins in muscadine grape are 3,5-diglucosides (*2*, *21*). To report the purity of the fractions, the amount of different anthocyanidins was expressed as a specific weight of diglucosides, following the methodology reported by Talcott and Lee (*2*, *21*).

Table 3 shows the major individual anthocyanidins in the anthocyanin fractions. Low levels of peonidin and malvidin were found in Carlos, with anthocyanidin diglucosides at 4.4% and 2.2%, respectively. The major anthocyanidins in Carlos were delphinidin, cyanidin, and petunidin. Except in Noble (with malvidin at 6.8%), only low levels (about 2%) of malvidin were found in Ison and Supreme. The two highest concentrations of

Table 3. Major Individual Anthocyanidins (Weight Percent) in the Anthocyanin Fraction after Acid Hydrolysis[a]

cultivars	delphinidin	cyanidin	petunidin	peonidin	malvidin	total antho-cyanidin
Carlos	21.8 ± 0.7[b]	10.6 ± 0.7	13.0 ± 0.4	4.4 ± 0.3	2.2 ± 0.1	51.9 ± 2.1
Ison	37.4 ± 0.3	24.1 ± 0.1	23.7 ± 0.2	8.2 ± 0.1	2.1 ± 0.0	95.5 ± 0.6
Noble	22.7 ± 0.5	13.5 ± 0.2	31.4 ± 0.4	18.9 ± 0.2	6.8 ± 0.1	93.3 ± 1.2
Supreme	25.3 ± 0.3	31.3 ± 0.2	21.2 ± 0.1	16.0 ± 0.1	2.3 ± 0.1	96.1 ± 0.7

[a] Values are averages of triplicate analyses ± standard error. [b] Amount of different anthocyanidins was expressed on the basis of a specific weight of anthocyanidin diglucosides.

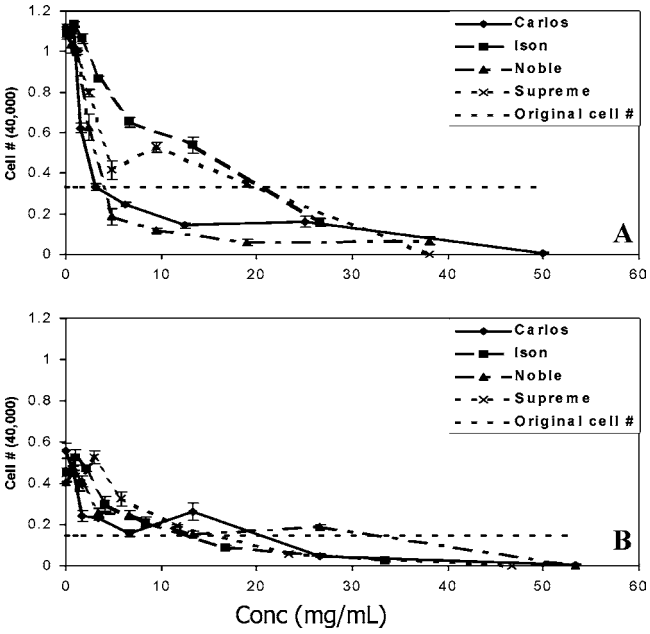

Figure 3. Inhibition of HT-29 and Caco-2 cancer cell population growth by crude extracts of muscadine grapes (mean ± SE, $n = 4$). The *x* axis is the concentration (mg/mL) of extracts in the culture medium. The original cell # is the cell number after 24-h incubation and before treatments were applied: (**A**) HT-29, (**B**) Caco-2.

anthocyanidins in Noble were delphinidin and petunidin, representing 22.7% and 31.4%, respectively. The contents of malvidin, cyanidin, and peonidin in Noble ranged from 6.8% to 18.9%. The contents of anthocyanidins identified were added up to represent the purity of the anthocyanin fractions. In general, more than 90% compounds in the whole dried weight of fractions were identified, representing 93.3%, 95.5%, and 96.1% in Noble, Ison, Supreme, respectively. In contrast, the identified anthocyanidin only accounted for 51.9% of the total weight in the fraction from Carlos.

Cell Viability. As expected, the crude extract showed lower inhibitory effects compared to the subsequent fraction preparations. Inhibition of HT-29 and Caco-2 cancer cell population growth by crude extracts of muscadine grapes is shown in **Figure 3**. Although the bioactivities in different cultivars varied with different cancer cell lines, lower IC_{50}s (meaning high inhibitory activity) were observed in Carlos and Noble in both HT-29 and Caco-2 cells. IC_{50} ranged from 1 to 2 mg/mL in Carlos and Noble, while the IC_{50}s for Ison and Supreme were significantly higher ($P < 0.01$). This was especially remarkable in HT-29, where 50% inhibition of cell population growth did not occur until the concentration reached 7 mg/mL of Ison crude extract.

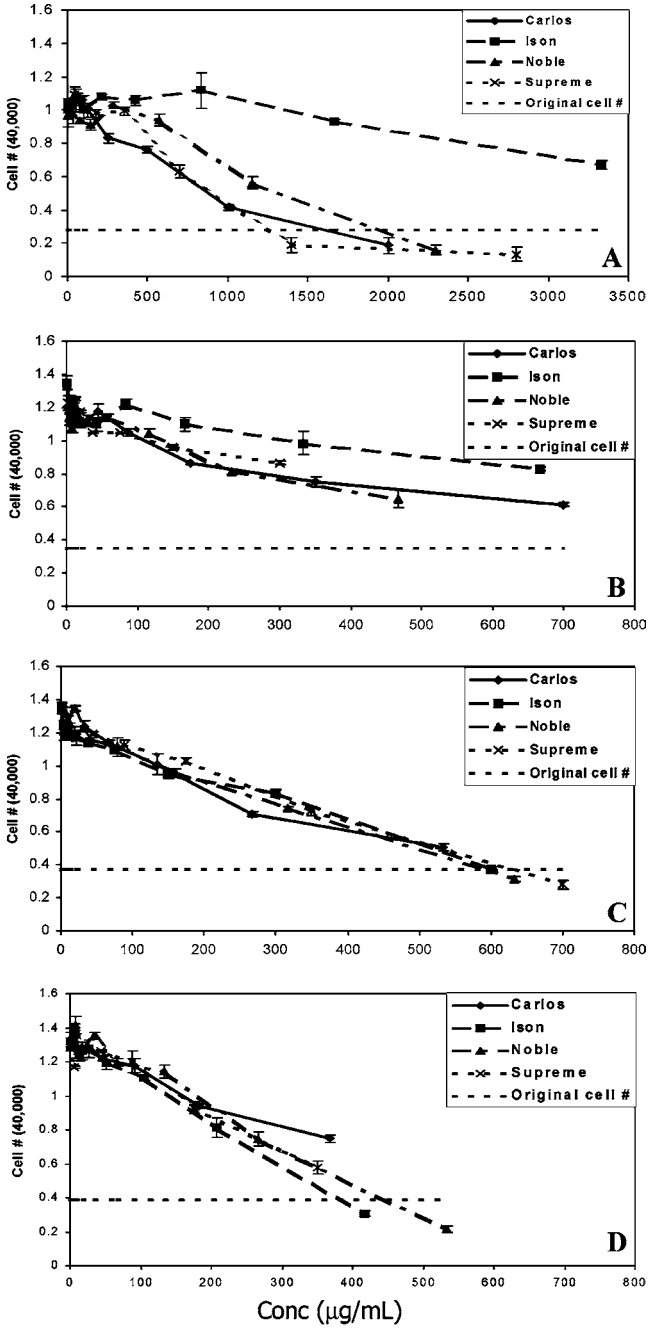

Figure 4. Inhibition of HT-29 cancer cell population growth by different fractions of muscadine grapes (mean ± SE, $n = 4$). The *x* axis is the concentration (μg/mL) of extracts in the culture medium. The original cell # is the cell number after 24-h incubation and before treatments were applied: (**A**) phenolic acid fractions, (**B**) flavonol fractions, (**C**) tannin/proanthocyanidin fractions, (**D**) anthocyanin fractions.

The inhibition of HT-29 cancer cell population growth by four different fractions of muscadine grapes is shown in **Figure 4**. In the phenolic acid fractions, 50% inhibition of cancer cell population growth was observed at 500−1000 μg/mL with Carlos, Noble, and Supreme (**Figure 4A**). In contrast, the phenolic acid fraction in Ison showed significantly lower ($P = 0.00012$) bioactivity than the other three cultivars. The IC_{50} was around 3000 μg/mL. This was in the effective range of the crude extract. Among all four fractions, the highest bioactivities were observed with the anthocyanin fractions (**Figure 4D**). The inhibitory effects were very close among the different cultivars, with the IC_{50} around 200 μg/mL. To more accurately present

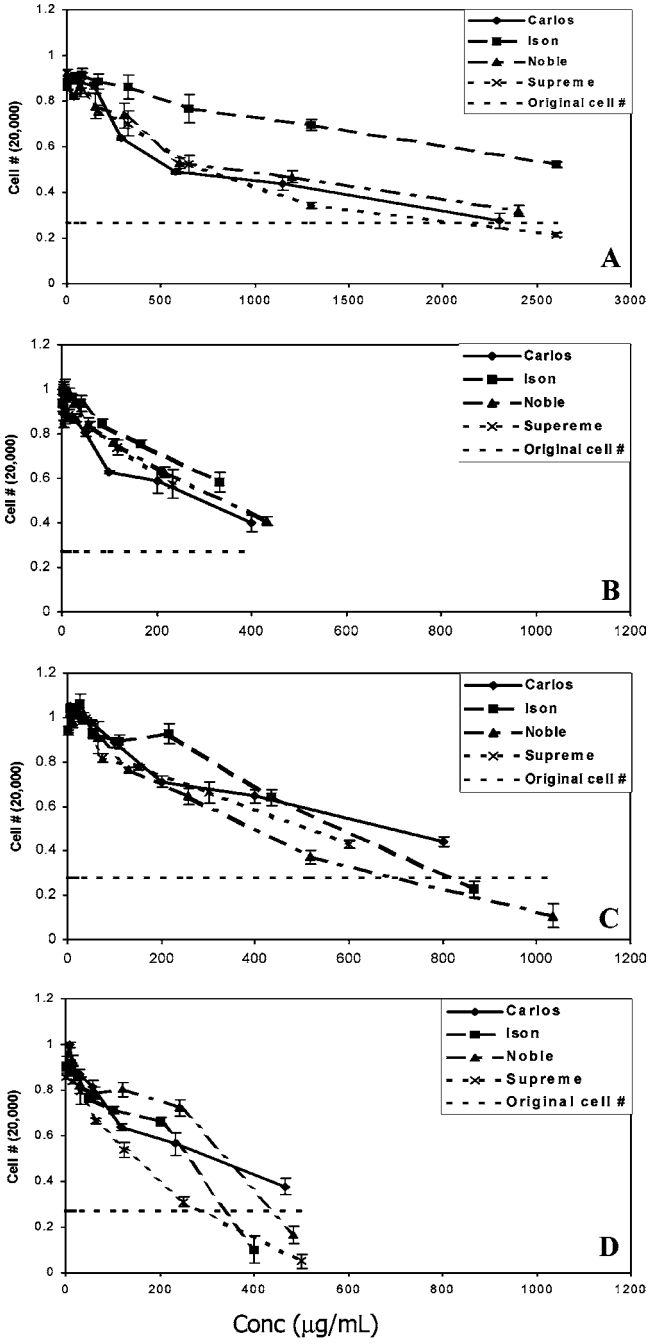

Figure 5. Inhibition of Caco-2 cancer cell population growth by different fractions of muscadine grapes (mean ± SE, $n = 4$). The *x* axis is the concentration (μg/mL) of extracts in the culture medium. The original cell # is the cell number after 24-h incubation and before treatments were applied: (**A**) phenolic acid fractions, (**B**) flavonol fractions, (**C**) tannin/proanthocyanidin fractions, (**D**) anthocyanin fractions.

the effective dosage level of anthocyanins, IC_{25} was also calculated. More than 25% inhibition of cell population growth (IC_{25}) was observed with about 100 μg/mL of anthocyanin fractions in all the four cultivars.

Intermediate bioactivities were observed in the flavonol and tannin fractions (**Figure 4B,C**). In tannins, a 50% inhibition was observed around 300 μg/mL, and the effective dosage levels were close for different cultivars. In the case of flavonols, the IC_{50} was around 300 μg/mL for Carlos, Ison, and Supreme. A significantly higher ($P = 0.0007$) IC_{50} (600 μg/mL) was observed with the flavonol fractions of Ison.

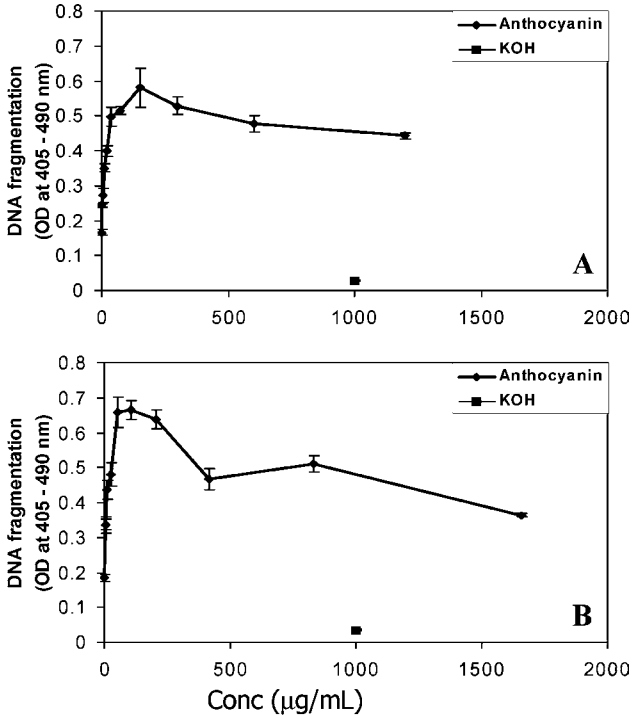

Figure 6. DNA fragmentation in HT-29 and Caco-2 associated with anthocyanin fractions (mean ± SE, $n = 3$): (**A**) anthocyanin fractions of Supreme in HT-29 and (**B**) anthocyanin fractions of Ison in Caco-2 cell line. KOH (1 M) was a control reagent to induce necrosis.

Similar trends of population growth inhibition were found in Caco-2 cells. Inhibition of Caco-2 cancer cell population growth by different fractions of muscadine grapes is shown in **Figure 5**. Among phenolic acid fractions, the lowest bioactivity was observed in Ison, with an IC_{50} around 2000 μg/mL, while the IC_{50} was around 500 μg/mL with the other three cultivars (**Figure 5A**). As in HT-29 cells, the anthocyanin fractions showed the highest bioactivities among the four fractions. The IC_{50} of the anthocyanin fractions ranged from 100 to 300 μg/mL. Unlike in HT-29 cells, where the inhibitory effects were close, Supreme showed the highest bioactivity, with an IC_{50} around 100 μg/mL. The lowest effect was observed in Noble, with an IC_{50} around 300 μg/mL (**Figure 5D**). In addition, the IC_{25} of anthocyanin fractions ranged from 50 to 150 μg/mL. Supreme showed the lowest IC_{25}, with 50 μg/mL, and the highest IC_{25} (150 μg/mL) was observed in Noble.

In flavonol fractions, Ison showed the lowest inhibitory effect, with an IC_{50} of 350 μg/mL (**Figure 5B**). This was similar to the HT-29 cell value. The IC_{50} for flavonol fractions in Caco-2 cells ranged from 200 to 350 μg/mL. With tannin fractions, the IC_{50} ranged from 300 to 500 μg/mL (**Figure 5C**).

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. Therefore, DNA fragmentation is the primary physiological characteristic of apoptosis. **Figure 6** shows the DNA fragmentation in HT-29 and Caco-2 cell lines resulting from the treatment with anthocyanin fractions. In HT-29 cells, a significant increase ($P = 0.0095$) in DNA fragmentation was observed at as low as 10 μg/mL of anthocyanin fraction from the Supreme cultivar. DNA fragmentation increased with increasing content of anthocyanins and reached its maximum

between the concentrations of 100 and 300 μg/mL, which was about 4 times that of the no-treatment control. Further increases in the anthocyanin concentration resulted in less increase in DNA fragmentation, although it was at least 2 times higher than the control in the evaluated dosage levels (300−1200 μg/mL). A similar trend was found in the Caco-2 cell line. A significant increase ($P < 0.001$) in DNA fragmentation was observed after treatment with anthocyanins from the Ison cultivar (**Figure 6B**). DNA fragmentation increased with increasing content of anthocyanins and reached its maximum in the range from 50 to 250 μg/mL, which was about 3 times the DNA fragmentation level of the no-treatment control. DNA fragmentation was about 2 times that of the control when the anthocyanin dosage was from 400 to 1600 μg/mL. The KOH treatment resulted in significantly lower ($P < 0.001$) levels of DNA fragmentation than control in both HT-29 and Caco-2 cells.

DISCUSSION

Most phenolics in muscadine are located in the skin and the seed, and the pulp has a very low level of polyphenols (*1*, *22*). Skins are also the primary source of anthocyanins. Therefore, polyphenols were only extracted from the skins of muscadine grapes in this study. Since ellagic acid is not normally found in other grape species (*23*, *24*), a few studies have been done on its evaluation in muscadine grape. Talcott and Lee (*2*) reported that ellagic acid content (102 mg/L) was much higher than myricetin, quercetin, and kaempferol (21.3−50.5 mg/L) in Noble juice. Our previous work found that ellagic acid was the most abundant (6.2−22.2 mg/100 g) phenolic compound in muscadine skin (*1*). In the current study, high amounts of ellagic acid were found not only in the phenolic acid fraction but also in flavonol fractions. The levels of myricetin, quercetin, and kaempferol in the flavonol fractions in the current study were also in agreement with previous reports (*1*, *2*).

Many recent studies have focused on the concentration of resveratrol in wine, probably because of the highly publicized "French paradox" (*22*). High contents of resveratrol are found in muscadine grapes (a few micrograms/gram of whole grape) (*1*, *22*). This is in agreement with the results of the current study (2.4−7.1% of the dry weight of flavonol fractions), although the unit is different.

As a bronze-skinned grape, Carlos had very low anthocyanin content (*1*), and this may be part of the reason that a low yield and low purity of the anthocyanin fraction were observed in Carlos. Six anthocyanidins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin) have been reported in Noble juice with pelargonidin at a low concentration (*2*). In the current study, exactly five anthocyanidin diglucosides (Dp-3,5-diglucoside, Cy-3,5-diglucoside, Pt-3,5-diglucoside, Pn-3,5-diglucoside, Mv-3,5-diglucoside) were found in Noble (*21*). The two highest concentrations of anthocyanidins in the Noble fraction in our study were delphinidin and petunidin, representing 22.7% and 31.4%, respectively. These values are in agreement with a previous report (*25*) in which 35.5% of Dp-diglucoside and 26.9% of Pt-diglucoside were found in the total anthocyanins.

Although current literature about the origin and treatment of cancer is very complex and far from clear, scientists have generalized six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (*26*). Among these, deregulated cell proliferation and obligate compensatory suppression of apoptosis provide a minimal "platform" necessary to support further neoplastic progression. Targeting of these critical events should have potent and specific therapeutic consequences (*27*).

Many studies have documented the potential anticancer effects of ellagic acid, resveratrol, and flavonols (especially quercetin) (*6*, *28*). Ellagic acid at a concentration of 10^{-5} M induced G1 arrest, inhibited overall cell growth, and induced apoptosis in cervical carcinoma CaSki cells (*29*). In an animal study, both ellagic acid and quercetin significantly reduced tumor incidence, and ellagic acid was found to be a better chemopreventor than quercetin (*30*). More importantly, ellagic acid can synergistically potentiate the effects of quercetin (at 5 and 10 μM, respectively) in the reduction of proliferation and viability and the induction of apoptosis in human leukemia MOLT-4 cell (*9*). In a similar study, a synergistic interaction among ellagic acid, resveratrol, and quercetin was observed in the induction of apoptosis and alteration of cell number and viability (*10*). In the current study, flavonol fractions had relatively high contents of ellagic acid, resveratrol, and quercetin. Their high inhibitory activities (IC_{50}, 200−350 μg/mL) may be the result of the synergistic combination of these polyphenols.

As natural pigments, the potential anticancer activities of anthocyanins have been evaluated in a few studies (*31*, *32*). After separation of bilberry phenolic extract through column chromatography, Katsube et al. (*33*) found that the anthocyanin fraction showed the highest activity in antiproliferation and apoptosis induction in HL-60 human promyelocytic leukemia and HCT-116 colon carcinoma cells. Studies also showed that the bioactivities of different anthocyanin/anthocyanidin varied. Cyanidin and delphinidin were found to inhibit the growth of human tumor cells, in vitro, in the micromolar range, whereas malvidin was less active (*34*). It has been reported that delphinidin, cyanidin, and petunidin can induce apoptosis in the HL-60 human promyelocytic leukemia cell line, whereas pelargonidin, peonidin, and malvidin showed no induction effect (*31*). The anthocyanin fractions in the current study contained high levels of delphinidin, cyanidin, and petunidin and lower levels of peonidin and malvidin. No pelargonidin was detected in any of our fractions. This could be an ideal mixture with potential strong inhibitory effects on cancer cell population growth.

Our cell viability results are in good agreement with previous reports. Kang et al. (*35*) reported that anthocyanins and cyanidin reduced the growth of colon cancer cell lines HT-29 and HCT-116. The IC_{50} of anthocyanins and cyanidin was 780 and 63 μM (equivalent to 350 and 18 μg/mL) for HT-29 cells after 72 h of treatment. Few or no dead cells were found even at the highest tested level (1000 μM of anthocyanins). Also in the HT-29 cell line, Marko et al. (*36*) found that anthocyanidins significantly inhibited tumor cell growth. The IC_{50} ranged from 35 to 90 μM with delphinidin, malvidin, cyanidin, and peonidin. Pelargonidin exhibited the lowest growth inhibitory potential with an IC_{50} of about 200 μM. The IC_{50} of the anthocyanin fraction (with more than 90% purity of diglucosides) in HT-29 cells in the current study was around 200 μg/mL. This was lower than the anthocyanin dosage level reported by Kang et al. (*35*) but higher than the IC_{50} of anthocyanidin reported by Kang et al. (*35*) and Marko et al. (*36*).

Anthocyanin-rich extract (ARE, major component is cyanidin-3-galactoside) resulted in a 60% growth inhibition of HT-29 colon cancer cell lines at the level of 50 μg/mL (*37*). Meanwhile, less than 10% inhibition was observed in NCM-460 normal colon cells with the same concentration of ARE. In another study

conducted by the same group, HT-29 cell growth was inhibited about 50% after 48 h of exposure to 25 μg/mL of chokeberry ARE (*38*). This effective dosage level is close to the IC_{50} in our study (around 200 μg/mL in HT-29 and 100−300 μg/mL in Caco-2). The fact that the major components in their studies were monoglycosides instead of diglucosides could be one of the reasons why the IC_{50} values were different. This can be indicated by the reported trend that anthocyanidins have higher inhibitory activities than anthocyanins (*35*).

The mechanisms by which anthocyanins alter cell population growth are unclear. In the study of Malik et al. (*37*), ARE-treated cells showed a blockage at the G1/G0 and G2/M phase of the cell cycle. A reduction of cells in the G(1) phase and the appearance of a fraction of cells with a hypodiploid DNA content was reported to be associated with treatment of delphinidin in human uterine carcinoma and colon adenocarcinoma cells (*39*). In addition, research also showed that cyanidin and delphinidin or malvidin are potent epidermal growth factor receptor (EGFR) and phosphodiesterase (PDE) inhibitors and thereby interfere with different signaling cascades involved in the regulation of cell growth (*36*). The potential mechanism of inhibition to cancer cell population growth by the muscadine fractions in the current study needs further examination

Using in vitro cancer cell models to screen natural products can speed up the discovery of new anticancer drugs (*40*). Apoptosis-inducing agents are expected to be one of the ideal choices. Assessment of apoptosis caused by anthocyanins/anthocyanidins has been documented in many studies. Lazze et al. (*39*) reported that delphinidin induced apoptosis in uterine carcinoma and colon adenocarcinoma cells. The occurrence of apoptosis was confirmed by morphological and biochemical features, including nuclear condensation and fragmentation, annexin V staining, DNA laddering, and poly (ADP-ribose) polymerase-1-proteolysis. The significant DNA fragmentation caused by treatment with anthocyanin fractions in the current study has provided more evidence in this area. An increase in DNA fragmentation was observed with anthocyanin concentration as low as 10 μg/mL in the current study. Our DNA fragmentation results are in good agreement with previous studies. Fimognari et al. (*41*) observed that 12.5 μg/mL cyanidin-glucoside was sufficient to induce apoptosis in Jurka T-leukemia cells. Induction of necrosis was also observed at high dosage levels. In addition, a dose-dependent increase in DNA fragmentation was also observed in HL-60 cell, which reached a 2-fold increase at 200 μg/mL. In the current study, a maximum increase in DNA fragmentation was observed in the range from 50 to 300 μg/mL of anthocyanin fractions in HT-29 and Caco-2 cancer cells. Further increase of the anthocyanin concentration resulted in less increase of DNA fragmentation, although fewer cells survived at these high concentrations. This suggests that necrosis could occur at high anthocyanin concentrations although anthocyanin appeared to be good apoptosis induction agent. These phenomena have also been found in other antitumor chemicals, such as fostriecin (*42*). Identification of the effective dosage levels for apoptosis induction is a prerequisite for further clinical studies and future cancer prevention. Although further research is desirable, based on the results of the current study, we propose that the most probable effective dosage levels for apoptosis induction were from 10 to 300 μg/mL of anthocyanins in HT29 and from 10 to 250 μg/mL in Caco-2 cells. Beyond these levels necrosis could be the predominant effect. The IC_{50}s of anthocyanins in HT29 and Caco-2 were ∼200 and 100−300 μg/mL, respectively, thus almost/already exceeding the effective apoptosis induction levels. Meanwhile, the IC_{25}s of anthocyanin fractions (50−150 μg/mL) were almost unquestionably in the range of effective apoptosis induction dosages. In the healthy state, cell renewal/proliferation is balanced by cell death/apoptosis. During tumor development, a shift toward proliferation may alter the balance (*27*). Even minute effects on apoptosis induction and inhibition of cell population growth may help maintain balance, thereby decreasing the chance of cancer progression.

This study suggests that phenolic compounds in muscadine skins are potential ingredients for cancer prevention. In regular juice production, pomace (skin, pulp and seeds) is usually lost. Although seeds are difficult to incorporate into the juice production because of flavor and other issues, skins can be easily incorporated. Some methods have been suggested to remove seeds from muscadine pomace to produce a puree that can be used in many food products, such as jams and muffins (*22*). Further studies in this direction will be valuable.

In conclusion, our results suggest that polyphenols from skins of muscadine grapes can inhibit colon cancer cell population growth and induce apoptosis. Dietary intakes of muscadine grapes may have the potential to reduce colon cancer risk. Efforts should be made to conserve these active compounds during food processing.

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