EVALUATION OF ANTICANCER ACTIVITIES OF PHENOLIC COMPOUNDS IN BLUEBERRIES AND MUSCADINE GRAPES

by

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(Under the Direction of CASIMIR C. AKOH)

ABSTRACT

Research has shown that diets rich in phenolic compounds may be associated with lower risk of several chronic diseases including cancer. This study systematically evaluated the bioactivities of phenolic compounds in blueberries and muscadine grapes, and assessed their potential cell growth inhibition and apoptosis induction effects using two colon cancer cell lines (HT-29 and Caco-2), and one liver cancer cell line (HepG2). In addition, the absorption of blueberry anthocyanin extracts was evaluated using Caco-2 human intestinal cell monolayers.

Polyphenols in three blueberry cultivars (Briteblue, Tifblue and Powderblue), and four cultivars of muscadine (Carlos, Ison, Noble, and Supreme) were extracted and freeze dried. The extracts were further separated into phenolic acids, tannins, flavonols, and anthocyanins using a HLB cartridge and LH20 column. In both blueberries and muscadine grapes, some individual phenolic acids and flavonoids were identified by HPLC with more than 90% purity in anthocyanin fractions. The dried extracts and fractions were added to the cell culture medium to test for cell growth inhibition and induction of apoptosis. Polyphenols from both blueberries and muscadine grapes had significant inhibitory effects on cancer cell growth. The phenolic acid fraction showed relatively lower bioactivities with 50% inhibition at 0.5-3 μg/mL. The

intermediate bioactivities were observed in the flavonol and tannin fractions. The greatest inhibitory effect among all four fractions was from the anthocyanin fractions in the three cell lines. Cell growth was significantly inhibited more than 50% by the anthocyanin fractions at concentrations of 15-300 μg/mL. Anthocyanin fractions from blueberries and muscadine grapes also resulted in significant increase in DNA fragmentation, indicating the induction of apoptosis. The results of the transport/absorption study showed that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers although the transport/absorption efficiency was relatively low compared to other aglycone polyphenols. Delphinidin-glucopyranoside (Dp-glc) showed the lowest transport/absorption efficiency, and malvidin-glucopyranoside showed the highest transport/absorption efficiency. Our result indicates that more free hydroxyl groups and less OCH_3 group can decrease the bioavailability of anthocyanins. In addition, the results showed that glucose-based anthocyanins might have higher bioavailability than galactose-based anthocyanins. These findings suggest that blueberries and muscadine grapes intake may reduce cancer risk.

INDEX WORDS: Anthocyanins, Absorption, Apoptosis, Bioavailability, Blueberries, Cancer cell growth, Muscadine grapes, Polyphenols

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DEDICATION

To my wife, Xiaoli, whose love and support gave me strength and hope to make this dream come true.

To my father, Tengyun Yi; my mother, Xinlan Wang; and my elder sister, Weixin Yi, for their endless love …

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Blueberry belongs to the *Vacciniaceae*, a subfamily of the *Ericaceae*. This subfamily has been further divided into the subgenera *Batodendron*, *Euvaccinium, Oxycoccus*, and *Cyanococcus*. The true blueberries belong to the ancient genus *Vaccinium*, subgenus *Cyanococcus*. Blueberries have been considered to be one of the fruits with the highest antioxidant potentials (*1*-*3*). Some studies have evaluated the bioactivities of blueberry. Bomser et al. (*4*) found that the ethyl acetate extracts and further hexane/chloroform subfraction of lowbush blueberry (*V. angustifolium* Ait) significantly induced quinone reductase (QR) activity, an enzyme involved in Phase II xenobiotic detoxification. Smith et al. (*5*) also evaluated the bioactivity of wild blueberry (*V. angustifolium* Ait). They found that the crude 70% acetone extract (flavonoid-rich) exhibited a clear, significant induction of QR activity.

Studies with a systematic evaluation of anticancer activity (antiproliferation and induction of apoptosis) of phenolic compounds in blueberry are very limited. In addition, reports have shown that the contents of phenolic compounds in blueberries vary widely (*1*). This makes the direct evaluation of the bioactivities of different varieties critical.

The genus *Vitis* belongs to the botanical family *Vitaceae* (vine family). The genus 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. The term bunch grape refers to the fact that these cultivars have berries that are borne 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. 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 (*6, 7*). Muscadine is distinguishable from most other grape varieties in that it has high content of ellagic acid and anthocyanin 3,5-diglucosides (*8, 9*). Currently, many researchers are focusing on the health benefit of dietary polyphenols, because of their potential antioxidative, antiinflammatory, and anticarcinogenic activities. Unfortunately, very few studies have been done to evaluate the potential health benefits of muscadine grapes. Any information on the potential anticancer benefits of muscadine grapes will be highly valuable.

Despite the statement that anthocyanins have potential in cancer prevention and other health benefit, a significant gap exists between what has been shown in many in vitro studies and what can be achieved under in vivo condition. One of the key factors needed to correctly relate the in vitro study results to human disease outcomes is information about bioavailability and metabolism (*10*). Although a few studies have been conducted to evaluate the bioavailability of anthocyanins (*11-14*), the information on absorption and metabolism is still very limited.

Information on the bioavailability of pure compounds is a good start to clarify the bioavailability of complex mixtures of phytochemicals (*15*). The combination of phytochemicals in fruits and vegetables is thought to be critical to their powerful antioxidant and anticancer activity (*16, 17*). Similarly, information on the bioavailability of complex mixtures of phytochemicals is essential. Thus, studies on the bioavailability of phenolic compounds such as anthocyanins based on specific crops is a good way to provide direct and valuable information on their absorption.

Seven chapters compose this dissertation including the introduction, summary and conclusions. The second chapter presents a literature review of topics related to blueberries and muscadine grapes, their phenolic compounds, absorptions by human body and potential anticancer activities. The third chapter evaluated the bioactivities of phenolic compounds in blueberries, assessed the potential antiproliferative and apoptosis induction effects on colon cancer cells. The fourth chapter evaluated the potential anticancer activities of polyphenols in different cultivars of muscadine grapes using colon cancer cell lines, and identified the active compounds. Systematical evaluation of the bioactivities of phenolic compounds in blueberries and muscadine grapes using a liver cancer cell line was presented in Chapter 5. The sixth chapter evaluated the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers, and investigated the effects of different aglycones, sugar moieties, and chemical structure on bioavailability of different types of anthocyanins.

The seventh chapter summarized Chapters 3,4, 5, and 6, and presents general conclusions.

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CHAPTER 2

LITERATURE REVIEW

Cancer

Cancer refers to a group of disorders and not a single disease. It is characterized by uncontrolled cell growth and by the ability of tumor cells to metastasize—to invade neighboring tissues and spread to other areas of the body. Cancers may be categorized based on the functions/locations of the cells from which they originate. The following terms are commonly used to distinguish tumors of different origin. Carcinoma- a tumor derived from epithelial cells, those cells that line the surface of our skin and organs. Our digestive tract and airways are also lined with epithelial cells. This is the most common cancer type and represents about 80-90% of all cancer cases reported. Sarcoma- a tumor derived from muscle, bone, cartilage, fat or connective tissues. Leukemia- a cancer derived from white blood cells or their precursors. Lymphoma- a cancer of bone marrow derived cells that affects the lymphatic system. Myelomas- a cancer involving the white blood cells responsible for the production of antibodies.

Hepatocellular carcinoma is one of the most common cancers worldwide. Although it is infrequent in developed countries, rising incidence of heptacellular carcinoma in the United States and other developed countries has been reported (*1, 2*). Colorectal cancer is the second leading cause of cancer death in North America and Europe, and the fourth most common form of cancer worldwide (*3*). 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 (*4, 5*). Phenolic

compounds could inhibit colon cancer development in human intestines before they are absorbed and are detected in the plasma.

In the healthy state, cell renewal/proliferation is balanced by cell death/apoptosis. During tumor development a shift towards proliferation may alter the balance. Deregulated cell proliferation and suppression of apoptosis provides a minimal 'platform' for further neoplastic progression. Scientists have stated that targeting of these critical events should have potent and specific therapeutic consequences (*6*). In other words, even minute effects on apoptosis induction and cell antiproliferation may help maintain balance, thereby decreasing the chance of cancer progression. Apoptosis is the term for programmed cell death characterized by specific morphologic and biochemical properties. Apoptotic cells typically undergo a series of structural changes: blebbing of the plasma membrane; condensation of the cytoplasm and nuclei; and cellular fragmentation into apoptotic bodies, membrane-bound structures containing condensed cytoplasm, some intact organelles, and nuclear fragments. 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.

Phenolic compounds

Phenolic compounds or polyphenols are plant secondary metabolites which possess in common an aromatic ring bearing one or more hydroxyl groups. Up to now, there are more than 8000 natural phenolic compounds that have been identified. Phenolic compounds are classified according to structural complexity and biosynthetic origin. The first modern monograph on phenolic compounds (*7*) included the following classifications: simple phenols, phenolic acids,

phenylacetic acids; cinnamic acids, coumarins, isocoumarins, chromones; lignans; 10 groups of flavonoids; lignins; tannins; benzophenones, xanthones and stilbenes; quinones; and betacyanins.

Phenolic acids and derivatives are simple compounds compared to other polyphenols. The term "phenolic acid" can be applied to a large variety of compounds bearing at least one phenolic hydroxyl group and one carboxyl function. It is, however, a common practice to use this terminology for only a limited number of products such as benzoic and cinnamic acid derivatives. Some authors (*8*) prefer a subdivision into phenolic acids, and phenol aldehydes (C6—C1 compounds), the C6—C2 compounds (such as hydroxyphenylacetic acids and acetophenones) and the C6—C3 compounds (phenylpropanoids, hydroxycinnamic acids). Some of the most commonly existing phenolic acids are salicylic, p-hydroxy benzoic, protocatechuic (3,4-dihydroxybenzoic acid), vanillic, gallic, syringic, gentisic, caffeine, and ferulic acid.

Of the most interesting structure among all the phenolic compounds is C6--C3--C6. It is the structural type encountered most often in phenolic compounds. It forms the backbone for all flavonoids (Figure 2.1), neoflavonoids and condensed tannins and, in a modified form, for isoflavonoids.

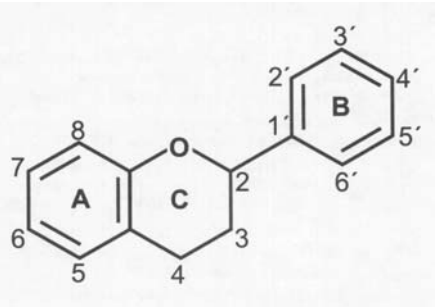


Figure 2.1. Basic structure of flavonoids (From reference (*9*)).

Flavonoids can be further divided into 13 classes, with more than 5000 compounds described by 1990 (*10*).

Six of the most common types of flavonoids are described here.

i) Flavones: They are named from flavone. Several flavones that lack hydroxyl groups in the B ring are known; flavones can have extra hydroxyl group in the A ring. Some of the most encountered examples are chrysin, apigenin, luteolin, diosmetin, apiin, acacetin, scutellarein, chrysoeriol, tricetin, and tricin.

ii) Flavanones: They differ from flavones in having the double bond in the 2,3 position replaced by a sigle bond. E.g., hesperidin, pinocembrin, alpinetin, pinostrobin, strobopinin, cryptostrobin, glabranin, liquiritigenin, naringenin, pruning, naringin, isosakuranetin, sakuranetin, farrerol, matteucinol, butin, eriodictyol, homoeriodictyol, hesperetin, and citromitin.

iii) Flavonols: These differ from flavones only in having a 3-hydroxyl substituent. E.g., quercetin, myricetin, kaempferol, galangin, fisetin, astragalin, morin, herbacetin, quercitrin, rutin, rhamnetin, isorhamnetin, myricitrin, quercetagetin, and gossypetin.

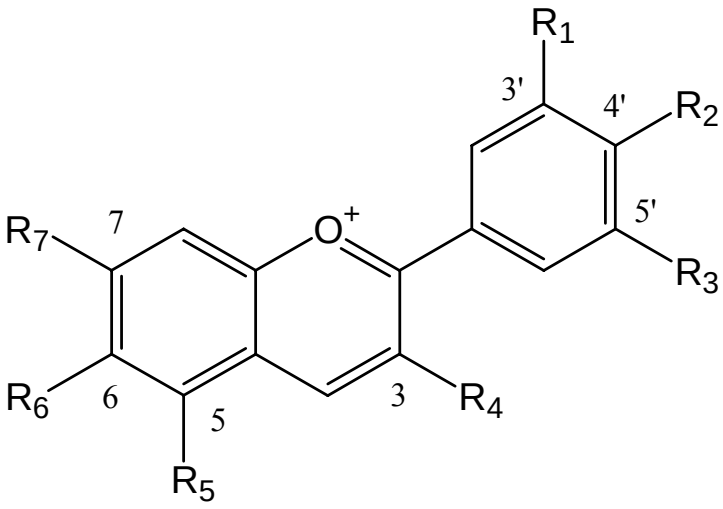
iv) 3-Hydroxyflavanones or dihydroflavonols or flavanonols: They differ from flavanones only in having a 3-hydroxyl substituent. E.g., taxifolin, 7-hydroxy-dihydroflavonol, pinobanksin, strobobanksin, alpinone, garbanzol, aromadendrin, engelitin, fustin, astilbin, dihydromorin, dihydrorobinetin, and dihydrosyringetin.

v) Flavanols (usually mean flavan-3-ols): Flavanols do not have carbonyl group in the 4 position compared with flavonols, and do not have the double bond in the 2,3 position. E.g., catechin, epicatechin, and epigallocatechin.

vi) Anthocyanidins: They have flavylium cation structurally related to the flavonols. E.g., pelargonidin, malvidin, cyanidin, petunidin, peonidin, and delphinidin.

Although belonging to flavonoids according to some researchers, isoflavonoids bear a unique pattern. In the isoflavonoids the carbon skeleton has become a 3-phenylbenzopyran-4-one in which it is no longer possible to detect a $C_6C_3C_6$ pattern (it appears as $C_6C_{2+1}C_6$). Isoflavanones are isomeric with flavanones, and isoflavones are isomeric with flavones. Some examples of isoflavones (genistein and daidzein) occur in legumes.

Anthocyanins are natural pigments present in fruits and vegetables. They are water soluble glycosylated and/or acylated flavonoids derivatives that are the source of most red, pink, purple, and blue colors in plant parts. The aglycone is called anthocyanidin. There are six types of anthocyanidins (Figure 2.2.).



Anthocyanidin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Delphinidin	-OH	-OH	-OH	-OH	-OH	-H	-OH
Cyanidin	-OH	-OH	-H	-OH	-OH	-H	-OH
Petunidin	$-OCH_3$	-OH	-OH	-OH	-OH	-H	-OH
Pelargonidin	-H	-OH	-H	-OH	-OH	-H	-OH
Peonidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	-OH
Malvidin	$-OCH_3$	-OH	$-OCH_3$	-OH	-OH	-H	-OH

Figure 2.2. Basic chemical structures of the major anthocyanins.

Absorption of phenolic compounds

A few researchers have reviewed the absorption and bioavailability of polyphenolic compounds (*11*). A commonly accepted concept is that the polyphenols are absorbed in the intestine by passive diffusion. For this to occur, the glycosylated polyphenols need to be converted to the aglycone by glycosidases in the food or gastrointestinal mucosa, or from the colon microflora. For example, polyphenol glucosides are hydrolyzed by human β-glucosidase in the intestine, whereas polyphenol rhamnosides need to be hydrolyzed by microflora α-rhamnosidases in the colon. There is also evidence for the direct absorption of quercetin-4'-O-glucoside, which have been detected in human plasma after ingestion of food containing these compounds (*12, 13*). It has been suggested that a glucose transport system may be involved in the absorption of quercetin-3-O-glucoside, which was more bioavailable than quercetin in rats (*14*). Quercetin 3-O-rutinoside (rutin) was much less bioavailable than quercetin aglycone in this study. The bioavailability of quercetin glucoside was also much higher than quercetin rutinoside in humans, suggesting that the glucoside is actively absorbed in the small intestine (*14*).

It was originally believed that anthocyanins have to be hydrolyzed to aglycone before they can be absorbed. Only recently, several studies have reported the absorption of anthocyanins as intact forms (*13, 15*). Cyanidin-3-glucoside and cyanidin-3,5-diglucoside have been reported to be incorporated into human plasma with their structures intact (*13*). Fifteen minutes after an oral supplementation of a mixture of 320 mg of cyanidin-3-glucoside (Cy-g) and 40 mg of cyanidin-3,5-diglucoside (Cy-dg) per kg of body weight, rats showed an increased level up to a maximum of 1563 μg (3490 nmol) of Cy-g(cyanidin-3-glucoside)/L and 195 μg (320 nmol) of Cy-dg/L in the plasma. In human plasma, 30 min after intake (2.7 mg of Cy-g and 0.25

mg of Cy-dg/kg of body weight), an average of 11 μg (24 nmol) of Cy-g/L and a trace of Cy-dg were found.

The nature of aglycone of the anthocyanin could influence the bioavailability (*16, 17*). Using animal model, Wu et al. (*17*) reported that Pg-glc had a much higher total urinary excretion than cyanidin-based anthocyanins. McGhie et al. (*16*) also reported the relative concentrations of Dp-based anthocyanins were lower than those of Mv-based anthocyanins in the urine of rats and humans. The authors suggest that this may be a result of the greater number of hydroxyl groups in Dp or the greater hydrophobic nature of Mv that facilitated increased portioning into cells and tissues.

Sugar moieties may influence the absorption of anthocyanins although the mechanisms are far from clear. Higher absorption percentage of anthocyanin glucosides in rabbit urine, was observed than anthocyanin rutinosides after 2 h, and this trend turned into opposite effect after 10-24 h (*18*). The authors suggested the possible reason could be that the anthocyanin glucosides in plasma increase and decrease more quickly than anthocyanin rutinosides. Some studies reported that monoglucosides of flavonoids/quercetin can be transported across the apical membrane of enterocytes by Sodium Dependent Glucose Transporter SGLT1 (*19, 20*). Milbury et al. (*21*) have suggested that the absorption of anthocyanins in their unchanged glycosylated forms may indicate the involvement of the glucose transport receptors, since quercetin and anthocyanidins (aglycones of anthocyanins) share a similar basic flavonoid structure.

Bioactivities of phenolic compounds

Phenolic Acids and Their Derivatives. The inhibitory effect of topically applied caffeic acid, ferulic acid, chlorogenic acid, and curcumin on tumor promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA) has been demonstrated (22). Caffeic acid, ellagic acid,

chlorogenic acid, and ferulic acid (0.02-0.05% in the diet) also inhibited 4-nitroquinoline-1-oxide (4-NQO)-induced tongue carcinogenesis in rats (23). More recent studies indicate that curcumin (0.2% and 0.6% in the diet), administered to azoxymethane (AOM)-treated rats during the promotion/progression stage, inhibited colon tumorigenesis (24). Curcumin (0.1% in the diet) also inhibited intestinal tumorigenesis in mice (25).

Catechins and Related Compounds. The inhibitory activity of catechins, especially (-)-epigallocatechin gallate (EGCG), against carcinogenesis were discovered from tea, since EGCG is the most abundant catechin in tea. The anticancer activity in tea has been demonstrated in different animal models for organ sites such as skin, lung, esophagus, stomach, liver, small intestine, pancreas, colon, bladder, and mammary gland (*11*). The inhibition of EGCG against skin, stomach, colon, and lung carcinogenesis (*26-28*), as well as the growth of human prostate and breast tumors in athymic mice have been reported (*29*, *30*).

An epidemiological study conducted by Yamane et al. (*28*) showed a lower risk of gastric cancer among people who consume a large amount of green tea. (-)-Epigallocatechin gallate (EGCG), one of the main constituents of green tea, inhibited tumor promotion by teleocidin in a two-stage carcinogenesis experiment with the use of mouse skin. The inhibitory effect of EGCG on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced carcinogenesis of the glandular stomach in rats was examined. The percentage of tumor-bearing rats in the group treated with MNNG plus EGCG was 31%, compared to 62% in the MNNG group. The difference was statistically significant ($P < 0.05$).

Quercetin and Other Flavonoids. When given i.p., quercetin and apigenin inhibited melanoma cell (B16-BL6) growth and metastatic potential in syngenetic mice (31). Feeding rats with quercetin or chalcone and 2-hydroxychalcone (0.05% in the diet), during either the

initiation or promotion stage, inhibited 4-NQO-induced carcinoma formation in the tongue. These compounds also decreased cell proliferation and polyamine levels (32). Dietary quercetin inhibited DMBA-induced tumorigenesis in hamster buccal pouch (33). Quercetin and ellagic acid, when given during the initiation stage, also inhibited DEN-induced lung tumorigenesis in mice (34).

Treatment of colon cancer cell lines and primary human colorectal tumors with quercetin (10 μM) reduced the level of Ras protein (*35*). In another study, quercetin and luteolin (20 μM) inhibited the proliferation and induced apoptosis of skin cancer cell lines A431 and other cell lines (*36*).

Resveratrol and Other Grape Constituents. Because of its presence in red wine, the biological activity of resveratrol has received considerable attention. When resveratrol (1-25 μmol) was topically applied together with TPA to mice in a two-stage carcinogenesis model, marked inhibition of the tumor incidence and multiplicity was observed. Resveratrol administration (i.p., 1 mg/kg/day) to rats inhibited the growth of innoculated Yoshida AH-130 ascites hepatoma (37).

A polyphenolic fraction from grape seeds, which contain catechins, procyanidins, and procyanidin gallates, inhibited TPA-promoted skin tumorigenesis in mice previously treated with DMBA (38). A diet enriched with red wine solids (solids from 750 ml of red wine per kg diet), which contained catechins, gallic acid, and other polyphenols, delayed the onset of tumors in the HTLV-1 transgenic mouse (39, 40).

Anthocyanin. Anthocyanin and related flavonoid phytochemicals are considered to be responsible for a range of unique and broad-spectrum health benefits (41). Anthocyanins not only possess antioxidant activity but also mediate other physiological functions related to cancer

suppression (42). The growth inhibitory effects of anthocyanins in K562 leukemia and HCT-15 carcinoma cells are greater than those of other phenolic components, such as flavonols and flavanones (43). Anthocyanin fractions from red soybeans and red beans inhibit the growth of HCT-15 human colon carcinoma cells in vitro and prolong the survival of Balb/C mice bearing syngeneic Meth A tumor cells (44). Extracts of berries of the Vaccinium species inhibit the induction of ornithine decarboxylase activity by the tumor promoter phorbol 12-myristate-13-acetate (TPA) (45).

Synergistic effects. Conney et al. (*46*) investigated the effect of curcumin on differentiation in the human promyelocytic HL-60 leukemia cell model system. It was found, although curcumin alone had little or no effect on cellular differentiation, when it was combined with all-trans retinoic acid or 1-alpha, 25-dihydroxyvitamin D3, a synergistic effect was observed. The author suggested that many dietary chemicals in fruits, vegetables, and other edible plants can prevent cancer by synergisms. Mertens (*47*) investigated low-dose combination of some phenolic compounds on viability and cell cycle kinetics in MOLT-4 cells. Quercetin (10 μM) caused an increase in dead cells, and a slight decrease in cells in the S phase. Apoptosis was confirmed by fluorescent microscopy. Ellagic acid (10 μM) had no effect, while quercetin and ellagic acid together (5 μM each) resulted in massive cell death. Resveratrol (10 μM) resulted in slight changes in cell cycle. The combination of resveratrol and ellagic acid (5 μM each) did not affect the number of dead cells compared to ellagic acid alone, yet reduced cells in the G0/G1 phase, and arrested cells in S phase more than what resveratrol alone did. The author suggested that combinations of polyphenols affect the cell cycle beyond what can be explained by the effects of the single compounds.

There is a large body of literature on the polyphenolic composition and content of plant foods and beverages (*48*). Because of the complexity of this wide group of plant metabolites, however, many polyphenols remain unidentified. Moreover, it is difficult to compare data within the literature, owing to the lack of agreement on an appropriate method to analyze the different types or families of polyphenolic compounds. As a result, information in the literature on the content and composition of polyphenols in plant foods is not only incomplete but sometimes also contradictory and difficult to compare. In addition, because of the synergism, it will be a high risk to predict the bioactivities of phenolic compounds based only on their contents in the fruits and other food systems. Direct and systematic evaluation of the potential health benefits based on specific fruits and food systems will be highly valuable.

Botanical characteristics of blueberries and muscadine grapes

Blueberries. Blueberry belongs to the *Vacciniaceae*, a subfamily of the *Ericaceae*. This subfamily has been further divided into the subgenera *Batodendron*, *Euvaccinium, Oxycoccus*, and *Cyanococcus*. The true blueberries belong to the ancient genus *Vaccinium*, subgenus *Cyanococcus*.

In general, blueberries can be classified as highbush, lowbush, and rabbiteye. The cultivated highbush blueberry, which range in height from 1.5 to 7 m, has been developed primarily from two species, *V. corymbosum* L. and *V. australe* Small. The term "lowbush blueberry" includes several species, eg. *V. myrtilloides* Michaux, *V. angustifolium* Aiton, *V. lamarcki*i Camp., and *V. brittonii* Porter. The rabbiteye blueberry (*V. ashei* Reade), which can attain heights of ~10 m, are found throughout most of northern Florida, southern Alabama, and Georgia. Some of the cultivated types of rabbiteye blueberry are: Tifblue, Woodard, Climax,

Briteblue, Bluebelle, Delite, Southland, Brightwell, Premier, Powderblue, Tifblue, Centurion, Beckyblue, Aliceblue, and Baldwin.

Muscadine grapes. The genus *Vitis* belongs to the botanical family *Vitaceae* (vine family). The genus 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. The term bunch grape refers to the fact that these cultivars have berries that are borne in a cluster and have a concentrated harvest period during which the entire cluster, or bunch, is harvested as a uniformally ripe intact unit. In contrast, muscadine cultivars are harvested as individual berries that ripen over an extended harvest period. The American bunch grape cultivars, such as 'Concord' or 'Niagara,' are often thought to be derived mostly from the native *V. labrusca* L. species commonly know as the "fox grape." Some researchers point out that most are hybrids among a number of native species. Many of them also contain some of the European type *V. vinifera* L. germplasm. Most muscadine grape cultivars are hybrids derived from the principal muscadine species *V. rotundifolia* Michx.

Background information on phenolic compounds in blueberries and muscadine grapes

Blueberries. Blueberries have been considered to be one of the fruits with the highest antioxidant potentials (*49-51*). Some studies have evaluated the bioactivities of blueberry. Bomser et al. (*45*) found that the ethyl acetate extracts and further hexane/chloroform subfraction of lowbush blueberry (*V. angustifolium* Ait) significantly induced quinone reductase (QR) activity, an enzyme involved in Phase II xenobiotic detoxification. In the same study, the crude extracts significantly inhibited the induction of ornithine decarboxylase (ODC). Smith et al. (*52*) also evaluated the bioactivity of wild blueberry (*V. angustifolium* Ait). They found that the crude 70% acetone extract (flavonoid-rich) exhibited clear, significant induction of QR activity.

In an evaluation of the bioactivity of lowbush blueberry (*Vaccinium angustifolium*), and highbush blueberry (*Vaccinium corymbosum*), Katsube et al. (*42*) found that the ethanol extract significantly inhibited proliferation of HL60 human promyelocytic leukemia cells and HCT116 human colon carcinoma cells in the range from 2 to 6 mg/mL.

Studies with a systematic evaluation of anticancer activity (antiproliferation and induction of apoptosis) of phenolic compounds in blueberry are very limited. In addition, reports have shown that the contents of phenolic compounds in blueberries vary widely. Our previous study evaluated the phenolic contents of different cultivars of blueberry, including rabbiteye (*Vaccinium ashei* Reade) and southern highbush (*Vaccinium corymbosum L.* Hybrids) blueberries and found significant differences in polyphenol contents among different cultivars (*49*). This makes the direct evaluation of the bioactivities of different varieties critical. The total anthocyanin contents in rabbiteye have been reported to be about 100 mg/100g FW, and the flavonoids ranged from 33 to 387 mg/100g FW (*49*). Our objectives in this study were to systematically evaluate bioactivities of phenolic compounds in rabbiteye blueberries, to assess the potential antiproliferative and apoptosis induction effects on colon and liver cancer cells, and identify the possible active components.

Muscadine grapes. 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 (*53, 54*). Muscadine is distinguishable from most other grapes variety in that it has high content of ellagic acid and anthocyanin 3,5-diglucosides (*55, 56*). Currently, many researchers are focusing on the health benefit of dietary polyphenols, because of their potential antioxidative, antiinflammatory, and anticarcinogenic activities (*11, 57, 58*). 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 (*59*). Unfortunately, very few studies have been done to evaluate the potential health benefits of muscadine grapes (*60, 61*). Any information on the potential anticancer benefits of muscadine grapes will be highly valuable. The objectives in the study were to systematically evaluate the potential anticancer activities of polyphenols in different cultivars of muscadine grapes using colon and liver cancer cell lines, and to identify the active compounds.

Anthocyanin absorption. Despite the statement that anthocyanins have potential in cancer prevention and other health benefit, a significant gap exists between what has been shown in many in vitro studies and what can be achieved under in vivo condition. One of the key factors needed to correctly relate the in vitro study results to human disease outcomes is information about bioavailability and metabolism (*62*). Although a few studies have been conducted to evaluate the bioavailability of anthocyanins (*13, 15-17*), the information on absorption and metabolism is still very limited.

Compared to human and animal models that are highly complex and may be confounded by factors such as chemical instability and inadequate analytical methodology, it is easy to control the different parameters in in vitro study. In vitro assay is simple, more convenient, and less expensive. Caco-2 cells have been the most extensively characterized and useful in vitro model in the field of drug permeability and absorption (*63-65*). The differentiated cell monolayers have been used for the study of unidirectional transport of phytochemicals such as quercetin, epicatechin, and proanthocyanidin (*66-69*), and many valuable information have been obtained using this model system.

Information on the bioavailability of pure compounds is a good start to clarify the bioavailability of complex mixtures of phytochemicals (*70*). The combination of phytochemicals in fruits and vegetables is thought to be critical to their powerful antioxidant and anticancer activity (*71, 72*). Similarly, information on the bioavailability of complex mixtures of phytochemicals is essential. Thus, studies on the bioavailability of phenolic compounds such as anthocyanins based on specific crops is a good way to provide direct and valuable information on their absorption. The objectives of the study were to evaluate the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers, and to investigate the effects of different aglycones, sugar moieties, and chemical structure on bioavailability of different types of anthocyanins.

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CHAPTER 3

PHENOLIC COMPOUNDS FROM BLUEBERRIES CAN INHIBIT COLON CANCER CELL PROLIFERATION AND INDUCE APOPTOSIS

Yi, W.; Fischer, J.; Krewer, G.; and Akoh, C.C. (2005). Journal of Agricultural and Food Chemistry 53 (18): 7320-7329.

Abstract

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

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

INTRODUCTION

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

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

Blueberries (belong to the Vacciniaceae) can be classified as highbush, lowbush, and rabbiteye. They have been considered to be one of the fruits with the highest antioxidant potentials (*17-19*). Some studies have evaluated the bioactivities of blueberry. Bomser et al. (*20*) found that the ethyl acetate extracts and further hexane/chloroform subfraction of lowbush blueberry (*V. angustifolium* Ait) significantly induced quinone reductase (QR) activity, an enzyme involved in Phase II xenobiotic detoxification. In the same study, the crude extracts significantly inhibited the induction of ornithine decarboxylase (ODC). Smith et al. (*21*) also evaluated the bioactivity of wild blueberry (*V. angustifolium* Ait). They found that the crude 70% acetone extract (flavonoid-rich) exhibited clear, significant induction of QR activity. In an evaluation of the bioactivity of lowbush blueberry (*Vaccinium angustifolium*), and highbush blueberry (*Vaccinium corymbosum*), Katsube et al. (*22*) found that the ethanol extract significantly inhibited proliferation of HL60 human promyelocytic leukemia cells and HCT116 human colon carcinoma cells in the range from 2 to 6 mg/mL.

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

blueberries, to assess the potential antiproliferative and apoptosis induction effects on colon cancer cells, and to identify the possible active components.

MATERIALS AND METHODS

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

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

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

Total polyphenols measurement. Total phenolics were measured according to the Folin-Ciocalteu reagent method (*25*). The extract/fractions were dissolved in 80% methanol and 20 μL of sample solution was introduced in a test tube, 1.0 mL of Folin-Ciocalteu reagent and 0.8 mL of sodium carbonate (7.5%) were added, and the contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a Shimadzu 300 UV-vis

spectrophotometer (Shimadzu UV-1601, Norcross, GA). The total phenolic content was expressed as gallic acid equivalents (GAE), using a standard curve generated with 50, 100, 200, 300, and 400 mg/L of gallic acid.

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

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

50% solvent C. Phenolic compounds were detected at wavelengths of 260, 280, 320, and 360 nm.

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

Cell cultures. Two cancer cell lines were purchased from ATCC (Manassas, VA). 1) HT-29 human colon; colorectal adenocarcinoma cultured in ATCC Medium: McCoy's 5a medium with 1.5 mM L-glutamine, 90%; fetal bovine serum, 10% and 2) Caco-2: human colon; colorectal adenocarcinoma cultured in ATCC Medium: Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 80%; fetal bovine serum, 20%. Cancer cells were cultured in the specific medium in an incubator with 5% CO_2 under 37 ^{o}C. Medium was changed 2 to 3 times per week.

Cell antiproliferation assay. After digestion with tripsin-EDTA, uniform amounts (~ 1 × 10^4 in the case of HT-29 and ~5,000 in Caco-2) of cells in growth media were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation at 37 ^{o}C in 5% CO_2, the growth medium was replaced with 100 μL media containing different concentrations of fruit extracts/fractions. Eight concentrations of extracts/fractions in 2 times dilution factor were applied. Depending on different fractions, the highest concentrations in crude extract and phenolic acid fractions were a few milligrams per milliliter and the highest concentrations in the rest of the fractions were around a few hundred milligrams per milliliter. The subsequent

concentrations were ½, ¼ …1/256 of the highest. Control cultures received everything but the fruit extracts/fractions, and blank wells contained 100 µL of growth medium and extract/fraction without cells. Except for anthocyanin fractions that were water soluble, dimethylsufoxide (DMSO) was added initially to the extracts/fractions to help dissolve the sample. The final DMSO content was 0.25%. Therefore, the control for these treatments also contained 0.25% DMSO. None of the extracts/fractions added changed the pH (.7.2) of the culture medium (data not shown). After 48 h of incubation, cell proliferation was determined using the ATCC MTT Cell Proliferation Assay at 570-655 nm with a Bio-Rad Model 680 Microplate Reader (Hercules, CA). To better explain the antiproliferation results, the inhibition of cell proliferation was calculated on the basis of the following formula:

Inhibition percentage = (cell no. in control - cell # in treatment)/(cell no. in control – original cell no. before the addition of extract or carrier) X 100%.

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

Detection of apoptosis. An early event in apoptosis is DNA fragmentation and release and activation of an endogenous endonuclease. The endonuclease cleaves double stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosome is tightly complexed with the core histones and is therefore protected from cleavage. DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA using a Cell Death detection ELISA kit (Boehringer Mannheim, Roche). Briefly, the cytosolic fraction (13,000 g supernatant) of cancer cells (approximately 1 x 10^5 of HT-29 or 5 x 10^4 of Caco-2) after treatment with different concentrations of anthocyanin extract was used as antigen source in a sandwich ELISA with a

primary anti-histone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. The DNA fragmentation was measured at 405 –490 nm using a Bio-Rad Model 680 Microplate Reader.

Statistical analysis. The bioactivities of different fractions in 3 cultivars were statistically compared. Statistical analysis was conducted by General Linear Model (GLM) followed by Duncan's multiple range test at α=0.05 (*26*).

RESULTS

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

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

polyphenolic content of fraction). However, some amount of catechin was found in the phenolic acid fractions of all three cultivars.

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

The highest purity of isolated fractions was observed in the anthocyanin fractions. Major individual anthocyanins in the anthocyanin fractions are presented in Table 3. Figure 1 shows the analytical HPLC chromatogram of blueberry anthocyanin fractions. Ten major peaks and 3 small peaks were observed in all three cultivars (Figure 1 B-D). Seven different anthocyanins were identified based on the retention time and characteristic spectra. The total weights of the identified anthocyanins were added to obtain the percentage of total identified anthocyanins in the total weight of whole fraction. More than 86% of whole fractions were identified in Tifblue and Powderblue, and about 75% were identified in Briteblue. Since some of the peaks (Figure 1) could not be identified, acid hydrolysis was conducted to evaluate the anthocyanidin profile. Five standards (Dp-Glc, Cy-Gal, Pt-Glc, Pn-Gal, and Mv-Glc) were hydrolyzed in water bath and run in HPLC to yield the characteristic retention times and spectra. Figure 2 shows the peaks of the 5 anthocyanidins. The anthocyanidin profiles of 3 fractions were obtained through acid hydrolysis (Figure 2 B-D). Table 4 shows the individual anthocyanidins in the anthocyanin fractions after acid hydrolysis. The amount of different anthocyanidins was expressed based on specific weight of anthocyanins: Dp-Glc, Cy-Gal, Pt-Glc, Pn-Gal, Mv-Glc, because most of the anthocyanins in blueberry are monoglycosides. The weight percentages were then calculated to assess the purity of different anthocyanin fractions. The highest purity was obtained in Tifblue

and Powderblue with ~98% anthocyanidin monoglycosides. About 89% of anthocyanidin monoglycosides were observed in Briteblue fraction. In general, five different anthocyanidins (Dp, Cy, Pt, Pn, and Mv) were identified and no other peak was found in any of the three cultivars (Figure 2).

Cell antiproliferation. The inhibition of HT-29 and Caco-2 cancer cell proliferation by crude extracts of blueberries is shown in Figure 3. In both cell lines, the IC_{50} in the crude extract ranged from 1000 to 3000 μg/mL. The Briteblue crude extract showed the highest antiproliferation activity among the 3 cultivars in both HT-29 and Caco-2 cells. The bioactivities of different blueberry cultivars varied with different cancer cell lines. The highest IC_{50} in HT-29 was observed in Powderblue, while in Caco-2, the highest IC_{50} was found in Tifblue among all three cultivars.

All four fractions, phenolic acids, flavonols, tannins or procyanidins, and anthocyanins showed significantly higher bioactivities, probably because of their higher purity, compared to the crude extracts. The inhibition of HT-29 cancer cell proliferation by different fractions is shown in Figure 4. The phenolic acid fraction showed the lowest antiproliferation activities among the 4 fractions with an IC_{50} around 1000 μg/mL (Figure 4A). The anthocyanin fraction showed the highest antiproliferation activity among the 4 fractions (Figure 4D). For Briteblue anthocyanins, the IC_{50} was around 25 μg/mL. This was significantly lower when compared with Tifblue and Powderblue although the anthocyanin content of the Briteblue fraction was lower than the other two cultivars (Table 4). The IC_{50} for Tifblue and Powderblue was ~ 50 μg/mL. Intermediate bioactivities were observed in tannin and flavonol fractions. The IC_{50} for the tannin fractions and flavonol fractions ranged from 70 to 100 μg/mL. In the tannin fractions, Briteblue showed the highest bioactivity among the three cultivars, although their total polyphenolic

contents were very close (Table 1). No significant difference was observed among the flavonol fractions from different cultivars.

A similar trend in the antiproliferation activities of the 4 fractions was found in Caco-2 cells (Figure 5). Again, the phenolic acid fraction showed the lowest antiproliferation activities among the 4 fractions, while the anthocyanin fraction showed the highest antiproliferation activity. The lowest IC_{50} (highest bioactivity) was observed at about 15 μg/mL anthocyanin fraction in Caco-2 cells. Significant inhibition effects were observed with as low as 1μg/mL anthocyanins. No significant difference was found in the anthocyanin fractions of the 3 cultivars, unlike in the HT-29 cells for which Briteblue anthocyanin showed the highest bioactivities. In the flavonol fractions, Briteblue showed the highest antiproliferation activities with an IC_{50} at ~50 μg/mL. In the procyanidin fraction, all of the 3 cultivars showed similar bioactivities with an IC_{50} at ~100 μg/mL (Figure 5C).

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

Apoptosis. DNA fragmentation in HT-29 and Caco-2 cells after treatment with anthocyanin fractions is shown in Figure 6. From Figure 6A, we can see that anthocyanins resulted in 3 to 7 times increase in DNA fragmentation compared with no treatment control. Since DNA fragmentation is the primary physiological characteristic of apoptosis, our results suggest that anthocyanin can induce programmed cell death in HT-29 colon cancer cells. The highest DNA fragmentation level was observed around 40 μg/mL, representing a 7 times increase compared to the control. Further increases of anthocyanin did not result in increased

level of DNA fragmentation, although very few live cells existed at those high dosage levels (Figure 4D). Similar results were obtained in Caco-2 cell lines when treated with the Tifblue anthocyanin fraction (Figure 6B). Anthocyanin treatment resulted in a 2-4 times increase in DNA fragmentation. The highest DNA fragmentation level was observed with ~80 μg/mL anthocyanin fraction. A further increase in anthocyanin level slowed DNA fragmentation. As a control reagent, KOH resulted in significant decrease in DNA fragmentation compared with no treatment.

DISCUSSION

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

The profile of anthocyanins in the current study are in good agreement with previous results (*30-32*). Five anthocyanidins (Dp, Cy, Pt, Pn, Mv) were reported in Tifblue and Powderblue cultivars by Ballington et al. (*31*). This is a perfect match with the anthocyanidin profile of the anthocyanin fractions in the current study. Although three peaks could not be identified because we did not have the standards, all of the seven major anthocyanins identified matched with previous reports. Our hydrolyzed products (aglycons) were in good concurrence

with individual anthocyanin measurements except in the Briteblue cultivar where ~50% Pn was lost. More malvidin was found after hydrolysis. This indicates that some unidentified peaks were malvidin monoglycosides. From the studies of Ballington et al. (*31*), the other two could be Mv-gal and Mv-ara.

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

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

Our antiproliferation and apoptosis induction results are in good agreement with several previous studies. In an evaluation of the bioactivities of bilberry, Katsube et al. (*22*) stated that it was anthocyanins in the bilberry extract that inhibited the growth of HL-60 human leukemia cells through apoptosis. They also found that the inhibitory effects of malvidin and delphinidin were greater than those of the flavonols. In the current study, we cannot state that the anticancer activities of flavonols were lower than those of anthocyanins in rabbiteye blueberry since the purity of flavonol fractions was much lower than that of the anthocyanin fractions. Instead, we can suggest that anthocyanin was one of the major components that inhibited colon cancer cell proliferation and induced apoptosis. For a comparison of effective dosage levels, in a study by

Katsube et al. (*22*), 2-6 mg/mL of an ethanol extract of selected berries (including blueberry) resulted in a significant inhibition of HL-60 and HCT116 cell growth. Anthocyanin fractions obtained by semi-preparative HPLC in the same study resulted in higher levels of cancer cell inhibition and apoptosis at 300 μg/mL. This is in good agreement with our findings for HT-29 and Caco-2 cells in the current study. The IC_{50} of crude blueberry extracts ranged from 1000 to 3000 μg/mL, and the IC_{50} of anthocyanin fractions ranged from 15 to 50 μg/mL. The lower effective dosage level in the current study could be because we used different cancer cell lines. In another study using chokeberry, anthocyanin-rich extracts (AREs) inhibited HT-29 cell growth about 50% after 48 h of exposure at 25 μg/mL (*34*). This is very close to the IC_{50} level in anthocyanin fractions we observed in the current study. In the study by Zhao et al. (*34*) the AREs contained 737 mg of total polyphenolics and 102 mg of monomeric anthocyanin per gram of extract. The purity of anthocyanin fractions in the current studies was about 90%.

In the current study, 70-100 μg/mL of procyanidin fractions inhibited HT-29 and Caco-2 cell growth by ~50%. These effective dosage levels were close to those reported for low bush (wild) blueberries (*35*). In low bush blueberry (*V. angustifolium* Ait.), proanthocyanidin-rich fractions exhibited significant antiproliferation activities in human prostate (LNCaP) and murine liver (Hepa 1c1c7) cancer cell lines (*35*). The evaluated concentration of proanthocyanidin-rich fractions was 20 μg/mL. The authors stated that there was a significant positive correlation between proanthocyanidin content of different fractions and antiproliferation activity.

The cancer prevention effects of Cy-glc were evaluated in human promyelocytic leukemia cell line HL-60 and Jurkat T-leukemia cells (*36*). IC_{50} was 174.9 μg/mL Cy-glc in Jurkat cells, with a higher effective concentration in HL-60 cells after 24/30 h of exposure. Although the calculation formula was slightly different from that used in the current study, we

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

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

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

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

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

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

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Table 3.1. The total polyphenolic content of different extracts/fractions from blueberries (unit: weight percent)[a].

Cultivars	Crude extract	Phenolic acids	Flavonols	Tannins
Briteblue	6.0±0.5 [b]	14.9±1.1	76.6±1.5	87.9±0.9
Tifblue	6.4±0.3	15.1±0.9	86.5±0.6	85.4±2.5
Powderblue	4.9±0.2	12.4±1.2	75.3±1.1	86.9±3.3

[a] Total polyphenols were expressed as gallic acid equivalents (GAE), and the total polyphenolic contents percentage was calculated based on: GAE/fraction weight X 100%.

[b] Values are averages of triplicate analyses ± standard error.

Table 3.2. Individual phenolic acids and flavonoids in different fractions of blueberries (unit: weight percent).

Cultivars	Phenolic acids				Flavonoids			
	Gallic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Catechin	Epicatechin	Quercetin	Kaempferol
				Phenolic acid fractions				
Briteblue	0.4±0.0[a] (2.4±0.1)	3.2±0.0 (21.7±0.1)	2.5±0.0 (17.0±0.1)	ND	2.3±0.1 (15.1±0.5)	ND[b]	ND	ND
Tifblue	3.2±0.0 (21.4±0.2)	1.1±0.0 (7.5±0.1)	0.3±0.0 (1.7±0.1)	0.4±0.0 (2.3±0.1)	1.9±0.1 (12.4±0.4)	ND	ND	ND
Powderblue	0.8±0.0 (6.5±0.1)	1.3±0.0 (10.3±0.2)	ND	0.4±0.0 (3.2±0.1)	2.8±0.1 (22.5±0.4)	ND	ND	ND
				Flavonol fractions				
Briteblue	0.7±0.1 (1.0±0.1)	0.7±0.1 (0.9±0.1)	0.6±0.1 (0.7±0.1)	ND	ND	ND	5.7±0.3 (7.5±0.4)	2.2±0.2 (2.9±0.3)
Tifblue	0.6±0.1 (0.7±0.1)	0.3±0.0 (0.3±0.0)	ND	0.3±0.0 (0.3±0.0)	ND	1.4±0.1 (1.7±0.1)	4.4±0.2 (5.1±0.3)	3.4±0.2 (3.9±0.2)
Powderblue	0.8±0.1 (1.1±0.1)	0.9±0.1 (1.2±0.1)	ND	0.6±0.0 (0.8±0.1)	ND	ND	8.5±0.3 (11.3±0.3)	5.9±0.3 (7.8±0.4)

[a] Data are percentage by weight of individual compounds in the whole weight of specific fraction. Data in parenthesis are percentage of individual compounds in the total polyphenols of specific fraction. Values are averages of triplicate analyses ± standard error.

[b] ND - Not detected.

Table 3.3. Major individual anthocyanins in the anthocyanin fractions (unit: weight percent) [a].

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

[a] Values are averages of triplicate analyses ± standard error. Dp-Glc (Delphinidin 3-O-β-glucopyranoside), Cy-Gal (Cyanidin 3-O-β-galactopyranoside), Cy-Glc (Cyanidin 3-O-β-glucopyranoside), Pt-Glc (Petunidin 3-O-β-glucopyranoside), Pn-Gal (Peonidin 3-O-β-galactopyranoside), Pn-Glc (Peonidin 3-O-β-glucopyranoside), and Mv-Glc (Malvidin 3-O-β-glucopyranoside).

Table 3.4. Major individual anthocyanidins in the anthocyanin fraction after acid hydrolysis (unit: weight percent) [a].

Cultivars	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Total anthocyanidins
Briteblue	18.6±0.9 [b]	10.0±0.4	30.3±0.5	9.1±1.3	21.4±0.6	89.3±3.2
Tifblue	12.6±0.8	17.5±1.1	22.1±0.8	25.2±1.2	20.0±0.7	97.4±4.1
Powderblue	20.0±0.3	21.2±0.6	26.2±0.7	15.5±0.9	16.8±1.1	98.7±3.3

[a] Values are averages of triplicate analyses ± standard error.

[b] Amount of different anthocyanidins was expressed based on specific weight of anthocyanins.

Figure 3.1. Chromatogram of analytical HPLC of blueberry anthocyanins fractions. A. Anthocyanin standards; B. Briteblue fraction; C. Tifblue fraction; D. Powderblue fraction. 1) Dp-Glc, 2) Cy-Gal, 3) Cy-Glc, 4) Pt-Glc, 5) Pn-Gal, 6) Pn-Glc, 7) Mv-Glc, 8) Pn-Ara. a) – f) unidentified peaks.

Figure 3.2. Chromatogram of analytical HPLC of blueberry anthocyanins fractions after acid hydrolysis. A. Anthocyanidin standards; B. Briteblue fraction; C. Tifblue fraction; D. Powderblue fraction. 1) Delphinidin, 2) Cyanidin, 3) Petunidin, 4) Peonidin, 5) Malvidin.

Figure 3.3. Inhibition of HT-29 and Caco-2 cancer cell proliferation by crude extracts in blueberries (mean ± SD, n = 8). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. A. HT-29; B. Caco-2.

Figure 3.4. Inhibition of HT-29 cancer cell proliferation by different fractions of blueberries (mean ± SD, n = 8). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. A. Phenolic acid fraction; B. Flavonol fraction; C. Tannin fraction; D. Anthocyanin fraction.

Figure 3.5. Inhibition of Caco-2 cancer cell proliferation by different fractions of blueberries (mean ± SD, n = 8). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. A. Phenolic acid fraction; B. Flavonol fraction; C. Tannin fraction; D. Anthocyanin fraction.

Figure 3.6. DNA fragmentation in HT-29 and Caco-2 cells associated with anthocyanin fractions (mean ± SD, n = 3). Axis x stands for the concentration (μg/mL) of extracts in the

culture medium. A. Anthocyanin fractions of Briteblue in HT-29 cells; B. Anthocyanin fractions of Tifblue in Caco-2 cells, 1 mol/L of KOH was used as a control reagent to induce necrosis.

Figure 3.1.

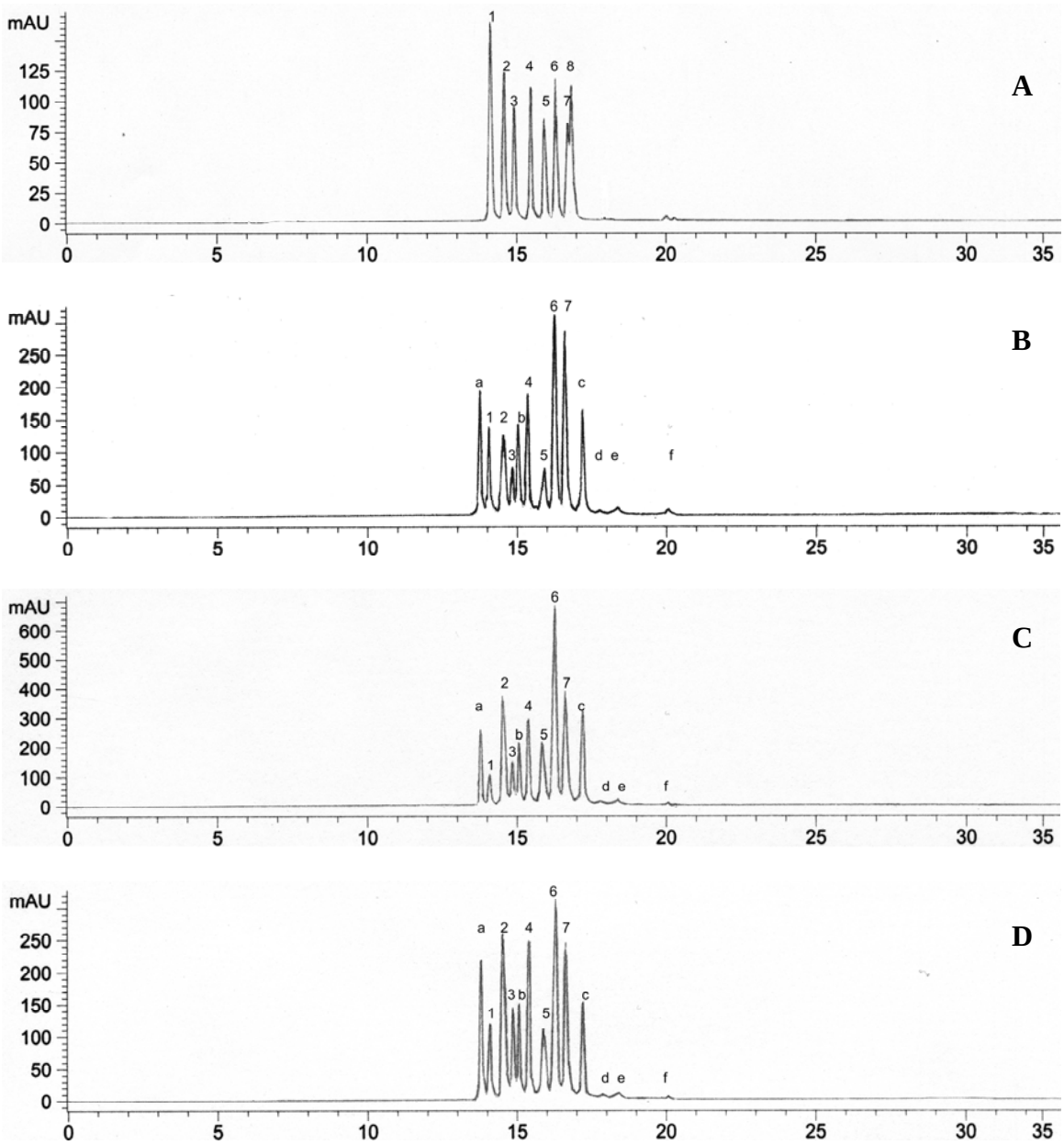


Figure 3.2.

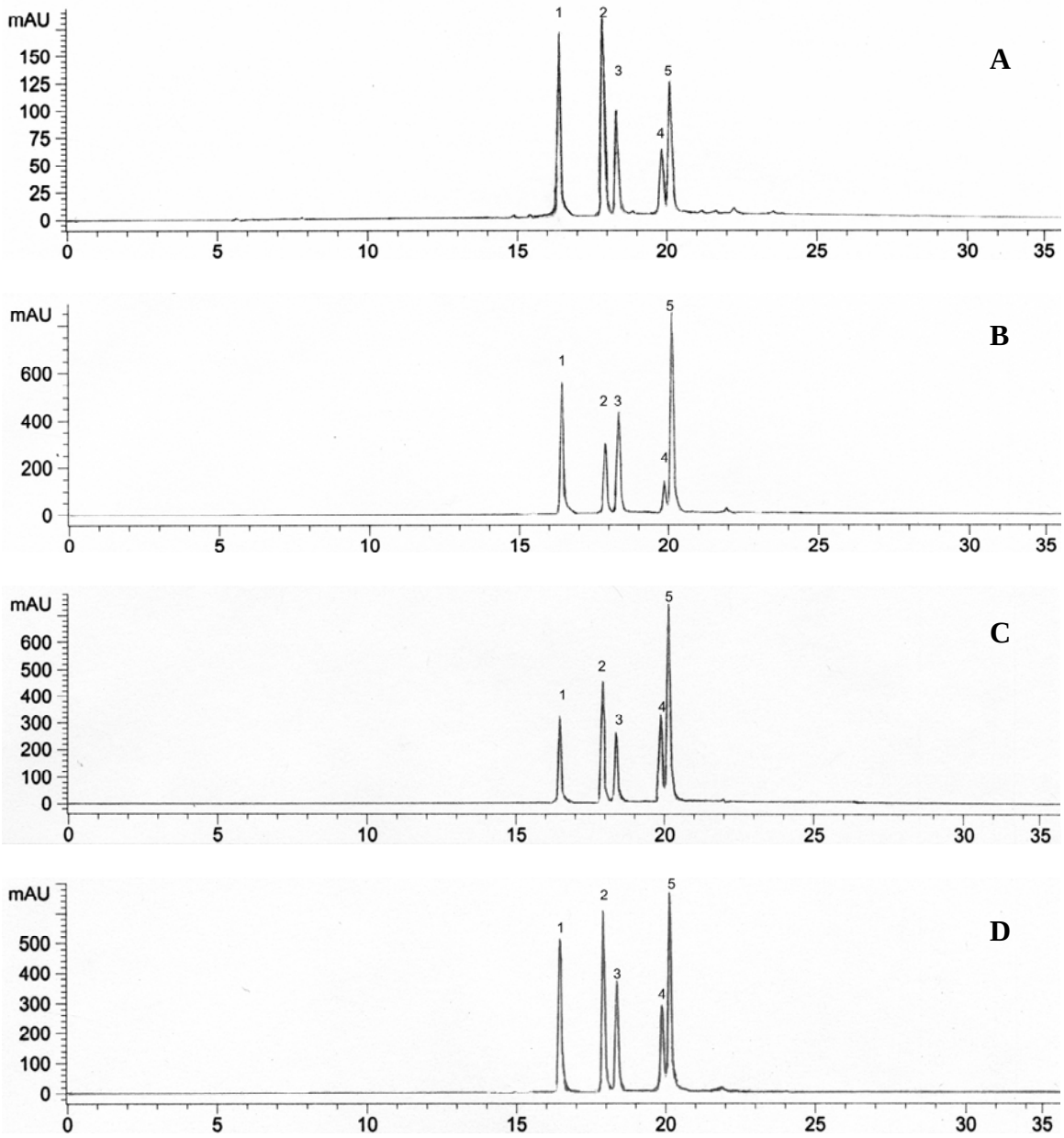


Figure 3.3.

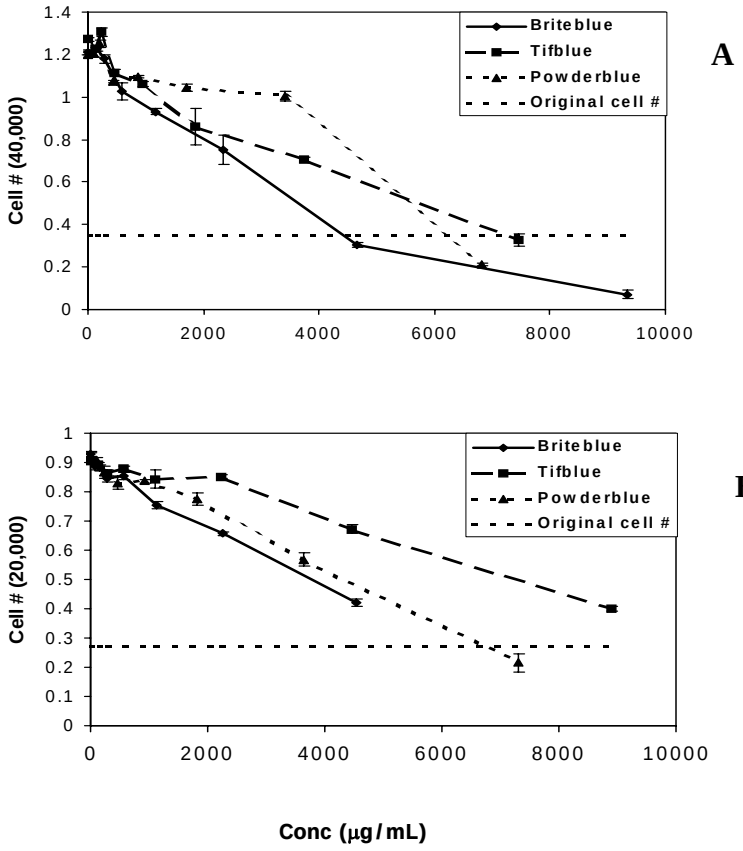

Briteblue
Tifblue
Powderblue
Original cell #
Cell # (40,000)
Cell # (20,000)
Conc (µg/mL)
A
B

Figure 3.4

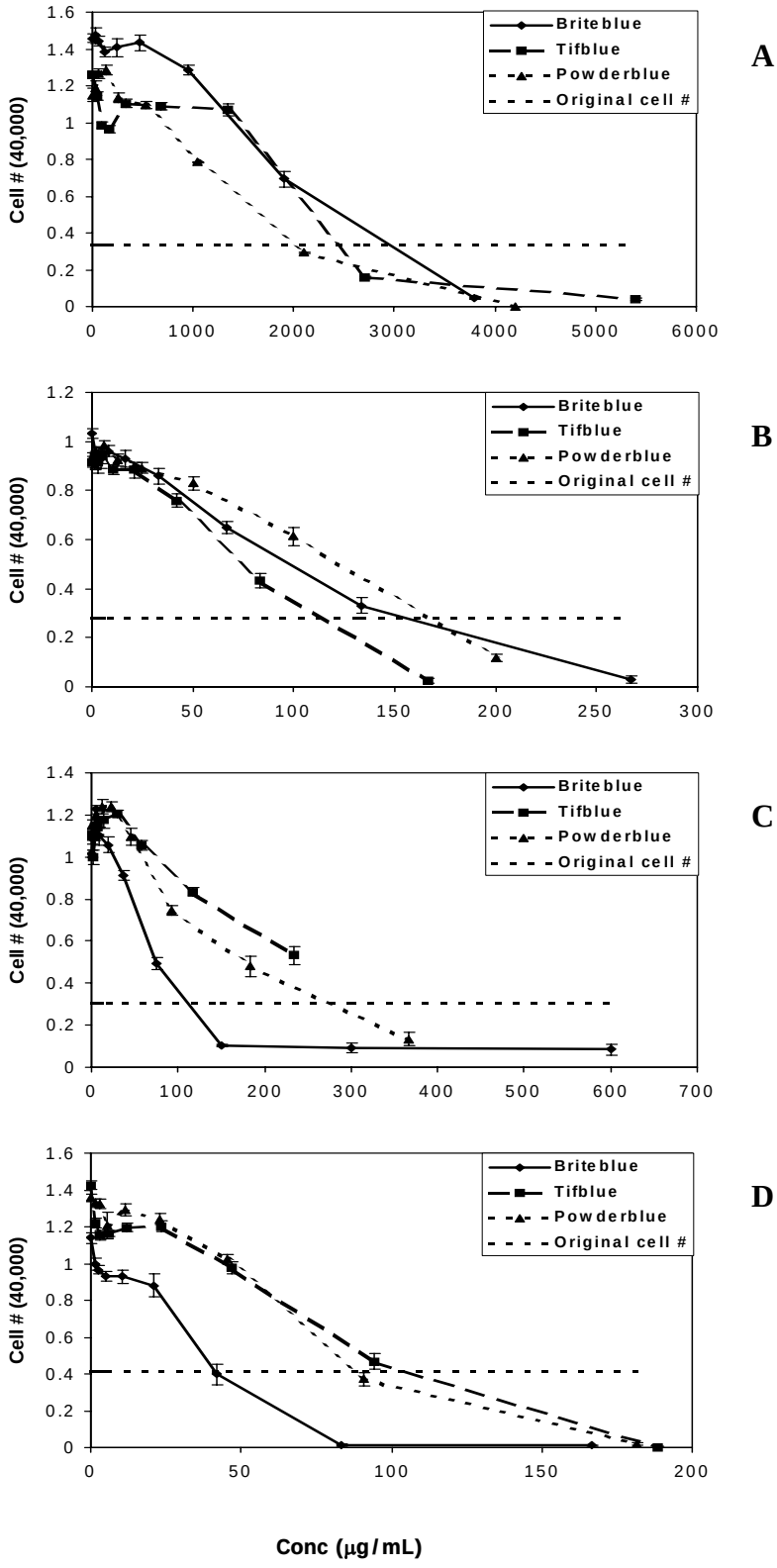

Briteblue
Tifblue
Powderblue
Original cell #
Cell # (40,000)
A
B
C
D
Conc (µg/mL)

Figure 3.5.

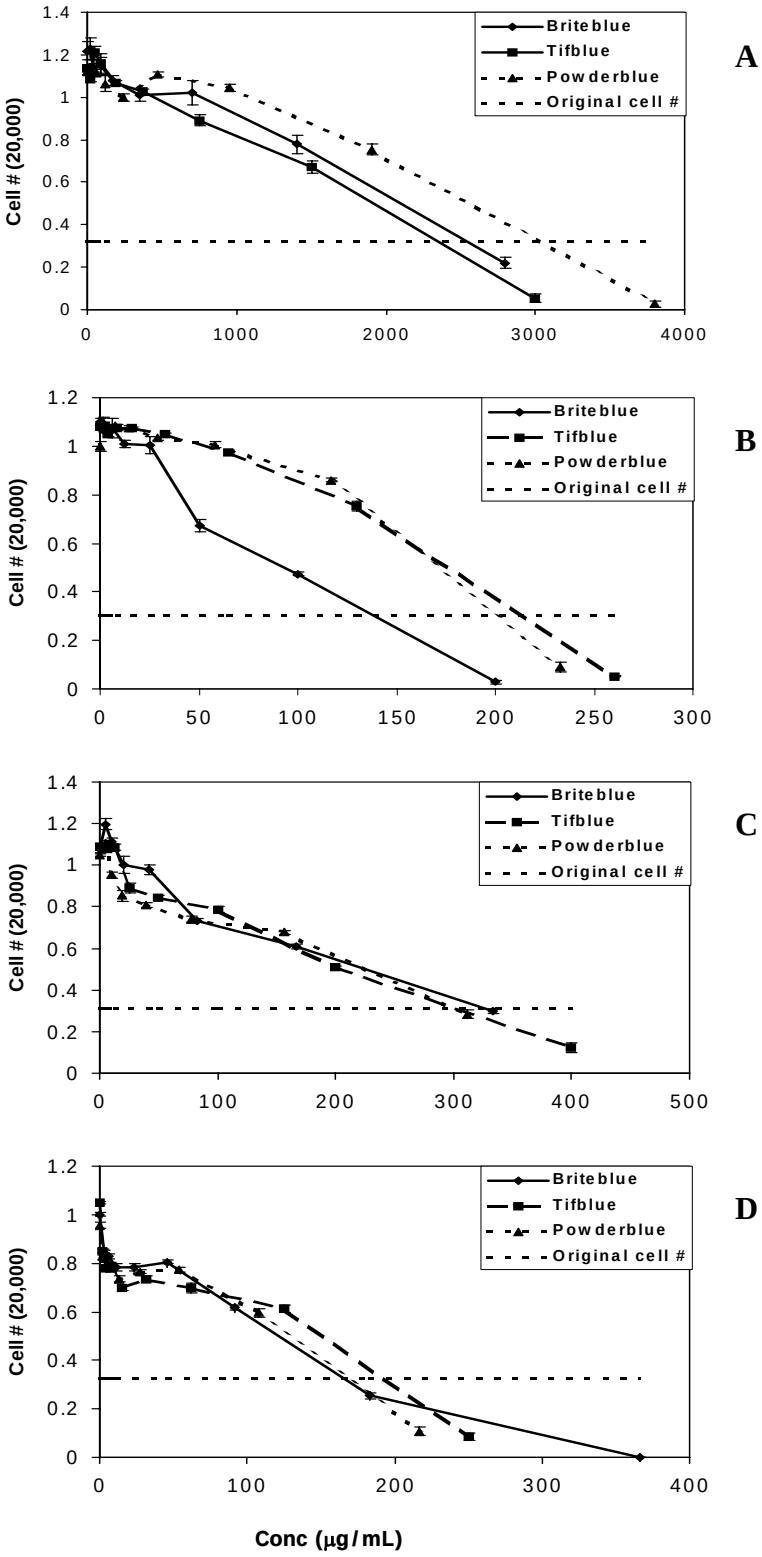
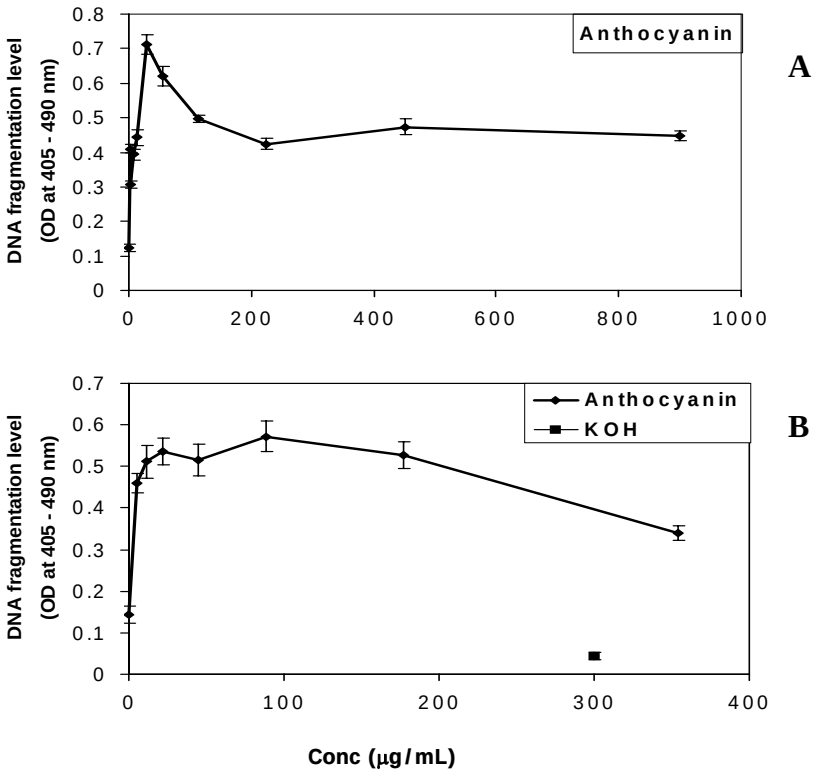

Briteblue
Tifblue
Powderblue
Original cell #
Cell # (20,000)
A
B
C
D
Conc (μg/mL)

Figure 3.6.


Anthocyanin
DNA fragmentation level (OD at 405 - 490 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
200
400
600
800
1000
A
Anthocyanin
KOH
DNA fragmentation level (OD at 405 - 490 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
B
Conc (μg/mL)

CHAPTER 4

STUDY OF ANTICANCER ACTIVITIES OF MUSCADINE GRAPE PHENOLICS IN VITRO

Yi, W.; Fischer, J.; and Akoh, C.C. (2005). Journal of Agricultural and Food Chemistry 53 (22): 8804-8812.

Abstract

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 the 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.

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, dimethylsufoxide (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 ^{o}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), 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 5 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 ^{o}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 ^{o}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 ^{o}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 x 250 mm column was used with column temperature at 40 ^{o}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: 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 post-run 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); Solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradients were: 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 post-run 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, 90%, fetal bovine serum, 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 non-essential amino acids, and 1.0 mM sodium pyruvate, 80%; fetal bovine serum, 20%. Cancer cells were grown in an incubator with 5% CO_2 at 37 ^{o}C. Medium was changed two to three times per week.

Cell Viability Assay. Uniform amounts of cells in growth media (~ 1×10^4 in the case of HT-29 and ~5,000 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 ^{o}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:

% inhibition = (cell no. in control – cell no. in treatment)/(cell no. in control – original cell no. before addition of extract or carrier) X 100%.

IC50 is the extract concentration under which a 50% inhibition of cell population growth occurred. IC50 was calculated by interpolation from dose-response curves. With anthocyanin fractions, IC25 (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, ~ 1 x 10^5 HT-29 or 5 x 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 x 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 ^{o}C for 90 min, the conjugate solution (a secondary anti-DNA antibody coupled to peroxidase) was added. After incubation at 20 ^{o}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 $\forall = 0.05$ (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) X 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%.

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) X 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. 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. 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 2 B, C, D**, and **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*). In order to report the purity of the fractions, the amount of different anthocyanidins was expressed as 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 anthocyanidins in Noble

were delphinidin and petunidin, representing 22.7% and 31.4%, respectively. The contents of malvidin, cyaniding, 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 IC50s (meaning high inhibitory activity) were observed in Carlos and Noble in both HT-29 and Caco-2 cells. IC50 ranged from 1 - 2 mg/mL in Carlos and Noble, while the IC50s 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.

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 4 A**). In contrast, the phenolic acid fraction in Ison showed significantly lower ($P=0.00012$) bioactivity than the other 3 cultivars. The IC50 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 4 D**). The inhibitory effects were very close among the different cultivars with the IC50 around 200 μg/mL. In order to more

accurately present the effective dosage level of anthocyanins, IC25 was also calculated. More than 25% inhibition of cell population growth (IC25) 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 4 B, 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 IC50 was around 300 μg/mL for Carlos, Ison, and Supreme. A significantly higher (P=0.0007) IC50 (600 μg/mL) was observed with the flavonol fractions of Ison.

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 IC50 around 2000 μg/mL, while the IC50 was around 500 μg/mL with the rest of 3 cultivars (**Figure 5 A**). As in HT-29 cells, the anthocyanin fractions showed the highest bioactivities among the four fractions. The IC50 of the anthocyanin fractions ranged from 100 – 300 μg/mL. Unlike in HT-29 cells, where the inhibitory effects were close, Supreme showed the highest bioactivity with an IC50 around 100 μg/mL. The lowest effect was observed in Noble with an IC50 around 300 μg/mL (**Figure 5 D**). In addition, the IC25 of anthocyanin fractions ranged from 50 – 150 μg/mL. Supreme showed the lowest IC25 with 50 μg/mL, and the highest IC25 (150 μg/mL) was observed in Noble.

In flavonol fractions, Ison showed the lowest inhibitory effect with an IC50 of 350 μg/mL (**Figure 5 B**). This was similar to the HT-29 cell value. The IC50 for flavonol fractions in Caco-2 cells ranged from 200 – 350 μg/mL. With tannin fractions, the IC50 ranged from 300 – 500 μg/mL (**Figure 5 C**).

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 100–300 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 control in the evaluated dosage levels (300 – 1200 μg/mL). A similar trend was found in Caco-2 cell line. A significant increase ($P<0.001$) in DNA fragmentation was observed after treatment with anthocyanins from the Ison cultivar (**Figure 6 B**). 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 microgram/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 5 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 provides 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 (IC50, 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, petunidin, and lower level 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 IC50 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 IC50 ranged from 35 – 90 μM with delphinidin, malvidin, cyanidin, and peonidin. Pelargonidin exhibited the lowest growth inhibitory potential with an IC50 of about 200 μM. The IC50 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 IC50 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 IC50 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 IC50 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 G1/G0 and G2/M phase of the cell cycle. A reduction of cells in 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 were potent epidermal growth factor receptor (EGFR) and phosphodiesterase (PDE) inhibitor, 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 – 300 μg/mL of anthocyanins in HT29, and 10 – 250 μg/mL in Caco-2 cells. Beyond these levels necrosis could

be the predominant effect. The IC50s 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 IC25s 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 towards 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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Table 4.1. The 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) X 100%. Results are averages of triplicate analyses ± standard error.

[b] Values without parenthesis represent total phenolic content percentages. Values in parenthesis 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) X 100%. Results are averages of triplicate analyses ± standard error.

Table 4.2. 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

Table 4.3. Major Individual Anthocyanidins (Weight Percent) in the Anthocyanin Fraction After Acid Hydrolysis [a].

Cultivars	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Total anthocyanidin
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 specific weight of anthocyanidin diglucosides.

Figure 4.1. Procedures of fractionation of different phenolic compounds.

Figure 4.2. 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.

Figure 4.3. Inhibition of HT-29 and Caco-2 cancer cell population growth by crude extracts of muscadine grapes (mean ± SE, $n = 4$). Axis x stands for the concentration (mg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) HT-29; (B) Caco-2.

Figure 4.4. Inhibition of HT-29 cancer cell population growth by different fractions of muscadine grapes (mean ± SE, $n = 4$). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) phenolic acid fraction; (B) flavonol fractions; (C) tannin/proanthocyanidin fractions; (D) anthocyanin fractions.

Figure 4.5. Inhibition of Caco-2 cancer cell population growth by different fractions of muscadine grapes (mean ± SE, $n = 4$). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) phenolic acid fraction; (B) flavonol fractions; (C) tannin/proanthocyanidin fractions; (D) anthocyanin fractions.

Figure 4.6. DNA fragmentation in HT-29 and Caco-2 associated with anthocyanin fractions (mean ± SE, $n = 3$). (A) Anthocyanin fractions of Supreme in HT-29. 1 M of KOH was a control reagent to induce necrosis; (B) Anthocyanin fractions of Ison in Caco-2 cell line. KOH (1 M) was a control reagent to induce necrosis.

Figure 4.1.

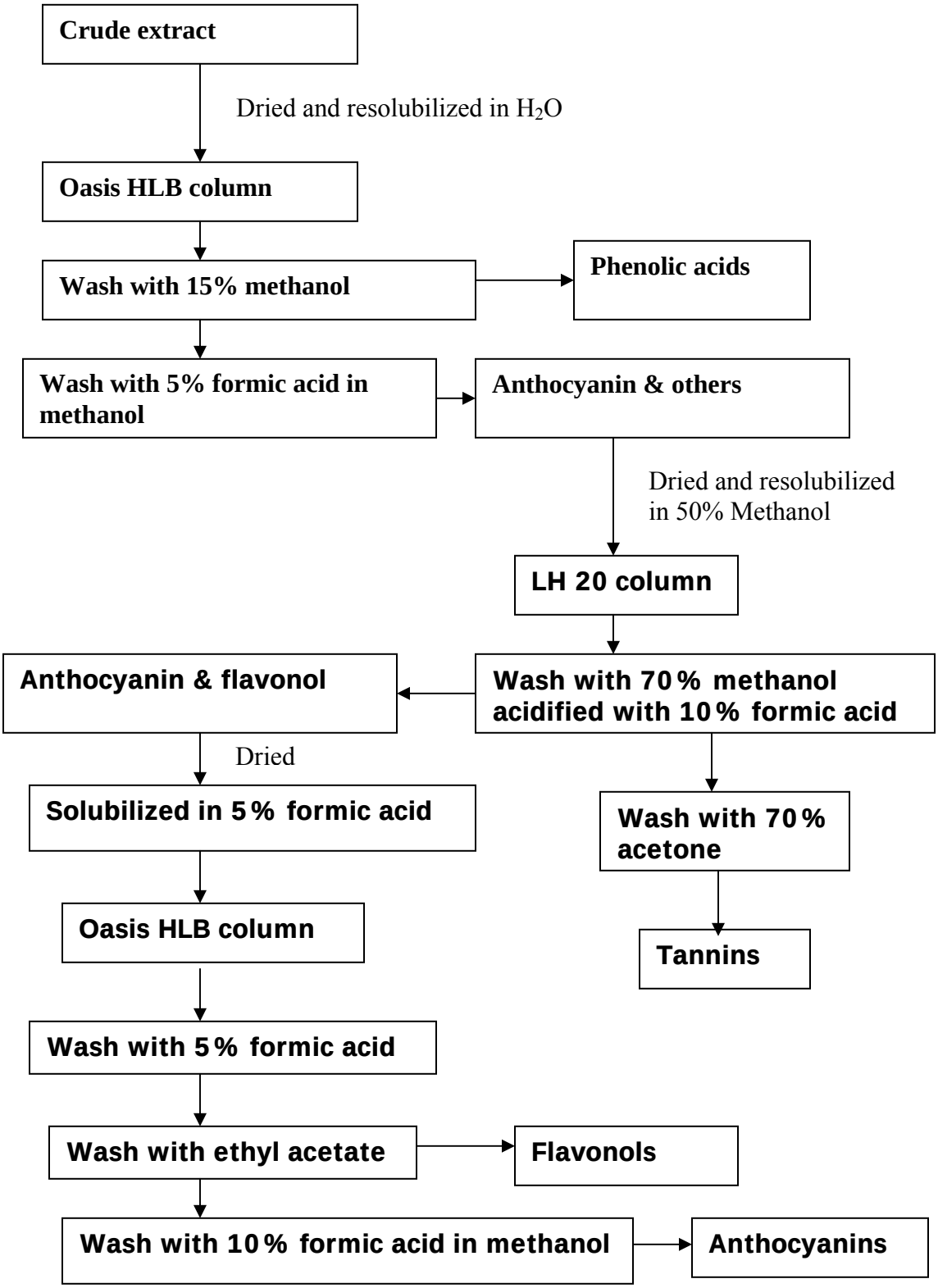

Crude extract
Dried and resolubilized in H_2O
Oasis HLB column
Wash with 15% methanol
Phenolic acids
Wash with 5% formic acid in methanol
Anthocyanin & others
Dried and resolubilized in 50% Methanol
LH 20 column
Anthocyanin & flavonol
Wash with 70% methanol acidified with 10% formic acid
Dried
Solubilized in 5% formic acid
Wash with 70% acetone
Oasis HLB column
Tannins
Wash with 5% formic acid
Wash with ethyl acetate
Flavonols
Wash with 10% formic acid in methanol
Anthocyanins

Figure 4.2.

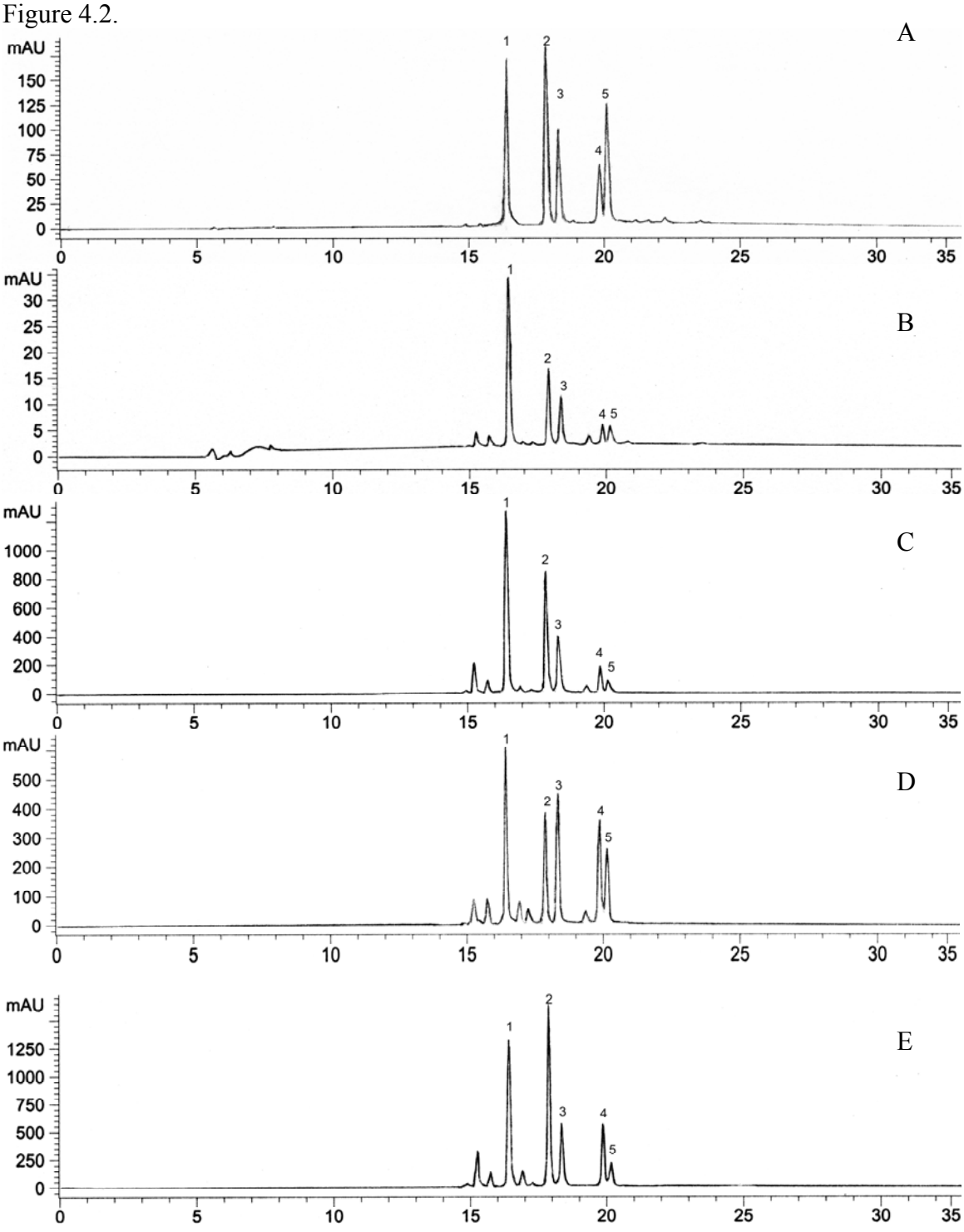
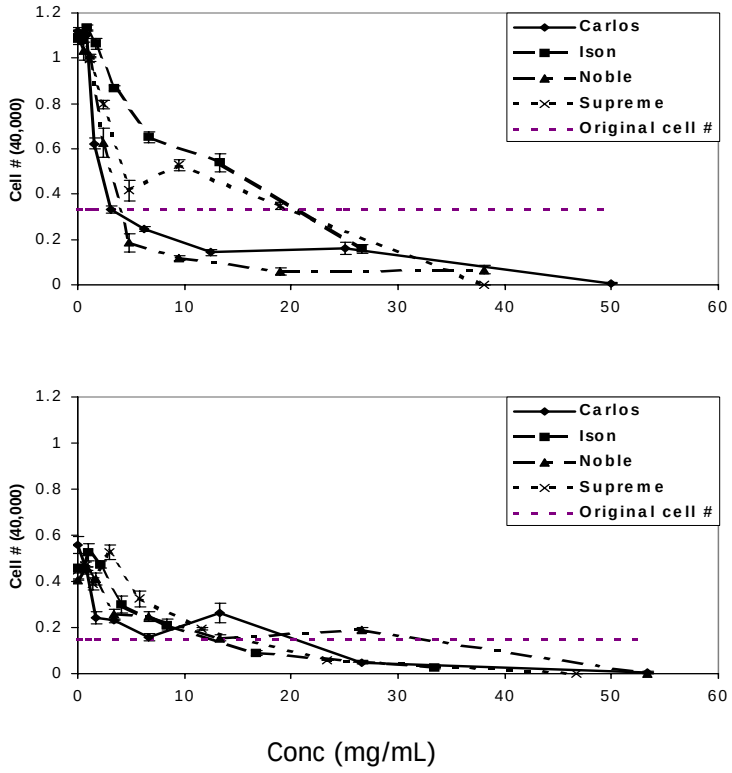

A
mAU
150
125
100
75
50
25
0
1
2
3
4
5
0
5
10
15
20
25
30
35
B
mAU
30
25
20
15
10
5
0
1
2
3
4 5
C
mAU
1000
800
600
400
200
0
D
mAU
500
400
300
200
100
0
E
mAU
1250
1000
750
500
250
0
Time (min)

Figure 4.3.


Carlos
Ison
Noble
Supreme
Original cell #
Cell # (40,000)
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Conc (mg/mL)

A

B

Figure 4.4.

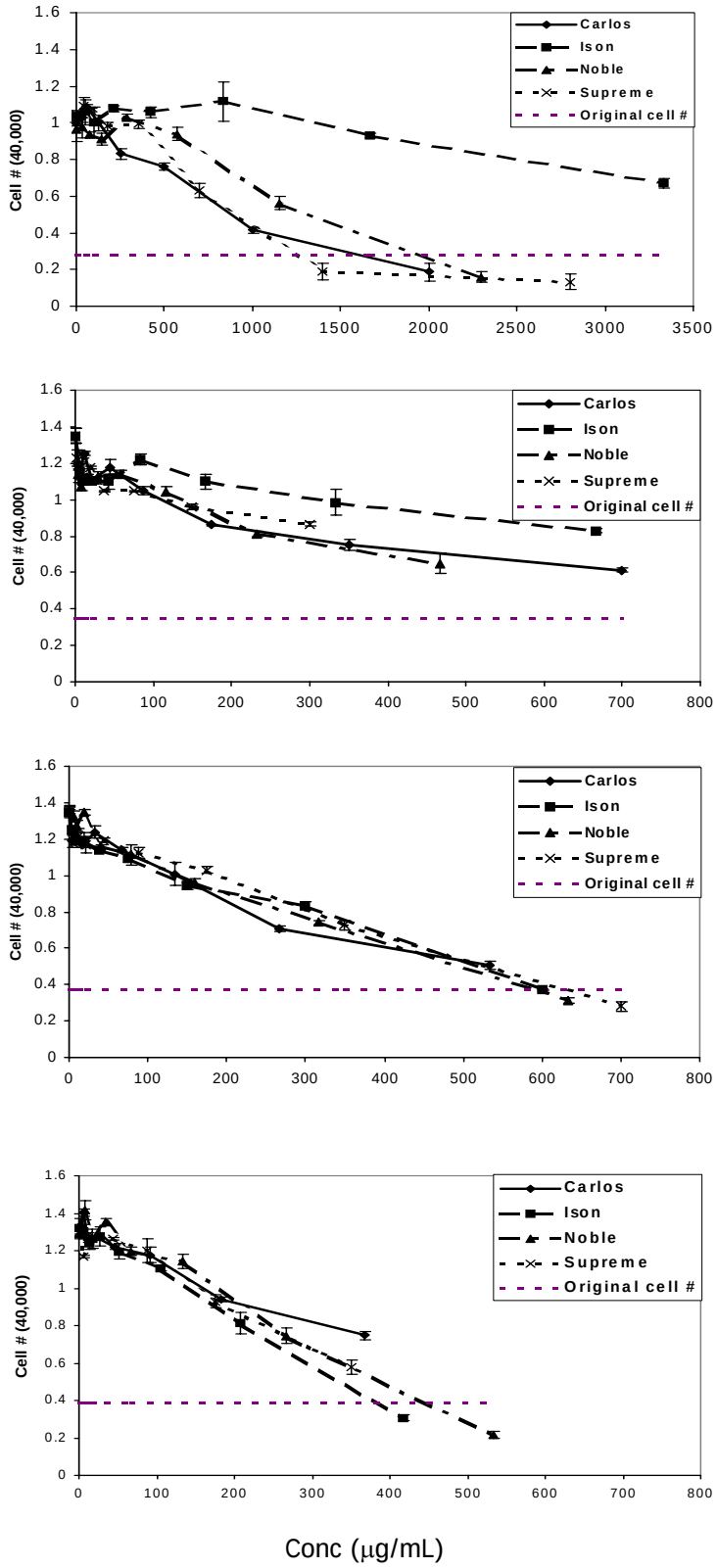

Carlos
Ison
Noble
Supreme
Original cell #
Cell # (40,000)
A
B
C
D
Conc (μg/mL)

Figure 4.5.

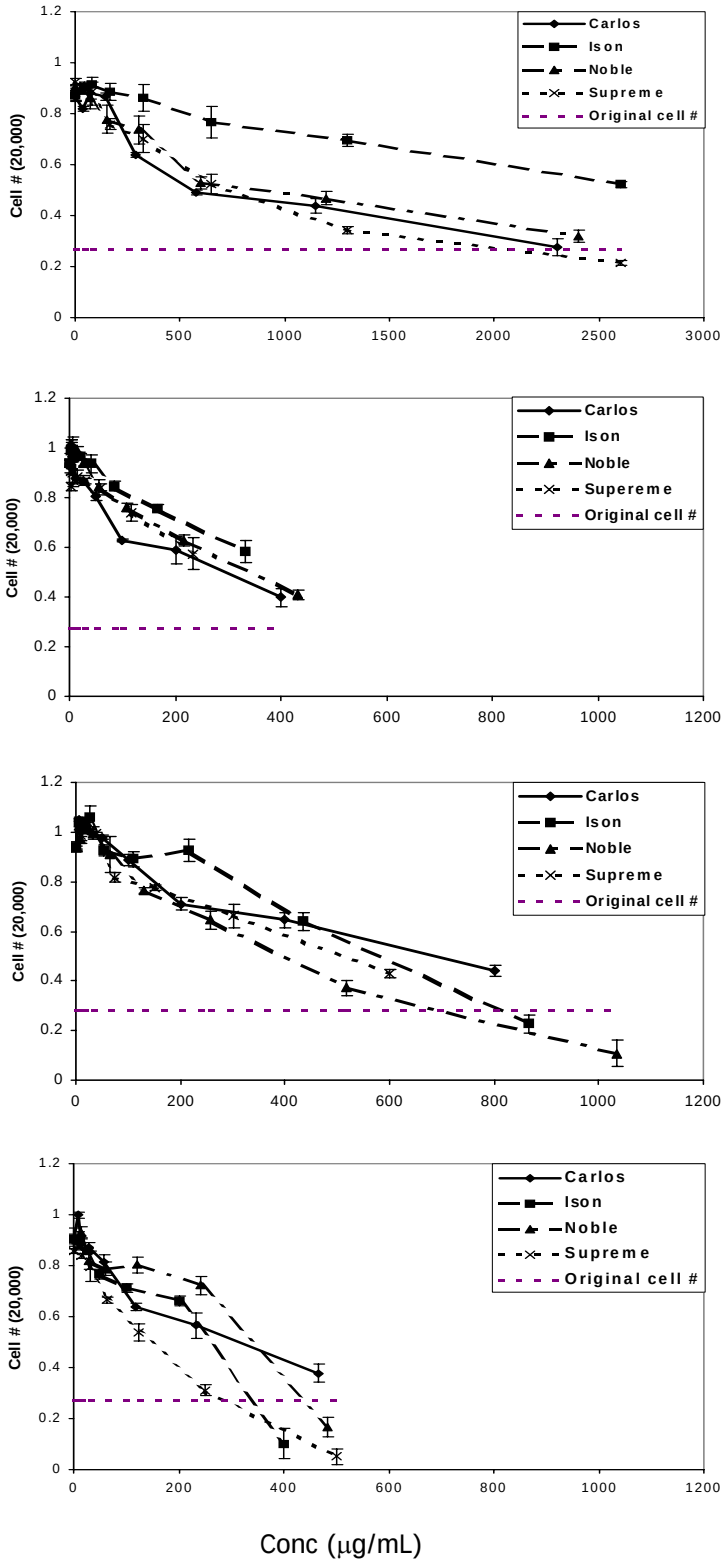
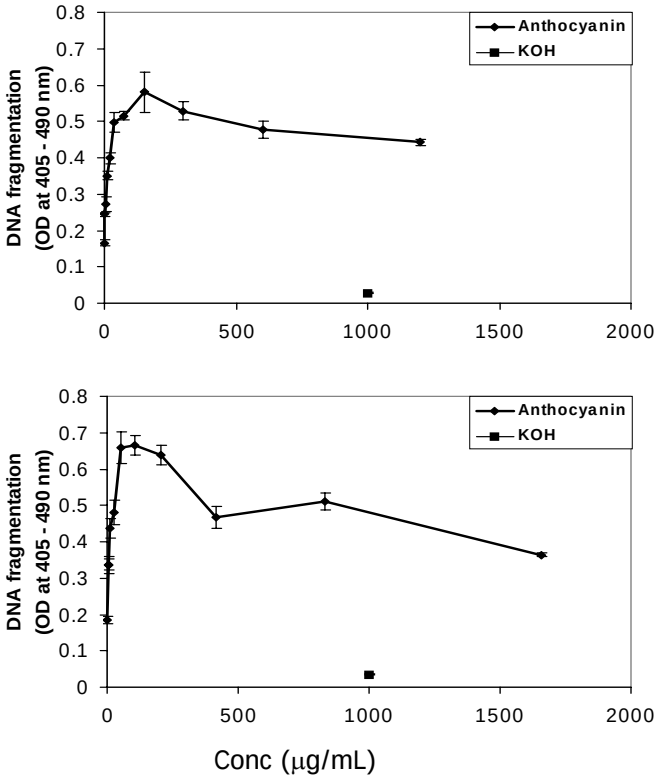

A
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (20,000)
B
Carlos
Ison
Noble
Supereme
Original cell #
Cell # (20,000)
C
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (20,000)
D
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (20,000)
Conc (μg/mL)

Figure 4.6.


Anthocyanin
KOH
DNA fragmentation (OD at 405 - 490 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
A
Anthocyanin
KOH
DNA fragmentation (OD at 405 - 490 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
Conc (μg/mL)
B

CHAPTER 5

EFFECTS OF PHENOLIC COMPOUNDS IN BLUEBERRIES AND MUSCADINE GRAPES ON HEPG2 CELL VIABILITY AND APOPTOSIS

Yi, W.; Akoh, C.C.; Fischer, J.; and Krewer, G. Submitted to Food Research International 09/02/2005.

Abstract

Although blueberries and muscadine grapes have high contents of polyphenols, few studies have been conducted to assess their potential effects on cancer cells. The objective of this study was to systematically evaluate the effects of different fractions of phenolic compounds in blueberries and muscadine grapes on HepG2 liver cancer cell viability and apoptosis. Three cultivars of blueberries ('Briteblue', 'Tifblue' and 'Powderblue') and four cultivars of muscadine ('Carlos', 'Ison', 'Noble', and 'Supreme') were assessed in this study. Polyphenols were extracted and further separated into phenolic acids, tannins, flavonols, and anthocyanins using a HLB cartridge and LH20 column. The phenolic acid fractions of muscadine grapes and blueberries showed a 50% inhibition of HepG2 cell population growth at the level of 1-2 mg/mL. The greatest inhibitory effects were observed from the anthocyanin fractions with 50% inhibitions of cancer cell population growth at concentrations of 70-150 and 100-300 μg/mL in blueberries and muscadine grapes, respectively. The flavonol and tannin fractions showed intermediate activities. In addition, DNA fragmentation was measured by using a Cell Death Detection ELISA kit to assess the induction of apoptosis. The anthocyanin fraction resulted in a 2 to 4 fold increase in DNA fragmentation compared to control in both muscadine grapes and blueberries. These findings of inhibition of cancer cell growth and induction of apoptosis suggest that blueberries and muscadine grapes may contribute to reduction in liver cancer risk.

Keywords: Apoptosis; Blueberries; Cancer; Cell viability; Muscadine grapes; Polyphenol

1. Introduction

Epidemiological studies have suggested that an inverse association exists between consumption of vegetables and fruits and the risk of human cancers at many body sites (*1*). Phenolic compounds, including phenolic acids and flavonoids, are especially promising candidates for cancer prevention (*2*). Much information is available on the reported inhibitory effects of specific plant phenolic compounds and extracts on mutagenesis and carcinogenesis (*3*). Yet, the potential ability of polyphenol combinations to prevent cancer progression has not been adequately studied. Scientists have suggested that it appears extremely unlikely that any one substance is responsible for all of the associations seen between plant foods and cancer prevention because of the great variety of dietary flavonoids and many types of potential mechanisms reported (*4*). It has been suggested that the combination of phytochemicals in fruits and vegetables is crucial for their potential anticancer activities (*5*). Studies based on individual crops will provide valuable information to further clarify their possible health benefit.

Blueberries belong to the family *Vacciniaceae*, and can be classified as highbush, lowbush, and rabbiteye. They have been considered one of the fruits with the highest antioxidant potentials, and a few studies have evaluated their anticancer activities (*6-8*). However, few studies have been done on their potential effects on liver cancer cells. Muscadine grapes belong to the botanical family *Vitaceae* (vine family). They are harvested as individual berries that ripen over an extended harvest period. They are different from the regular bunch grapes in that they have 2 more somatic chromosomes. The phytochemical profiles of muscadine have been documented by a few studies (*9, 10*). Unfortunately, very few studies have been done to evaluate their potential cancer prevention benefits. Any information on the potential anticancer benefits of muscadine grapes will be valuable.

As a well-differentiated transformed cell line, HepG2 cells have been widely used and considered a good model for liver cancer research (*11*). Using a HepG2 cell model, studies have been done to evaluate the bioactivities of fruits including cranberry, apple, red grape, strawberry, peach, lemon, pear, banana, orange, grapefruit, and pineapple (*3, 5*). The objectives of our current report were to systematically evaluate the potential anticancer activities of polyphenols in rabbiteye blueberries and muscadine grapes using a HepG2 cell model, and to assess their potential effects on cancer cell growth and apoptosis.

2. Materials and Methods

2.1. Chemicals and reagents

Pure standards of gallic acid, *p*-hydroxybenzoic acid, (+)-catechin, caffeic acid, (-)-epicatechin, *p*-coumaric acid, ferulic acid, ellagic acid, quercetin, kaempferol, and trans-resveratrol were purchased from Sigma (St. Louis, MO). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards were: Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), Mv-Glc (Malvidin 3-*O*-β-glucopyranoside), and Pn-Ara (Peonidin 3-*O*-α-arabinopyranoside). Folin-Ciocalteu reagent, dimethylsufoxide (DMSO), and Vanillin were purchased from Sigma (St. Louis, MO). Acetone, acetonitrile, methanol, *O*-phosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), sulfuric acid, 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). The human hepatocellular carcinoma HepG2 cell line was purchased from ATCC (Manassas, VA).

2.2. Sample collection

Mature blueberries and muscadine grapes were harvested from the field in 2004. The blueberry cultivars collected were Briteblue (Alapaha, GA), Tifblue (Alma, GA), and Powderblue (Chula, GA). Four cultivars of muscadine grapes were collected from Paulk Vineyards (Wray, GA), which include one bronze fruited (Carlos) and three purple fruited (Ison, Noble, and Supreme). Samples were frozen, and stored at –40 ^{o}C until use.

2.3. Extraction and fractionation

Polyphenolic fractions were obtained using a modified procedure reported by Youdim, McDonald, Kalt, and Joseph (*12*) and Oszmianski, Ramos, and Bourzeix (*13*). Crude extracts of blueberries and muscadine grapes were obtained through homogenization of whole blueberries and muscadine skins in acetone:methanol:water:formic acid (40:40:20:0.1, v/v/v/v). Crude extracts were freeze-dried using a UNITOP 600L freeze dryer (Virtis, Gardiner, New York), resolubilized in water, and applied to an activated Oasis HLB cartridge (Waters Corporation, Milford, MA). The 15% methanol fraction contained the phenolic acids, and the acidified methanol eluted the anthocyanins and other components of interest. The fraction containing the anthocyanins was dried again and resolubilized in 50% methanol in water and applied to a Sephadex LH20 column (Amersham Biosciences AB, Uppsala, Sweden). The column was then washed with 70% methanol acidified with 10% formic acid to elute anthocyanins and flavonols. The LH20 column was then washed with 70% acetone to elute the tannins or procyanidins. After freeze drying the anthocyanins and flavonol fraction, the fraction was resolubilized in 5% formic acid in water and applied to the second Oasis HLB cartridge. The cartridge was washed with 5%

formic acid, followed by ethyl acetate and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols and the acidified methanol eluted the anthocyanins. All fractions were freeze-dried and resolubilized for the cell viability assay. Extraction and fractionation were repeated 5 times and the fractions were pooled together to obtain sufficient amount for the bioassay.

2.4. Analytical measurement of phenolic compounds

2.4.1. Total polyphenols measurement

Total phenolics were measured according to the Folin-Ciocalteu reagent method (*14*). The extracts/fractions were dissolved in 80% methanol. Sample solutions were mixed with Folin-Ciocalteu reagent and sodium carbonate and allowed to stand for 30 min. Absorption at 765 nm was measured in a Shimadzu 300 UV-vis spectrophotometer (Shimadzu UV-1601, Norcross, GA). The total polyphenolic 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.

2.4.2. Proanthocyanidin measurement

Vanillin Assay was conducted to measure the contents of proanthocyanidins (*8, 15*). Briefly, tannin fractions were dissolved in methanol, and 250 μL of sample solutions were 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 ^{o}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.

2.4.3. Hydrolysis

For the phenolic acids and flavonoids analysis, fractions were dissolved in methanol containing 1.2 N HCl (40 mL methanol + 10 mL 6 N HCl). The samples were then placed in a water-bath at 80 ^{o}C while shaking at 200 rpm for 2 h for acid hydrolysis of phenolic glycosides to aglycones. For the anthocyanin hydrolysis, fractions were dissolved in 50% methanol solution containing 2 N HCl (50 mL methanol + 33 mL water + 17 mL 37% HCl). Samples were placed in a water-bath at 90 ^{o}C with shaking at 200 rpm for 1 h for acid hydrolysis of anthocyanins to anthocyanidins.

2.4.4. 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 in blueberries and muscadine grapes fractions, procedures previously reported by our laboratory were used (*6, 9*). Column: Beckman ultrasphere C18 ODS 4.6 x 250 mm and column temperature: 40 ^{o}C. Phenolic compounds were detected at wavelengths of 260, 280, 313, 320, and 360 nm.

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

2.5. Cell cultures

The human hepatocellular carcinoma HepG2 cancer cells were 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 non-essential amino acids, 1.0 mM sodium

pyruvate (90%), and 10% fetal bovine serum. Cells were incubated under 37 ^{o}C with 5% CO_2. Medium was changed 2 to 3 times per week.

2.6. Cell viability assay

After digestion with trypsin-EDTA, uniform amounts (~ 2×10^4) of HepG2 cells in growth media were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation at 37 ^{o}C in 5% CO_2, the growth medium was replaced with 100 μL media containing different concentrations of fruit extracts/fractions. Eight concentrations of each extract/fraction were applied. Depending on different fractions, the highest concentration of crude extracts and phenolic acid fractions was a few mg/mL and the highest concentration for the rest of the fractions was around a few hundred μg/mL. The subsequent concentrations were ½, ¼ …… 1/256 of the highest. Control cultures received everything but the fruit extracts/fractions, and blank wells contained 100 μL of growth medium and extract/fraction without cells. Except for anthocyanin fractions that were water soluble, DMSO was added initially to the extracts/fractions to help dissolve the sample. The final DMSO content was 0.25%. Therefore, the control for these treatments also contained 0.25% DMSO. None of the extracts/fractions changed the pH (.7.2) of the culture medium (data not shown). After 48 h of incubation, cell population growth was determined using the ATCC MTT Cell Proliferation Assay at 570-655 nm with 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 (*16*). To better explain the inhibitory results, the inhibition of cell population growth was calculated based on the following formula:

Inhibition percentage = (Cell # in control - Cell # in treatment)/(Cell # in control – original cell # before the extract or carrier was added) X 100%.

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

2.7. Detection of apoptosis

DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA using a Cell Death detection ELISA kit (Boehringer Mannheim, Roche) following the procedure recommended by Roche. Briefly, the cytosolic fraction (13,000 x g supernatant) of approximately 2 x 10^5 HepG2 cells after treatment with different concentrations of anthocyanin extract was used as antigen source in a sandwich ELISA with a primary anti-histone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. As a control reagent to induce necrosis, 1 M of KOH (instead of anthocyanins) was applied to the cancer cells. After incubation at 20 ^oC for 90 min, the conjugate solution (a secondary anti-DNA antibody coupled to peroxidase) was added. After incubation at 20 ^oC 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.

2.8. Statistical analysis

Statistical analysis was conducted using the General Linear Model (SAS 8.2, SAS Inst., Inc., 1999). The bioactivities of different fractions in different cultivars were compared by Duncan's multiple range test at $\forall = 0.05$. Correlation and regression was conducted to evaluate the relationship between total polyphenol contents and inhibitory activity. Pearson correlations were used to assess the relationship between inhibitory activity and the respective anthocyanidin constituent concentrations.

3. Results

3.1. Extraction and fractionation

Table 5.1 shows the total polyphenolic contents (weight percent) of different extracts/fractions of blueberries and muscadine grapes. The total polyphenolic contents percentage was calculated based on: GAE/(fraction weight) X 100%. In both blueberries and muscadine grapes, the crude extracts had significantly lower total polyphenolic contents than the remaining 3 fractions. The total polyphenolic contents in the crude extract of muscadine grapes (7.9 - 9.3%) were higher than those in blueberries (4.9 - 6.4%). Except for Ison, the total polyphenolic contents in the phenolic acid fractions of muscadine grapes were significantly higher than those in blueberries. Similar results were observed with the flavonol fractions and the tannin fractions in both blueberries and muscadine grapes representing from 75.3 to 93.2% of total polyphenolic contents

The proanthocyanidin estimations of tannin fractions in blueberries and muscadine grapes are shown in Table 5.2. The proanthocyanidin content percentage was calculated as CAE/(fraction weight) X 100%. The proanthocyanidin contents in the tannin fractions of

blueberries ranged from 69.3 to 81.7%, while the contents in muscadine grapes ranged from 64.6 to 81.6%.

The contents of individual phenolic acids and flavonoids in different fractions of blueberries are shown in Table 5.3. In the phenolic acid fractions, no ferulic acids were detected in Briteblue and no coumaric acids were detected in Powderblue. Besides gallic, caffeic, *p*-coumaric, and ferulic acids, some amount of catechin was found in the phenolic acid fractions. Major flavonols found in the flavonol fractions were quercetin and kaempferol. The contents of quercetin and kaempferol ranged from 4.4 to 8.5%, and from 2.2 to 5.9%, respectively. Table 5.4 shows the individual phenolic acids and flavonoids in the phenolic acid fractions and the flavonol fractions of muscadine grapes. The major identified compound in the phenolic acid fraction was ellagic acid ranging from 1.8% in Ison to 4.3% in Noble. Ellagic acid, resveratrol, myricetin, quercetin, and kaempferol were found in the flavonol fractions. The levels of myricetin ranged from 2.6 to 4.1%, and quercetin ranged from 2.7 to 5.0% of fraction dry weight. Unlike in blueberries, the flavonol fractions of muscadine grapes had high levels of resveratrol content ranging from 2.4 to 7.1%.

Compared with other fractions, the anthocyanin fraction had very high purity in both blueberries and muscadine grapes (Table 5.5, 5.6). The major individual anthocyanins in the anthocyanin fraction of blueberries are presented in Table 5.5. The percentage of total anthocyanins was obtained by summing up all of the individual anthocyanins identified. The identified anthocyanins accounted for more than 86% (in weight) in Tifblue anthocyanin and Powderblue anthocyanin fractions, and about 75% were identified in the Briteblue anthocyanin fraction. Besides anthocyanins, the anthocyanidin contents were evaluated after acid hydrolysis. Table 5.6 shows the major anthocyanidins in the anthocyanin fractions of blueberries and

muscadine grapes after hydrolysis. The amount of different anthocyanidins was expressed based on specific weight of anthocyanins: Delphinidin (Dp-Glc), Cyanidin (Cy-Gal), Petunidin (Pt-Glc), Peonidin (Pn-Gal), Malvidin (Mv-Glc), since most of the anthocyanins in blueberry are monoglycosides. It has been reported that the predominant anthocyanins in muscadine grape are 3,5-diglucosides (*10, 17*). Therefore, the amount of different anthocyanidins in the muscadine fraction was expressed as specific weight of diglucosides. The predominant anthocyanidins in the fractions of blueberries and muscadine grapes were delphinidin, cyanidin, petunidin, peonidin, and malvidin. The total anthocyanidin contents in blueberry fractions ranged from 89.3 to 98.7%, and the total anthocyanidin contents in muscadine fractions ranged from 51.9% (Carlos) to 96.1% (Supreme).

3.2. Cell viability

As expected, the crude extract showed lower inhibitory effects compared to the subsequent fractions. Figure 5.1 shows the inhibition of HepG2 cancer cell population growth by crude extracts and phenolic acid fractions of blueberries. The IC50s of crude extracts ranged from 2000 μg/mL with Briteblue to around 3000 μg/mL with Tifblue and Powderblue. Phenolic acid fractions resulted in 50% inhibition of HepG2 cell population growth at a concentration of about 2000 μg/mL, and the IC50 was close for all three blueberries cultivars.

Inhibition of HepG2 cancer cell population growth by flavonol, tannin, and anthocyanin fractions of blueberries is shown in Figure 5.2. Among all four fractions, the highest bioactivities were observed with the anthocyanin fractions. The IC50 of anthocyanin fraction of Tifblue was around 150 μg/mL, and a 50% inhibition of HepG2 cell population growth was observed at around 70 μg/mL of anthocyanin fractions with Briteblue and Powderblue. The bioactivities of the flavonol and tannin fractions were intermediate compared with phenolic acid

fraction and anthocyanin fraction. The IC50 of flavonol fractions ranged from around 130 μg/mL (Briteblue and Powderblue) to 170 μg/mL (Tifblue). The IC50 of tannin fractions ranged from 110 to 210 μg/mL, and Powderblue showed significantly higher inhibitory activity (IC50 around 110 μg/mL) compared with the other two cultivars.

Similar trends on cancer cell population growth were found with muscadine grapes (Figures 5.3 and 5.4). Inhibition of HepG2 cancer cell population growth by crude extract and phenolic acid fraction of muscadine grapes is shown in Figure 5.3. As with blueberries, crude extracts of muscadine grapes exhibited lower activity compared with the four fractions. With crude extracts, Carlos showed the highest bioactivity among all four cultivars with IC50 of 1500 μg/mL. The IC50 of phenolic acid fraction ranged from 1100 to 1700 μg/mL and the bioactivities of the four muscadine cultivars were close.

Figure 5.4 shows the inhibition of HepG2 cancer cell population growth by flavonol, tannin, and anthocyanin fractions of muscadine grapes. As with blueberries, the anthocyanin fraction had the highest inhibitory activity among all fractions. The IC50 of anthocyanin fractions varied with different cultivars. Supreme showed the highest bioactivity with an IC50 around 130 μg/mL, while Noble had lowest inhibitory effect with an IC50 around 340 μg/mL. The anthocyanin fractions of Carlos and Ison showed intermediate IC50. In the case of flavonol fractions, Carlos had the highest activity with IC50 around 300 μg/mL, and the IC50 of the other 3 cultivars ranged from 400 to 500 μg/mL. In contrast to the flavonol fractions, the tannin fraction of Carlos showed the lowest inhibitory activity among all four cultivars (Figure 5.4 B).

3.3. Apoptosis

An early event in apoptosis is DNA fragmentation and release and activation of an endogenous endonuclease. DNA fragmentation is the primary physiological characteristic of

apoptosis. Figure 5.5 shows the DNA fragmentation in HepG2 cells associated with anthocyanin fractions. Briteblue blueberry anthocyanin fraction resulted in a significant increase in DNA fragmentation. At a level of around 100 μg/mL, DNA fragmentation reached its maximum, which was about 3 – 4 times of the no treatment control. Further increases in the anthocyanin concentration resulted in lower level of DNA fragmentation (Figure 5.5 A), suggesting that necrosis may occur above 100 μg/mL. Similarly, a significant increase in DNA fragmentation was observed after treatment with anthocyanins from the Noble muscadine grapes. A maximum increase in DNA fragmentation was observed from 200 to 700 μg/mL, which was about 3 times the DNA fragmentation level of the no treatment control. Levels of Noble anthocyanin fractions above 200 – 700 μg/mL resulted in less DNA fragmentation. In contrast, the KOH treatment, which caused necrosis, resulted in low levels of DNA fragmentation control.

3.4. Correlation between inhibitory activity and phenolic compound concentrations

Figure 5.6 shows the correlation between total polyphenol concentrations and the IC50 in Hep G2 cancer cells. Significant linear correlations ($P < 0.01$) were observed. The highest correlation ($r = -0.965$) was observed with blueberries, while a lower correlation ($r = -0.667$) was observed when the results of blueberries and muscadine grapes were combined (Figure 6 C).

Pearson correlations were used to examine the relationships between the individual anthocyanidin contents and the inhibition of cell population growth (IC50). Tables 5.7, 5.8, 5.9 show the correlation matrix for IC50 and different anthocyanidin constituents in blueberries, muscadine grapes, and both blueberries and muscadine grapes, respectively. With blueberries, the IC50 showed a negative correlation ($r = -0.981$ and -0.872, respectively, $P<0.01$) with delphinidin and petunidin, indicating that increasing concentration will increase growth inhibition; meanwhile a positive correlation ($P<0.01$) was observed between IC50 and peonidin

(Table 5.7). In muscadine grapes, a negative correlation was observed between IC50 and cyanidin; meanwhile petunidin and malvidin positively correlated with IC50 (Table 5.8). When the data of both blueberries and muscadine grapes were pooled together, a very low correlation was observed. Only malvidin showed a significant correlation (r = -0.584) with IC50 (Table 5.9).

4. Discussion

The origin and treatment of cancer is very complex, yet recent studies have shown that deregulated cell proliferation and suppression of apoptosis provides a minimal 'platform' necessary to support further neoplastic progression. Targeting of these critical events should have potent and specific therapeutic consequences (*18*). A few studies have evaluated the inhibitory effects of phenolic compounds on HepG2 cancer cell growth. Ramos et al. (*11*) studied the effects of pure polyphenols (quercetin, chlorogenic acid, and epicatechin) and fruit extracts (strawberry and plum) on HepG2 cell population growth and apoptosis. They found that quercetin was the most active with an IC50 of 87 μM (about 26 μg/mL). The estimated IC50 of strawberry and plum extract was 0.6 mg/mL and 1.5 mg/mL. In another study, Yeh, Huang, and Yen (*19*) reported that no significant effect on the cell growth of HepG2 cells was observed until the concentration of phenolic acids reached 40 μM. At 50 μM, maximum inhibition was found with 70% ferulic acid, 13% gentisic acid, and 6% gallic acid, and no effects were found with p-hydroxybenzoic acid or p-coumaric acid. This is in agreement with the current study where only low bioactivity was observed with phenolic acid fractions from both blueberries and muscadine grapes. The other possible reason for the low bioactivity of these fractions in the current study could be because of its relatively low total polyphenol purity compared with the other fractions.

A good correlation between total polyphenols and IC50 only existed in blueberries in the current study. The lowest correlation was observed when the data of blueberries and muscadine grapes were pooled together. This is probably because of the differences in the profile of phenolic compounds in blueberries and muscadine grapes. Total polyphenols alone obviously cannot explain the bioactivity differences among the various fractions. Except for phenolic acid fractions in muscadine which showed higher bioactivity, blueberry fractions had higher inhibitory potentials than muscadine fractions although the total polyphenols contents and proanthocyanidin contents were close.

As natural pigments, the potential anticancer activities of anthocyanins have been evaluated in several studies (*20, 21*). Kang, Seeram, Nair, and Bourquin (*22*) reported that cyanidin and tart cherry anthocyanins reduced the growth of HT29 colon cancer cells. The IC50s of anthocyanins and cyanidin were 780 and 63 μM (equivalent to 350 and 18 μg/mL). In another study, cyanidin and delphinidin were found to inhibit the growth of human lung and vulva tumor cells in the μM range, whereas malvidin was less active, and Cy-3-gal and Mv-3-glc were least active (*23*). The author stated that the sugar residues in position 3 decreased the growth-inhibitory properties. Further, studies have shown that the potency of apoptosis induction of anthocyanidins is associated with the number of OH groups at the B-ring, and the ortho-dihydroxyphenyl structure at the B-ring is essential for apoptosis action (*20*). The biological activities of anthocyanins appear to increase with a decreasing number of sugar units and/or with an increasing number of hydroxyl groups on their aglycone forms (*24*). In the current study, blueberry anthocyanins showed higher bioactivity than anthocyanin fractions of muscadine grapes, and the difference cannot be explained by the molar differences of the anthocyanidins (because of the different molecular weights of mono- and diglycosides). The

possible reasons could be: 1) monoglycosides in blueberry anthocyanins were more active than diglycosides in muscadine grapes; and 2) differences in the anthocyanidin profiles of these two crops.

The effects of anthocyanidin on HepG2 cell viability have been reported by Yeh and Yen (*25*). The estimated IC50 of cyanidin, delphinidin, and malvidin were 18.4, 10.8, and 50.4 μM (equivalent to 5, 3, and 17 μg/mL), respectively. These results are lower than the IC50 (ranging from 70 μg/mL to a few hundred μg/mL) of anthocyanin fractions in the current study. This is in agreement with the theory that anthocyanidins are more active than anthocyanins.

Since the purity of anthocyanin fractions of both blueberries and muscadine grapes are very high, we correlated the individual anthocyanidin concentrations with the IC50. In blueberries, a negative correlation was observed in delphinidin and petunidin, meaning they are more active than the rest. In muscadine grapes, cyanidin negatively correlated with IC50. These results are in agreement with the study by Meiers et al. (*23*). The interesting phenomenon is that petunidin negatively correlated with IC50 in blueberries, while positively correlated with it in muscadine grapes. One explanation could be that their attached sugar units are different. It is also important to note that the positive correlation doesn't mean the anthocyanidin can increase cell population growth; instead it indicates that it is less active than the rest of the anthocyanidins.

Most of previous reports on the bioactivity of individual anthocyanins were conducted using anthocyanidin. As Hou et al. (*21*) have pointed out, most of the results on the biological activities of anthocyanins were from anthocyanidins probably due to the fact that anthocyanidins are easier to prepare than anthocyanins. Whether the naturally occurring anthocyanins will also affect the same molecular mechanisms needs further study. Our cell viability inhibitory results

are based on naturally existing anthocyanins, and will provide useful information for future studies in this area.

Another interesting phenomenon in the current study is that slightly higher cell population growth was observed at very low concentrations of flavonol fractions of both blueberries and muscadine (Figure 2 A and Figure 4 A), although no statistical differences were observed. Similar results were obtained with flavonoids in rat hepatoma cells (*26*). In a study by Watjen et al. (*26*), protective effects against H_2O_2-induced cytotoxicity and apoptosis were detected at concentrations as low as 10-25 μM; meanwhile these flavonoids induced cytotoxicity and apoptosis at concentrations between 50 and 250 μM. Identification of the effective concentration is critical. Watjen et al. (*26*) suggested the antiapoptotic actions of phenolic compounds may protect against neurodegenerative diseases, whereas their proapoptotic actions can be used for cancer prevention.

Apoptosis, or programmed cell death, allows the organism to tightly control cell numbers and tissue size (*27*). Apoptosis-inducing agents are expected to be ideal anticancer drugs. Many cancer drugs eliminate tumor cells by inducing apoptosis (*24*). The effect of anthocyanins/anthocyanidins on apoptosis has been documented in many studies. Fimognari, Berti, Nusse, Cantelli-Forti, and Hrelia (*28*) observed that cyanidin-glucoside induced apoptosis in Jurka T-leukemia cells at a concentration as low as 12.5 μg/mL. In addition, a 2 times increase in DNA fragmentation was observed in HL-60 cells at a concentration of 200 μg/mL. These results are in good agreement with the current study in which a maximum (3-4 times) increase in DNA fragmentation was observed with 100 μg/mL of blueberry anthocyanins (monoglycosides). In both blueberries and muscadine grapes, DNA fragmentation reached maximum around a few hundred μg/mL. Further increases in anthocyanin concentration resulted

in less DNA fragmentation compared to control, although fewer cells survived at these high concentrations. This suggests that necrosis could occur at high anthocyanin concentrations. A similar phenomenon was reported with delphinidin in HL-60 cells where the best concentration for apoptosis induction was 100 μM for 6 h (*24*). These phenomena have also been found with antitumor chemicals, such as fostriecin (*29*). Therefore, identification of effective anthocyanin concentrations for apoptosis induction is a prerequisite for future clinical studies.

It is important to note that more information about bioavailability and metabolism is required in order to correctly relate the *in vitro* study results to human disease outcomes, where exposure to polyphenols is chronic and at relatively low concentrations (*30*). Although most polyphenols are absorbed to some extent, the data on the bioavailability of phenolic compounds is still limited (*31, 32*). Further studies are required to evaluate the bioavailability, metabolism and beneficial mechanism of phenolic compounds in blueberries and muscadine grapes before their potential health benefit can be fully determined. Animal studies are currently ongoing in our laboratory for further clarification of the bioactivities of these phenolic compounds.

In conclusion, our study found that phenolic compounds in blueberries and muscadine grapes could inhibit HepG2 liver cancer cell population growth and induce apoptosis. Dietary intakes of these fruits may have potential to reduce liver cancer.

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Table 5.1. Total polyphenolic content of different extracts/fractions from blueberries and muscadine grapes (unit: wt%)[a].

Cultivars	Crude extract	Phenolic acid fraction	Flavonol fraction	Tannin fraction
		Blueberries		
Briteblue	6.0±0.5 [b]	14.9±1.1	76.6±1.5	87.9±0.9
Tifblue	6.4±0.3	15.1±0.9	86.5±0.6	85.4±2.5
Powderblue	4.9±0.2	12.4±1.2	75.3±1.1	86.9±3.3
		Muscadine		
Carlos	8.9±0.2 [b]	24.9±0.4	76.3±2.2	85.1±4.4
Ison	8.2±0.3	14.3±0.3	86.0±1.4	86.1±4.0
Noble	7.9±0.3	25.8±0.3	82.4±2.1	93.2±2.5
Supreme	9.3±0.2	27.4±0.7	86.1±1.5	90.5±3.2

[a] Total polyphenols were expressed as gallic acid equivalents (GAE), and the total polyphenolic contents percentage was calculated based on: GAE/(fraction weight) X 100%.

[b] Values are averages of triplicate analyses ± standard error.

Table 5.2. Proanthocyanidin estimation of tannin fractions in different cultivars of blueberries and muscadine grapes (unit: wt%) [a].

Tannin fraction of blueberries				
Cultivars	Briteblue	Tifblue	Powderblue	-
Proanthocyanidin content	69.3±0.7 [b]	79.1±1.4	81.7±1.2	-

Tannin fraction of muscadine grapes				
Cultivars	Carlos	Ison	Noble	Supreme
Proanthocyanidin content	64.6±2.2 [b]	67.8±1.5	74.7±2.4	81.6±2.9

[a] The proanthocyanidin estimations were expressed as catechin equivalents (CAE), and the proanthocyanidin content percentage was calculated based on: CAE/(fraction weight) X 100%.

[b] Values are averages of triplicate analyses ± standard error.

Table 5.3. Individual phenolic acids and flavonoids in different fractions of blueberries (unit: wt%).

Cultivars	Gallic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Catechin	Epicatechin	Quercetin	Kaempferol
				Phenolic acid fractions				
Briteblue	0.4±0.0[a]	3.2±0.0	2.5±0.0	ND[b]	2.3±0.1	ND	ND	ND
Tifblue	3.2±0.0	1.1±0.0	0.3±0.0	0.4±0.0	1.9±0.1	ND	ND	ND
Powderblue	0.8±0.0	1.3±0.0	ND	0.4±0.0	2.8±0.1	ND	ND	ND
				Flavonol fractions				
Briteblue	0.7±0.1	0.7±0.1	0.6±0.1	ND	ND	ND	5.7±0.3	2.2±0.2
Tifblue	0.6±0.1	0.3±0.0	ND	0.3±0.0	ND	1.4±0.1	4.4±0.2	3.4±0.2
Powderblue	0.8±0.1	0.9±0.1	ND	0.6±0.0	ND	ND	8.5±0.3	5.9±0.3

[a] Data are percentage by weight of individual compounds in the whole weight of specific fraction. Values are averages of triplicate analyses ± standard error.

[b] ND - Not detected.

Table 5.4. Individual phenolic acids and flavonoids in different fractions of muscadine grapes (unit: wt%) [a] .

Cultivars	Ellagic acid	Resveratrol	Myricetin	Quercetin	Kaempferol
			Phenolic acid fraction		
Carlos	4.2±0.1	0.4±0.0	ND [b]	0.4±0.0	ND
Ison	1.8±0.1	0.4±0.0	0.3±0.0	1.1±0.0	ND
Noble	4.3±0.3	0.3±0.0	0.9±0.0	1.1±0.0	ND
Supreme	3.4±0.1	0.2±0.0	ND	1.2±0.0	ND
			Flavonol fraction		
Carlos	5.9±0.2	7.1±0.1	3.6±0.1	5.0±0.1	1.3±0.0
Ison	6.6±0.5	2.4±0.1	2.6±0.1	2.9±0.1	1.7±0.1
Noble	8.1±0.6	4.0±0.1	4.1±0.1	2.7±0.2	1.7±0.1
Supreme	4.1±0.4	2.5±0.1	3.0±0.3	3.5±0.2	0.8±0.0

[a] Data are percentage by weight of individual compounds in the whole weight of specific fraction. Values are averages of triplicate analyses ± standard error.

[b] ND- Not detected.

Table 5.5. Major individual anthocyanins in the blueberry anthocyanin fractions (unit: wt%) [a].

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

[a] Values are averages of triplicate analyses ± standard error. Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), and Mv-Glc (Malvidin 3-*O*-β-glucopyranoside).

Table 5.6. Major individual anthocyanidins in the anthocyanin fraction of blueberries and muscadine after acid hydrolysis (unit: wt%)[a].

Cultivars	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Total anthocyanidins
			Blueberries			
Briteblue	18.6±0.9 [b]	10.0±0.4	30.3±0.5	9.1±1.3	21.4±0.6	89.3±3.2
Tifblue	12.6±0.8	17.5±1.1	22.1±0.8	25.2±1.2	20.0±0.7	97.4±4.1
Powderblue	20.0±0.3	21.2±0.6	26.2±0.7	15.5±0.9	16.8±1.1	98.7±3.3
			Muscadine grapes			
Carlos	21.8±0.7 [c]	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 based on specific weight of anthocyanins. Delphinidin (Dp-Glc), Cyanidin (Cy-Gal), Petunidin (Pt-Glc), Peonidin (Pn-Gal), Malvidin (Mv-Glc).

[c] Amount of different anthocyanidins was expressed based on specific weight of anthocyanidin diglucosides.

Table 5.7. Correlation matrix for IC50 and different anthocyanidin constituents in blueberries.

	IC50	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
IC50	1					
Delphinidin	-0.981[a]**	1				
	(0.000)					
Cyanidin	0.206	-0.014	1			
	(0.595)	(0.971)				
Petunidin	-0.872**	0.763*	-0.657*	1		
	(0.002)	(0.017)	(0.054)			
Peonidin	0.924**	-0.834**	0.564	-0.993**	1	
	(0.000)	(0.005)	(0.114)	(0.000)		
Malvidin	0.206	-0.391	-0.915**	0.297	-0.183	1
	(0.594)	(0.299)	(0.001)	(0.438)	(0.638)	

[a] The value is Pearson correlation coefficient r, and the value in parenthesis is P value.

* Correlation is significant at $\alpha = 0.05$.

** Correlation is significant at $\alpha = 0.01$.

Table 5.8. Correlation matrix for IC50 and different anthocyanidin constituents in muscadine grapes.

	IC50	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
IC50	1					
Delphinidin	-0.095[a]	1				
	(0.770)					
Cyanidin	-0.635*	0.483	1			
	(0.027)	(0.112)				
Petunidin	0.690*	0.156	0.099	1		
	(0.013)	(0.628)	(0.759)			
Peonidin	0.328	-0.251	0.291	0.809**	1	
	(0.299)	(0.431)	(0.360)	(0.001)		
Malvidin	0.862**	-0.403	-0.433	0.793**	0.713**	1
	(0.000)	(0.194)	(0.160)	(0.002)	(0.009)	

[a] The value is Pearson correlation coefficient r, and the value in parenthesis is P value

* Correlation is significant at $\alpha = 0.05$.

** Correlation is significant at $\alpha = 0.01$.

Table 5.9. Correlation matrix for IC50 and different anthocyanidin constituents in both blueberries and muscadine grapes [a].

	IC50	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
IC50	1					
Delphinidin	0.364[b]	1				
	(0.105)					
Cyanidin	-0.149	0.450*	1			
	(0.518)	(0.041)				
Petunidin	0.048	-0.050	-0.115	1		
	(0.836)	(0.828)	(0.620)			
Peonidin	0.058	-0.516**	0.237	0.335	1	
	(0.802)	(0.017)	(0.301)	(0.138)		
Malvidin	-0.584**	-0.729**	-0.372*	0.467*	0.413*	1
	(0.005)	(0.000)	(0.097)	(0.033)	(0.063)	

[a] Correlation results were obtained when the data of blueberries and muscadine grapes were pooled together.

[b] The value is Pearson correlation coefficient r, and the value in parenthesis is P value

* Correlation is significant at $\alpha = 0.05$.

** Correlation is significant at $\alpha = 0.01$.

Fig. 5.1. Inhibition of HepG2 cancer cell population growth by crude extract and phenolic acid fraction of blueberries (mean ± SE, n = 4). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) Crude extract; (B) Phenolic acid fraction.

Fig. 5.2. Inhibition of HepG2 cancer cell population growth by different fractions of blueberries (mean ± SE, n = 4). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) Flavonol fraction; (B) Tannin fraction; (C) Anthocyanin fraction.

Fig. 5.3. Inhibition of HepG2 cancer cell population growth by crude extract and phenolic acid fraction of muscadine grapes (mean ± SE, n = 4). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) Crude extract; (B) Phenolic acid fraction.

Fig. 5.4. Inhibition of HepG2 cancer cell population growth by different fractions of muscadine grapes (mean ± SE, n = 4). Axis x stands for the concentration (μg/mL) of extracts in the culture medium. Original cell # is the cell number after 24 h incubation and before treatments were applied. (A) Flavonol fraction; (B) Tannin fraction; (C) Anthocyanin fraction.

Fig. 5.5. DNA fragmentation in HepG2 cells associated with anthocyanin fractions (mean ± SE, n = 3). (A) Anthocyanin fraction of Briteblue blueberry, and 1 M of KOH was a control reagent to induce necrosis; (B) Anthocyanin fraction of Noble muscadine grape, and 1 M of KOH was a control reagent to induce necrosis.

Fig. 5.6. Correlation between total polyphenol concentrations and the IC50 in HepG2 cancer cells. (A) Correlation was conducted based on the results of different fractions in blueberries (n = 12); (B) Correlation was conducted based on the results of different fractions in muscadine

grapes (n=16); (C) Correlation was conducted after the results of different fractions in both blueberries and muscadine grapes were pooled together (n=28).

Figure 5.1.

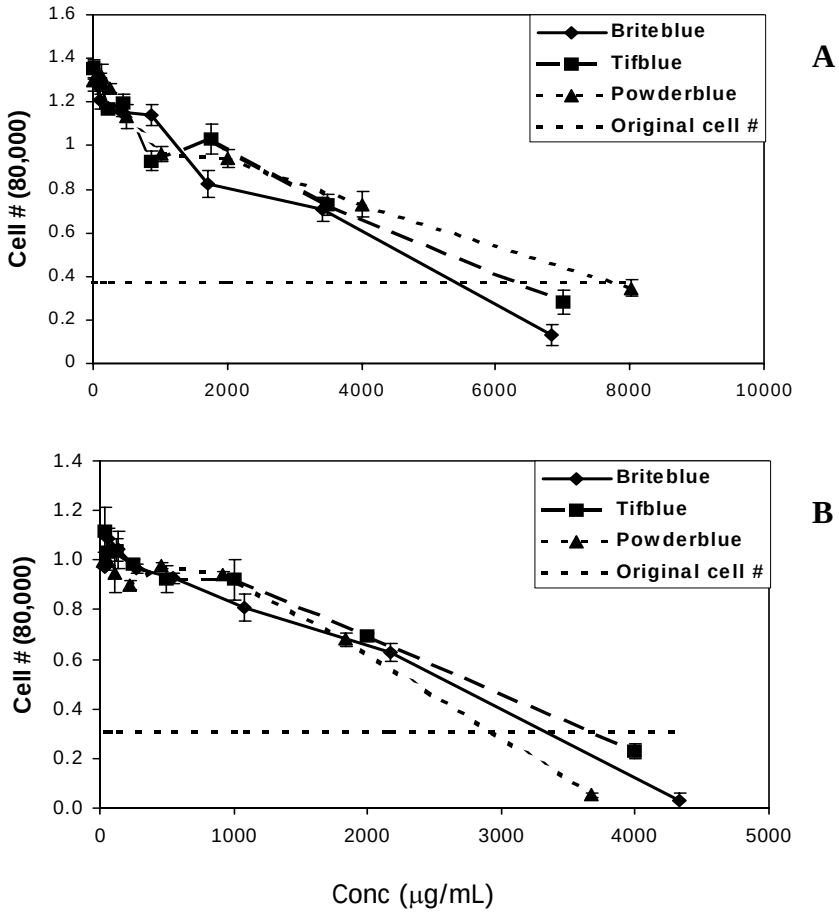

A
Briteblue
Tifblue
Powderblue
Original cell #
Cell # (80,000)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2000
4000
6000
8000
10000
B
Briteblue
Tifblue
Powderblue
Original cell #
Cell # (80,000)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
Conc (μg/mL)

Figure 5.2.

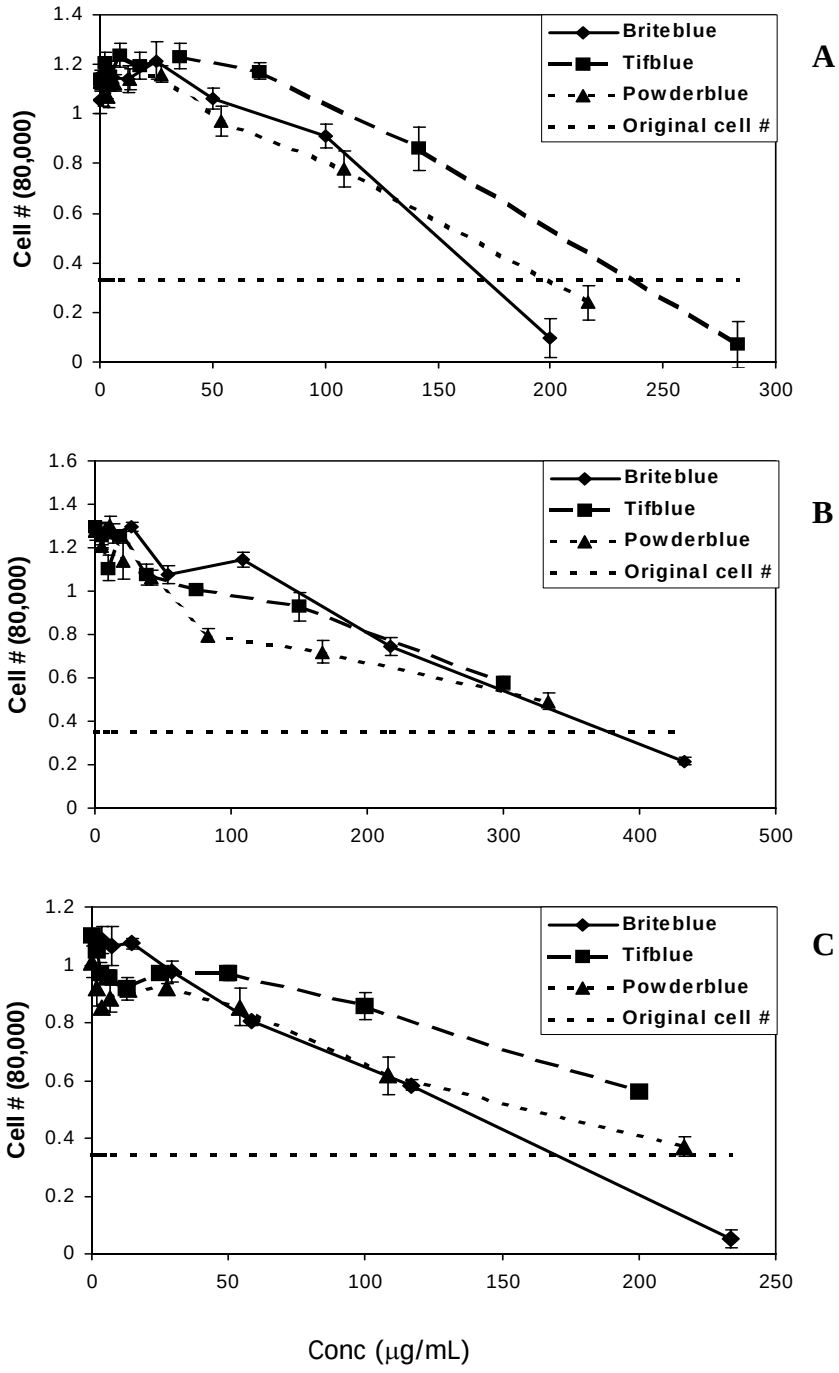

A
B
C
Briteblue
Tifblue
Powderblue
Original cell #
Cell # (80,000)
Conc (μg/mL)

Figure 5.3.

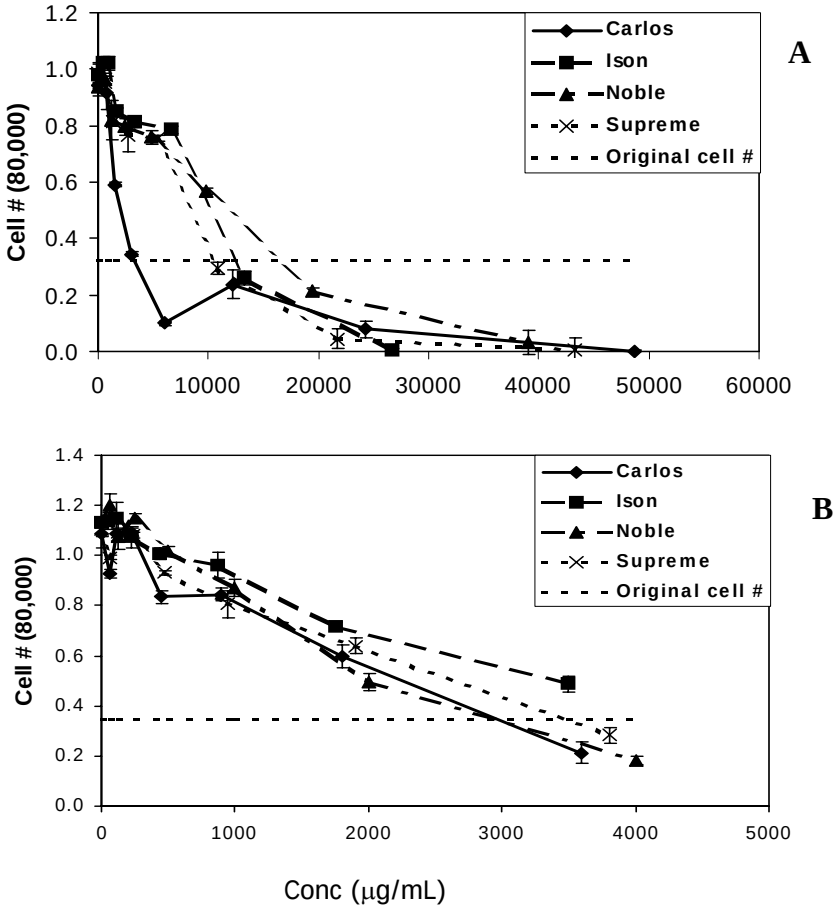

A
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (80,000)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10000
20000
30000
40000
50000
60000
B
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (80,000)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
Conc (μg/mL)

Figure 5.4.

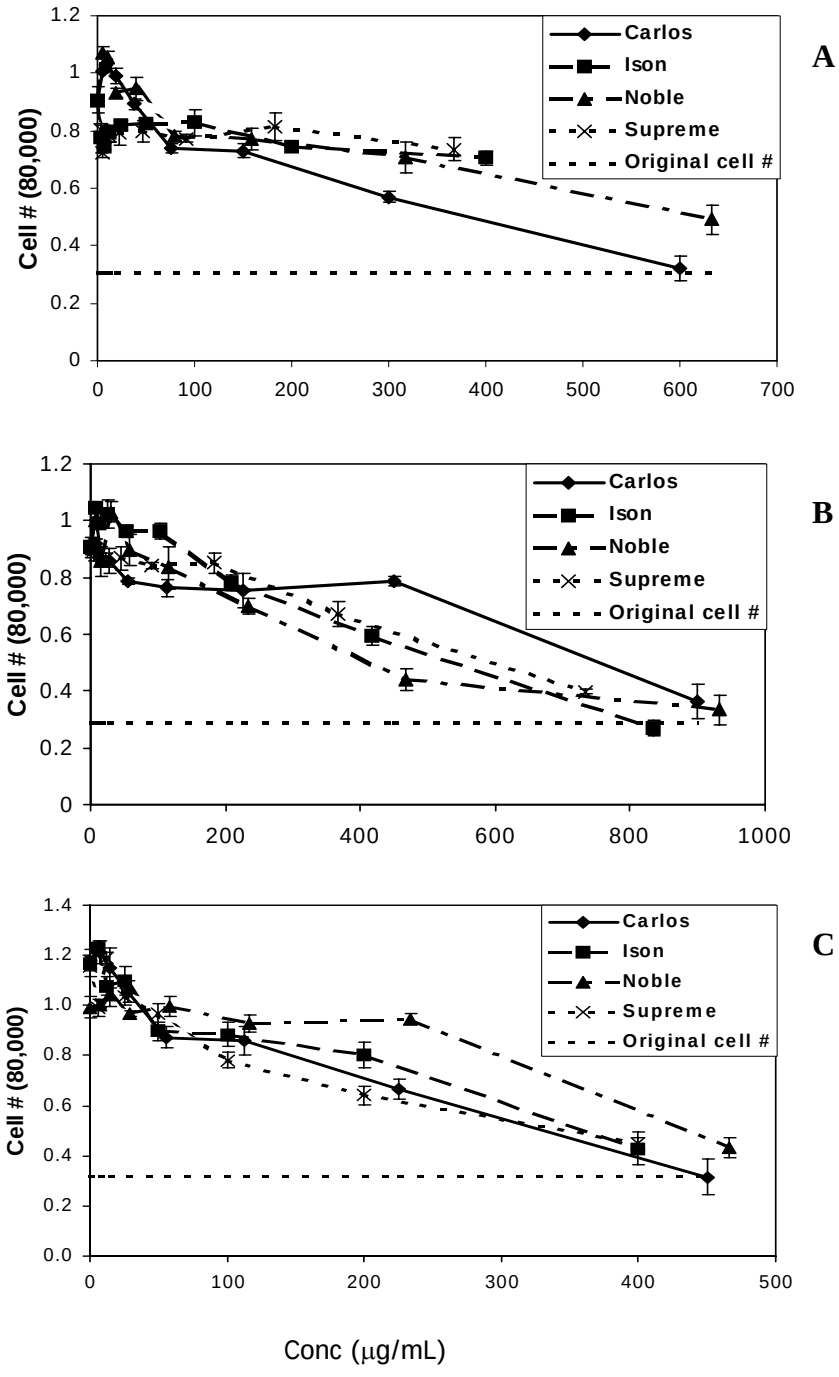

A
Carlos
Ison
Noble
Supreme
Original cell #
Cell # (80,000)
B
C
Conc (μg/mL)

Figure 5.5.

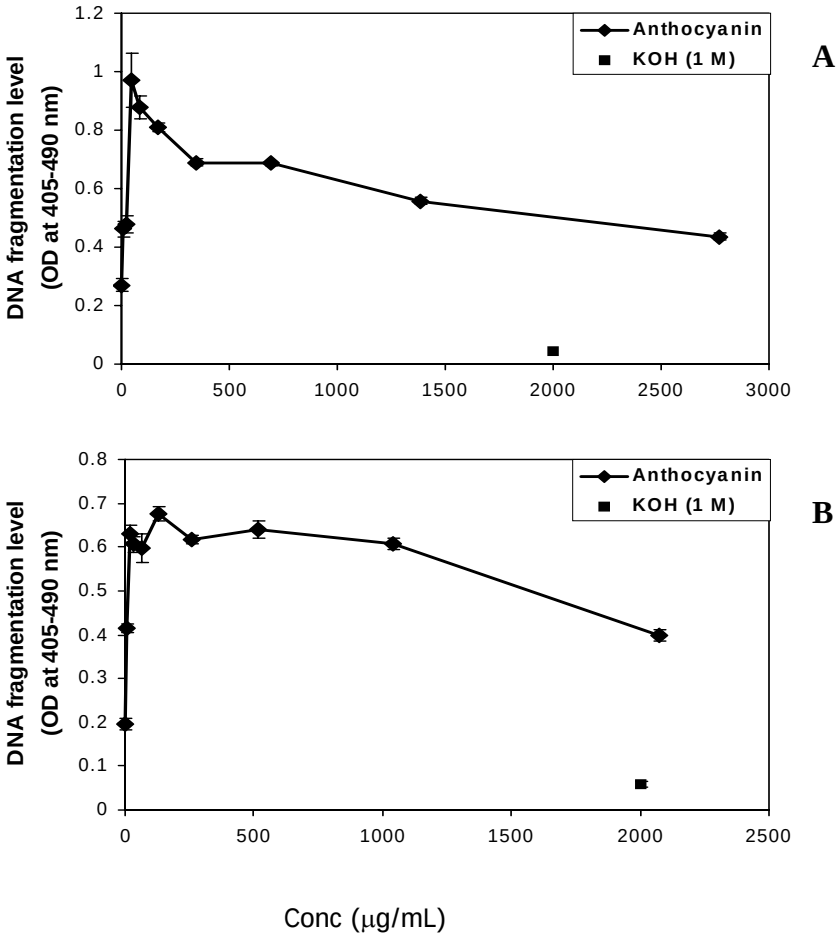
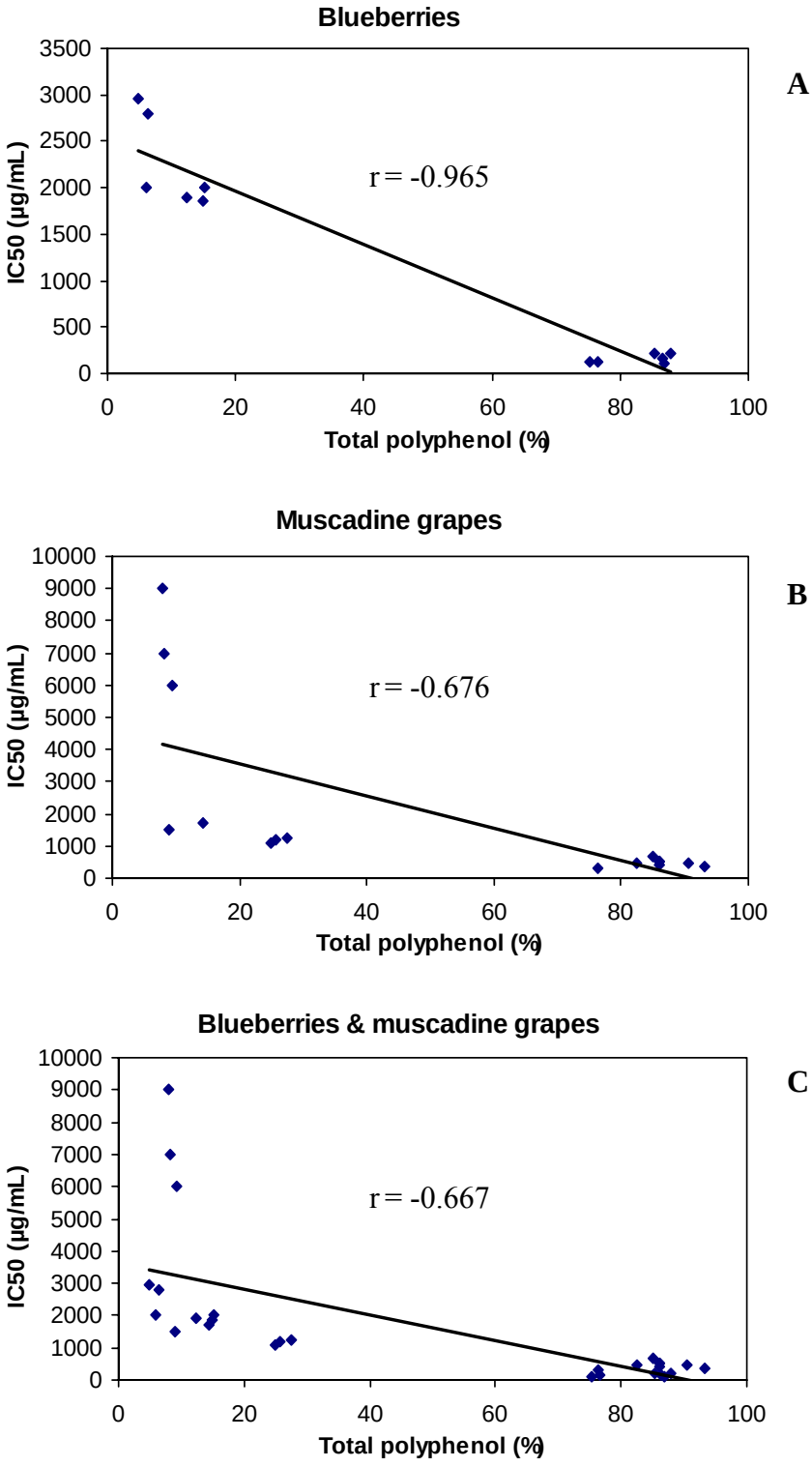

DNA fragmentation level (OD at 405-490 nm)
Anthocyanin
KOH (1 M)
A
B
Conc (μg/mL)

Figure 5.6.


Blueberries
IC50 (µg/mL)
Total polyphenol (%)
r = -0.965
A
Muscadine grapes
IC50 (µg/mL)
Total polyphenol (%)
r = -0.676
B
Blueberries & muscadine grapes
IC50 (µg/mL)
Total polyphenol (%)
r = -0.667
C

CHAPTER 6

ABSORPTION OF ANTHOCYANINS FROM BLUEBERRY EXTRACTS BY CACO-2 HUMAN INTESTINAL CELL MONOLAYERS

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Abstract

Recent studies have shown that dietary polyphenols may contribute to the prevention of cardiovascular disease and cancer. Anthocyanins from different plant sources including blueberries have been shown to possess potential anticancer activities. One of the key factors needed to correctly relate the in vitro study results to human disease outcomes is information about bioavailability. The objectives of the current study were to evaluate the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers, and to investigate the effects of different aglycones, sugar moieties, and chemical structure on bioavailability of different types of anthocyanins. The results of this study showed that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers although the transport/absorption efficiency was relatively low compared to other aglycone polyphenols. The transport efficiency of anthocyanins averaged ~3-4% [less than 1% in delphinidin glucoside (Dp-glc)]. No significant difference in transport/absorption efficiency was observed among three blueberry cultivars. The observed trends among different anthocyanins generally agreed well with some published in vivo results. Dp-glc showed the lowest transport/absorption efficiency, and malvidin glucoside (Mv-glc) showed the highest transport/absorption efficiency. Our result indicates that more free hydroxyl groups and less OCH_3 groups can decrease the bioavailability of anthocyanins. In addition, cyanindin glucoside (Cy-glc) showed significantly higher transport efficiency than cyanidin galactoside (Cy-gal), and peonidin glucoside (Pn-glc) showed significantly higher transport efficiency than peonidin galactoside (Pn-gal), indicating that glucose-based anthocyanins have higher bioavailability than galactose-based anthocyanins.

KEYWORDS: absorption; aglycone; anthocyanins; bioavailability; blueberry; Caco-2 monolayer; sugar moieties

INTRODUCTION

Flavonoids and anthocyanins are naturally occurring phenolic compounds that are present in fruits and vegetables. Recent studies have shown that dietary polyphenols may contribute to the prevention of cardiovascular diseases and cancer (*1, 2*). Dietary consumption of anthocyanins has been reported to be higher than other flavonoids such as quercetin, probably because of their widespread distribution and occurrence in fruits and vegetables (*3-5*).

Anthocyanins are potent antioxidants. Fruits and vegetables with high anthocyanin contents generally have higher antioxidant capacity than other fruits and vegetables (*6-8*). Anthocyanins from different plant sources may also have potential anticancer activities (*9-11*). A few studies have reported the anticancer activities of blueberries, which are a rich source of anthocyanins (*12-14*). Our previous studies (*15*) evaluated the effects of polyphenol extracts on colon cancer cell growth and apoptosis, and found that anthocyanin fractions showed the greatest bioactivity among all four fractions evaluated. It was, thus, suggested that anthocyanins were one of the major components that inhibited colon cancer cell proliferation and induced apoptosis.

Despite the statement that anthocyanins have potential in cancer prevention and other health benefits, a significant gap exists between what has been shown in many in vitro studies and what can be achieved under in vivo conditions. One of the key factors needed to correctly relate in vitro study results to human disease outcomes is information about bioavailability and metabolism (*16*). Although a few studies have been conducted to evaluate the bioavailability of anthocyanins (*17-20*), the information on absorption and metabolism is still very limited.

Compared to human and animal models that are highly complex and may be confounded by factors such as chemical instability and inadequate analytical methodology, it is easy to control these parameters in an in vitro studies. An in vitro assay is simple, more convenient, and

less expensive. Caco-2 cells have been the most extensively characterized and useful in vitro model in the field of drug permeability and absorption (*21-23*). The differentiated cell monolayers have been used for the study of unidirectional transport of phytochemicals such as quercetin, epicatechin, and proanthocyanidin (*24-27*), and much valuable information has been obtained using this model system.

Information on the bioavailability of pure compounds is a good start to clarify the bioavailability of complex mixtures of phytochemicals (*28*). The combination of phytochemicals in fruits and vegetables is thought to be critical to their powerful antioxidant and anticancer activity (*29, 30*). Similarly, information on the bioavailability of complex mixtures of phytochemicals is essential. Thus, studies on the bioavailability of phenolic compounds such as anthocyanins from specific crops are a good way to provide direct and valuable information about their absorption. The objectives of the current study were to evaluate the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers, and to investigate the effects of different aglycones, sugar moieties, and chemical structure on the bioavailability of different types of anthocyanins.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, methanol, *O*-phosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), formic acid, water (HPLC grade), and Hank's Balanced Salts Solution (HBSS) were purchased from Fisher Scientific (Norcross, GA). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards were 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). Caco-2 human colorectal adenocarcinoma cells were purchased from ATCC (Manassas, VA). Blueberries were collected from the field in 2004. The blueberry cultivars collected were Briteblue (Alapaha, GA), Tifblue (Alma, GA), and Powderblue (Chula, GA). All cultivars were grown with irrigation or under conditions of adequate rainfall. Samples were frozen, and stored at –40 ^{o}C until use.

Extraction and Fractionation. Anthocyanin fractions were obtained using a modified procedure of Youdim et al. (*31*) and Oszmianski et al. (*32*), which we previously reported (*15*). Briefly, 100 g of blueberries were homogenized in 300 mL of acetone:methanol:water:formic acid (40:40:20:0.1, v/v/v/v). 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), washed with water, 15% methanol in water and finally with methanol acidified with 5% formic acid. The acidified methanol eluted the anthocyanins and other flavonoids. The fraction containing the anthocyanins was dried again 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. After freeze-drying the anthocyanin and flavonol fraction, the fraction was 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 acidified methanol eluted the anthocyanins which we used for further analysis.

HPLC Analysis. Anthocyanin measurements were conducted following a procedure previously reported in our laboratory (*15*). HPLC was performed with a Hewlett-Packard (Avondale, PA), model 1100 liquid chromatograph with quaternary pumps and a diode array

UV-visible detector. 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 gradient system was: 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 post-run with HPLC-grade water. Anthocyanins were detected at 520 nm.

Cell cultures. Caco-2 human colon cancer cells were cultured in ATCC Medium: Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 80%; fetal bovine serum, 20%. Cancer cells were grown in an incubator with 5% CO_2 and 95% humidity at 37 ^{o}C. Medium was changed 2 to 3 times per week.

Transport/Absorption. Transport experiments were carried out using Transwell inserts (polycarbonate membrane, 0.4 μm pore size, 24 mm diameter, Corning Incorporated, Corning, NY). Inserts were placed in 6-well plates. Caco-2 cells (passage 30-50) were seeded at 1.5 x $10^5/cm^2$ on the membrane inserts with 1.5 ml medium in the apical/luminal side and 2.5 ml medium in the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 20-26 days post seeding by changing the medium three times a week. Transepithelial electrical resistance (TEER) of cells grown in the Transwell was measured using the Millicell-ERS voltmeter (Millipore Co., Billerica, MA). Only monolayers with TEER value higher than 400 Ω cm^2 were used for experiments. TEER values were also obtained after completion of transport experiments.

Medium was removed and the cells were washed with HBSS (pH=7.4, 37^{o}C). Anthocyanin extract solutions in HBSS (approximately 50 μg/mL) were added to the apical side of the cells. Transepithelial transport were followed as a function of time. At 0, 1, and 2 h, the

anthocyanin concentrations at apical and basolateral sides were sampled, cooled in an ice bath, and measured using HPLC. The cellular absorption of anthocyanins was also measured following the procedure below. The cell membranes were washed 3 times with HBSS (pH=7.4, 37^{o}C), and then removed/tore off from the insert. Anthocyanins were extracted using methanol with 5% formic acid. Cells were sonicated for 15 min and centrifuged at 2000 X g. The supernatant was collected, and cells were rinsed 2 more times with acidified methanol, and centrifuged again at 2000 X g. The supernatant solutions were evaporated under nitrogen, and reconstituted in 600 μL of methanol with 5% formic acid following the methodology reported by Boyer et al. (*28*). The extract solutions were then injected into HPLC.

Statistical analysis. The transport and absorption efficiencies of different anthocyanins and different anthocyanin extracts from blueberry cultivars were statistically compared. Statistical analysis was conducted using the General Linear Model (GLM) followed by Duncan's multiple range test at α=0.05.

RESULTS

Highly purified anthocyanin extracts (with ~90% purity of identified anthocyanins) were obtained from three cultivars of blueberries. Seven monoglycoside anthocyanins were identified: Dp-glc, Cy-gal, Cy-glc, Pt-glc, Pn-gal, Pn-glc, and Mv-glc. Their structures are shown in Figure 6.1 in the flavylium cation form. Our previous reports (*15*) have shown additional detail on the anthocyanin extracts. The anthocyanin extracts were dissolved in HBSS, and added to the apical side of the cell monolayers. Transport of anthocyanin fractions across the Caco-2 monolayers was studied in apical to basolateral direction. Figure 6.2 shows the chromatogram of analytical HPLC of Tifblue anthocyanins in the basolateral solutions after a 2 h incubation. Four peaks (a-d) could not be identified because of a lack of standards. Figure 6.3 shows the anthocyanin

concentrations in the apical solutions after incubation with Caco-2 monolayers over time. A drastic decrease was observed in the 7 anthocyanins from all three cultivars. Total anthocyanins refers to the total amount of all seven anthocyanins identified. In Briteblue, the total anthocyanins decreased from 28.7 to 19.0 μg/mL (66% retained) in 1 h, and 13.3 μg/mL (46% retained) in 2 h. In the case of the seven individual Briteblue anthocyanins, the concentration in the apical solution decreased over time. The concentration after 1 h incubation ranged from 49 to 66% of those at 0 h, and the concentration after 2 h incubation ranged from 29 to 50%. Similarly, in Tifblue, the total anthocyanins decreased from 48.2 to 33.0 μg/mL (68% retained) in 1 h, and 24.5 μg/mL (51% retained) in 2 h. The concentrations of seven individual Tifblue anthocyanins after 1 h incubation ranged from 50 to 75% of those at 0 h, and the concentration after 2 h incubation ranged from 35 to 59%. In Powderblue, the total anthocyanins decreased from 41.1 to 24.6 μg/mL (60% retained) in 1 h, and 19.8 μg/mL (48% retained) in 2 h. The concentrations of seven individual Powderblue anthocyanins after 1 h incubation ranged from 44 to 71% of those at 0 h, and the concentration after 2 h incubation ranged from 29 to 60%.

All of the seven major anthocyanins in the extracts were detected in the basolateral solutions on the basis of retention time and characteristic spectra. The anthocyanin concentration in the basolateral solutions after incubation with Caco-2 monolayers over time is shown in Figure 6.4. In general, the concentration of anthocyanins increased with incubation time. In Briteblue, the total amount of identified anthocyanins reached 1.22 μg/mL after 1 h incubation and 1.66 μg/mL after 2 h. Among the seven individual anthocyanins, Pt-glc and Pn-glc showed the highest concentration in the basolateral solutions with 0.50 and 0.41 μg/mL after 2 h incubation, respectively. The lowest concentration (0.06 μg/mL) was observed in Dp-glc. In Tifblue, the total anthocyanins in the basolateral solution reached 1.79 and 2.57 μg/mL after 1 h

and 2 h incubation periods, respectively. As in Briteblue, Pt-glc and Pn-glc showed the highest concentration among the seven anthocyanins with 0.62 and 0.71 μg/mL after 2 h incubation, respectively. Dp-glc was the lowest with concentration at 0.06 μg/mL. For Powderblue, the total anthocyanins in the basolateral solution reached 0.95 and 2.12 μg/mL after 1 h and 2 h incubation period, respectively. Similarly, the highest concentration among the seven anthocyanins was observed in Pt-glc and Pn-glc, and the lowest in Dp-glc.

Because Pt-glc and Pn-glc possessed two of the highest contents of anthocyanins in the apical solutions, the transport of anthocyanins across the cell monolayers could be related to the original concentrations of individual anthocyanins in the apical solution. Therefore, transport efficiency percentages were calculated. Calculation was based on: (anthocyanin concentrations at the basolateral side over time) / (anthocyanin concentrations at the apical side at 0 h) x 100%. Table 6.1 shows the transport efficiency percentages of anthocyanins from the apical to the basolateral side of cell monolayers. The transport efficiency of the total anthocyanins from different cultivars of blueberry extracts was also calculated. Statistical analysis showed no significant difference among the three cultivars in the transport efficiency of total anthocyanins except that Powderblue extract showed significantly lower (1.61%) transport efficiency than the other two cultivars after 1 h incubation. In the case of individual anthocyanins, Dp-glc showed the lowest transport efficiency (ranging from 0.72 - 1.18%) in all three cultivars. In Tifblue and Powderblue, Pn-gal, Pn-glc, and Mv-glc showed the highest transport efficiency after 2 h incubation, with transport efficiency ranging from 4.15 - 4.72%. In Briteblue, Pn-glc and Mv-glc showed the highest transport efficiency with 5.17% and 6.03% after 2 h incubation, respectively. Unlike in Tifblue and Powderblue, the transport efficiency of Pn-gal (4.18%) was not one of the highest.

In Briteblue extract, Cy-glc showed significantly higher transport efficiency (4.58%) than Cy-gal (3.94%); similarly Pn-glc (5.17%) showed significantly higher transport efficiency than Pn-gal (4.18%). The difference is less significant at 1 h than at 2 h incubation (Table 6.1). Similar trends were observed in Tifblue and Powderblue extract. Cy-glc showed significantly higher transport efficiency (4.01 and 4.18%) than Cy-gal (3.29 and 3.28% in Tifblue and Powderblue, respectively). Meanwhile, Pn-glc showed higher transport efficiency than Pn-gal in both Tifblue and Powderblue, yet statistical analysis did not identify the significant difference, probably because of the relatively high standard deviations.

The direct absorption of anthocyanins by Caco-2 cells was also evaluated. The anthocyanins absorbed by cells were extracted and reconstituted in 600 μL of methanol with 5% formic acid. Figure 6.5 shows the anthocyanin concentrations absorbed by cells over time. The concentration of anthocyanins in Caco-2 cells increased with incubation time. In Briteblue, the total amount of identified anthocyanins was 0.06 μg/mL after 1 h incubation and 0.16 μg/mL after 2 h. In Tifblue, the total anthocyanins in the cells were 0.13 and 0.24 μg/mL after 1 h and 2 h incubation, respectively. In Powderblue, the total anthocyanins were 0.12 and 0.22 μg/mL after 1 h and 2 h incubation periods, respectively. Dp-glc was not detected in any of the three treatments. Compared with other anthocyanins, Pt-glc and Pn-glc exhibited the highest contents. As in transport efficiency, absorption efficiency was calculated. The calculation was based on: (anthocyanin concentrations extracted from Caco-2 cells over time) / (anthocyanin concentrations at the apical side at 0 h) x 100%. Table 6.2 shows the absorption efficiency of anthocyanins by Caco-2 cells. As in transport efficiency, Pn-gal, Pn-glc, and Mv-glc showed the highest absorption efficiency among all of the seven anthocyanins. The absorption efficiency after 2 h incubation ranged from 0.26 - 0.28% in Briteblue, 0.21 - 0.23% in Tifblue, and 0.23 -

0.29% in Powderblue. Cy-glc showed significantly higher absorption efficiency than Cy-gal. The absorption efficiency of Cy-glc and Cy-gal after a 2 h incubation were 0.24% versus 0.17% in Briteblue, 0.19% versus 0.13% in Tifblue, and 0.23% versus 0.16% in Powderblue. Although similar trends were observed in Pn-glc and Pn-gal, statistical analysis did not show significant differences.

DISCUSSIONS

It has been reported that Caco-2 cells can undergo spontaneous differentiation in culture conditions and exhibit the characteristics of mature enterocytes (*33*). The cell surface facing the top medium develops a brush borders that resembles the luminal membrane of the intestinal epithelium. The cell surface attaching to the permeable membrane and facing the bottom medium develops into the basolateral membrane. In the current study, we evaluated the absorption of purified blueberry anthocyanin extracts using a Caco-2 cell model. We found that blueberry anthocyanins could be transported through the Caco-2 cell monolayers in intact glycone forms.

It was originally believed that anthocyanins needed to be hydrolyzed to an aglycone form before they can be absorbed. Only recently, several studies have reported the absorption of anthocyanins as intact forms (*18, 19*). Our results showed that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers, although the transport/absorption efficiency was relatively low compared with some aglycone polyphenols. Using a Caco-2 cell model, Steensma et al. (*34*) observed 10-15 μM (~3-4 μg/mL) of genistein and daidzein in the basolateral side, and 10 times lower absorption of the glycosides genistin and daidzin. In the current study, the anthocyanin concentration observed in the basolateral solution ranged from less than 0.1 to 0.7 μg/mL depending on different individual anthocyanins and the original

concentration. In case of transport rates, 30-40% of genistein and daidzein at the apical side was transported to the basolateral side in 6 h (*34*). In our study, the transport efficiency of anthocyanins averaged ~3-4% (less than 1% in Dp-glc). This low absorption rate probably resulted from the poor lipophilic properties of anthocyanins.

The nature of aglycone of the anthocyanin could influence their bioavailability (*17, 20*). Using an animal model, Wu et al. (*17*) reported that Pg-glc had a much higher total urinary excretion than cyanidin-based anthocyanins. McGhie et al. (*20*) also reported the relative concentrations of Dp-based anthocyanins were lower than those of Mv-based anthocyanins in the urine of rats and humans. The authors suggested that this may be a result of the greater number of hydroxyl groups in Dp or the greater hydrophobic nature of Mv that facilitated increased portioning into cells and tissues. This result is in good agreement with our results. In our current study, Dp-glc showed lowest transport/absorption efficiency, and Mv-glc showed highest transport/absorption efficiency.

Free hydroxyl groups in flavonoids can hinder transport in Caco-2 cell monolayers (*35*). Ollila et al. (*36*) stated that polyhydroxylated flavonoids show longer retention delays in membranes and this is most likely due to hydrogen bond formation between the flavonoids hydroxyl groups and polar groups of the lipid molecules at the lipid/water interface. In the current study, Dp-glc (which has 6 hydroxyl groups in Dp) showed the lowest transport efficiency in all three cultivars. In Tifblue and Powderblue, Pn-gal, Pn-glc, and Mv-glc showed the highest transport efficiency. Both Pn and Mv have 4 hydroxyl groups, the lowest among all the anthocyanins evaluated in the current study. In addition, Dp has no OCH_3 group, while Pn has 1 and Mv has 2 OCH_3 group. Our results provided further evidence that aglycone structure,

especially hydrophilic and hydrophobic groups are important for the bioavailability of anthocyanins.

In the current study, Cy-glc showed significantly higher transport efficiency than Cy-gal, and Pn-glc showed significantly higher transport efficiency than Pn-gal. In terms of absorption efficiency, Cy-glc showed significantly higher efficiency than Cy-gal, yet, no significant differences were found between Pn-glc and Pn-gal. Sugar moieties may influence the absorption of anthocyanins although the mechanisms are far from clear. Higher absorption percentage of anthocyanin glucosides in rabbit urine was observed than anthocyanin rutinosides after 2 h, and this trend turned into an opposite effect after 10-24 h (*37*). The authors suggested the possible reason could be that the anthocyanin glucosides in plasma increase and decrease more quickly than anthocyanin rutinosides. Some studies reported that monoglucosides of flavonoids/quercetin can be transported across the apical membrane of enterocytes by the Sodium Dependent Glucose Transporter SGLT1 (*38, 39*). Mailleau et al. (*40*) reported that SGLT1 activities rapidly increased from day 12 up to day 20 post seeding of Caco-2 cells. In addition, quercetin-glc uptake in Caco-2 cells was inhibited by adding glucose (*38*). Milbury et al. (*41*) have suggested that the absorption of anthocyanins in their unchanged glycosylated forms may indicate the involvement of the glucose transport receptors, since quercetin and anthocyanidins (aglycones of anthocyanins) share a similar basic flavonoid structure. The reason that no significant differences were found between Pn-glc and Pn-gal in the absorption efficiency could be that they already reached maximum absorption capacity.

Anthocyanins can undergo reversible structural transformations and dramatic changes in color with changes in pH. More importantly, anthocyanins are unstable at pH 7.0. Kang et al. (*10*) reported that anthocyanins and cyanidin could spontaneously degrade to chalcone and

benzoic acid derivatives at pH 7.0 and 37 ^{o}C. The fast decrease of anthocyanin in the apical solution and relatively low concentrations in the basolateral side could also be due to the degradation that may occur under the culture conditions. Our experiment showed that more than 30% loss could occur at 37 ^{o}C at neutral pH (data not shown). This could be another reason why low absorption and excretion of anthocyanins were observed compared with other flavonoids (*18*). It is worthy to note that not only anthocyanins in their original form, but anthocyanin degradation products/metabolites may also be the active forms responsible for their bioactivities (*9*). Scalbert et al. (*42*) suggested that the active compounds may not be the native polyphenols found in food, which are most often tested in in vitro studies; they are more likely to be metabolites. Although there have been recently some reports about metabolites of anthocyanins, the metabolism process is too complicated and far from clear (*3, 17, 18*). Further studies are essential in this area.

In the current study, we used HBSS at pH 7.4, since this is the widely accepted buffered pH in Caco-2 monolayer (*43*). Although the pH in the cellular interstice and blood is ~7.4, the pH in the upper GI tract under fasting conditions ranges from 5.0 to 6.5, and the acidic microclimate operating just above the epithelial cell layer has been reported to be 5.8-6.3 (*44*). Modification of this typical method such as using pH-gradient condition will be helpful. This is especially important for anthocyanins studies since their stability is low under neutral and high pH.

A Caco-2 cell model can only serve as a one-way screen such that compounds with high permeability in this model are typically well absorbed in vivo, yet it cannot be assumed that compounds with low permeability are poorly absorbed in vivo (*21*). Using a Caco-2 cell model,

we have shown that anthocyanins can be transported across cell monolayers. However, whether there are other transport mechanisms involved will require further studies.

In conclusion, the results of this study demonstrated that anthocyanins from blueberries can be transported through Caco-2 cell monolayers, although the transport/absorption efficiency was relatively low compared with some aglycone polyphenols. No significant difference in transport/absorption efficiency was observed among the three blueberry cultivars. The transport efficiency of anthocyanins averaged ~3-4% (less than 1% in Dp-glc). This low absorption rate probably resulted from the poor lipophilic properties of anthocyanins. The observed trend among different anthocyanins is in general agreement with some existing in vivo reports. Dp-glc showed the lowest transport/absorption efficiency and Mv-glc showed the highest transport/absorption efficiency. We proposed that more free hydroxyl groups and less OCH_3 group in anthocyanins may decrease their bioavailability in vivo. In addition, Cy-glc showed significantly higher transport efficiency than Cy-gal, and Pn-glc showed significantly higher transport efficiency than Pn-gal, indicating that glucose-based anthocyanins have higher bioavailability than galactose-based anthocyanins. The Caco-2 cell monolayer is a good model system to investigate the impact of molecular structure on bioavailability of anthocyanins, especially when some parameters are difficult to control under in vivo conditions.

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Table 6.1. Transport efficiency of anthocyanins from apical to basolateral side of Caco-2 cell monolayer [a].

Anthocyanins	Briteblue		Tifblue		Powderblue	
	1 h	2 h	1 h	2 h	1 h	2 h
Dp-glc	1.07 a [c]	0.89 a	1.18 a	1.07 a	0.72 a	0.92 a
Cy-gal	3.10 b	3.94 b	2.39 b	3.29 b	1.54 b	3.28 b
Cy-glc	3.28 bc	4.58 c	2.77 bc	4.01 c	1.93 c	4.18 c
Pt-glc	3.44 bc	4.44 bc	2.63 bc	3.67 bc	1.86 bc	3.93 c
Pn-gal	3.01 b	4.18 bc	2.82 bc	4.15 cd	1.74 bc	4.21 c
Pn-glc	3.47 bc	5.17 d	2.96 c	4.54 d	1.89 c	4.41 cd
Mv-glc	3.74 c	6.03 e	2.94 c	4.56 d	2.01 c	4.72 d
Total anthocyanins [b]	3.10 B [d]	4.20 C	2.60 B	3.73 C	1.61 A	3.61 C

[a] Transport efficiency percentages was calculated based on: (anthocyanin concentrations at the basolateral side over time) / (anthocyanin concentrations at the apical side at 0 h) x 100%. Values are averages of triplicates.

[b] Total anthocyanins refers to the total amount of all seven anthocyanins identified.

[c] Means within the same column followed by the same lower case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

[d] Means within the same row followed by the same upper case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

Table 6.2. Absorption efficiency of anthocyanins by Caco-2 cells [a].

Anthocyanins	Briteblue		Tifblue		Powderblue	
	1 h	2 h	1 h	2 h	1 h	2 h
Dp-glc	0 a [c]	0 a	0 a	0 a	0 a	0 a
Cy-gal	0 a	0.17 b	0.07 b	0.13 b	0.08 b	0.16 b
Cy-glc	0 a	0.24 c	0.11 cd	0.19 cd	0.10 c	0.23 cd
Pt-glc	0.11 b	0.23 c	0.09 c	0.18 c	0.11 c	0.20 c
Pn-gal	0 a	0.26 cd	0.12 d	0.21 d	0.13 d	0.23 cd
Pn-glc	0.13 c	0.27 cd	0.11 cd	0.22 d	0.14 d	0.25 d
Mv-glc	0.14 d	0.28 d	0.13 e	0.23 d	0.16 e	0.29 e
Total anthocyanins [b]	0.07 A [d]	0.20 B	0.09 A	0.17 B	0.10 A	0.19 B

[a] Absorption efficiency was calculated based on: (anthocyanin concentrations extracted from Caco-2 cells over time) / (anthocyanin concentrations at the apical side at 0 h) x 100%. Values are averages of triplicates.

[b] Total anthocyanins means the total amount of all seven anthocyanins identified.

[c] Means within the same column followed by the same lower case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

[d] Means within the same row followed by the same upper case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

Figure 6.1. The anthocyanin structure. The anthocyanin shown here is in the flavylium cation form.

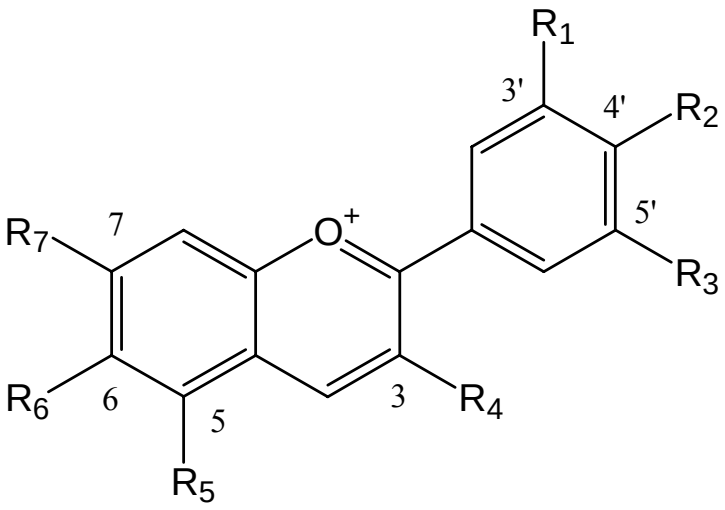
Figure 6.2. The chromatogram of analytical HPLC of Tifblue berry anthocyanins in the basolateral solutions after 2 h incubation. A. Anthocyanin standards; B. Tifblue berry anthocyanins in the basolateral solutions. 1) Dp-glc, 2) Cy-gal, 3) Cy-glc, 4) Pt-glc, 5) Pn-gal, 6) Pn-glc, 7) Mv-glc, 8) Pn-ara. a) – d) unidentified peaks.

Figure 6.3. The anthocyanin concentrations at the apical side of cell monolayer over time (mean ± SD, n = 3). Total means the total amount of all seven anthocyanins identified. A, Briteblue; B, Tifblue; C, Powderblue.

Figure 6.4. The anthocyanin concentrations at the basolateral side of cell monolayer over time (mean ± SD, n = 3). Total means the total amount of all seven anthocyanins identified. A, Briteblue; B, Tifblue; C, Powderblue.

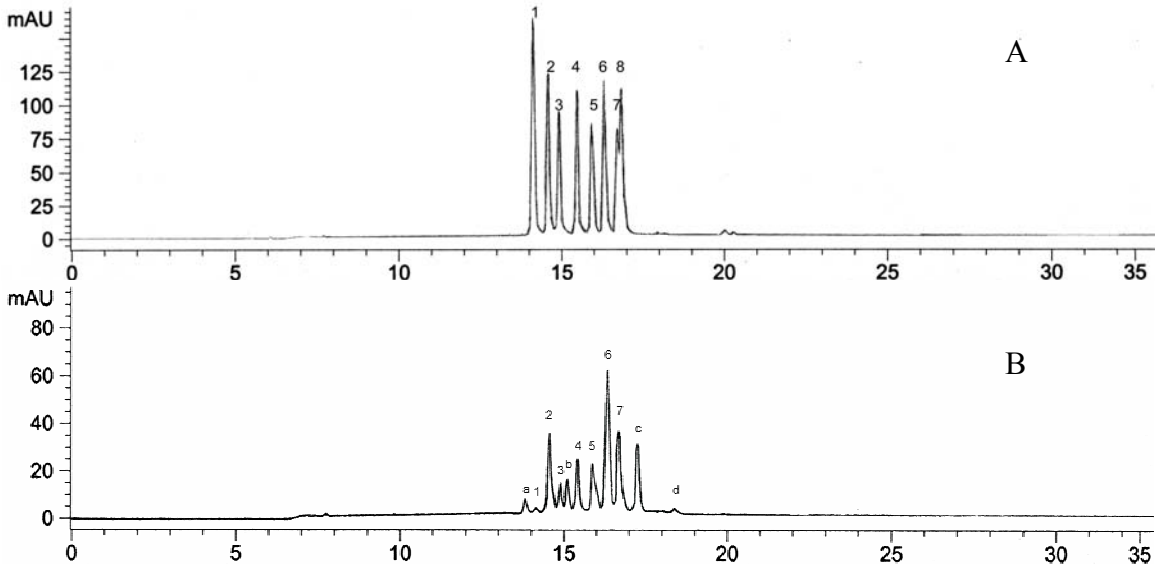
Figure 6.5. The anthocyanin concentrations absorbed by Caco-2 cells over time (mean ± SD, n = 3). Total means the total amount of all seven anthocyanins identified. A, Briteblue; B, Tifblue; C, Powderblue.

Figure 6.1.



Anthocyanin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Dp-glc	-OH	-OH	-OH	-O-glc	-OH	-H	-OH
Cy-gal	-OH	-OH	-H	-O-gal	-OH	-H	-OH
Cy-glc	-OH	-OH	-H	-O-glc	-OH	-H	-OH
Pt-glc	$-OCH_3$	-OH	-OH	-O-glc	-OH	-H	-OH
Pn-gal	$-OCH_3$	-OH	-H	-O-gal	-OH	-H	-OH
Pn-glc	$-OCH_3$	-OH	-H	-O-glc	-OH	-H	-OH
Mv-glc	$-OCH_3$	-OH	$-OCH_3$	-O-glc	-OH	-H	-OH

Figure 6.2.


mAU
125
100
75
50
25
0
1
2
3
4
5
6
7
8
A
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30
35
mAU
80
60
40
20
0
a
1
2
3
b
4
5
6
7
c
d
B
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5
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25
30
35

Figure 6.3.

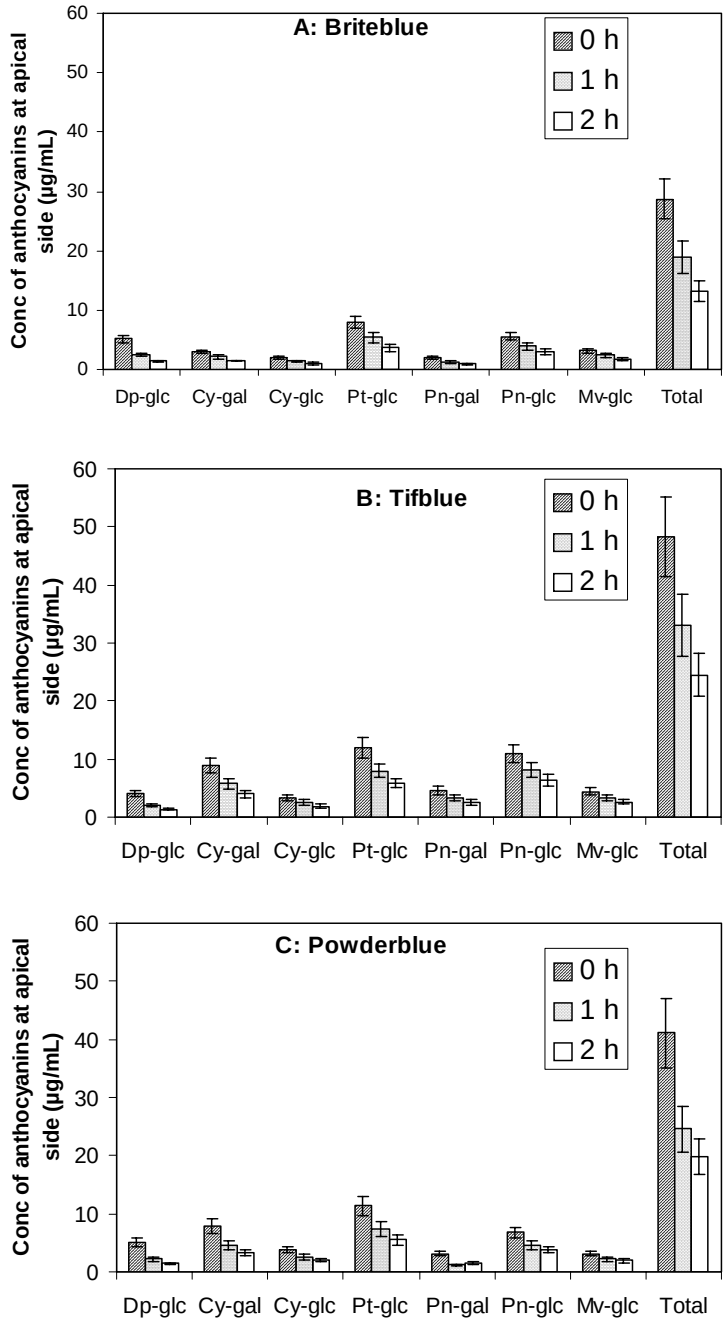

A: Briteblue
B: Tifblue
C: Powderblue
0 h
1 h
2 h
Conc of anthocyanins at apical side (µg/mL)
0
10
20
30
40
50
60
Dp-glc
Cy-gal
Cy-glc
Pt-glc
Pn-gal
Pn-glc
Mv-glc
Total

Figure 6.4.

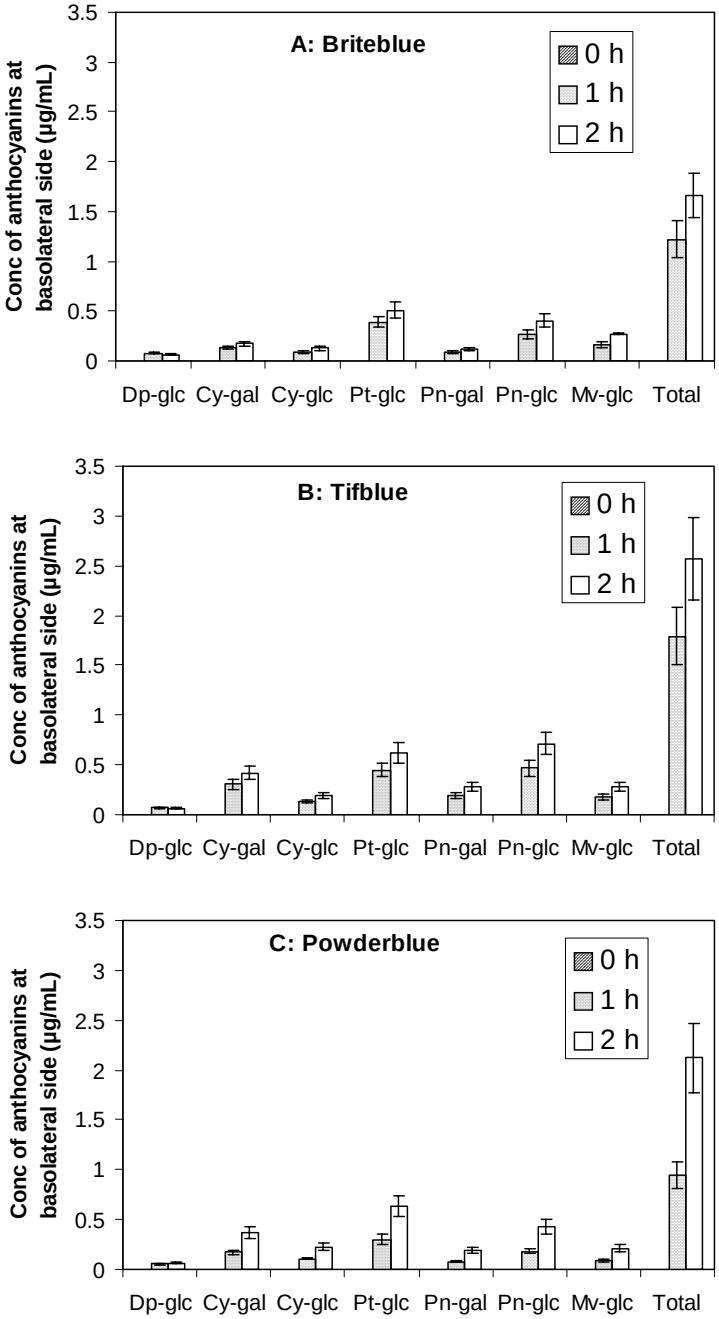
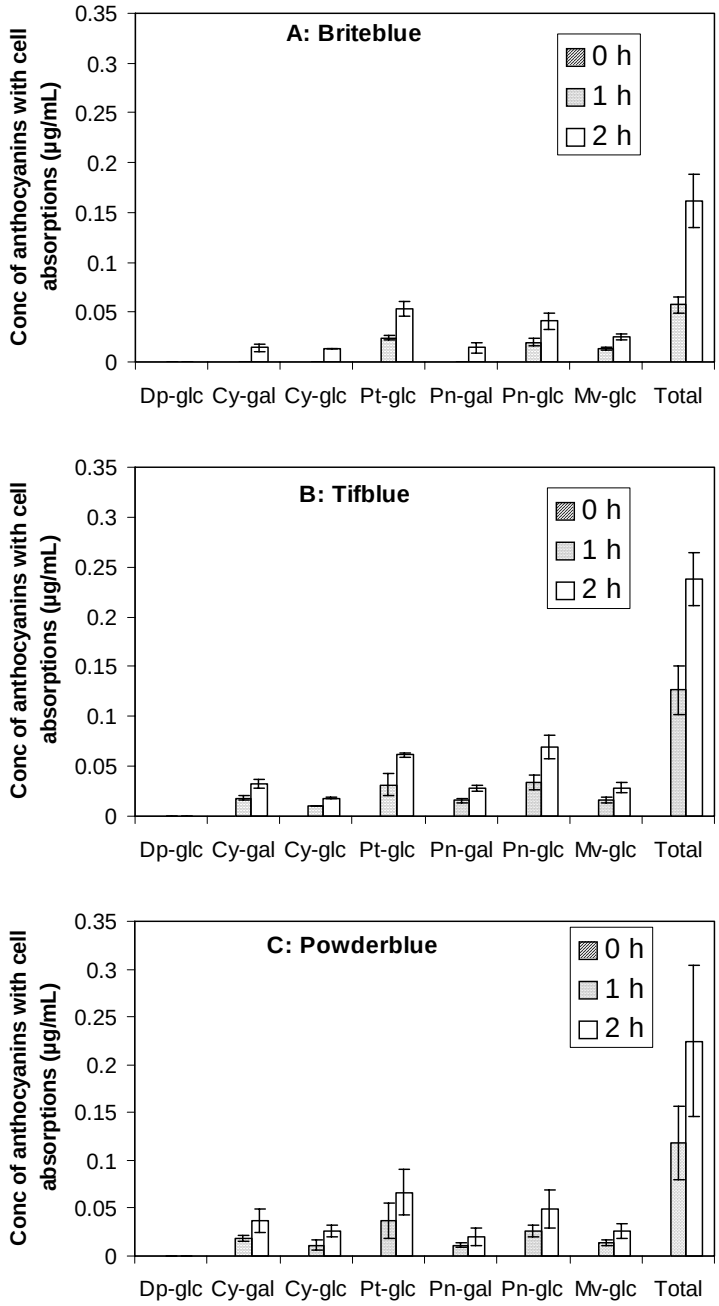

A: Briteblue
B: Tifblue
C: Powderblue
Conc of anthocyanins at basolateral side (µg/mL)
0 h
1 h
2 h
Dp-glc
Cy-gal
Cy-glc
Pt-glc
Pn-gal
Pn-glc
Mv-glc
Total
0
0.5
1
1.5
2
2.5
3
3.5

Figure 6.5.


A: Briteblue
B: Tifblue
C: Powderblue
Conc of anthocyanins with cell absorptions (µg/mL)
0 h
1 h
2 h
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Dp-glc
Cy-gal
Cy-glc
Pt-glc
Pn-gal
Pn-glc
Mv-glc
Total

CHAPTER 7

SUMMARY AND CONCLUSIONS

Polyphenols in three blueberry cultivars (Briteblue, Tifblue and Powderblue), and four cultivars of muscadine (Carlos, Ison, Noble, and Supreme) were extracted and freeze dried. The extracts were further separated into phenolic acids, tannins, flavonols, and anthocyanins using a HLB cartridge and LH20 column. Some individual phenolic acids and flavonoids were identified by HPLC with more than 90% purity in anthocyanin fractions.

In blueberries, flavonol and tannin fractions resulted in a 50% inhibition of cell growth at concentrations of 70-100 and 50-100 μg/mL in HT-29 and Caco-2 cells, respectively. The phenolic acid fractions showed relatively lower bioactivities with 50% inhibition at ~1000 μg/mL. The greatest inhibitory effect among all four fractions was from the anthocyanin fractions. Both HT-29 and Caco-2 cell growth were significantly inhibited more than 50% by the anthocyanin fractions at concentrations of 15-50 μg/mL. DNA fragmentation was measured by using a Cell Death Detection ELISA kit to assess the induction of apoptosis. Anthocyanin fractions resulted in 2-7 times increase in DNA fragmentation, indicating the induction of apoptosis.

A similar trend was found in muscadine grapes. The phenolic acid fractions showed a 50% inhibition at the level of 0.5–3 mg/mL to colon cancer cell growth in HT-29 and Caco-2 cells. 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 a 2–4 times increase in DNA fragmentation, indicating the induction of apoptosis.

In liver cancer cell model, the phenolic acid fractions of muscadine grapes and blueberries showed a 50% inhibition of HepG2 cell population growth at the level of 1-2 mg/mL. The greatest inhibitory effects were observed from the anthocyanin fractions with 50% inhibitions of cancer cell population growth at concentrations of 70-150 and 100-300 μg/mL in blueberries and muscadine grapes, respectively. The flavonol and tannin fractions showed intermediate activities. In addition, DNA fragmentation was measured to assess the induction of apoptosis. The anthocyanin fraction resulted in a 2 to 4 fold increase in DNA fragmentation compared to control in both muscadine grapes and blueberries.

In the anthocyanin bioavailability study, the results demonstrated that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers although the transport/absorption efficiency was relatively low compared with some aglycone polyphenols. The transport efficiency of anthocyanins averaged ~3-4% (less than 1% in Dp-glc). This low absorption rate probably resulted from the poor lipophilic properties of anthocyanins. The observed trend among different anthocyanins is in general agreement with some existing in vivo reports. Dp-glc showed lowest transport/absorption efficiency and Mv-glc showed highest transport/absorption efficiency. We proposed that more free hydroxyl groups and less OCH_3 group in anthocyanins may have decreased their bioavailability in vivo. In addition, Cy-glc showed significantly higher transport efficiency than Cy-gal, and Pn-glc showed significantly higher transport efficiency than Pn-gal, indicating glucose-based anthocyanins have higher bioavailability than galactose-based anthocyanins. Caco-2 cell monolayer is a good model system to investigate the impact of molecular structure on bioavailability of anthocyanins, especially when some parameters are difficult to control under in vivo conditions.

In conclusion, our study found that phenolic compounds in rabbiteye blueberries and muscadine grapes could inhibit colon and liver cancer cell growth and induce cancer cell apoptosis. Anthocyanins from blueberries could be absorbed and transported through human intestinal models. Further studies are required to clarify the mechanisms and to evaluate the bioavailability and metabolism of phenolic compounds in blueberries and muscadine grapes under both in vitro and in vivo conditions. Large scale human clinical trial studies are essential before it can be determined if intake of blueberries/muscadine grapes can reduce cancer risk.