JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Induction of Apoptosis in Cancer Cells by Bilberry (*Vaccinium myrtillus*) and the Anthocyanins

NAOMI KATSUBE,† KEIKO IWASHITA,‡ TOJIRO TSUSHIDA,‡ KOJI YAMAKI,‡ AND MASUKO KOBORI*,‡

Fruit Processing Research Center, AOHATA Corporation, Takehara, Hiroshima 729-2392, Japan, and National Food Research Institute, Tsukuba, Ibaraki 305-8642, Japan

Among ethanol extracts of 10 edible berries, bilberry extract was found to be the most effective at inhibiting the growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells in vitro. Bilberry extract induced apoptotic cell bodies and nucleosomal DNA fragmentation in HL60 cells. The proportion of apoptotic cells induced by bilberry extract in HCT116 was much lower than that in HL60 cells, and DNA fragmentation was not induced in the former. Of the extracts tested, that from bilberry contained the largest amounts of phenolic compounds, including anthocyanins, and showed the greatest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Pure delphinidin and malvidin, like the glycosides isolated from the bilberry extract, induced apoptosis in HL60 cells. These results indicate that the bilberry extract and the anthocyanins, bearing delphinidin or malvidin as the aglycon, inhibit the growth of HL60 cells through the induction of apoptosis. Only pure delphinidin and the glycoside isolated from the bilberry extract, but not malvidin and the glycoside, inhibited the growth of HCT116 cells.

KEYWORDS: Berries; bilberry; anthocyanins; apoptosis; HL60 human leukemia cells; HCT116 human colon carcinoma cells

INTRODUCTION

Berries are rich sources of the naturally occurring phenolic pigments, the anthocyanins. Anthocyanins and other phenolic compounds possess antioxidant activity, which is considered to be an important physiological function (*1*−*5*). Antioxidants prevent the formation of highly reactive lipid peroxidation products and reduce the deleterious effects of reactive oxygen species (*6*). Dietary intake of natural phenolic antioxidants has been suggested to contribute to the prevention of heart disease and cancer (*7*, *8*). Among fruits and vegetables, berry extracts have relatively high antioxidant activity, which is well correlated with their content of anthocyanin and total phenolic compounds (*9*−*11*).

Anthocyanins not only possess antioxidant activity but also mediate other physiological functions related to cancer suppression (*5*, *12*−*15*). 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 flavanons (*12*). 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 (*13*). Extracts of berries of the *Vaccinium* species inhibit the induction of ornithine decarboxylase activity by the tumor promoter phorbol 12-myristate 13-acetate (TPA) (*16*). Thus, berries and the anthocyanins contained by them are thought to be promising functional foods for reducing risks of cancer and other diseases.

Apoptosis is one of the major mechanisms of cancer suppression (*17*, *18*). The flavonoids, such as quercetin, apigenin, and phloretin, inhibit cancer cell growth through the induction of apoptosis (*19*−*22*). However, the apoptosis-inducing properties of anthocyanins in cancer cells have not yet been reported. Here, we investigated the growth inhibitory effects of 10 edible berry extracts on two different cancer cell lines. Bilberry extract was found to be most effective for inhibiting HL60 human leukemia and HCT116 human colon carcinoma cells in vitro. Bilberry extracts and anthocyanins purified from them induced apoptosis in HL60 cells but little in HCT116 cells. These results suggest that the growth inhibitory and apoptosis-inducing effects of bilberry and the anthocyanins in cancer cells are crucial pathways for cancer prevention.

MATERIALS AND METHODS

Materials. Lowbush blueberry (*Vaccinium angustifolium*), highbush blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium oxycoccos*), raspberry (*Rubus idaeus*), and strawberry (*Fragaria* x *ananassa*) were purchased from Nishimoto Trading Co. Ltd. (Kobe, Japan). Black currant (*Ribes nigrum*) and red currant (*Ribes sativum*) were obtained from Toyota Tsusho Co. (Nagoya, Japan). Blackberry (*Rubus mesogaeus*) was obtained from Q&B Foods Inc. (Irwindale, CA) Bilberry (*Vaccinium myrtillus*) and cowberry (*Vaccinium vitis-idaea*) were

* To whom correspondence should be addressed. Tel.: +81-298-38-8055. Fax: +81-298-38-7996. E-mail: kobori@nfri.affrc.go.jp.
† AOHATA Corp.
‡ National Food Research Institute.

10.1021/jf025781x CCC: $25.00
Published on Web 11/26/2002

purchased from Japan Agricultural Products Marketing Inc. (Tokyo, Japan) and Kawasho Co. (Tokyo, Japan), respectively. Each type of berry was transported frozen and stored at −20 °C until used for experiments. Anthocyanidins (delphinidin, malvidin, peonidin, cyanidin, and pelargonidin) and flavonols (myricetin, quercetin, and kaempferol) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Preparation of Ethanol Extracts of Berries. Frozen berries (100 g) were homogenized in 400 mL of ethanol and centrifuged at 10000*g* for 15 min. The supernatants were filtered through No. 5B filter paper (Kiriyama, Tokyo, Japan) and concentrated by rotary evaporation at 37 °C. The extracts were then lyophilized and tested as berry ethanol extracts. The amount of berry extract is given as dry weight.

Cells and Cell Culture. HL60 human promyelocytic leukemia cells (JCRB0085) were provided by the Human Science Research Resources Bank (Osaka, Japan). HCT116 human colon carcinoma cells (ATCC CCL247) were purchased from Dainippon Pharmaceutical Corp. (Osaka, Japan). HL60 and HCT116 cells were maintained in PRMI1640 medium (Invitrogen Corp., Carlsbad, CA) and McCoy's 5A medium (Invitrogen), respectively. Cells were cultured at 37 °C in 5% CO_2 in air in medium supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Inc., Aurora, OH).

Treatment of Cells with Berry Extracts, Fractions, or Phenolic Components. HL60 cells were plated in medium at a density of 1×10^5 cells/mL in 24-well plates (Falcon 353047, Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). Each of the samples (berry extracts, bilberry fractions, and the phenolic components) was dissolved in 50% ethanol and added to the medium in each well. The concentrations of the berry extracts and bilberry fractions are given as milligrams dry weight per milliliter of medium. Cells were then grown for 6, 24, or 48 h in the absence or in the presence of test sample. HCT116 cells were plated in medium at a density of 5×10^4 cells/mL in 24-well plates and allowed to adhere for 24 h. The medium was then changed to fresh medium containing the test sample, and the cells were further cultured for 24 or 48 h. Thereafter, the cells were harvested, and the viable cell number was counted or induction of apoptosis was assessed. Viable cells were counted in a hemocytometer by trypan blue exclusion, and the viable cell number is expressed as arithmetic means ± standard deviation (SD) of triplicate determinations, as shown in the figures.

Determination of Nuclear Morphology. Cells were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan), and the nuclei were stained with 1 mM bisbenzimide (Hoechst) 33258 (Wako Pure Chemical Industries, Osaka, Japan) in PBS. The stained cells were observed under an incident-light fluorescence microscope (Olympus AX70 plus BX-FLA, Olympus Optical Co., Ltd., Tokyo, Japan).

DNA Extraction and Agarose Gel Electrophoresis. Cells (approximately 4×10^5 cells) were lysed in 20 μL of lysis buffer [50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS], incubated with 0.5 mg/mL RNase (Sigma Chemical Co., St. Louis, MO) at 50 °C for 30 min, and then incubated with 0.5 mg/mL Proteinase K (Sigma Chemical) at 50 °C for 60 min. The samples were electrophoresed in Tris−borate buffer (pH 8.0) on a 2% agarose gel, and the DNA was stained with ethidium bromide (*23*).

Measurement of Total Phenolic Compounds and Anthocyanin Contents. Total phenolic compounds in berry ethanol extract were determined by the Folin−Ciocalteu method (*24*) and expressed as milligrams of D-catechin equivalents per gram of extract. Anthocyanins in berry ethanol extract were analyzed by the HPLC method of Ando et al. (*25*). HPLC (Waters Millennium PDA software, Waters Co., Milford, MA) was performed on a LiChroCART column (Lichrospher100 RP18, 250 mm × 4.6 mm i.d., 5 μm, Merk, Darmstadt, Germany) at a column temperature of 40 °C. Conditions for the mobile phase were as follows: solvent A, 1.5% H_3PO_4/H_2O (v/v); solvent B, 1.5% H_3PO_4/20% AcOH/50% CH_3CN/28.5% H_2O (v/v/v/v); gradient, %B initial to 10 min (10%), 30 min (60%); flow rate, 1 mL/min. Anthocyanins were detected at 530 and 280 nm using a diode array detector. Total anthocyanin content in berry ethanol extract was calculated from the peak area at 530 nm absorbance and expressed as milligrams of cyanidin equivalent per gram of extract.

Measurement of DPPH Radical Scavenging Activity (*26*). Each berry ethanol extract was dissolved in 80% ethanol at a concentration of 100 mg dry wt/mL. The extract was then further diluted to 1:100 or 1:50 with 80% ethanol. Three hundred microliters of the diluted extract was added to 900 μL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution [133 μM DPPH (Nakarai Tesque, Co., Kyoto, Japan), 67 μM MES buffer (pH6.0), 6.7% ethanol], mixed well, and left to stand for 20 min, after which the absorbance of the resulting solution was measured at 520 nm. The DPPH radical scavenging activity was compared with that of Trolox (Sigma-Aldrich, Inc.,) (*27*).

Fractionation of Anthocyanin and Other Phenolic Fractions from Bilberry Extract by OASIS HLB Column Chromatography. An OASIS HLB column (gel volume 20 mL, Waters Co.) was washed with ethanol and pre-equilibrated with distilled water. The bilberry ethanol extract (196 mg dry wt) was applied to the column. The column was then washed with distilled water (0% methanol) and eluted with 20, 40, 60, 80, and 100% methanol. Eluate was collected in 5-mL aliquots in glass tubes, and the phenolic components were identified at 530 and 280 nm by a spectrophotometer. The small fractions were then combined into six fractions, that is, 0, 20, 40, 60, 80, and 100% methanol-eluted fractions. The fractions were dried by a vacuum centrifugal concentrator (Taitec, Tokyo, Japan). The yields of the fractions were 17.0 (0%), 1.7 (20%), 8.7 (40%), 2.0 (60%), 1.5 (80%), and 2.9 mg (100%), respectively. The fractions were dissolved in 50% methanol for testing their growth inhibitory effects on cancer cells.

Fractionation of Anthocyanins from Bilberry Extract. The bilberry ethanol extract (5 g) was applied to a YMC ODS gel A120−150 column (450 mm × 20 mm i.d., YMC Co., Ltd., Kyoto, Japan) and washed with distilled water. The anthocyanins and other phenolic components were eluted with 80% methanol and dried by a vacuum centrifugal concentrator. The eluted fraction was dissolved in 80% methanol at a concentration of 50 μg/μL. The eluted fraction (30 μL) was then applied to an HPLC column (Inertsil ODS-3 column, 250 mm × 20 mm i.d., GL Sciences Inc., Tokyo, Japan) at a column temperature of 40 °C and eluted under the following conditions: solvent A, 10% formic acid/H_2O (v/v); solvent B, 10% formic acid/25% CH_3CN/65% H_2O (v/v/v/v); gradient, %B initial to 10 min (20%), 30 min (40%), 60 min (85%); flow rate, 7 mL/min. The eluate was collected for 20 s (2.3 mL) per fraction in glass tubes. Anthocyanins and other phenolic components were detected at 520 and 280 nm using a diode array detector (SPD-M10AVP). The small fractions were combined into 16 fractions, as shown in **Figure 8A**. The fractions were then concentrated by a vacuum centrifugal concentrator. The concentrated fractions were applied to a Sep-Pak C18 column (Waters Co.), washed with distilled water, and eluted with 100% methanol. The fractions were then dried by a vacuum centrifugal concentrator. The HPLC was performed 32 times under the same conditions. The total yields of the fractions were 9.3 mg (fraction 1), 11.1 mg (fraction 2), 9.3 mg (fraction 3), 7.7 mg (fraction 4), 8.2 mg (fraction 5), 18.7 mg (fraction 6), 7.3 mg (fraction 7), 5.3 mg (fraction 8), 4.7 mg (fraction 9), 4.2 mg (fraction 10), 3.7 mg (fraction 11), 5.1 ng (fraction 12), 5.2 mg (fraction 13), 2.4 mg (fraction 14), 3.5 mg (fraction 15), and 4.3 mg (fraction 16), respectively. The anthocyanins were identified by comparing the HPLC elution pattern at 530 nm with that previously reported (*28*) and with LC−MS spectra.

Fractions 3 and 5 were dried by a vacuum centrifuge evaporator and dissolved in 5 mL of distilled water, and 3 mL of concentrated HCl was added. The fractions were then concentrated again by the vacuum centrifuge evaporator. During the concentration, delphinidin-galactoside (fraction 3) and delphinidin-glucoside (fraction 5) were recrystallized from HCl−H_2O. The purity of the isolated delphinidin-galactoside and delphinidin-glucoside was more than 99% by HPLC.

LC−MS Analysis of Anthocyanins in Bilberry Extract. The analysis was performed on a Hitachi M-1200AP LC−MS system (Hitachi, Tokyo, Japan) using a YMC-PacK ODS-A column (250 mm × 4.6 mm i.d., YMC Co., Ltd.). HPLC conditions were as follows: solvent A, 1% formic acid/H_2O (v/v); solvent B, 1% formic acid/25% CH_3CN/74% H_2O (v/v/v); gradient, %B initial to 10 min (10%), 30 min (60%); column temperature, 40 °C; flow rate, 1 mL/min. Anthocyanins and other phenolic components were detected at 280 nm using a UV detector (L-7400, Hitachi). MS parameters were as

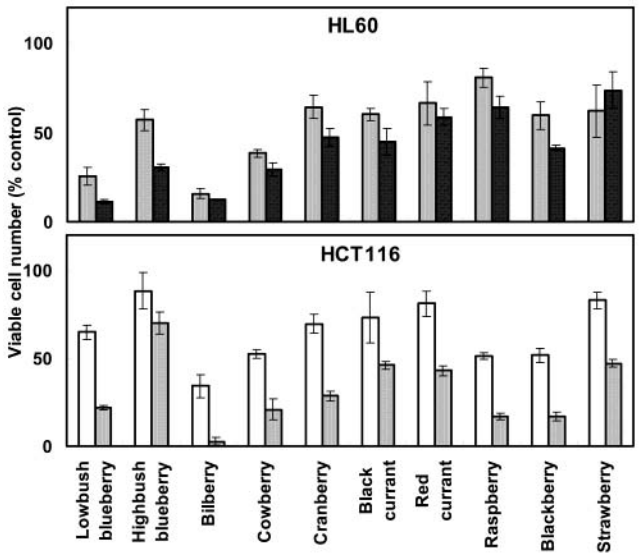


Figure 1. Effects of berry ethanol extracts on the growth of HL60 and HCT 116 cells. HL60 cells were incubated with 4 or 6 mg/mL berry ethanol extract for 24 h. HCT 116 cells were incubated with 2 or 4 mg/mL berry extract for 48 h. Each value is the mean ± SD of triplicate cultures. White bars, 2 mg/mL; gray bars, 4 mg/mL; black bars, 6 mg/mL.

follows: ionization mode, APC+; nebulizer temperature, 200 °C; desolvator, 400 °C; scan rate, 192.17 amu/s; *m*/*z* acquisition from 200 to 800.

RESULTS

Growth Inhibitory Effects of Berry Ethanol Extracts on Cancer Cells. Berry ethanol extracts were added to the culture medium of HL60 human leukemia cells and incubated for 24 h. The berry extracts inhibited the growth of HL60 cells at a concentration of 4 or 6 mg dry wt/mL (**Figure 1**). HCT116 human colon carcinoma cells were preincubated for 24 h to allow adhesion to the plate. The berry extracts were then added to the medium and the cells incubated for 48 h. The berry extracts inhibited the growth of HCT116 cells at a concentration of 2 or 4 mg dry wt/mL (**Figure 1**). Most of the berry extracts inhibited the HCT116 cells completely at a concentration 6 mg/mL after 48 h of incubation (data not shown). Bilberry extract was the most effective among the 10 ethanol extracts at inhibiting the growth of these cancer cell lines. The bilberry extract decreased the number of viable HL60 cells by 84 and 88% at a concentration of 4 and 6 mg/mL, respectively, and the number of viable HCT116 cells by 66% and 97% at 2 and 4 mg/mL, respectively (**Figure 1**). The cancer cell lines were then incubated with 0.5, 1, 2, and 4 mg/mL bilberry extract for 24 and 48 h. HL60 cell growth was inhibited over this range of concentrations following 24 and 48 h of incubation (**Figure 2**). In contrast, the growth of HCT116 cells was not inhibited by 0.5−2 mg/mL bilberry extract after 24 h, but when the incubation time was extended to 48 h, inhibition was observed at 1−4 mg/mL of the bilberry extract (**Figure 2**).

Apoptosis-Inducing Effect of Bilberry Ethanol Extract on Cancer Cells. After 6 h of incubation with 4 mg/mL bilberry extract, apoptotic cell bodies and nuclear fragmentation were observed in HL60 cells (**Figure 3**). The bilberry extract induced nucleosomal DNA fragmentation typical of apoptosis in these cells in a dose-dependent manner after 24 h of treatment (**Figure 4**). These results document that the bilberry ethanol extract induced apoptosis in HL60 cells. Apoptotic cells with fragmented nuclei were also observed in HCT116 cells treated with

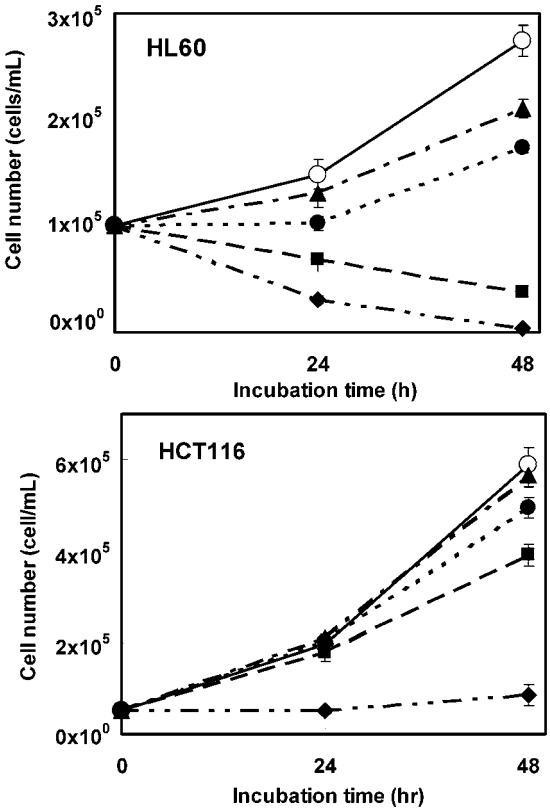


Figure 2. Effect of bilberry extract on the growth of HL60 and HCT116 cells. Cells were incubated with the bilberry extract for 24 and 48 h. Each value is the mean ± SD of triplicate cultures. ○, None; ▲, 0.5 mg/mL; ●, 1.0 mg/mL; ■, 2.0 mg/mL; ◆, 4.0 mg/mL.

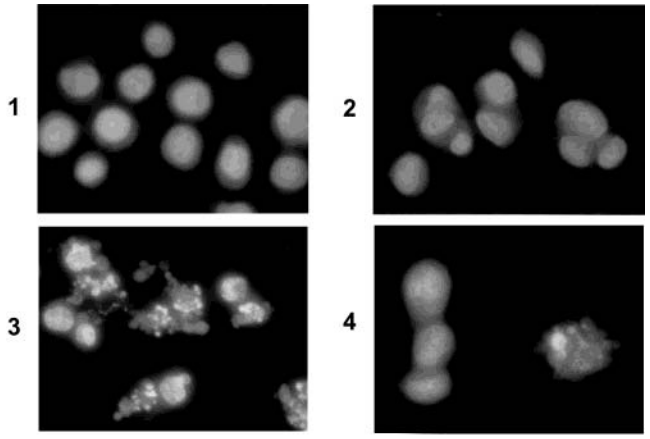


Figure 3. Nuclear morphology of HL60 and HCT116 cells. HL60 and HCT116 cells were treated with or without 4 mg/mL bilberry extract and then stained with Hoechst 33258. 1, HL60 cells; 2, HCT116 cells; 3, HL60 cells treated with 4 mg/mL bilberry extract for 6 h; 4, HCT116 cells treated with 4 mg/mL bilberry extract for 24 h.

the 4 mg/mL bilberry extract, but the proportion of apoptotic cells was much lower than that in HL60 (**Figure 3**). In the HCT116 cells, the nucleosomal DNA fragmentation was not observed on agarose gel electrophoresis after treatment with bilberry extract for 48 h. Thus, bilberry extract induced significant levels of apoptosis in HL60 cells but not in HCT116 cells.

Growth Inhibitory Effects of Bilberry Fractions Separated by OASIS HLB Column Chromatography on Cancer Cells. **Table 1** shows the total phenolic and anthocyanin content and

Table 1. DPPH Radical Scavenging Activity and Total Phenolic and Anthocyanin Contents in Berry Extracts[a]

	total phenolics (mg/g)	anthocyanin (mg/g)	DPPH radical scavenging activity [μmol of Trolox/g or (mg of phenolics)]
lowbush blueberry	35.9 ± 0.4	12.1 ± 0.5	178.1 ± 5.4 (5.0 ± 0.5)
highbush blueberry	26.4 ± 0.4	6.3 ± 0.4	128.4 ± 8.2 (4.9 ± 1.5)
bilberry	55.1 ± 1.0	26.3 ± 1.5	287.9 ± 10.3 (5.2 ± 0.4)
cowberry	35.4 ± 0.1	6.1 ± 0.4	196.9 ± 6.4 (5.6 ± 1.1)
cranberry	20.1 ± 0.4	3.1 ± 0.2	92.9 ± 2.3 (4.6 ± 0.9)
black currant	40.9 ± 0.7	15.3 ± 0.7	200.3 ± 3.3 (4.9 ± 0.2)
red currant	13.0 ± 0.1	2.3 ± 0.1	71.3 ± 3.4 (5.5 ± 1.5)
raspberry	39.0 ± 0.6	4.4 ± 0.2	208.0 ± 8.5 (5.3 ± 2.2)
blackberry	42.5 ± 0.3	10.0 ± 0.6	238.5 ± 5.9 (5.6 ± 0.7)
strawberry	22.5 ± 0.2	2.4 ± 0.2	121.6 ± 4.5 (5.4 ± 2.5)

[a] Each value is the mean ± SD of triplicate experiments.

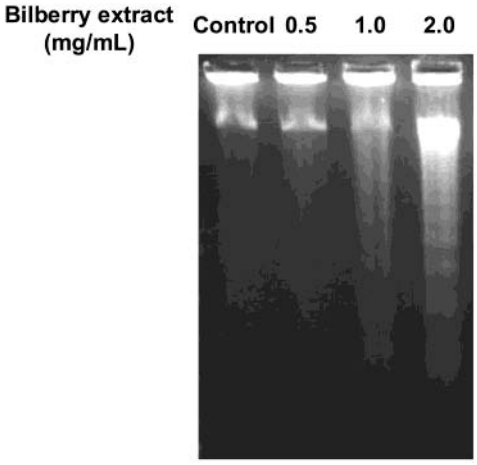


Figure 4. Analysis of DNA fragmentation patterns by agarose gel electrophoresis. DNA was extracted from HL60 cells treated with bilberry extract for 24 h.

DPPH radical scavenging activity of the berry extracts. Phenolic components were most abundant in the bilberry extract, which also possessed the greatest DPPH radical scavenging ability. The anthocyanin content of bilberry extract was also higher than in the other extracts. We next fractionated the bilberry extract into six fractions by OASIS HLB column chromatography (**Figure 5A**) and examined the growth inhibitory effects of these fractions. Most of the anthocyanins were eluted with 40% methanol, and the 20−40% methanol fraction markedly inhibited the growth of HL60 and HCT116 cells (**Figure 5A**,**B**). This fraction decreased the viable cell number by 94% in HL60 cells and 99% in HCT116 cells at a concentration of 400 μg dry wt/mL (**Figure 5B**). The growth inhibitory effects of the other fractions were much lower than those of the anthocyanin-rich 20−40% methanol fraction (**Figure 5B**). These results suggest that it is the anthocyanins contained in the bilberry extract which inhibit HL60 and HCT116 cancer cell growth and which induce apoptosis in HL60 cells.

Growth Inhibitory and Apoptosis-Inducing Effects of Pure Anthocyanidins on Cancer Cells. Figure 6A shows the structures of anthocyanidins contained in the berries. Some flavonoids, such as quercetin and apigenin, were reported to induce apoptosis in cancer cells at a concentration of about 100 μM or less (*19*, *20*). Therefore, we determined the growth inhibitory and apoptosis-inducing effects of the anthocyanidins, pelargonidin, cyanidin, peonidin, delphinidin, and malvidin, on HL60 and HCT116 cells at 50, 100, and 200 μM. Delphinidin markedly inhibited the growth of HL60 and HCT116 cells over a concentration range of 50−200 μM. The number of viable

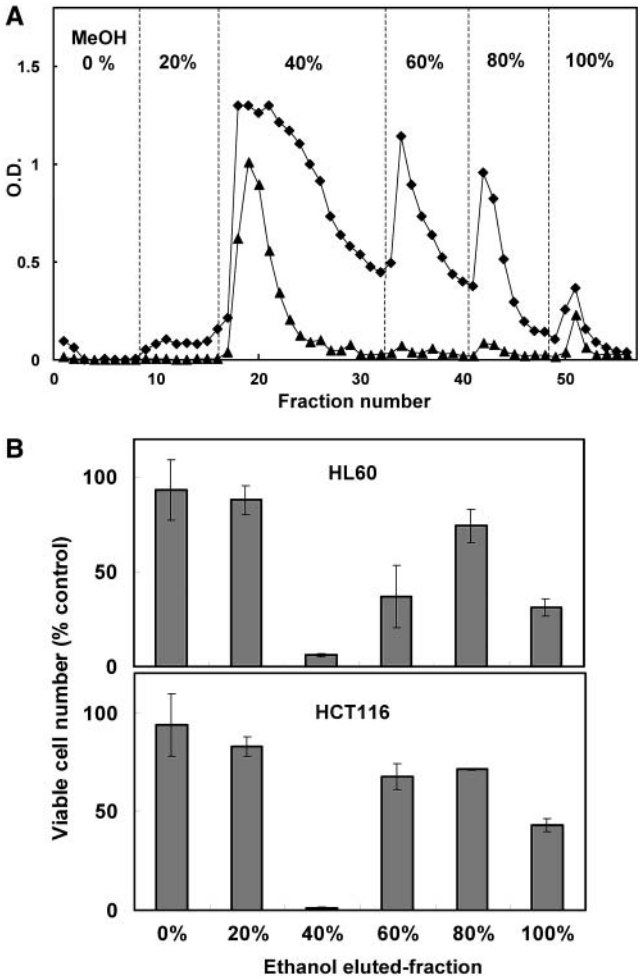
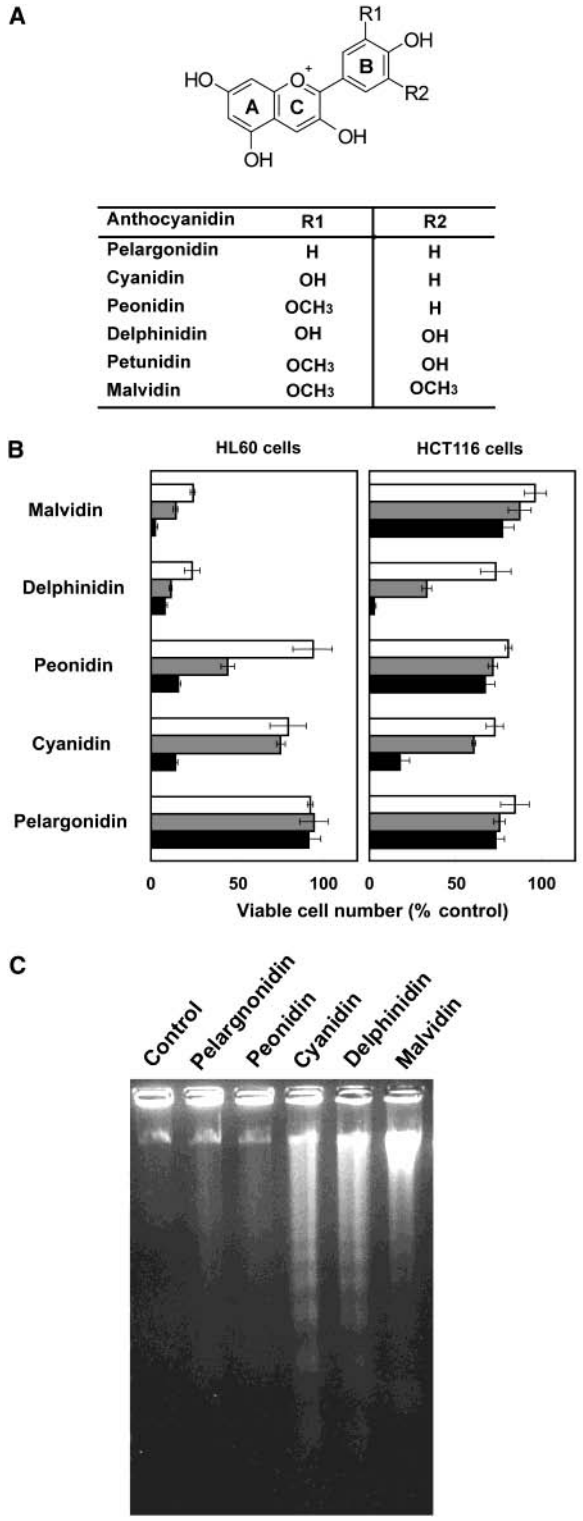


Figure 5. Fractionation of anthocyanin and other phenolic fractions from bilberry ethanol extract and their effects on the growth of HL60 and HCT116 cells. (A) Elution patterns of anthocyanins (530 nm, ◆) and polyphenols (280 mm, ▲) after OASIS HLB column chromatography. The bilberry extract was fractionated into five fractions [0 (0), 0–20 (20), 20–40 (40), 40–60 (60), 60–80 (80), and 80–100% (100) ethanol-eluted fractions]. (B) Effects of the bilberry fractions on the growth of HL60 and HCT116 cells. HL60 cells were incubated for 24 h with 400 μg/mL bilberry ethanol-eluted fractions after OASIS HLB column chromatography. HCT116 cells were incubated for 48 h with 400 μg/mL of the bilberry fractions. Each value is the mean ± SD of triplicate cultures.

HL60 and HCT116 cells was reduced by 100 μM delphinidin to 12% and 36% of the control, respectively (**Figure 6B**). Malvidin more selectively inhibited growth of HL60 rather than HCT116 cells. Thus, 50−200 μM malvidin reduced the number



Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 6. Growth inhibitory and apoptosis-inducing effects of anthocyanidins on cancer cells. (A) Structures of anthocyanidins contained in berries. (B) Effects of anthocyanidins on the growth of HL60 and HCT116 cells. Anthocyanidins were incubated with HL60 cells for 24 h or with HCT116 cells for 48 h. White bars, 50 μM; gray bars, 100 μM; black bars, 200 μM. Each value is the mean ± SD of triplicate cultures. (C) Analysis of DNA fragmentation patterns by agarose gel electrophoresis. DNA was extracted from HL60 cells treated with 200 μM anthocyanidins for 24 h.

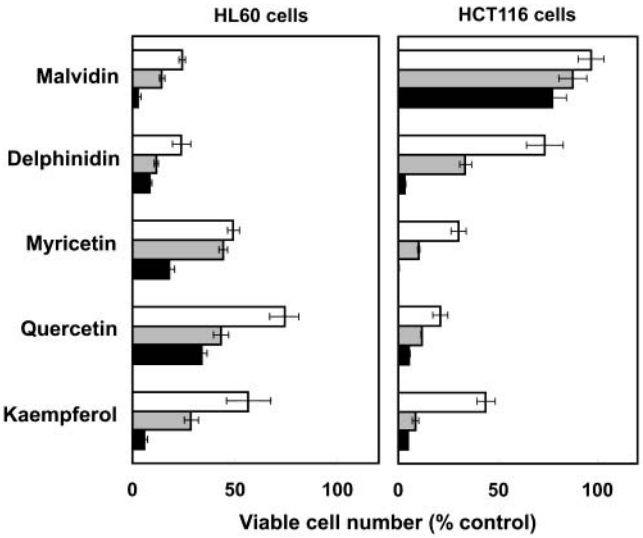


Figure 7. Effects of flavonols on growth of HL60 and HCT116 cells. The flavonoids were incubated with HL60 cells for 24 h or HCT116 cells for 48 h. White bars, 50 μM; gray bars, 100 μM; black bars, 200 μM. Each value is the mean ± SD of triplicate cultures.

of viable HL60 cells to 3−25% of the control, whereas at 200 μM this value was only 78% of the control for HCT116 (**Figure 6B**). Similarly, the growth inhibitory effect of peonidin was high in HL60 but low in HCT116 cells. Peonidin reduced the viable HL60 cell number to 20% of the control only when we increased the concentration to 400 μM (data not shown). In contrast, cyanidin reduced the number both of HL60 and HCT116 viable cells by 82−85% of the control at 200 μM (**Figure 6B**). Pelargonidin, however, had no effect on the number of viable HL60 cells but slightly decreased HCT116 viable cell numbers at 50−200 μM (**Figure 6B**).

Induction of apoptotic cell bodies and nuclear fragmentation was observed after 6 h of treatment with 200 μM malvidin, delphinidin, or cyanidin, but not peonidin or pelargonidin (data not shown). Malvidin, delphinidin, and cyanidin, but not peonidin and pelargonidin, induced nucleosomal DNA fragmentation in HL60 cells (**Figure 6C**). Induction of apoptosis in HCT116 cells was minimal following treatment with 200 μM pure anthocyanidins.

Comparison of Growth Inhibitory Effects of Anthocyanidins with Flavonols on HL60 and HCT116 Cancer Cells. The flavonols myricetin, quercetin, and kaempferol were reported to be constituents of edible berries and to induce apoptosis in cancer cells (*19*, *20*, *29*). The growth inhibitory effects of malvidin and delphinidin in cancer cells were compared with those of flavonols. Myricetin, quercetin, and kaempferol inhibited the growth of HL60 and HCT116 cells at the concentration range 50−200 μM (**Figure 7**). The inhibitory effects of malvidin and delphinidin were greater than those of the flavonols in HL60 cells (**Figure 7**). In contrast, the latter were more effective than the anthocyanidins in inhibiting HCT116 cells (**Figure 7**). Bilberry has been reported to contain flavonols but only at about 1% of the anthocyanin content (3.3 mg of cyanidin equiv/g fresh wt) (*29*). The growth inhibitory and apoptosis-inducing effects of bilberry on cancer cells are therefore likely to be attributable to the anthocyanins.

Growth Inhibitory and Apoptosis-Inducing Effects on HL60 and HCT116 Cancer Cells of Bilberry Anthocyanins Separated by HPLC. The anthocyanins in bilberry extract were analyzed by HPLC, and the major peaks at 530 and 280 nm were fractionated as shown in **Figure 8A**. The anthocyanins were identified by their LC−MS spectra. The anthocyanin or polyphenol contents of the fractions were purified to greater than 95% by HPLC. We then determined the effects of the

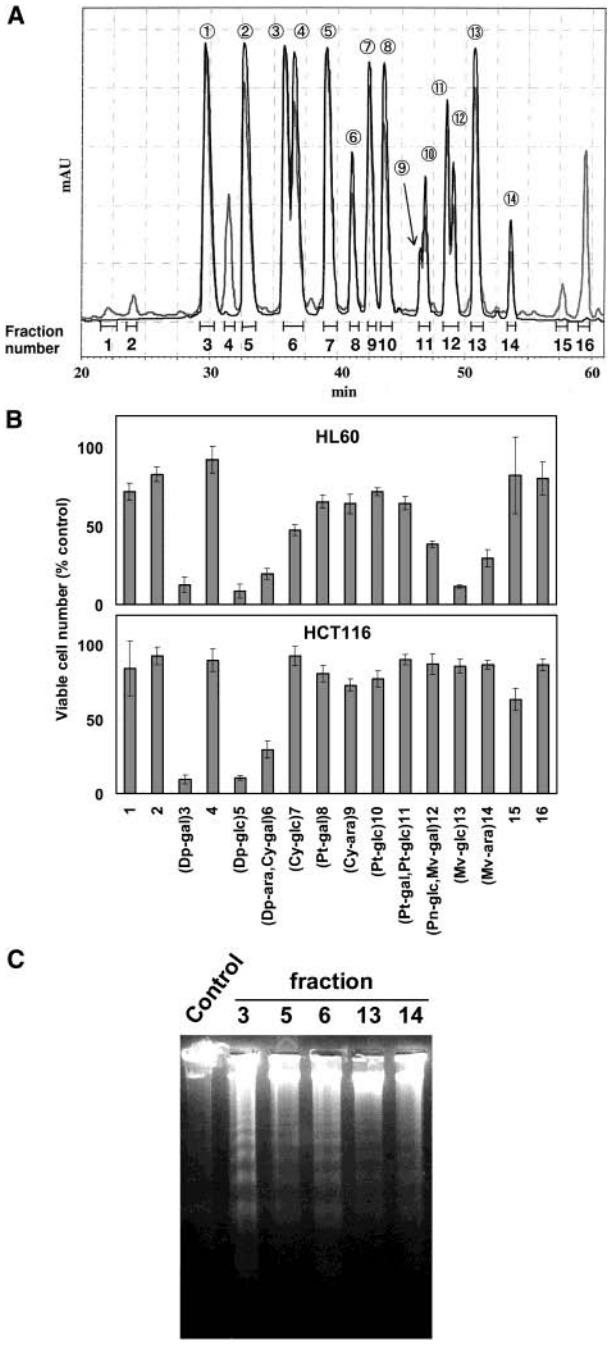


Figure 8. Fractionation of anthocyanins from bilberry extract by HPLC and their effects on growth inhibition and apoptosis induction of cancer cells. (A) Elution patterns of anthocyanins (530 nm, black line) and polyphenols (280 nm, gray line) after HPLC (Inertsil ODS-3 column, 20 × 250 mm). Anthocyanins were identified by LC–MS analysis (circled numbers 1–14). The eluate was fractionated into aliquots of 20 s each. The small fractions were collected and reconstituted into 16 fractions, which contain one or two peaks detected at 530 or 280 nm, as shown in the chart. Key to circled numbers: 1, Dp-gal; 2, Dp-glc; 3, Cy-gal; 4, Dp-ara; 5, Cy-glc; 6, Pt-gal; 7, Cy-ara; 8, Pt-glc; 9, Pn-gal; 10, Pt-ara; 11, Pn-glc; 12, Mv-gal; 13, Mv-glc; 14, Mv-ara. Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; glc, 3-glucoside; gal, 3-galactoside; ara, 3-arabinoside. (B) Effects of fractions separated by HPLC on the growth of HL60 and HCT116 cells. Three hundred microgram per milliliter fractions were incubated with HL60 for 24 h or HCT116 cells for 48 h. Each value is the mean ± SD of triplicate cultures. Abbreviations are as defined in panel A. (C) Analysis of DNA fragmentation patterns by agarose gel electrophoresis. DNA was extracted from HL60 cells treated with the fractions separated by HPLC (300 μg/mL) for 24 h.

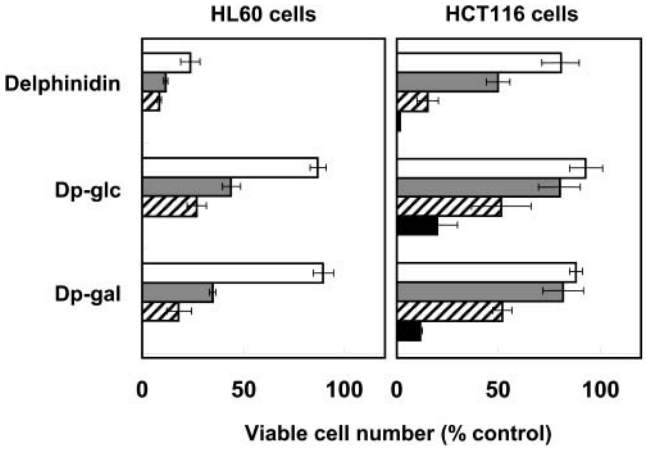


Figure 9. Effects of the delphinidin-glycosides purified from bilberry extract on the growth of HL60 and HCT116 cells. The delphinidin-glycosides were incubated with HL60 cells for 24 h and HCT 116 cells for 48 h. Dp-glc; delphinidin-3-glucoside, Dp-gal; delphinidin-3-galactoside. White bars, 50 μM; gray bars, 100 μM; striped bars, 200 μM; black bars, 400 μM. Each value is the mean ± SD of triplicate cultures.

bilberry anthocyanin fractions on the viability of HL60 and HCT116 cells. Three hundred micrograms dry weight per milliliter bilberry fractions were incubated with HL60 cells for 24 h or with HCT116 cells for 48 h. The anthocyanin fractions possessed stronger HL60 cell growth inhibition activity than nonanthocyanin polyphenol fractions (**Figure 8B**). Fractions 3, 5, and 6 contained delphinidin-glycosides, and fractions 12, 13, and 14 included malvidin-glycosides (**Figure 8A**,**B**). These fractions markedly reduced HL60 viability compared with certain other anthocyanin fractions containing cyanidin-, petunidin-, and peonidin-glycosides (**Figure 8B**). In contrast, the anthocyanin fractions including malvidin-glycosides did not decrease the viability of HCT116 cells (**Figure 8B**). However, the delphinidin-glycoside-containing fractions 3, 5, and 6 did significantly reduce the number of viable HCT116 cells (**Figure 8B**).

Figure 8C shows the induction of nucleosomal DNA fragmentation by fractions 3, 5, 6, 13, and 14. The anthocyanin fractions containing delphinidin- and malvidin-glycosides induced apoptosis in HL60 cells.

Delphinidin-3-galactoside and delphinidin-3-glucoside were further purified from fractions 3 and 5 by recrystallization from $HCl-H_2O$. The purity of both of these delphinidin-glycosides was greater than 99% by HPLC (data not shown). The growth inhibitory effects of the purified delphinidin-glycosides on HL60 and HCT116 cells were somewhat lower than those of the aglycon delphinidin (**Figure 9**).

DISCUSSION

An ethanol extract of bilberry, rich in anthocyanins, inhibited the growth of HL60 human leukemia cells through the induction of apoptosis. Comparing anthocyanidins and the anthocyanins isolated from bilberry extract indicated that it was the anthocyanins, mainly delphinidin- and malvidin-glycosides, in the bilberry extract which caused apoptosis in the HL60 cells. The bilberry ethanol extract and certain anthocyanins also inhibited the growth of HCT116 human colon carcinoma cells. However, HCT116 cells were less sensitive to apoptosis induction by anthocyanins than HL60 cells. It is known that apoptosis can be induced in HCT116 by camptothecin, albeit not readily, and that the apoptotic effects of DNA-damaging agents are blocked

in HCT116 cells (*30*). The apoptosis-inducing pathway of anthocyanins may be blocked in HCT116 cells, too.

We also examined the effect of bilberry on the growth of normal human dermal fibroblast (HNDF) cells. The bilberry extract was applied to an OASIS HLB column, washed with distilled water, and eluted with 100% methanol. The adsorbed fraction decreased the number of viable HNDF cells to 71% of the control at 200 μg/mL. At the same concentration, this fraction decreased the number of viable HCT116 cells to 37% of the control. Thus, bilberry extract inhibited normal fibroblast cell growth, although the inhibitory effect was less than that observed with HCT116 cancer cells. Tumors proliferate beyond the constraints limiting growth in normal tissue, and most existing cancer drugs crudely interfere with DNA synthesis and cell division (*17*). Berries and the anthocyanins probably inhibit the growth of highly proliferating cells but not with specificity solely for cancer cells. However, such a broad effect may contribute to the suppression of various types of cancer.

Here we first demonstrated the apoptosis-inducing effects of anthocyanins on HL60 leukemia cells. The flavonols myricetin, quercetin, and kaempferol, contained in bilberry, were previously reported to be inducers of apoptosis in cancer cells. The effects of malvidin and delphinidin were stronger than those of the flavonols on HL60 cells. As the content of flavonols is very low in bilberry, apoptosis induced by the bilberry extract was most likely mainly due to the anthocyanins. Among the different berries, bilberry contained the greatest amount of anthocyanins, and the ratio of the content of cyanidin−, delphinidin−, and malvidin-glycosides was about 30:36:13. Our results suggest that the bilberry extract had the strongest inhibitory effects on cancer cell growth because it contained large amounts of anthocyanins, especially delphinidin- and malvidin-glycosides.

The anthocyanins cyanidin-3-glucoside and cyanidin-3,5-diglucoside have been reported to be incorporated into human plasma with their structures maintained (*31*). Our results showed that not only the aglycons anthocyanidins but also the glycosides anthocyanins inhibited cancer cell growth and induced apoptosis in cancer cells. Thus, berries such as bilberry containing a large amount of anthocyanins are likely to suppress cancer through the induction of apoptosis and/or inhibition of cancer cell growth in vivo. Recently, the plasma levels of some antioxidants, such as ascorbic acid and α- and β-carotene, were reported to be increased by daily fruit and vegetable intake (*32*). The consumption of berries may also contribute to maintaining plasma levels of anthocyanins, which possess antioxidant activity and inhibitory effects on cancer cell growth. Berries are therefore promising functional foods for reducing cancer risk because of their apoptosis-inducing effect, antioxidant activity, and other anticancer effects.

ACKNOWLEDGMENT

We thank Dr. Shioka Hamamatsu and Ms. Sachiko Hoshino (National Food Research Institute) for technical support with the LC−MS analysis, and Dr. Toshihiko Shoji (Asahi Beer Co.) for valuable discussions.

LITERATURE CITED

(1) Moyer, R. A.; Hummer, K. M.; Finn, C. E.; Frei, B.; Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519−525.

(2) Liu, M.; Li, X. Q.; Weber, C.; Lee, C. Y.; Brown J.; Liu R. H. Antioxidant and antiproliferative activities of raspberries. *J. Agric. Food Chem.* **2002**, *50*, 2926−2930.

(3) Sellappan, S.; Akoh, C. C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georhia-grown blueberries and blackberries. *J. Agric. Food Chem.* **2002**, *50*, 2432−2438.

(4) Wada, L.; Ou B. Antioxidant activity and phenolic content of Oregon caneberries. *J. Agric. Food Chem.* **2002**, *50*, 3495−3500.

(5) Wang, J.; Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-γ-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 850−857.

(6) R-Tortosa, C. R.; Andersen, O. M.; Gardner, P. T.; Morrice, P. C.; Wood, S. G.; Duthie, S. J.; Collins, A. R.; Duthie, G. G. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biol. Med.* **2001**, *31*, 1033−1037.

(7) Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915−7922.

(8) Ames, B. N. Dietary carcinogens and anticarcinogens. *Science* **1983**, *221*, 1256−1263.

(9) Heinonen, I. M.; Meyer A. S.; Frankel, E. N. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. *J. Agric. Food Chem.* **1998**, *46*, 4107−4112.

(10) Connor, A. M.; Luby, J. J.; Hancock, J. F.; Berkheimer, S.; Hanson, E. J. Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *J. Agric. Food Chem.* **2002**, *50*, 893−898.

(11) Pior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.

(12) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, *13*, 590−594.

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

(14) Meiers, S.; Kemeny, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958−962.

(15) Nagase, H.; Sasaki, K.; Kito, H.; Haga, A.; Sato, T. Inhibitiory effect of delphinidin from *Solanum melogena* on human fibrosarcoma HT-1080 invasiveness in vitro. *Planta Med.* **1998**, *64*, 216−219.

(16) Bomser, J.; Madhavi, D. L.; Singletary, K.; Smith, M. A. L. In vitro anticancer activity of fruit extracts from *Vaccinium* species. *Planta Med.* **1996**, *62*, 212−216.

(17) Evan, G. I.; Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. *Nature* **2001**, *411*, 342−348.

(18) Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. Apoptosis: A link between cancer genetics and chemotherapy. *Cell* **2002**, *108*, 153−164.

(19) Kunts, S.; Wenzel, U.; Daniel, H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur. J. Nutr.* **1999**, *38*, 133−142.

(20) Wang, I. K.; Lin-Shiau S. Y.; Lin, J. K. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukemia HL-60 cells. *Eur. J. Cancer* **1999**, *35*, 1517−1525.

(21) Kobori, M.; Iwashita, K.; Shinmoto, H.; Tsushida, T. Phloretin-induced apoptosis in B16 melanoma 4A5 and HL60 human leukemia cells. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 719−725.

(22) Iwashita, M.; Kobori, M.; Yamaki, K.; Tsushida, T. Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1813−1820.

(23) Waring, P. DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. *J. Biol. Chem.* **1990**, *265*, 14476−14480.
(24) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
(25) Ando, T.; Tatsuzawa, F.; Saito, N.; Takahashi, M.; Tsunashima, Y.; Numajiri, H.; Watanabe, H.; Kokubo, H.; Hara, R.; Seki, H.; Hashimoto, G. Differences in the floral anthocyanin content of petunias and *Petunia exserta*. *Phytochemistry* **2000**, *54*, 495−501.
(26) Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* **1998**, *36*, 2090−2097.
(27) Zafrilla, P.; Ferreres, F.; Tomas-Barberan, F. A. Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. *J. Agric. Food Chem.* **2001**, *49*, 3651−3665.
(28) Mazza, G.; Miniati, E. Small Fruits VIII. Whortleberries. In *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, FL, 1993; pp 112−116.
(29) Hakkinen, S. H.; Karenlampi, S. O.; Heinonen, I. M.; Mykkanen, H. M.; Torrronen, A. R. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 2274−2279.
(30) Han, Z.; Wei, W.; Dunaway S.; Darnowski, J. W.; Calabresi, P.; Sedivy, J.; Hendrickson, E. A.; Balan, K. V.; Pantazis, P.; Wyche, J. H. Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. *J. Biol. Chem.* **2002**, *277*, 17154−17160.
(31) Miyazawa, T.; Nakagawa, K.; Kudo, M.; Muraishi, K.; Someya, K. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. *J. Agric. Food Chem.* **1999**, *47*, 1083−1091.
(32) John, J. H.; Ziebland, S.; Yudkin, P.; Roe, L. S.; Neil, H. A. W. Effects of fruit and vegetable consumption on plasma antioxidant concentrations and blood pressure: a randomized controlled trial. *Lancet* **2002**, *359*, 1969−1974.

Received for review July 1, 2002. Revised manuscript received September 5, 2002. Accepted September 6, 2002.

JF025781X