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Berry Phenolic Extracts Modulate the Expression of p21^{WAF1} and Bax but Not Bcl-2 in HT-29 Colon Cancer Cells

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Previous studies have shown that anthocyanin-rich berry extracts inhibit the growth of cancer cells in vitro. The objective of this study was to compare the effects of berry extracts containing different phenolic profiles on cell viability and expression of markers of cell proliferation and apoptosis in human colon cancer HT-29 cells. Berry extracts were prepared with methanol extraction, and contents of the main phenolic compounds were analyzed using HPLC. Anthocyanins were the predominant phenolic compounds in bilberry, black currant, and lingonberry extracts and ellagitannins in cloudberry extract, whereas both were present in raspberry and strawberry extracts. Cells were exposed to 0−60 mg/mL of extracts, and the cell growth inhibition was determined after 24 h. The degree of cell growth inhibition was as follows: bilberry > black currant > cloudberry > lingonberry > raspberry > strawberry. A 14-fold increase in the expression of p21^{WAF1}, an inhibitor of cell proliferation and a member of the cyclin kinase inhibitors, was seen in cells exposed to cloudberry extract compared to other berry treatments (2.7−7-fold increase). The pro-apoptosis marker, Bax, was increased 1.3-fold only in cloudberry- and bilberry-treated cells, whereas the pro-survival marker, Bcl-2, was detected only in control cells. The results demonstrate that berry extracts inhibit cancer cell proliferation mainly via the p21^{WAF1} pathway. Cloudberry, despite its very low anthocyanin content, was a potent inhibitor of cell proliferation. Therefore, it is concluded that, in addition to anthocyanins, also other phenolic or nonphenolic phytochemicals are responsible for the antiproliferative activity of berries.

KEYWORDS: Anthocyanins; antiproliferation; phenolic compounds; colon cancer; HT-29; p21^{WAF1}; Bax; Bcl-2; cell growth; cell cycle arrest; cloudberry; bilberry; raspberry; black currant; strawberry; lingonberry

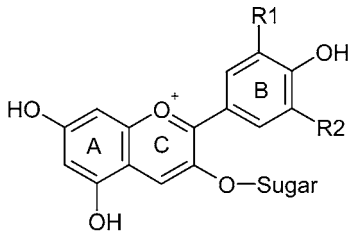
INTRODUCTION

Epidemiological studies consistently indicate that diets rich in fruits and vegetables and lower in red meat are associated with a reduced risk of several cancers, including colon cancer (*1*, *2*).

Certain dietary constituents such as plant-derived phenolic compounds manifest many beneficial effects and can potentially inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages (*3*). Phenolic compounds, especially anthocyanins and other flavonoids, have shown a potential role in colon cancer prevention (*4*−*6*).

Berries contain a variety of phenolic compounds such as flavonoids, phenolic acids, and tannins and are a rich source of anthocyanins, flavonols, and ellagitannins (*7*−*11*), and they contribute to the dietary intake of these phenolics.

Investigations into the role of anthocyanins (**Figure 1**) and 10 different berry extracts on cancer cells have found anthocyanin-rich bilberry extracts to be most effective in inhibiting the growth of HL 60 human leukemia and HCT 116 human colon carcinoma cells in vitro (*5*). Additionally, commercially prepared grape, bilberry, and chokeberry anthocyanin-rich extracts have been shown to inhibit the growth of HT-29 colon cancer cells and have little effect on the growth of the nontumorigenic colonic NCM460 cells (*12*, *13*). Cranberry



Name	R1	R2
Pelargonidin 3-glycoside	H	H
Cyanidin 3-glycoside	H	OH
Delphinidin 3-glycoside	OH	OH
Peonidin 3-glycoside	H	OCH_3
Petunidin 3-glycoside	OH	OCH_3
Malvidin 3-glycoside	OCH_3	OCH_3

Figure 1. Chemical structures of anthocyanins.

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extract and its flavonoid fractions have also been shown to inhibit the proliferation of human tumor cell lines (*14*, *15*). The effects of two anthocyanidins, delphinidin and cyanidin, have also been tested, and the compounds were found to cause cell cycle perturbations and apoptosis in different human cell lines (*16*).

Apoptosis is regulated in part by Bcl-2 genes, which promote cell survival, and Bax overexpression enhances apoptosis (*17*). However, the broad-acting cyclin-dependent kinase inhibitor, $p21^{WAF1}$, occupies a central position in cell cycle regulation, integrating genotoxic insults into growth arrest and apoptotic signaling pathways that ultimately determine cell fate (*18*).

The objective of this study was to compare the antiproliferative potential of berry extracts containing different profiles of phenolic compounds (anthocyanins, flavonols, and ellagitannins) in human colon cancer HT-29 cells. We sought to determine if cloudberry extracts, containing a low anthocyanin content, show properties similar to berry extracts with higher anthocyanin content on cell growth and markers of cell proliferation and apoptosis. The balance between the expression of the two cell death regulatory genes, Bcl-2 and Bax, and levels of $p21^{WAF1}$ in HT-29 cells were therefore investigated.

MATERIALS AND METHODS

Standards. Cyanidin 3-*O*-glucoside and anthocyanin mixtures were obtained from Polyphenols AS (Sandnes, Norway). The anthocyanin mixtures contained either 6 anthocyanidin glycosides, representing 3-*O*-glucosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, or 15 anthocyanidin glycosides, representing 3-*O*-galactosides, 3-*O*-glucosides, and 3-*O*-arabinosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin. Rutin (quercetin 3-*O*-rhamnosylglucoside), (−)-epicatechin, chlorogenic acid, *p*-hydroxybenzoic acid, gallic acid, ellagic acid, and camptothecin were obtained from Sigma (St. Louis, MO).

Berry Phenolic Extraction. Berries (all domestic) were purchased from market stalls and one wholesaler (Pakkasmarja Ltd.) in the Kuopio area of Finland during the summers of 2003 and 2004. Berries were separated from stalks and leaves when necessary. One pooled sample was prepared representing 2−10 subsamples of each berry. The subsamples (0.5−2.0 kg) with identical amounts (usually 100 g) of each were added to the pool. The pooled samples were frozen and stored at −20 °C until analyzed. Frozen (100 g) bilberry (*Vaccinium myrtillus*), black currant (*Ribes nigrum*), cloudberry (*Rubus chamaemorus*), lingonberry (*Vaccinium vitis-idaea*), red raspberry (*Rubus idaeus*), and strawberry (*Fragaria* × *ananassa*) were homogenized using a blender. The homogenized berry samples (10 g) were extracted with 15 mL of 100% methanol by vortexing for at least 1 min followed by centrifugation at 3500*g* for 10 min to pellet the debris. The supernatants were transferred to a rotary evaporator flask. The extraction procedure was repeated three times before the methanol was removed using a Laborota 4000 efficient rotary evaporator at 30 °C (Heidolph, Schwabach, Germany) under vacuum (Vacuubrand PC 2001 Vario, Wertheim, Germany). The semidried samples were then reconstituted in a final volume of 5 mL of Milli-Q water. The concentration of stock berry extract is expressed so that 1 mL of extract solution is extracted from 2 g of fresh berries. The extracts were filtered (0.45 μm, Pall Life Sciences, Ann Arbor, MI) prior to analysis by HPLC and used for cell proliferation assays, DNA fragmentation, and gene expression studies. The procedure presented above is insufficient to extract all of the phenolic compounds from the berries, but it efficiently extracts most of the flavonoids and simple phenolics (*19*, *20*) as well as ellagitannins (*11*). Aliquots (500 μL) of each berry extract were stored at −20 °C.

HPLC Analysis. Contents of anthocyanins, flavonols, and ellagitannins in berry extracts were analyzed with high-performance liquid chromatography (HPLC). For the analysis of anthocyanins and flavonols, a 10 or 25 μL injection of the filtrates was separated on a LiChroCart Purospher Star RP-18e column (250 × 4.6 mm i.d., 5 μm; 4 × 4 mm guard column; Merck, Darmstadt, Germany) using a HP 1100 series HPLC (Waldbronn Analytical Division, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a diode array detector linked to a HP ChemStation data handling system.

The analysis of anthocyanins was performed using 8.5% aqueous formic acid as eluent A and acetonitrile/methanol 85:15 (v/v, HPLC grade) as eluent B. The flow rates of the mobile phase were 0.85 mL/min for 0−12 min and 0.7 mL/min for 13−88 min. The gradient used was as follows: 0−2 min, from 4 to 6% B; 2−4 min, from 6 to 8% B; 4−13 min, from 8 to 9% B; 13−20 min, from 9 to 10% B; 20−40 min, from 10 to 11% B; 40−53 min, 11% B; 53−65 min, from 11 to 19% B; 65−81 min, from 19 to 35% B; 81−84 min, from 35 to 80% B, followed by an isocratic elution with 80% B for 4 min and then returning to the initial conditions for 6 min before the next injection. Anthocyanins were detected at 520 nm.

Flavonols were analyzed using 0.25% aqueous formic acid as eluent A and acetonitrile/methanol 85:15 (v/v, HPLC grade) as eluent B. The flow rates of the mobile phase were 0.4 mL/min for 0−9 min, 0.4−0.6 mL/min for 9−14 min, and 0.6 mL/min for 14−68 min. The gradient used was as follows: 0−6 min, from 16 to 18% B; 6−9 min, 18% B; 9−14 min, from 18 to 20% B; 14−30 min, 20% B; 30−42 min, from 20 to 26% B; 42−50 min, from 26 to 29% B; 50−52 min, from 29 to 42% B; 52−62 min, 42% B; 62−66 min, from 42 to 54% B; 66−68 min, from 54 to 80% B, and then returning to the initial conditions for 6 min before the next injection. Flavonols were detected at 360 nm.

Identification of anthocyanins and flavonols was based on reference compounds (UV−vis spectra and retention times), the literature (*21*−*24*), and our previous studies (*10*, *11*, *25*, *26*). Quantification of anthocyanins was carried out using relevant anthocyanidin 3-*O*-glucosides as external standards, whereas rutin (quercetin 3-*O*-rhamnosylglucoside) was used for quantification of flavonols. The concentrations are expressed as micrograms per milliliter for the weight of the aglycone.

Ellagitannins were determined as ellagic acid equivalents after acid hydrolysis using a slightly modified method optimized by Häkkinen and co-workers (*9*). Reconstituted berry extracts (0.5 mL of raspberry and cloudberry, 1.0 mL of other berries) were refluxed for 20 h at 85 °C in 70% methanol containing 2 M HCl. After hydrolysis, the solution was cooled at room temperature and made up to 50 mL with methanol. Prior to HPLC analysis, the samples were filtered through a 0.45 μm syringe filter (Pall Life Sciences). A 10 or 20 μL injection of the filtrates was separated on a LiChroCart Purospher RP-18e column (125 × 3 mm i.d., 5 μm; 4 × 4 mm guard column; Merck) using the HPLC equipment described above. The mobile phase consisted of aqueous 1% formic acid (eluent A) and acetonitrile/methanol 85:15 (v/v, HPLC grade) (eluent B), and the elution gradient was as follows: 0−20 min, from 5 to 30% B; 20−30 min, from 30 to 90% B, followed by an isocratic elution for 5 min with 90% B and then returning to the initial conditions for 5 min before the next injection. The flow rate of the mobile phase was 0.5 mL/min. Ellagic acid and its derivatives were detected at 254 nm, quantified using ellagic acid as the external standard, and expressed as ellagic acid equivalents (micrograms per milliliter).

Cell Culture. HT-29 cells (passage 143) were purchased from the European Collection of Cell Cultures (ECACC) and used between passage 150 and 180. Cells were cultured and passaged in McCoy's 5A supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (Sigma). The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were passaged at preconfluent densities by the use of 0.25% trypsin−EDTA solution (Sigma).

Cell Proliferation Assay. For determination of cell proliferation, HT-29 cells were seeded at a density of 5000 cells per well (25 cells/μL) into 96-well cell culture plates (Nalge Nunc International, Rochester, NY) and allowed to adhere for 30 h. The medium was then replaced with fresh culture medium (200 μL) containing the extracts (0−60 mg/mL) or pure compounds [0−100 μg/mL, mixture of 15 anthocyanidin 3-*O*-glycosides, cyanidin 3-glucoside, rutin, (−)-epicatechin, chlorogenic acid, *p*-hydroxybenzoic acid, gallic acid, and ellagic acid], and cells were allowed to grow for another 24 h. Controls were always treated with the same amount of H_2O or dimethyl sulfoxide

(DMSO) as used in the corresponding experiments. Total cell counts were determined using SYTOX-Green, which becomes fluorescent after DNA binding. Each experiment was performed with four replicates of the same berry extract and repeated three times. The results are expressed as means ± SEM.

Following incubation, the medium was removed using an aspirator. Cell lysis solution (95 μL of 1% Triton X-100 in 0.9% NaCl) was then added and incubated at room temperature for 10 min. The background fluorescence was then measured prior to the addition of 5 μL of 10 μM SYTOX-Green nucleic acid stain (Molecular Probes, Invitrogen). The cells were incubated with the dye at room temperature for 30 min with shaking at 300 rpm (Titramax 100, Heidolph, Germany) to allow binding to the DNA. Fluorescence measurements were performed using a Wallac Victor 1420 multilabel counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA) with excitation at 485 nm and emission at 535 nm for 1 s.

DNA Fragmentation Assay. HT-29 cells were seeded at a density of 1×10^6 in 6-well cell culture plates and allowed to adhere for 24 h. The medium was then replaced with fresh culture medium (4 mL) containing the berry extracts (0−60 mg/mL) or 10 μM camptothecin (positive control), and cells were grown for another 48 h. Thereafter, the medium containing the unattached cells was collected along with the trypsinized cells. The cells were centrifuged for 10 min at 500*g*, and cell pellets were washed with 1 mL of phosphate buffer solution (PBS) and transferred to a 1.5 mL microfuge tube. The cells were then centrifuged for 1 min at 16000*g*, and the cell pellet was washed again with 0.5 mL of PBS. The PBS was removed, and cell pellets were lysed by the addition of 0.5 mL of lysis buffer [10 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8), 0.5% Triton X-100, 200 μg/mL proteinase K] and incubated at 37 °C for 30 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was then added to the samples, mixed, and centrifuged at 16000*g* for 5 min at 4 °C. The upper phase containing the DNA was transferred to a new 1.5 mL microfuge tube, and $^1/_{10}$ volume of 3 M sodium acetate, pH 5.5, and an equal volume of isopropanol were added. DNA was precipitated overnight at −80 °C. Following centrifugation at 16000*g* for 15 min at 4 °C, the DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 μL of TE (Tris−EDTA) buffer (pH 8) containing 100 μg/mL RNase A (Qiagen Inc., Valencia, CA) and incubated at 37 °C for 30 min. The DNA was separated on a 1% agarose gel containing ethidium bromide and visualized by UV illumination.

RNA Isolation, cDNA Synthesis, and Real-time RT-PCR. For RT-PCR analysis of $p21^{WAF1}$, Bax, and Bcl-2 mRNA levels, HT-29 cells were seeded at a density of 2.5×10^5 cells/mL/well into 12-well cell culture plates and allowed to adhere for 24 h. The medium was then replaced with fresh culture medium (1 mL) containing the berry extracts, and cells were grown for another 24 h prior to total RNA extraction. Controls were always treated with the same amount of H_2O as used in the corresponding experiments. Total cell counts were determined using a hemacytometer to calculate the percentage of cell growth inhibition, and trypan blue exclusion assay was used to determine cell viability at the end of the treatment period.

Total cellular RNA was extracted using the Mini RNA Isolation Kit II (Zymo Research, Orange, CA), according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcriptase reactions to generate cDNA templates were performed using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA), according to the manufacturer's protocol. Quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems). Primers and probes specific for each gene were obtained from the Assay-on-Demand collection (Applied Biosystems). The assays were $p21^{WAF1}$ (cyclin-dependent kinase inhibitor 1A, CDKN1A) (Hs00355782_m1), Bax (Hs00180269_m1), Bcl-2 (Hs00153350_m1), and 18S (Hs99999901_s1). Each sample had a final volume of 20 μL containing 6 ng of cDNA, 1× primer/probe mix (0.4 mM each of the forward and reverse PCR primers and 0.1 mM of the TaqMan probe), and 1× TaqMan Universal PCR Master Mix (Applied Biosystems). Temperature conditions consisted of a step of 5 min at 95 °C, followed by 60 °C for 1 min and 95 °C for 15 s for total of 40 cycles. Data were collected during each extension phase of the PCR reaction and analyzed with the SDS software package (Applied Biosystems). Threshold cycles were determined for each gene. A parallel standard curve using 72, 24, 12, 4, and 2 ng (in triplicate) of cDNA synthesized from each treated sample was generated for each gene. This standard curve was used to determine the relative concentration of RNA in each sample by comparison using methods described in ABI Prism User Bulletin 2 (Applied Biosystems). The level of expression of $p21^{WAF1}$, Bax, or Bcl-2 mRNA was given as relative copy number normalized against the mean of the housekeeping gene, 18S ribosomal RNA, mRNA expression, and shown as mean ± SEM.

Statistics. For cell proliferation assays, one-way ANOVA with Dunnett's *t* post hoc test was used. For gene expression assays, one-way ANOVA with Dunnett's *C* post hoc test was used. SPSS 11.5 for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis.

Table 1. Concentrations of Anthocyanins, Flavonols, and Ellagitannins in Berry Extracts[a,b]

	content ± SEM (μg/mL)		
	anthocyanins	flavonols	ellagitannins
cloudberry	26 ± 1	32 ± 1	1040 ± 33
strawberry	610 ± 4	11 ± 1	280 ± 23
raspberry	880 ± 6	8 ± 1	1410 ± 160
lingonberry	1310 ± 16	430 ± 17	nd[c]
black currant	4770 ± 51	290 ± 6	nd
bilberry	12800 ± 370	290 ± 16	nd

[a] Contents of anthocyanins and flavonols are expressed as the weight of the aglycone moieties. [b] Contents of ellagitannins are expressed as ellagic acid equivalents. [c] nd, below detection limit.

RESULTS

Phenolic Profiles of Berry Extracts. The concentrations of anthocyanins, flavonols, and ellagitannins in berry extracts are presented in **Table 1**. The anthocyanin and flavonol contents in berry extracts varied from 26 to 12800 μg/mL and from 8 to 430 μg/mL, respectively. Extraction of bilberries provided the most anthocyanin-rich extract, whereas the highest flavonol concentration was observed in lingonberry extract. Ellagitannins were detected only in cloudberry, raspberry, and strawberry extracts, and the concentrations were 1040, 1410, and 280 μg/mL, respectively. The studied berry extracts can be put in order based on the total sum of the phenolics analyzed as follows: bilberry > black currant > raspberry > lingonberry > cloudberry > strawberry.

The anthocyanin profiles of berry extracts are shown in **Figure 2**. In cloudberry, lingonberry, and raspberry extracts, cyanidin glycosides were the main anthocyanins. Malvidin, peonidin, and petunidin glycosides were present only in bilberry extract, whereas mainly pelargonidin glycosides were observed in strawberry extract. Black currant extract contained equal quantities of delphinidin and cyanidin glycosides.

Effects of Berry Extracts on Cell Proliferation. The berry extracts decreased the proliferation of HT-29 cells in a concentration-dependent manner, and the degrees of inhibition were different for each berry extract (**Figure 3**). Only bilberry showed significant ($p < 0.001$) growth inhibition at 10 mg/mL (30% cell reduction). Black currant, which contained about 3 times less anthocyanin than bilberry (**Table 1**), showed significant ($p < 0.05$) growth inhibition at 20 mg/mL (20% cell reduction). Cloudberry seemed to be as effective as black currant in inhibiting 40% cell growth at 40 mg/mL, although the anthocyanin concentration of the extract was very low. Lingonberry extract inhibited 30% of cell growth at 40 mg/mL and had 50% inhibition at 60 mg/mL. The least effective berries were raspberry and strawberry, which inhibited cell growth by

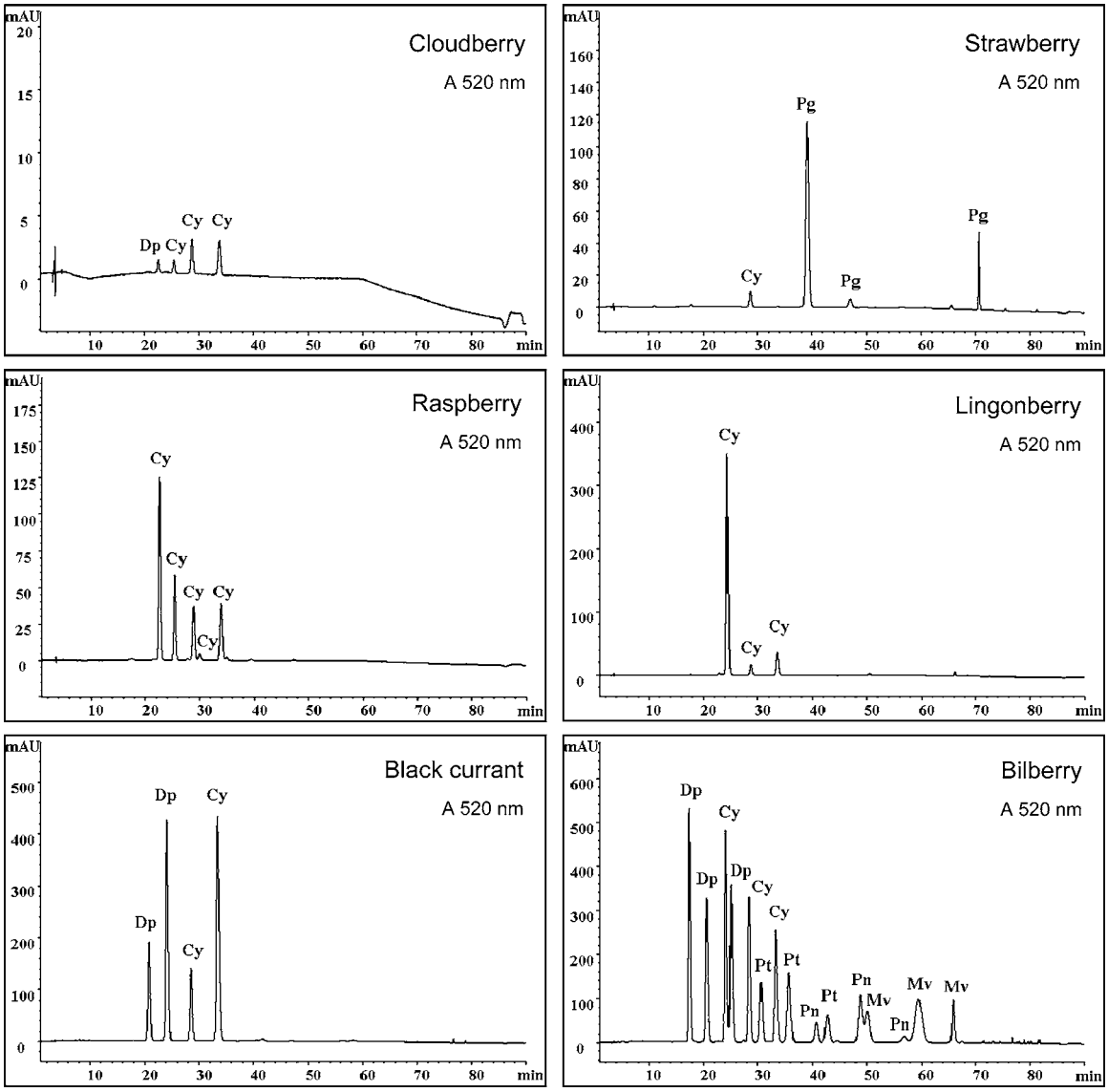

Figure 2. Chromatographic data of anthocyanins in berry extracts. Anthocyanins were detected at 520 nm and identified as follows: Dp, delphinidin derivative; Cy, cyanidin derivative; Pt, petunidin derivative; Pg, pelargonidin derivative; Pn, peonidin derivative; Mv, malvidin derivative.

20−30% at 60 mg/mL. Cell growth inhibition was sustained for up to 36 h (data not shown), and >90% of cells were viable as determined by trypan blue exclusion assay, which indicated cytostatic inhibition.

Effects of Pure Phenolic Compounds on Cell Proliferation. The mixture of 15 anthocyanidin 3-*O*-glycosides, cyanidin 3-glucoside, ellagic acid, and gallic acid decreased the proliferation of HT-29 cells in a concentration-dependent manner, whereas rutin had no cell growth inhibitory effect on HT-29 cells (**Figure 4**). The cell growth inhibitory effect of pure anthocyanins was significant at concentrations $\geq 75\ \mu$g/mL. No significant cell growth inhibitory effect was found for HT-29 cells exposed to either (−)-epicatechin, chlorogenic acid, or *p*-hydroxybenzoic acid (data not shown).

DNA Fragmentation. To determine the effect of anthocyanin content on DNA fragmentation, HT-29 cells were exposed to bilberry extracts (high anthocyanin content) and cloudberry extracts (low anthocyanin content) for 48 h. **Figure 5**, panels **A** and **B**, shows the agarose electrophoretic pattern of HT-29 cell DNA fragmentation following exposure to varying concentrations of cloudberry and bilberry extract, respectively. Cloudberry and bilberry extract had no effect on DNA fragmentation at concentrations up to 10 mg/mL; however, cells exposed to 20−60 mg/mL bilberry extract showed pronounced fragmentation of DNA. On the other hand, cloudberry extracts at 20 mg/mL caused only slight fragmentation of DNA and were only more pronounced when cells were exposed to 40 and 60 mg/mL cloudberry extract.

RT-PCR Analysis of p21^{WAF1}, Bax, and Bcl-2 mRNA Levels. RT-PCR was performed after 24 h of incubation with berry extracts, and levels of mRNA expression were determined. To evaluate the mechanism by which cell proliferation was inhibited by the berry extracts, we assessed the expression of p21^{WAF1}. Levels of p21^{WAF1} mRNA expression were found to be increased significantly ($p < 0.001$) following exposure to berry extracts, and mRNA levels were different for each berry treatment (**Figure 6**). The fold change in p21^{WAF1} was not dependent on the extent of cell growth inhibition (25−45%) by the berry extracts. Cells exposed to 10 mg/mL bilberry extract (40% cell growth inhibition) had a 7-fold increase in p21^{WAF1} expression, whereas 2.7-, 14-, and 5.5-fold increases in p21^{WAF1} were observed in cells exposed to 40 mg/mL of black currant (40% cell growth inhibition), cloudberry (45% cell growth inhibition), and lingonberry (35% cell growth inhibition) extracts, respectively. For cells exposed to 60 mg/mL raspberry (35% cell growth inhibition) and strawberry extracts (25% cell

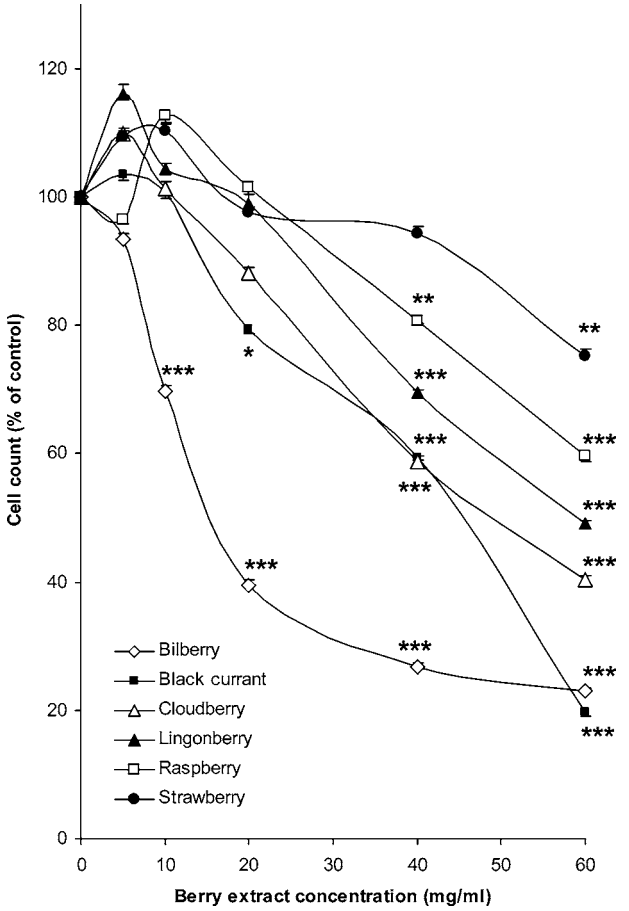

Figure 3. Growth inhibition of HT-29 cells by different berry extracts. HT-29 cells were incubated with 0–60 mg/mL berry extract for 24 h. Total cell numbers were determined after 24 h of incubation using the nucleic acid stain SYTOX-Green. Data are expressed as percentage of untreated cells and represent the means ± SEM ($n = 12$) of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significant difference compared to untreated controls based on one-way ANOVA with Dunnett's *t* post-hoc test.

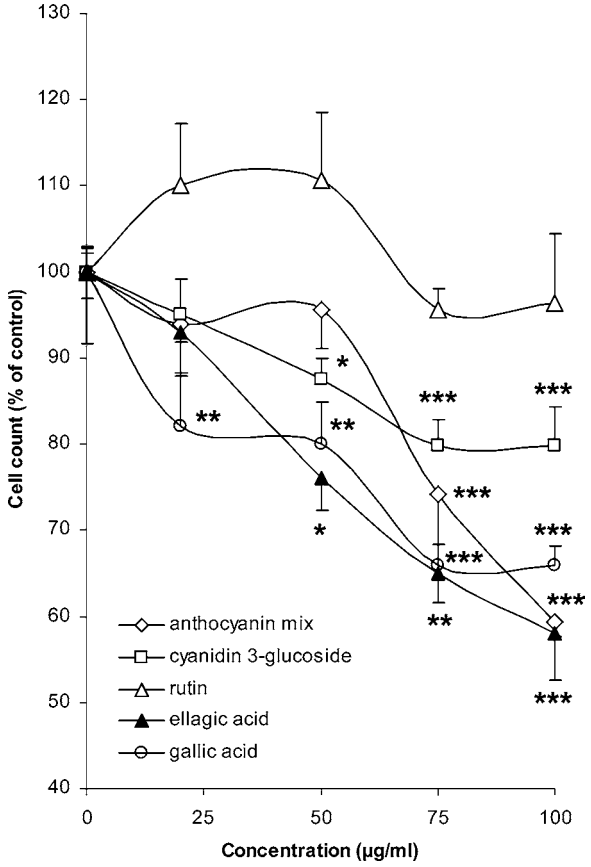

Figure 4. Growth inhibition of HT-29 cells by pure phenolics. HT-29 cells were incubated with 0–100 μg/mL pure phenolic compounds for 24 h. Total cell numbers were determined after 24 h of incubation using the nucleic acid stain SYTOX-Green. Data are expressed as percentage of untreated cells and represent the means ± SEM ($n = 4$) of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significant difference compared to untreated controls based on one-way ANOVA with Dunnett's *t* post-hoc test.

growth inhibition), the levels of $p21^{WAF1}$ mRNA levels were increased by 5.8- and 3-fold, respectively.

Bax mRNA expression was detected in all cells exposed to berry extracts; however, significant differences ($p < 0.01$) were found only in cells exposed to 40 mg/mL cloudberry extract and 10 mg/mL bilberry extract. Both berry extracts increased Bax mRNA expression by 1.3-fold. The other berry extracts showed no difference from the control samples. Levels of Bcl-2 mRNA were undetectable in the majority of treated samples. In control samples the signal for Bcl-2 mRNA was detected only after 36 PCR cycles. To assess the efficacy of the Bcl-2 assay, cDNA from peripheral blood mononuclear cells were used, and levels of Bcl-2 mRNA were detected after 24 PCR cycles (36 ng) and at concentrations as low as 0.5 ng (31 cycles). This compares to at least 34 PCR cycles being required to detect 72 ng of Bcl-2 mRNA in control HT-29 cells.

DISCUSSION

For comparative purposes, we chose to evaluate the effect of whole berry methanolic extracts on cell growth to determine the level of growth inhibition. The reported results are based on a mixed sample of fruit of each species, and horticultural factors may influence phenolic profiles and responses in these types of studies.

The phenolic profiles of the extracts are in good agreement with our previous studies (*10*, *11*). Anthocyanins dominated in bilberry, black currant, and lingonberry extracts, whereas ellagitannins were the major phenolic components in cloudberry extract. High or relatively high levels of both anthocyanins and ellagitannins were detected in raspberry and strawberry extracts. Flavonols were present in all extracts, but as a minor group compared to anthocyanins and ellagitannins. Methanol extraction recovers also other phenolic compounds such as flavan-3-ols and phenolic acids (*19*, *20*). Because they represent minor phenolic groups of berries (*10*, *11*), they were not quantified in the extracts. We also found that (−)-epicatechin, chlorogenic acid, and *p*-hydroxybenzoic acid as representatives of these phenolic groups had no effect on cell growth (data not shown).

All of the berry extracts studied decreased the proliferation of HT-29 cells, with the following degree of cell growth inhibition: bilberry > black currant > cloudberry > lingonberry > raspberry > strawberry. With the exception of cloudberry, the order of antiproliferative potency of the extracts correlates with their anthocyanin concentration.

We found bilberry extracts to be effective in inhibiting cell growth significantly at low concentration (10 mg/mL), whereas higher concentrations were required for other berries. The cell growth inhibition can be explained for bilberry extract at 10 mg/mL and for black currant extract at 40 mg/mL by the fact that anthocyanin levels at these concentrations were 64 and 96

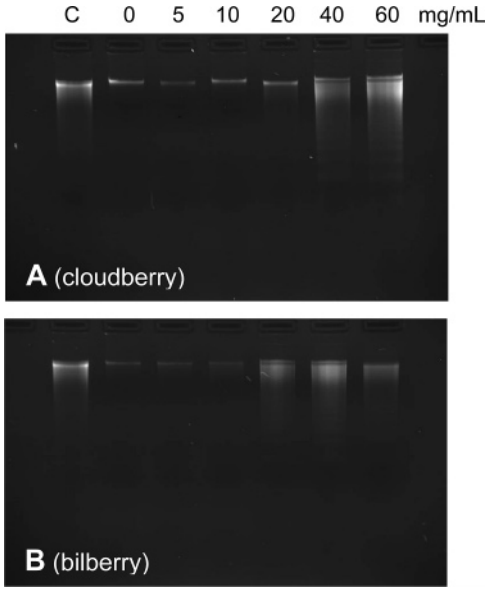

Figure 5. Effect of cloudberry (**A**) and bilberry (**B**) extract on DNA fragmentation as detected by agarose gel electrophoresis. HT-29 cells were treated with 0–60 mg/mL berry extracts for 48 h. C, camptothecin (positive control).

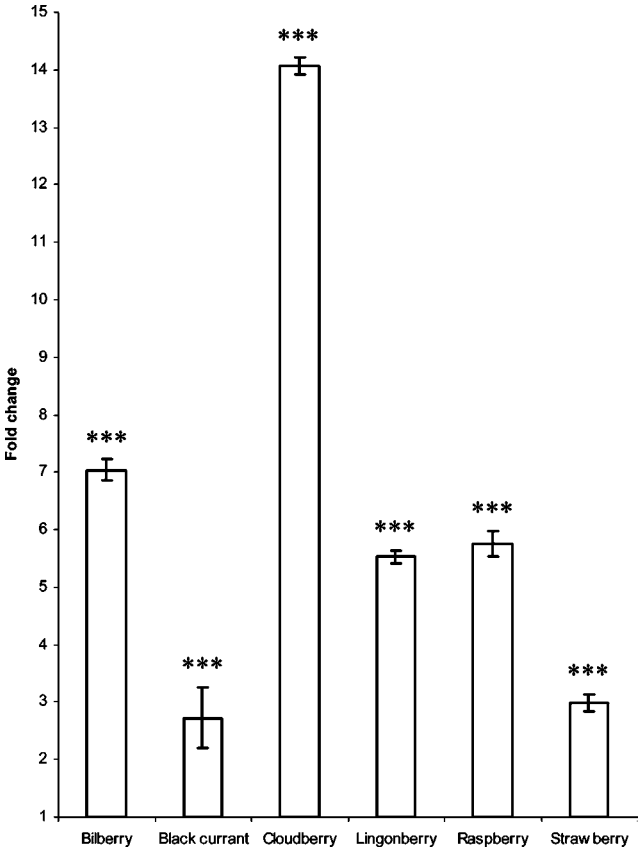

Figure 6. Fold change in expression of $p21^{WAF1}$ in HT-29 cells exposed to different berry extracts. Concentrations of berry extracts used were 10 mg/mL (bilberry), 40 mg/mL (black currant, cloudberry, and lingonberry), and 60 mg/mL (raspberry and strawberry). The level of $p21^{WAF1}$ is given as relative copy number normalized to the endogenous control 18S and represents the means ± SEM ($n = 3$) of two independent experiments. ***, $p < 0.001$, significant difference compared to untreated controls based on one-way ANOVA with Dunnett's *C* post-hoc test.

μg/mL, respectively, which resulted in the same percentage of growth inhibition of HT-29 cells exposed to pure anthocyanins at similar concentrations. These results are comparable to those studies that report anthocyanins as contributors to the inhibition of cell proliferation (*4*, *12*, *13*, *27*).

Our results, however, suggest that the anthocyanin composition of berry extracts is not an important factor in HT-29 cell growth inhibition. As shown in **Figure 2**, the anthocyanin compositions of the berry extracts are different. Strawberry, raspberry, and lingonberry extracts, however, resulted in the same inhibitory effect on cell growth (ca. 20%) at concentrations of 60, 40, and 30 mg/mL, respectively, representing the anthocyanin concentration of 20 μg/mL. In addition, bilberry and black currant extracts at concentrations of 15 and 40 mg/mL, respectively, have equal anthocyanin concentrations and inhibit the cell growth similarly. These findings support the fact that the inhibitory effect of berry extracts is based on the concentration rather than the composition of anthocyanins. Our study with cyanidin 3-glucoside and a mixture of 15 anthocyanins resulted in the same conclusion that the inhibitory effect of anthocyanins is not dependent on the anthocyanin structure. However, because our evidence was obtained from experiments with crude extracts, only one pure anthocyanin compound, and a mixture of several anthocyanins, no definite conclusion can be drawn on the role of anthocyanin composition. Our findings are also in slight contradiction with other studies (*13*, *27*, *28*). Zhao and co-workers (*13*) stated that the greater inhibition of HT-29 cell growth within 24 h by chokeberry anthocyanin-rich extract (ARE) as compared to grape or bilberry AREs may be related to the differences in the anthocyanin profiles of these extracts or to the presence of other phenolics. In chokeberry ARE, cyanidin 3-glycosides were the only anthocyanins present, whereas bilberry and grape extracts contained glycosides of five different anthocyanidins. Yi and co-workers (*27*) concluded that a mixture of high levels of delphinidin, cyanidin, and petunidin and lower levels of peonidin and malvidin could be an ideal mixture for cancer cell growth inhibition. In addition, it has been reported that the number of hydroxyl and methoxyl groups in the B ring of anthocyanidins has a strong effect on cancer cell growth inhibition (*28*).

Interestingly, Olsson and co-workers (*29*) noted that the anthocyanin fraction of blueberry extract inhibited proliferation of MCF-7 cells to a lower extent than the total extract and showed even lower inhibition against HT-29 cells, suggesting that the inhibitory effect is probably only partially due to the anthocyanins. This is supported by our own results when we compare black currant and cloudberry extracts used at 40 mg/mL. At this concentration, both berry extracts inhibited 40% of HT-29 cell growth, and this is not likely entirely due to the relatively low anthocyanin content in cloudberry extracts (0.5 μg/mL). One possibility may be the ellagitannins (*8*, *10*) in the cloudberry extracts, as noted by Seeram and co-workers (*30*), who found pomegranate ellagitannin fractions inhibiting the growth of HT-29 cells. Moreover, Liu and co-workers (*31*) studied four cultivars of raspberries and showed that phytochemicals other than anthocyanins from raspberries were responsible for the inhibition of HepG2 human liver cancer cells and that the pigment content was not a factor in the inhibition of cell proliferation. Cloudberry, like other ellagitannin-rich berries, contains also free gallic acid and ellagic acid and their derivatives (*11*), which in the present study were more effective than pure anthocyanins in inhibiting cell growth (**Figure 4**). However, the contribution of ellagitannins remains obscure, because raspberry extract with a higher concentration of ellagitannins (plus anthocyanins) was less effective than cloudberry in inhibiting cell growth. Differences in the ellagitannin

composition of raspberry and cloudberry could be one explanation for this discrepancy. However, the ellagitannin composition of cloudberry is not known.

It has been reported that flavonols have strong antiproliferative activities (*32*). In the present study, however, flavonols were mostly in glycosylated forms in berry extracts (*10*, *11*), which had no inhibitory effects on cancer cell growth in our study with rutin (quercetin glycoside) and in some previous studies (*33*, *34*). On this basis, and from the fact that the flavonol concentrations in our experiments were below 15 μg/mL, we can conclude that the inhibitory effect of berry extracts on HT-29 cell growth was not likely due to flavonols or flavonol glycosides.

Cancer cell growth is dependent on the balance between proliferation and apoptosis. We employed the DNA fragmentation assay to examine the effect of anthocyanin content on apoptosis. Cloudberry has minimal anthocyanins compared to bilberry; however, cloudberry extract was able to induce apoptosis similarly to bilberry extract. Furthermore, the degree of DNA fragmentation in HT-29 cells exposed to cloudberry and bilberry extract (**Figure 5**) is quite similar to the cell growth inhibition results (**Figure 3**). Significant inhibition of cell growth was observed only in cells exposed to 40 and 60 mg/mL cloudberry extract, and these concentrations also caused fragmentation of DNA. For bilberry, however, HT-29 cells only showed pronounced DNA fragmentation starting at 20 mg/mL, even though significant inhibition of cell growth was observed at 10 mg/mL. Interestingly, when HCT116 cells were treated with bilberry extracts for 48 h, no nucleosomal DNA fragmentation occurred (*5*).

One of the mechanisms of cell growth inhibition is cell cycle arrest. When cells were exposed to either anthocyanin-rich extracts (*12*) or ferulic acid from black raspberries (*35*) or strawberry and plum extracts (*36*), the main cell cycle blockage occurred at the G1/G0 and G2/M phases of the cell cycle. Cell cycle progression is regulated by the interaction between the cyclin-dependent kinases (CDKs) and cyclin, and this complex is negatively regulated by cyclin kinase inhibitors (CKIs). To evaluate this, the expression of $p21^{WAF1}$, an inhibitor of cell proliferation and member of the CKIs, was investigated. We found $p21^{WAF1}$ to be highly expressed (up to 14-fold) in HT-29 colon cancer cells following exposure to cloudberry extracts, and this high mRNA expression level was observed when cell growth was inhibited by 45%. In bilberry extracts, $p21^{WAF1}$ mRNA expression was increased only 7-fold when there was 40% cell growth inhibition. This is interesting considering that bilberry extract contains about 500 and 9 times more anthocyanins and flavonols, respectively, than cloudberry extract. The ellagic acid component of cloudberry may increase the expression of $p21^{WAF1}$, as has been shown to be the case in both cervical carcinoma (CaSki) cells (*37*) and human bladder cancer T24 cells (*38*).

We also investigated the expression of Bax and Bcl-2 mRNA expression to elucidate the mechanism involved in apoptosis. Gene expression analysis showed that the pro-apoptosis marker, Bax mRNA expression, was changed significantly only for cells treated with either cloudberry or bilberry extracts (1.3-fold increase for both berries). The increase in Bax occurred in cells exposed to 40 mg/mL cloudberry extract, a concentration at which DNA fragmentation was also observed. On the other hand, for cells exposed to bilberry extracts, both an increase in Bax and a significant inhibition of cell growth occurred at 10 mg/mL. Bcl-2 mRNA were detectable in control samples; however, the levels of Bcl-2 mRNA expression were quite low in HT-29 cells. The cycle threshold (Ct) values for Bcl-2 were around 36−39, indicative of low levels of expression; however, cells treated with berry extracts may also have down-regulated the expression of Bcl-2, resulting in the absence of mRNA expression in the majority of treated samples compared with the controls. Yu and co-workers (*39*) showed Bax mRNA to be up-regulated and Bcl-2 to be down-regulated in HT-29 cells by genistein, an isoflavone, in a dose-dependent manner. However, berries do not contain isoflavones and hence may not show actions similar to those of genistein. Interestingly, it has been reported that levels of Bcl-2 decreased in a dose-dependent manner in HT-29 cells treated with quercetin for 72 h, whereas Bax levels did not change (*33*).

In conclusion, the results from our study suggest that berry extracts exert their action mainly through cytostatic inhibition of cell growth via the CKI $p21^{WAF1}$ pathway and that cloudberry and bilberry extracts also induce apoptosis, indicated both by DNA fragmentation and by increased Bax mRNA expression. Cloudberry seems to be just as effective as, or better than, berries with higher anthocyanin content in inhibiting HT-29 cancer cell growth. A better understanding of how cloudberry phytochemicals inhibit tumor cell proliferation will enable them to be used more efficiently and perhaps synergistically with other antiproliferative agents (*15*).

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