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Inhibition of Cancer Cell Proliferation and Suppression of TNF-induced Activation of NFÎ B by Edible Berry Juice

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Abstract. *Background: Berries contain several phytochemicals, such as phenolic acids, proanthocyanidins, anthocyanins and other flavonoids. There has been growing interest in a variety of potential chemopreventive activities of edible berries. The potential chemopreventive activity of a variety of small berries cultivated or collected in the province of Québec, Canada were evaluated here. Materials and Methods: Strawberry, raspberry, black currant, red currant, white currant, gooseberry, high-bush blueberry, low-bush blueberry, velvet leaf blueberry, serviceberry, blackberry, black chokeberry, sea buckthorn and cranberry were evaluated for antioxidant capacity, anti-proliferative activity, anti-inflammatory activity, induction of apoptosis and cell cycle arrest. Results: The growth of various cancer cell lines, including those of stomach, prostate, intestine and breast, was strongly inhibited by raspberry, black currant, white currant, gooseberry, velvet leaf blueberry, low-bush blueberry, sea buckthorn and cranberry juice, but not (or only slightly) by strawberry, high-bush blueberry, serviceberry, red currant, or blackberry juice. No correlation was found between the anti-proliferative activity of berry juices and their antioxidant capacity ($p>0.05$). The inhibition of cancer cell proliferation by berry juices did not involve caspase-dependent apoptosis, but appeared to involve cell-cycle arrest, as evidenced by down-regulation of the expression of cdk4, cdk6, cyclin D1 and cyclin D3. Of the 13 berries tested, juice of 6 significantly inhibited the TNF-induced activation of COX-2 expression and activation of the nuclear transcription factor NFÎ B. Conclusion: These results illustrate that berry juices have striking differences in their potential chemopreventive activity and that the inclusion of a variety of berries in the diet might be useful for preventing the development of tumors.*

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Diets rich in fruits and vegetables have been associated with a reduced risk of cancer (1, 2). Many non-nutrient plant compounds known as phytochemicals have been identified and been found to exert anticancer activity, including numerous phenolic compounds (3). The cancer protective effects of fruits and vegetables have been suggested to be due to different mechanisms such as inhibition of carcinogen activation, stimulation of carcinogen detoxification, scavenging of free radical species, control of cell-cycle progression, inhibition of cell proliferation, induction of apoptosis, inhibition of the activity of oncogenes, inhibition of angiogenesis and metastasis, and inhibition of hormone or growth-factor activity (4-6). It has been established that the complex mixtures of phytochemicals present in fruits and vegetables are more effective than their individual constituents in preventing cancer, through both additive and synergistic effects (3, 7-9). For this reason, it is important to study potential anticancer activity of fruits and vegetables using whole extracts containing all phytochemicals, not only using purified molecules or fractions enriched in certain classes of molecules. This is exemplified by a recent study on cranberries where it was found that a total cranberry extract and a total polyphenol fraction were more antiproliferative against cancer cells than were other, more purified fractions, such as anthocyanins, proanthocyanidins and organic acids (10). Pomegranate juice was also found to have greater antiproliferative and antioxidant activities than its purified constituent polyphenols (7).

Edible berries constitute a very good source of several phytochemicals, such as phenolic acids, proanthocyanidins, anthocyanins and other flavonoids (11-15). Much of the research on berries has focused on chemical composition, particularly on their flavonoid and phenolic acid contents

(13, 16-20), and on antioxidant activity (11, 21-31). More recently, there has been growing interest in a variety of potential chemopreventive properties of berries, including inhibition of cancer cell proliferation (10, 22, 23, 32-34), antiangiogenic activity (35-40), cellular transformation (41, 42) and anti-inflammatory activity (43, 44). Unregulated cell proliferation and suppression of apoptosis are essential for cancer evolution and progression (45, 46). It would thus be of great interest to identify fruits, vegetables and their cultivars that possess antiproliferative and pro-apoptotic activities against tumor cells. Antiproliferative activity has been reported for several fruits and berries, including apples (47-49), strawberries (23, 48, 50, 51), raspberries (22, 52), blackberries (42), blueberries (22, 53, 54), currants (22), chokeberries (22), cranberries (10, 48, 55, 56) and cherries (33). However, most of these studies used either extraction methods which employed solvents and acids, or purified compounds. As part of our ongoing research on the chemopreventive potential of fruits and vegetables grown and consumed in the province of Québec, Canada, we have chosen to analyze whole juices instead of extracts. We think that this approach is the best for preserving synergistic effects of hydrosoluble phytochemicals and minimizing extraction-induced loss of active compounds as well as their deterioration by oxidation and degradation.

The objectives of this research were for 13 types of berry to (i) determine their antiproliferative activity against five cancer cell lines; (ii) determine their antioxidant capacity; (iii) identify potential correlations between antiproliferative and antioxidant activities; (iv) determine if apoptosis or cell-cycle arrest is involved in their antiproliferative activity; (v) identify berries that have anti-inflammatory activity *in vitro*.

Table I. *Identification of species and cultivars of fruits used in the experiments.*

Common name	Species	Cultivar or place of collection
Strawberry	*Fragaria ananassa*	Jewel
Raspberry	*Rubus idaeus*	Titan
Black current	*Ribes nigrum*	Titania, Ben Lomond, Tiben, Ben Nevis.
Red currant	*Ribes rubrum*	Unknown cultivar
White currant	*Ribes sativum*	Blanka
Gooseberry	*Ribes hirtellum*	Unknown cultivar, Pointe-au-Père (Québec, Canada)
High-bush blueberry	*Vaccinium corymbosum*	Patriot
Low-bush blueberry	*Vaccinium angustifolium*	Collected in the wild near St-Tite-des-Caps (Québec, Canada)
Velvet leaf blueberry	*Vaccinium myrtilloides*	Collected in the wild near St-Tite-des-Caps (Québec, Canada)
Serviceberry	*Amelanchier sanguinea*	Collected in the wild
Blackberry	*Rubus allegheniensis*	99,2
Black chokeberry	*Aronia melanocarpa*	Unknown
Sea buckthorn	*Hippophae rhamnoides*	Sunny
Cranberry	*Vaccinium macrocarpon*	Unknown cultivar

Materials and Methods

Plant materials. Thirteen different berries (see Table I) including strawberry (*Fragaria ananassa*), raspberry (*Rubus idaeus*), black currant (*Ribes nigrum*), red currant (*Ribes rubrum*), white currant (*Ribes sativum*), gooseberry (*Ribes hirtellum*), high-bush blueberry (*Vaccinium corymbosum*), low-bush blueberry (*Vaccinium angustifolia*), velvet leaf blueberry (*Vaccinium myrtilloides*), serviceberry (*Amelanchier sanguinea*), blackberry (*Rubus allegheniensis*), sea buckthorn (*Hippophae rhamnoides*) and cranberry (*Vaccinium macrocarpon*) were obtained from local farmers or collected in the wild.

Chemicals. Fluorescein sodium salt, 2'-azo-*bis* (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Etoposide was from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) was purchased from Biosource International (Camarillo, CA, USA). Beta-actin mouse monoclonal antibody was purchased from Sigma (St. Louis, MO, USA). Cdk4, cdk6, cyclin D1 and cyclin D3 monoclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). COX-2 mouse monoclonal antibody was from Cayman Chemical (Ann Arbor, MI, USA). Recombinant human TNFα was purchased from Calbiochem (San Diego, CA, USA).

Preparation of berry juices. Fresh berries were stored at 4˚C and processed within 24 h. Juices were prepared in a cold room by passing 100 g of berries through a domestic centrifugal juice extractor (Juiceman Professional series 210, model JM210C). The liquid obtained was clarified by centrifugation at 50,000 xg, 45 min at 4˚C. The supernatant was then sterilized by filtration through a 0.22-μm filter and aliquots were immediately frozen in liquid nitrogen.

Cell culture. Media were obtained from Life Technologies (Burlington, ON, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT, USA). Transferrin, pyruvate and insulin were obtained from Sigma-Aldrich (Oakville, ON, Canada). All cell lines were cultured at 37˚C under a humidified atmosphere containing 5% CO_2 in media containing 100 units/ml penicillin and 100 μg/ml streptomycin. AGS (stomach adenocarcinoma, ATCC CRL-1739) were cultured in F12-K medium containing 10% FBS; MCF-7 (mammary gland adenocarcinoma, ATCC HTB-22) were cultured in Minimum

Essential Medium (MEM) containing 0.01 mg/ml insulin and 10% FBS; PC-3 (prostatic adenocarcinoma, ATCC CRL-1435) were cultured in Ham's F12 containing 10% calf serum; Caco-2 (colorectal adenocarcinoma, ATCC CRL-2102) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 10 μg/ml transferrin and 1 mM pyruvate; MDA-MB-231 (mammary gland adenocarcinoma, ATCC HTB-26) were cultured in a-MEM containing 10% FBS and 10 μg/ml insulin.

Cancer cell proliferation assay. Cells were plated in 96-well plates at 5,000 cells/well in 200 μl medium containing 10% FBS and incubated at 37˚C under a humidified atmosphere containing 5% CO_2 for 24 h. The next day, the medium was removed and replaced by 100 μl fresh medium containing 10 % FCS and berry juice at 0, 10, 20, 30, 40 or 50 μl/ml. Cell viability was determined by assaying the mitochondrial activity of cells after 48-h incubation, with the highly sensitive WST-1 assay. Briefly, 10 μl of the tetrazolium salt WST-1 reagent was added to each well and the soluble formazan dye produced by metabolically active cells was monitored every minute for 30 min at 37˚C on a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA, USA).

Oxygen radical absorbance capacity (ORAC) assay. The ORAC-fluorescein assay was performed essentially as described elsewhere (57) with minor modifications. Briefly, 20 μl of antioxidant (juice or Trolox standards) and 120 μl of 0.117 μM fluorescein in 75 mM phosphate buffer, pH 7.4, were pipetted into the well of the microplate. The mixture was preincubated for 15 min at 37˚C and then 60 μl of 40 mM AAPH was added rapidly using an electronic multichannel pipette. Fluorescence (λ_{ex}=485 nm; λ_{em}=520 nm) was recorded every min for 80 min using a SpectraMAX™ Gemini fluorescence plate reader (Molecular Devices). Calibration solutions of Trolox (0.5-8 μM) were also tested with each assay. Data were exported from SoftMax Pro 5.0 software (Molecular Devices) to Excel 11.1.1 (Microsoft) for further calculations. The area under the fluorescence decay curve (AUC) was calculated as $AUC=1+f_1/f_0+ f_2/f_0+...+ f_{80}/f_0$ where fo is the initial fluorescence at t=0 and f_i the fluorescence at t=i. ORAC-FL values were expressed as Trolox equivalents by using a standard curve and regression analysis performed using Prism 4.0 software (GraphPad Software, San Diego, CA, USA).

Fluorimetric caspase-3 assay. Cells were grown to about 80% confluence and treated for 24 h with berry juices at 25 μl/ml or with etoposide (20 μM), which is known to induce apoptosis in a variety of cells. Cells were collected, washed in cold PBS and lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA, USA) for 20 min at 4˚C. The lysates were clarified by centrifugation at 16,000 xg for 20 min. Caspase activity was determined essentially as described elsewhere (58) by incubation with 50 μM fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC, caspase-3-specific) in assay buffer (50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA) on 96-well plates. The release of AFC (λ_{ex}=400nm, λ_{em}=505nm) was monitored for at least 20 min at 37˚C on a SpectraMAX™ Gemini fluorescence plate reader (Molecular Devices). Caspase activity was expressed as relative fluorescence unit (rfu) per second per μg of protein used in the assay.

Cell viability assays. Cells were grown to about 80% confluence in 6-well plates and treated for 24 h with various berry juices at 25 μl/ml. Adherent and nonadherent cells were collected and viability was assessed by mixing aliquots of cell suspensions with equal volumes of 0.4% trypan blue (GibcoBRL). Cells that accumulated the dye were considered to be dead.

Protein assay. Protein concentrations were determined using the bicinchoninic assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

Immunoblot analysis. Samples containing equal amounts of protein were prepared in sample buffer (62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% glycerol, 2% SDS, and 0.1% bromophenol blue), heated at 100˚C for 3 min and separated on 0.75 mm-thick, sodium dodecyl sulphate-polyacrylamide gels with a MINI-PROTEAN II apparatus (Bio-Rad, Mississauga, ON, Canada). Proteins were electroblotted onto 0.45 μm-pore diameter PVDF membranes (Roche Diagnostics, Laval, QC, Canada) in transfer buffer (96 mM glycine, 10 mM Tris and 15% methanol) for 1.5 h at 100V. Membranes were blocked overnight at 4˚C in Tris-buffered saline (TBS: 20 mM Tris-HCl (pH 7.5), 137 mM NaCl) containing 0.1% (v/v) Tween 20 and 3% BSA. Blots were incubated with primary antibodies in blocking buffer for 2 h at room temperature, followed by a 1 h incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in the same incubation medium. Immunoreactive bands were revealed with an enhanced chemiluminescence Western blotting kit (Renaissance, NEN™ Life Science, Boston, MA, USA) and SuperRX films (Fuji).

NFÎ B gene reporter assay. To determine TNF-induced NFÎ B-dependent reporter gene transcription, PC-3 cells were transiently transfected with pNFÎ B-SEAP (Clontech, Mountain View, CA, USA) using the Fugene 6 transfection reagent, according to the manufacturer's instructions (Roche Diagnostics). After 24 h of transfection, the cells were stimulated with 1 nM TNFα and the conditioned media was collected after 18 h. Secreted alkaline phosphatase (SEAP) activity in the conditioned media was measured *via* the hydrolysis of pNPP, as described by the manufacturer.

Results

Inhibition of cancer cell proliferation. Juices from 13 freshly harvested edible berries (see Table I) were prepared in a domestic juice extractor, clarified by centrifugation and sterilized by filtration. The resulting juices are thus similar to those that can prepared at home using similar equipment, except for the small amounts of insoluble matter removed by high speed centrifugation. These sterile juice preparations were stored in liquid nitrogen for up to 6 months without any noticeable loss of activity (not shown). The berry juices were tested against five different cancer cell lines representing the major forms of cancer in North America.

As shown in Figure 1, many juices reduced the proliferation of cancer cells but the extent of inhibition was different between the various berries. Raspberry, black

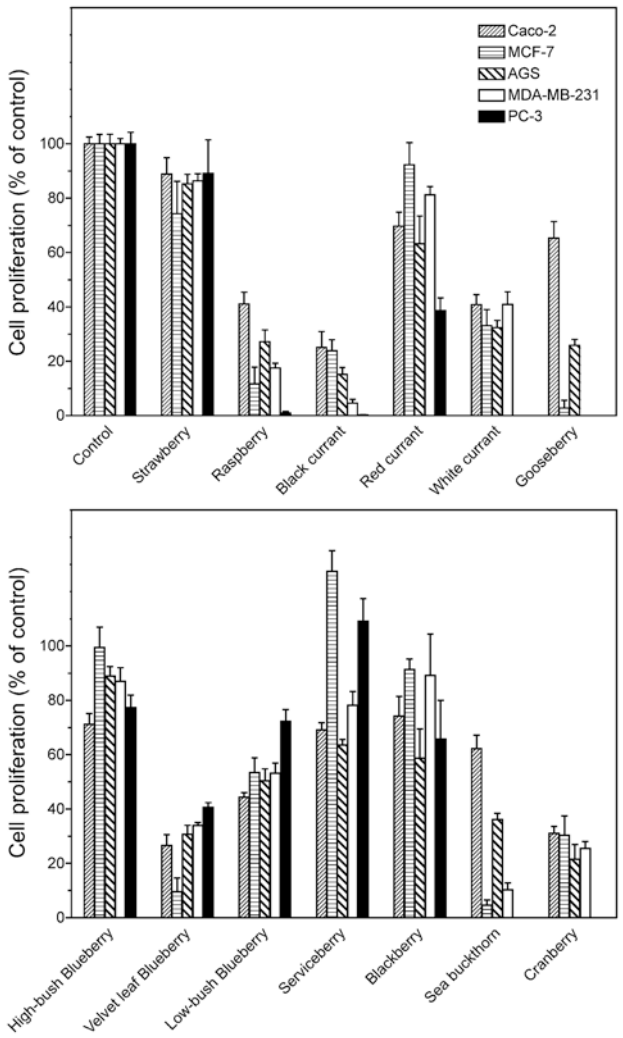

Figure 1. *Antiproliferative activities of berry juices against 5 human cancer cell lines. Cells were plated into 96-well dishes (5000 cells/well) 24 h before the addition of juices from the indicated berries at 50 μl/ml. After a 48-h incubation, cell proliferation was determined by the WST-1 assay, as described in the Materials and Methods section. Results are presented as mean±SEM, based on duplicate determinations in three independent experiments.*

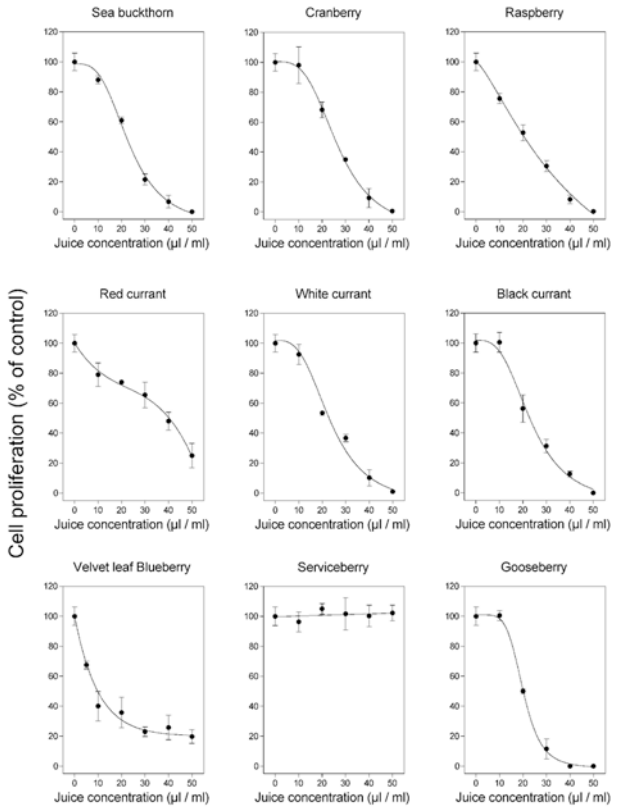

Figure 2. *Dose-dependent inhibition of prostate cancer cell proliferation by berry juices. PC-3 cells were plated into 96-well dishes (5000 cells/well) 24 h before the addition of juices from various indicated berries at 0, 10, 20, 30, 40, or 50 μl/ml. After 48-h incubation, cell proliferation was determined by the WST-1 assay, as described in the Materials and Methods section. Results are presented as mean±SEM, based on duplicate determinations in three independent experiments.*

currant, white currant, gooseberry, velvet leaf blueberry, sea buckthorn, and cranberry exhibited the greatest degree of inhibiting proliferation of most cancer cell lines. Low-bush blueberry juice was a moderate inhibitor (28-56% inhibition) while strawberry, red currant, serviceberry and blackberry were weak inhibitors of cancer cell proliferation. Some differences in sensitivity toward berry juices can be observed between cell lines; in particular, proliferation of the intestinal cancer cell line Caco-2 was much less susceptible to inhibition by gooseberry and sea buckthorn than were other cell lines. Conversely, proliferation of both the breast cancer cell line MDA-MB-231 and the prostate cancer line PC-3 were much more reduced by gooseberry, sea buckthorn and black currant juice than were other cell lines. To further characterize the antiproliferative activity of berries, 9 of 13 juices were tested on PC-3 cells and MDA-MB-231, using various concentrations. As shown in Figure 2, proliferation of PC-3 prostate cancer cells was reduced in a concentration-dependent manner by all berries tested, except for serviceberry. Similar results were obtained with the breast cancer cell line MDA-MB-231 (Figure 3); however these cells were less sensitive than PC-3 cells, as shown by the higher IC_{50} determined for each berry juice (Table II). PC-3 and MDA-MB-231 were particularly sensitive to velvet leaf blueberry with IC_{50} values of 10 and 25 μl/ml, respectively.

Antioxidant capacity and correlation with antiproliferative activity. The antioxidant capacity of berry juices was determined using the oxygen radical absorbance capacity (ORAC) assay (Figure 4). The range of ORAC values varied

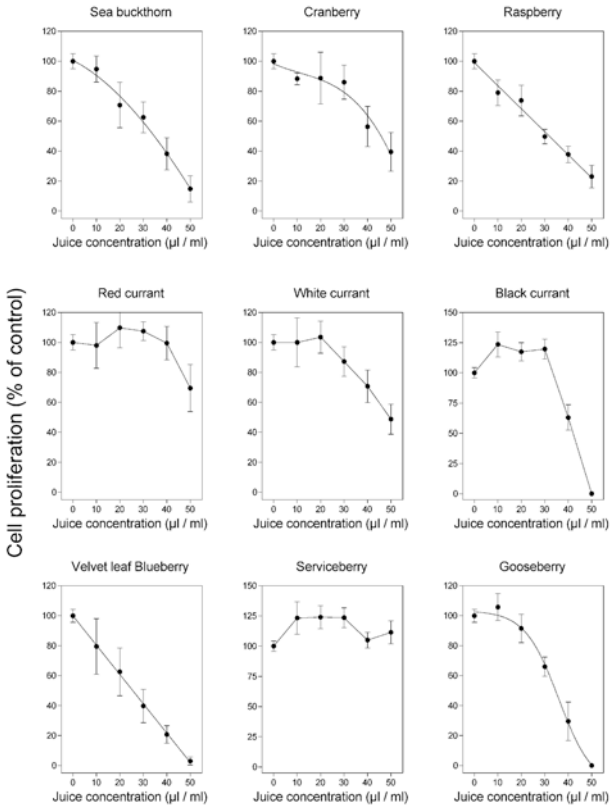

Figure 3. *Dose-dependent inhibition of breast cancer cell proliferation by berry juices. MDA-MB-231 cells were plated into 96-well dishes (5000 cells/well) 24 h before the addition of juices from various indicated berries at 0, 10, 20, 30, 40, or 50 µl/ml. After 48-h incubation, cell proliferation was determined by the WST-1 assay, as described in the Materials and Methods section. Results are presented as mean±SEM, based on duplicate determinations in three independent experiments.*

6-fold between cranberry (26.9 µmol TE/ml) and white currant (4.5 µmol TE/ml). As previously reported (14, 23, 24, 48), cranberry, strawberry and raspberry contain high levels of antioxidants, approximately 2-fold that of the three species of blueberries. Importantly, there is no correlation between the antioxidant capacity and the anti-proliferative activity for the 5 cell lines tested (R^2<0.008, p>0.05). For example, strawberry juice was a very weak (<15% at 50 µl/ml) inhibitor of cancer cell proliferation (Figure 1), but exibited the second highest ORAC value (22.6 µmol TE/ml) of the berries tested (Figure 4). Similarly, serviceberry juice was also a weak inhibitor of cancer cell proliferation (0-30% inhibition at 50 µl/ml) according to the data in Figures 1-3, but was one of the berries tested which had a relatively high antioxidant capacity (14.6 µmol TE/ml). In contrast, white currant juice had the lowest antioxidant capacity (4.4 µmol TE/ml) but was a very potent inhibitor of PC-3 proliferation (Figures 1 and 2) and was moderately inhibitory (50-68% inhibition at 50 µl/ml) for other cell lines tested (Figures 1 and 3).

Table II. *Inhibition of PC-3 and MDA-MB-231 cancer cell proliferation by 9 berry juices.*

	IC_{50} (µl/ml)	
Berry	PC-3	MDA-MB-231
Sea buckthorn	22	35
Cranberry	25	46
Raspberry	20	32
Red currant	40	>50
White currant	24	50
Black currant	23	35
Velvet leaf blueberry	10	25
Serviceberry	ND	ND
Gooseberry	20	35

ND: no effect at the highest concentration tested.

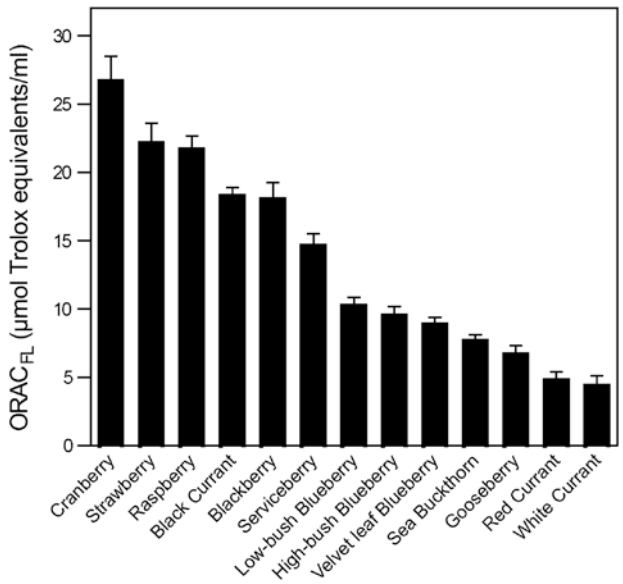

Figure 4. *Antioxidant capacity of small berry juices. Antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) assay, as described in the Materials and Methods section. ORAC values (means±SD), based on duplicate determinations from two distinct experiments, are expressed as µmol Trolox equivalent per ml of berry juice.*

Cell viability and caspase-dependent apoptosis. The inhibition of cell proliferation by berry juices, as determined using the metabolic assay with WST-1, could either be the result of inhibition of cell growth or a direct cytotoxic effect. To test whether cells were killed as opposed to cell growth being arrested, we treated PC-3 and MDA-MB-231 cell lines with various berry juices for 24 h, then counted dead and viable cells using Trypan blue dye and measured caspase-3 activity in cell lysates. As a control for the induction of caspase-dependent apoptosis we used the anticancer drug etoposide (59).

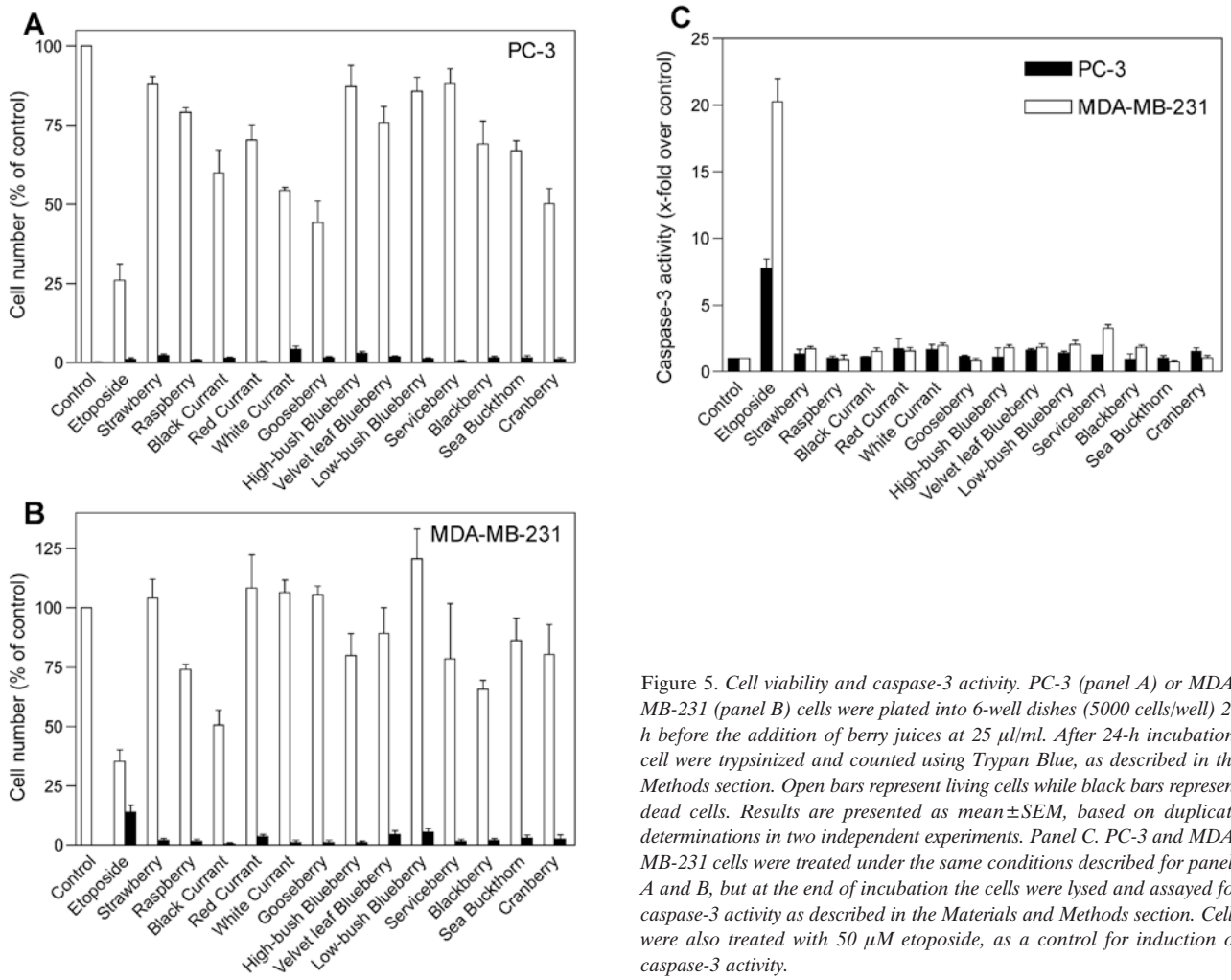

Figure 5. *Cell viability and caspase-3 activity. PC-3 (panel A) or MDA-MB-231 (panel B) cells were plated into 6-well dishes (5000 cells/well) 24 h before the addition of berry juices at 25 μl/ml. After 24-h incubation, cell were trypsinized and counted using Trypan Blue, as described in the Methods section. Open bars represent living cells while black bars represent dead cells. Results are presented as mean±SEM, based on duplicate determinations in two independent experiments. Panel C. PC-3 and MDA-MB-231 cells were treated under the same conditions described for panels A and B, but at the end of incubation the cells were lysed and assayed for caspase-3 activity as described in the Materials and Methods section. Cells were also treated with 50 μM etoposide, as a control for induction of caspase-3 activity.*

As shown in Figure 5, the PC-3 cell number was reduced by treatment with several berry juices, including raspberry, black currant, red currant, white currant, gooseberry, velvet leaf blueberry, blackberry, sea buckthorn, and cranberry. MDA-MB-231 cell number was significantly reduced by raspberry, black currant, high-bush blueberry, velvet leaf blueberry, blackberry, sea buckthorn and cranberry juices.

Caspase-3 activity was strongly induced in both PC-3 (7.8-fold induction) and MDA-MB-231 (20-fold induction) cell lines by etoposide but was not significantly changed by berry juices. These results suggest that berry juices inhibit cell proliferation rather than directly kill cells *via* caspase-dependent apoptosis.

Cell-cycle. To assess whether berry juices induce a cell-cycle arrest, PC-3 cells were synchronized in serum-free medium for 24 h and then treated for 24 h with berry juices at 25 μl/ml, and analyzed for the expression of key regulators of the cell-cycle (cdk4, cdk6, cyclin D1, and cyclin D3). As shown in Figure 6, gooseberry, sea buckthorn and cranberry juices induced a marked reduction in the expression of cdk4, cdk6, cyclin D1 and cyclin D3. The berry juices which did not inhibit cancer cell proliferation (strawberry, red currant, serviceberry and blackberry) had little or no inhibitory effect on the expression of cdk4 and cyclin D3. However, aside from strawberry and high-bush blueberry, all berry juices tested inhibited the expression of cyclin D1. Taken together, these results show that several berry juices can interfere in the cell-cycle and strongly suggest that cells are arrested in the G_1 phase.

Antiproliferative and antioxidant capacities of four varieties of black currant juice. One berry producer is currently growing, in the same field, 4 different varieties of black currants and we were interested to determine whether these were different in terms of antiproliferative activity and antioxidant capacity. For one cultivar (Titania), we were also able to test the effects of long term freezing (1 year).

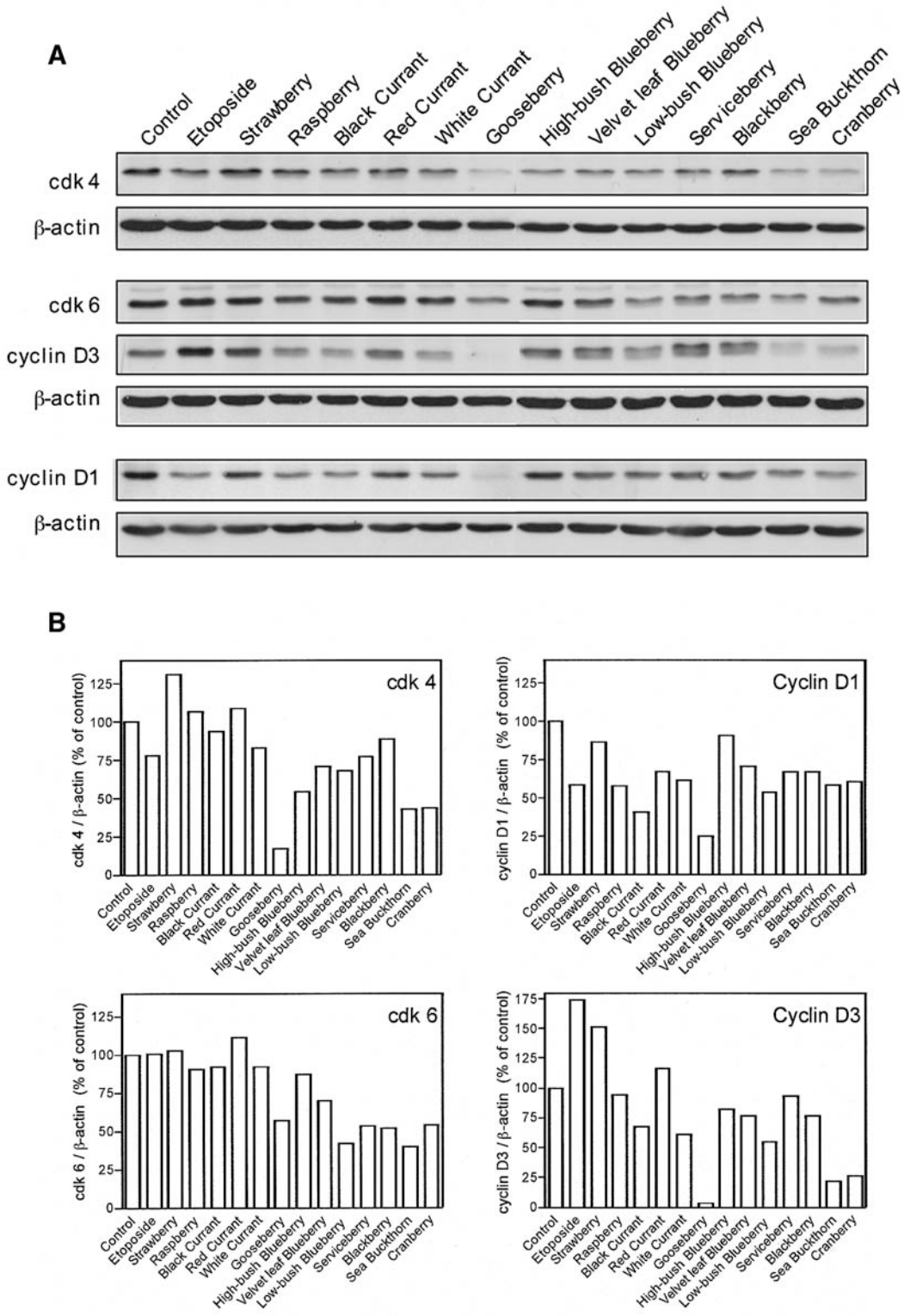

Figure 6. *Effect of berries on protein expression of cdks and cyclins. PC-3 cells were synchronized by serum deprivation for 24 h and then treated with various berry juices. After 24-h incubation, cell lysates were prepared and subjected to SDS-PAGE followed by immunoblot analysis for cdk4, cdk6, cyclin D1 and cyclin D3. All blots were subsequently reprobed for β-actin as a control for gel loading. A) The results shown here are from a representative experiment repeated twice with similar results. B) Immunoreactive protein bands detected by immunoblot analysis were quantified by densitometry.*

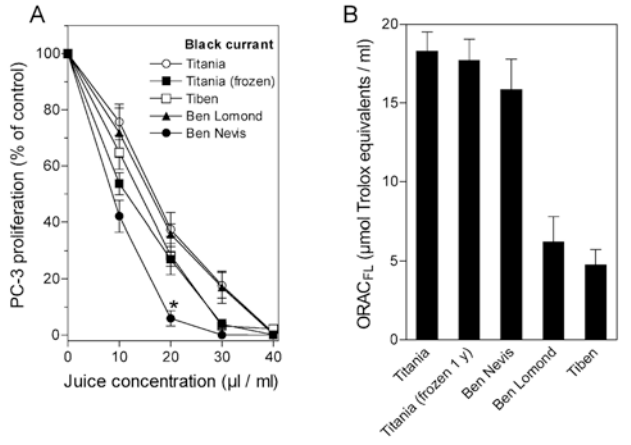

Figure 7. *Antiproliferative activity and antioxidant capacity of four black currant cultivars. A) PC-3 cells were plated into 96-well dishes (5,000 cells/well) 24 h before the addition of juices from various indicated black currant cultivars at 0, 10, 20, 30, or 40 µl/ml. After 48-h incubation, cell proliferation was determined by the WST-1 assay as described in the Material and Methods section. Results are presented as mean±SEM, based on duplicate determinations in three independent experiments. B) Antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) assay, as described in the Materials and Methods section. ORAC values (means±SD), based on duplicate determinations in two distinct experiments, are expressed as µmol Trolox equivalent per ml of black currant juice. Statistically significant differences are marked with an asterisk ($p<0.05$).*

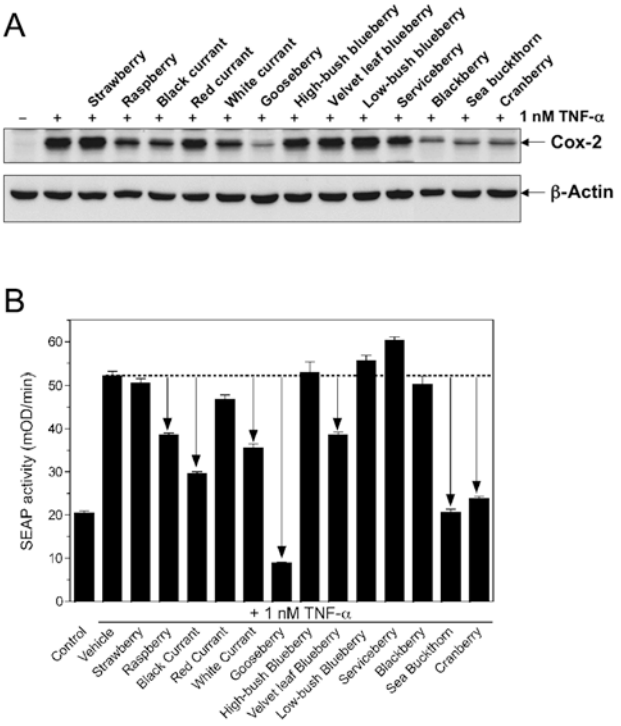

Figure 8. *Effect of berry juices on TNF-induced expression of COX-2 and on TNF-induced, NFÎ B-dependent SEAP reporter gene expression. A) PC-3 cells were pretreated with vehicle (water) or various berry juices at 25 µl/ml for 1 h, stimulated with 1 nM TNF- a for 24 h at 37˚C and then assayed for COX-2 expression using immunoblot analysis. PVDF membranes were reprobed for β-actin as a control for gel loading. B) PC-3 cells were transiently transfected with pNFÎ B-SEAP and treated with vehicle (water) or various berry juices at 25 µl/ml for 1 h, exposed to 1 nM TNF-α for 18 h and then assayed for alkaline phosphatase activity as described in Materials and Methods.*

As shown in Figure 7A, PC-3 cell proliferation was inhibited in a dose-dependent manner after incubation with each of the 4 cultivars of black currant. At 20 µl/ml, black currant juices inhibited cell proliferation to different extents: Titania: 62.5±5.9%; Titania frozen for 1 year: 73.0±2.5%; Ben Lomond: 64.0±3.4%; Tiben: 71.7±6.8%; Ben Nevis: 94.1±2.7%. The Ben Nevis cultivar was the most effective at inhibiting PC-3 cell proliferation and the degree of inhibition was significantly different ($p<0.05$) from that for other cultivars.

The antioxidant capacity of the black currant cultivars was determined using the ORAC assay (Figure 7B). As observed for other berries, there was no correlation between antiproliferative activity and antioxidant capacity ($R^2=0.029$, $p>0.05$). The ORAC value for the Ben Nevis cultivar (15.8±1.9 µmol TE/ml) was similar to that of the Titania cultivar (18.3±1.2 µmol TE/ml), although Ben Nevis was significantly more antiproliferative than the Titania cultivar. Ben Lomond and Tiben cultivars had the lowest ORAC values (6.2±1.6 and 4.8±1.0 µmol TE/ml, respectively), which represent a 3.0- to 3.8-fold difference with the Titania cultivar, although they inhibited PC-3 cell proliferation to the same extent. Long-term freezing of Titania black currant did not have any significant effect on either antiproliferative activity or antioxidant capacity.

Anti-inflammatory activity of berry juices. To investigate the potential anti-inflammatory effects of berry juices, we tested their ability to inhibit TNF-induced expression of COX-2 in PC-3 cells. As shown in Figure 8A, several berry juices inhibited the expression of COX-2 and can be ranked according to their inhibitory potency as: gooseberry (83%) > blackberry (73%) = sea buckthorn (73%) = cranberry (73%) > raspberry (43%) = black currant (43%) > white currant (49%) > serviceberry (38%) > high-bush blueberry (21%) > velvet-leaf blueberry (14%). As it is well established that the TNF-induced expression of COX-2 is mediated by the nuclear transcription factor NFÎ B, we tested the ability of berries to repress TNF-induced NFÎ B-dependent expression of a reporter gene. PC-3 cells transiently transfected with the NFÎ B-SEAP reporter construct were either untreated or pre-treated with berry juices and then stimulated with TNF. A 2.5-fold increase in SEAP activity was observed upon stimulation of the control cells with 1 nM TNF (Figure 8B). Among the 13 berries tested, seven juices significantly

repressed the TNF-induced, NFÎ B-dependent expression of SEAP. In order of potency they were: gooseberry (100%) > sea buckthorn (99%) > cranberry (89%) > black currant (72%) > white currant (53%) > raspberry (43%) = velvet leaf blueberry (43%). Results from the gene reporter assay (Figure 8B) are in agreement with those obtained by measuring COX-2 expression (Figure 8A), with the exception of blackberry juice, which only slightly inhibited the SEAP reporter activity (6%) but markedly inhibited TNF-induced expression of COX-2 (73%).

Discussion

In this study, we evaluated the potential anticancer effects of edible berries using juices made with a domestic extractor. The tested juices are thus very similar to those that consumers can make and drink at home everyday. Furthermore, these juices contain the vast majority of the berry hydrosoluble phytochemicals, allowing a fair assessment of their total anticancer activities as opposed to using purified molecules or fractions isolated from berries.

In two systematic screenings of total antioxidants in dietary plants (31) and common foods (60), it was found that there is more than a 1000-fold difference among antioxidant concentrations in various commonly consumed dietary plants and that berries contain the highest levels of antioxidants. Our study confirms that various berries display significant differences in antioxidant capacity. As shown previously (14, 31, 48, 61), cranberry, strawberry, raspberry, blackberry and black currant contain the highest antioxidant concentrations among tested berries.

It was previously reported (28) that low-bush blueberry had a higher antioxidant capacity than high-bush blueberry. In this study, using only one variety per species, we found that high-bush, low-bush and velvet leaf blueberry had very similar ORAC values. However, there were striking differences in the extent to which these three species of blueberry inhibited cancer cell proliferation. Velvet leaf blueberry was clearly a better inhibitor than high- or low-bush blueberry. These results indicate that the antiproliferative activity of blueberries is not correlated to their antioxidant content. This is in agreement with previous studies on eight strawberry cultivars (23), four raspberry cultivars (52) and 8 common fruits (48) where no correlation was found between the total antioxidant activity and the inhibition of HepG2 liver cancer cell proliferation. In a previous study on berries grown in Sweden (22), a correlation was found between the inhibition of cancer cell proliferation and vitamin C levels. However, in that study the correlation found was only moderately significant at the highest concentration of extracts tested, and a blueberry extract containing low levels of vitamin C was a strong inhibitor of HT29 and MCF-7 cancer cell proliferation (22). In this study we found that, among the 13 berries tested, the strongest inhibition of cancer cell proliferation was obtained with juices of velvet leaf blueberry, black currant, raspberry, gooseberry and cranberry, whereas strawberry, serviceberry, blackberry and high-bush blueberry had little or no inhibitory effect on 5 different cancer cell lines. Strawberry extracts have been reported to have antiproliferative activity (23, 48, 50, 51), but our results showed that the Jewel cultivar was a weak inhibitor of the proliferation of five different cancer cell lines, although it had high levels of antioxidants. In a previous study on the antioxidant and antiproliferative activities of eight strawberry cultivars, an extract from the Jewel cultivar was found to be antiproliferative against HepG2 (23). This discrepancy could be explained by the fact that the cancer cell lines used are not the same or it could be due to a higher concentration of phytochemicals in strawberry extracts compared to our juices. Similarly, we found that high-bush blueberry juice only slightly inhibited the proliferation of most cancer cell lines, whereas this species of blueberry has been reported to inhibit cancer cell proliferation (22, 62). In this case again, most studies used extracts of blueberry instead of juices, allowing the use of higher but less physiological concentrations of phytochemicals.

There was a striking difference between the antiproliferative activities of the three currant juices tested. White currant juice was a much stronger inhibitor of cancer cell proliferation than red currant, a somewhat unexpected result since berry color is conferred by polyphenolic compounds, which have been shown to be antiproliferative in a number of fruits and berries. The extent of the inhibition of cancer cell proliferation by blueberry juices from three different species was also quite different, although their antioxidant capacity was very similar. Velvet leaf blueberry, whose antiproliferative activity has not been tested before, was the greatest inhibitor.

We found that several berry juices induced a down-regulation of cdk4, cdk6, cyclin D1 and cyclin D3 expression in PC-3 cells, suggesting cell-cycle arrest in the G_1 phase of the cell-cycle. Pomegranate fruit extract was also reported to induce G_1 arrest in PC-3 cells through reduced expression of cdk2, cdk4, cdk6 and cyclins D1, D2, and E (63). A grape seed polyphenolic fraction rich in procyanidins was also shown to induce G_1 arrest in prostate carcinoma DU145 cells with reduced expression of cdk2, cdk4 and cyclin E (64). Resveratrol, a polyphenolic phytoalexin found in grapes, many other fruits, nuts and red wine, induced G_1 arrest in human epidermoid carcinoma A431 cells through an induction of WAF1/p21 that inhibited cdk2, cdk4, cdk6 and cyclins D1, D2 and E (65). Thus, inhibition of cancer cell proliferation through arrest in the G_1 phase of the cell-cycle seems to be a mechanism shared by many fruit and berry phytochemicals, particularly polyphenolic compounds.

Conclusion

We have shown that juice from berries inhibits the proliferation of 5 cancer cell lines and that the 13 berries tested display striking differences in their potential for chemoprevention. These antiproliferative activities were not correlated with antioxidant capacities and did not involve a significant activation of caspase-3, but seem to be due to cell-cycle arrest in the G_1 phase. The inclusion of berries in the diet, selected for their high anticancer activities, may contribute significantly towards preventing cancer in humans. Nonetheless, our results will have to be confirmed in animal models and, ultimately, in humans before any conclusion can be made on the potency of each individual berry.

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