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Inhibition of Cancer Cell Proliferation in Vitro by Fruit and Berry Extracts and Correlations with Antioxidant Levels

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The effects of 10 different extracts of fruits and berries on cell proliferation of colon cancer cells HT29 and breast cancer cells MCF-7 were investigated. The fruits and berries used were rosehips, blueberries, black currant, black chokeberries, apple, sea buckthorn, plum, lingonberries, cherries, and raspberries. The extracts decreased the proliferation of both colon cancer cells HT29 and breast cancer cells MCF-7, and the effect was concentration dependent. The inhibition effect for the highest concentration of the extracts varied 2−3-fold among the species, and it was in the ranges of 46−74% (average = 62%) for the HT29 cells and 24−68% (average = 52%) for the MCF-7 cells. There were great differences in the content of the analyzed antioxidants in the extracts. The level of the vitamin C content varied almost 100-fold, and the content of total carotenoids varied almost 150-fold among the species. Also in the composition and content of flavonols, hydroxycinnamic acids, anthocyanins, and phenolics were found great differences among the 10 species. The inhibition of cancer cell proliferation seen in these experiments correlated with levels of some carotenoids and with vitamin C levels, present at levels that can be found in human tissues. The same inhibition of cell proliferation could not be found by ascorbate standard alone. This correlation might indicate a synergistic effect of vitamin C and other substances. In MCF-7 cells, the anthocyanins may contribute to the inhibition of proliferation.

KEYWORDS: Ascorbate; dehydroascorbate; phenolics; hydroxycinnamic acids; ellagic acid; flavonol; anthocyanin; cancer cell proliferation; correlation

INTRODUCTION

The potential of specific food to lower the risk of cancer has received increased attention. Consistent and strong epidemiological evidence has shown that a high consumption of fruits and vegetables is associated with a reduced risk of cancers (*1*−*3*). Apart from being a primary food source of some essential nutrients, fruits and vegetables also contain a variety of phytochemicals, which might have potential beneficial health effects. Therefore, there has been a growing interest in understanding the reason for the cancer protective effect of fruits and vegetables and to identify the components with the anticarcinogenic effect. Compounds in plants with antioxidative activity have been given special interest. Antioxidants can scavenge reactive oxygen species, which might have the potential to damage cell components, such as DNA, proteins, and lipids. Oxidative damage might be involved in initiating events in cancer, and free radicals may help to induce the initiation of apoptosis (*4*). Furthermore, antioxidants may have other effects associated with a decreased risk of cancer by affecting the enzyme activity of cyclooxygenase-2 or inhibiting oncogene expression (*5*, *6*).

Cancer cell proliferation is important in the progression of a cancer tumor. Aberrations in the regulation of a number of key pathways controlling cell proliferation are necessary for establishment of all tumors. Unregulated cell proliferation together with suppressed apoptosis is the minimal common platform for cancer evolution and progression (*7*). Several antioxidants in fruits and vegetables have been suggested to contribute to the anticarcinogenic effect, and some, such as flavonoids and carotenoids, have also been shown to inhibit cancer cell proliferation in vitro (*8*, *9*). The vast majority of epidemiological studies have found protective effects of vitamin C (*10*), whereas plasma carotenoids have been found to inversely correlate with indices of lipid peroxidation and oxidative DNA damage (*11*). Flavonoids as well as ellagic acid have been shown to induce apoptosis in vitro (*12*, *13*).

In this study, the effects of 10 different extract of fruits and berries on cell proliferation of colon cancer cells HT29 and estrogen-receptor positive breast cancer cells MCF-7 were

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Table 1. Plant Materials Used in the Experiments: Species/Cultivar/Origin of the 10 Fruits and Berries

fruits/berries, common name	species	cultivar/collection place
rosehips	*Rosa villosa* × *villosa*	9024
blueberries	*Vaccinium corymbosum*	1046
black currant	*Ribes nigrum*	Sörbacks-gaard
black chokeberries	*Aronia melanocarpa*	Balsgård assortment, unknown origin
apple (peel)	*Malus domestica*	Karin Schneider
sea buckthorn	*Hippophaë rhamnoides*	Balsgård assortment, unknown origin
plum	*Prunus domestica*	Arbuznaja
lingonberries	*Vaccinium vitis-idaea*	Sweden, G,F-county
cherries	*Prunus avium*	0236B1
raspberries	*Rubus idaeus*	St. Walfrid

investigated. The different fruits and berries were chosen from previous knowledge of the content of the antioxidants in these species so that a variety of levels of the antioxidants would be represented. The extracts were analyzed for their contents of vitamin C, carotenoids, flavonoids, and phenolic acids, which have been suggested to have anticarcinogenic properties. The possible correlation of the content of these antioxidants and the effects on cancer cell proliferation was investigated.

MATERIALS AND METHODS

Plant Materials. Ten different fruits and berries, rosehips (*Rosa villosa*), blueberries (*Vaccinium corymbosum*), black currant (*Ribes nigrum*), black chokeberries (*Aronia melanocarpa*), apple (*Malus domestica*), sea buckthorn (*Hippophaë rhamnoides*), plum (*Prunus domestica*), lingonberries (*Vaccinium vitis-idaea*), cherries (*Prunus avium*), and raspberries (*Rubus idaeus*), were collected at Balsgård and Kivik research stations, Swedish University of Agricultural Sciences, in Sweden. The fruits and berries were at commercial harvest maturity stage and were of defined variety/clone, except for the black chokeberries, lingonberries, and sea buckthorn. The black chokeberries were from the Balsgård station assortment, the lingonberries were originally collected from wild populations in a forest district in Sweden, and the sea buckthorn was originally sampled from wild populations (**Table 1**). The samples were immediately after harvest placed cold in a box at the field. On the harvest day, debris such as leaves and twigs was removed, and the kernels were removed from the cherries, the rosehips, and the plum. Only the peel was used from the apples. The samples were frozen overnight at −20 °C and the next day stored at −80 °C until analysis or cell proliferation experiment.

Preparation of Extracts. Each sample of the fruits and berries was homogenized in ethanol/water 1:1 with 50 mM H_3PO_4 with an Ultraturrax (IKA T8). N_2 was blown though the extraction solution for 5 min, and the tubes were thereafter sealed and placed in darkness at 4 °C for 20 h for further extraction. The samples were centrifuged at 12000*g* and 4 °C for 10 min. Aliquots of the supernatants were stored at −80 °C before analysis with HPLC or use for cancer cell proliferation tests.

The extracts used for cell proliferation tests were evaporated to near dryness under N_2 and dissolved in ethanol/water 1:1.

Cancer Cell Proliferation Studies. Human colon cancer cells HT29 and estrogen receptor-positive breast cancer cells MCF-7 were obtained from the American Tissue Culture Collection (Rockville, MD) and incubated for 24 h at 37 °C in 95% air/5% CO_2. The cancer cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, and 10 μg/mL streptomycin. For the MCF-7 cells, 0.1 mM nonessential amino acids and bovine insulin (0.01 mg/mL) were also added. The cell proliferation rate was determined by the ability of the cells to cleave tetrazolium salt (WST-1, from Roche Diagnostics, Mannheim, Germany) to formazan (*14*). In brief, 2×10^4 cells in 0.2 mL medium was plated on a 96-well microplate and incubated for 24 h for attachment. The medium was then replaced with new medium of equal amount before the fruit or berry extracts were added. Four different concentrations of the extracts were used: 0.025, 0.05, 0.25, and 0.5% of plant dry matter of total weight in the wells (weight approximated to be equal to volume in the wells). An equal amount of solvent was added in the control wells. After 24 h of incubation, 20 μL of the reagent WST-1 was added, followed by incubation for 1 h for HT29 cells and for 3 h for MCF-7 cells. The formation of formazan was determined photometrically at 490 nm against 620 nm as a background by a microplate reader (Bio-Rad). Three replicates were used for each extract, and the proliferation tests were repeated on three different occasions.

Table 2. (A) Concentrations[a] of Anthocyanins Used in Cancer Proliferation Experiments from the 10 Fruits and Berries and from Three Anthocyanin Standards; (B) Relative Proportions of the Individual Anthocyanidins as Percent of Total Concentration of Anthocyanins

	(A) concn of anthocyanins			(B) rel proportions of anthocyanidins			
fruits/berries	high	middle	low	delphinidin	cyanidin	malvidin	others
rosehips	1	0.1	0.01	nd	100	nd	nd[b]
blueberries	350	35	3.5	29	5	31	35
black currant	320	32	3.2	52[c]	45[c]	nd	3
black chokeberries	180	18	1.8	nd	100	nd	nd
apple (peel)	40	4	0.4	nd	100	nd	nd
sea buckthorn	0	0	0	nd	nd	nd	nd
plum	44	4.4	0.44	nd	100	nd	nd
lingonberries	64	6.4	0.64	nd	96	nd	4
cherries	250	25	2.5	nd	100	nd	nd
raspberries	110	11	1.1	nd	100	nd	nd
delphinidin-3-g (D)	200	20		100			
cyanidin-3-g (C)	200	20			100		
malvidin-3-g (M)	200	20				100	
D + C + M	200	20		33	33	33	

[a] Concentrations presented are the final concentrations in the wells of the cell culture plates, in μg/mL. Results are based on three to four independent replicate samples. [b] Not detected. [c] Estimated value due to incomplete separation.

In addition, a fraction containing the anthocyanins from the total extracts, prepared as described below, was used for proliferation studies of HT29 and MCF-7 cells. The same method as for the total extracts was used, except that the medium with the extract was replaced with pure medium before the absorbance was measured at 450 nm to avoid the interfering absorbance of the anthocyanins in these higher concentrations of the substances. The final concentrations of the anthocyanins were 0.01−350 μg/mL (**Table 2**) in the wells, approximately reflecting the relative concentrations in the fruits and berries. Standards of delphinidin-3-glucoside, cyanidin-3-glucoside, malvidin-3-glucoside, and a mixture of these, of final concentrations of 20 or 200 μg/mL in the wells, were also used for proliferation studies.

Standards of ascorbic acid of final concentrations of 5−200 μg/mL in the wells were also used for proliferation studies according to the method described above.

Analysis of Antioxidants. The samples for ascorbate, ∼1.0 g, were homogenized with an Ultraturrax (IKA T8) in dim green light in a darkroom and extracted in 10 mL of 1.5% metaphosphoric acid. The samples were centrifuged at 16500*g* for 15 min at 4 °C. The supernatant was filtered through a C_{18} Sep-Pak column. The first 3 mL was discarded, and an aliquot of the following 1 mL was used for analysis by HPLC, as in the method of Wimalasiri and Wills (*15*), with some modifications. The HPLC system consisted of an Algilent 1100 pump, a Maraton autosampler with a rheodyne injector, and a Hewlett-Packard UV-1100 detector. A Waters carbohydrate analysis, 3.9 × 300 mm, column with a Waters C_{18} precolumn was used. The separation was performed with isocratic elution at 1.2 mL/min at room temperature for 5 min. The mobile phase was acetonitrile with 35% (v/v) 15 mM NH_4PO_4, adjusted with 1 M H_3PO_4 to pH 4.3. Detection was carried out at 248 nm. Integration and evaluation were performed with an Agilent Chemstation version 8.03. The peak of ascorbate in the samples was identified by comparing the retention time with ascorbate standard.

The dehydroascorbate concentration was determined by subtracting the ascorbate concentration from the total ascorbate concentration, obtained after the use of a reduction procedure (*16*).

The carotenoids in the extracts were analyzed according to the method of Khachik et al. (*17*), with some modifications. Extractions were made with lyophilized samples. The extracts were centrifuged at 10000*g* for 5 min at room temperature. Samples were analyzed immediately by HPLC (Kontron system) with a DAD440 detector. Evaluation was performed with Kromasystem 2000, using retention times and spectral data as compared by standards. *all-trans*-β-Carotene was used for quantification, and β-8-carotenal was used as internal standard.

The phenolics were analyzed according to modified methods of Madhavi et al. (*18*) and Schieber et al. (*19*). The ethanol/water extracts were evaporated with N_2 until near dryness and resolved in water, before an additional extraction with ethyl acetate three times. The ethyl acetate extract was evaporated to dryness and resolved in DMSO/MeOH/H_2O 25:10:15. Samples were then centrifuged at 10000*g* for 5 min before analysis on a Kontron HPLC system, equipped with a DAD440 detector. Evaluation was performed with Kromasystem 2000, using retention times and spectral data as compared by standards.

Total phenolics were determined according to the Folin−Ciocalteu method. Briefly, appropriate dilutions of the samples were mixed with the Folin−Ciocalteu reagent and neutralized with 15% sodium carbonate. The absorbance was measured after 120 min at 765 nm. Gallic acid was used as a standard, and results were expressed as mean (milligrams of gallic acid equivalents per gram of dry weight) for triplicate samples. Total anthocyanidins were determined according to a spectrophotometric method (*20*). Cyanidin-3-glucoside was used as a standard, and the results were expressed as milligram equivalents of standard per gram of dry weight for triplicate samples.

A fraction containing the anthocyanins was separated from the total extracts of the 10 fruits and berries as in ref *21* with some modifications. An extract of each of the fruits and berries was prepared as previously described, in ethanol/water 1:1 and 50 mM H_3PO_4. The total extract was diluted 1:6 with Milli-Q water. Fourteen milliliters of the diluted total extract was loaded on a solid phase extraction cartridge (Sep-Pak C18; 50/Bx), washed with 5 mL of Milli-Q water, and anthocyanins were eluted with 1 mL of 99.5% ethanol. The anthocyanin fractions from the different fruits and berries were evaporated to near dryness under nitrogen and dissolved in 50% ethanol. The anthocyanin fraction was analyzed by HPLC (Kontron system) with a DAD440 detector, using a Luna C18(2) column (150 × 4.6 mm, 5.7 μm). The mobile phase was a binary gradient with A, 2% (v/v) formic acid in H_2O, and B, 2% (v/v) formic acid in methanol, with a flow rate of 1 mL/min. The binary gradient was as follows: 0−37% B (0−60 min), 37−80% B (60−62 min), 80% B (62−64 min), 80−0% B (64−66 min), and 0% B (66−70 min). Spectra were collected between 200 and 600 nm, and detection was carried out at 530 nm. The anthocyanins were identified at HPLC by their retention times and spectral data as compared by standards.

Statistics. All values of antioxidants were based on three to four independent replicate samples for each fruit or berry species. The results presented are the mean ± SD. Results were analyzed with Origin software (Microcal Software Inc., Northampton, MA). Correlations between the levels of the different antioxidants and the cancer cell proliferation inhibition by the extracts were calculated by Pearson and Spearman correlation. In Spearman correlation the values were ranked, before the correlation between the two groups was calculated, using Minitab Statistical Software (Minitab Corp.).

RESULTS

Inhibition of Cancer Cell Proliferation. The extracts decreased the proliferation of both colon cancer cells HT29 and breast cancer cells MCF-7 (**Figure 1**). The inhibition of the proliferation was concentration dependent, and the degrees of inhibition were different for the different extracts. The HT29 cells were on average inhibited to a higher degree than the MCF-7 cells. The inhibition effect for the highest concentration of the extracts varied 2−3-fold among the species, and it was

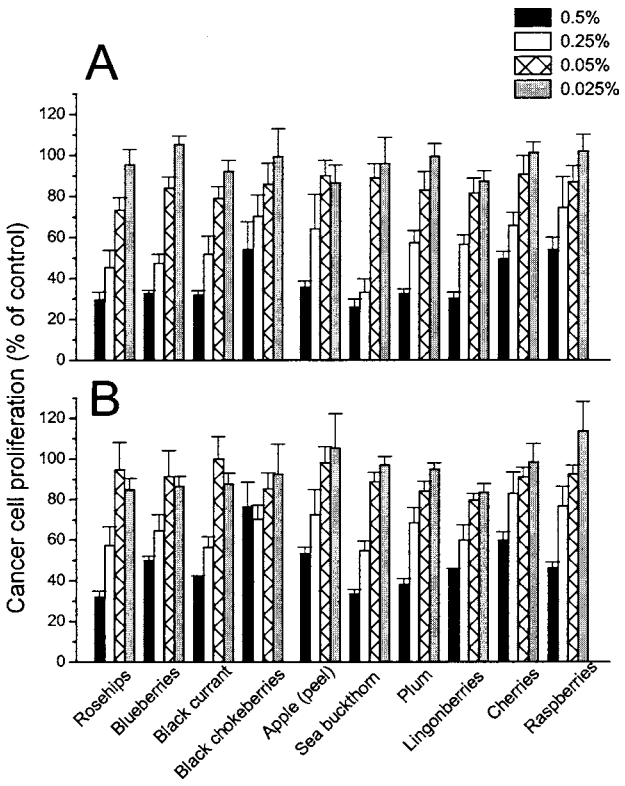

Figure 1. (**A**) Colon cancer cell HT29 and (**B**) breast cancer cell MCF-7 proliferation. Results are presented as means ± SD based on three independent replicates for each extract, and the proliferation tests were repeated on three different occasions.

in the ranges of 46−74% (average = 62%) for the HT29 cells and 24−68% (average = 52%) for the MCF-7 cells. There was a significant difference of the inhibition of cell proliferation in the concentrations between 0.5 and 0.25% for HT29 cells in rosehips, black currant, apples, prunes, and lingonberries and for MCF-7 cells in rosehips, black currant, black chokeberries, sea buckthorn, prunes, lingonberries, cherries, and raspberries. All fruits and berries showed significant difference, for both HT29 and MCF-7 cells, between the concentrations of 0.5−0.05 and 0.5−0.025%.

Rosehips, blueberries, and sea buckthorn had the highest inhibition effect for the proliferation of HT29 cells in the two highest concentrations of the extracts, whereas for MCF-7 rosehips, black currant, and sea buckthorn had the highest effect for these concentrations.

Cancer cell proliferation was inhibited to different extents by the fractions containing the anthocyanins of the extracts from each of the 10 fruits and berries (**Table 3**). In HT29, the anthocyanin fraction of the extract of black chokeberries, apple, rosehips, and raspberries and lingonberries decreased cell proliferation, whereas blueberries and cherries increased cell proliferation at the highest concentration. In MCF-7, the anthocyanin fraction of the extract of blueberries, black currant, and black chokeberries decreased cell proliferation, whereas apple and cherries increased cell proliferation. No significant effect on cell proliferation was found at the middle and low concentrations in either HT29 or MCF-7 cells (values not shown), except for the anthocyanin fraction of sea buckthorn, which decreased the proliferation of MCF-7 cells to 92% of control at the middle concentration. Standards of delphinidin-3-glucoside, cyanidin-3-glucoside, and malvidin-3-glucoside decreased cell proliferation of both HT29 and MCF-7 to a high extent at the concentration of 200 μg/mL in the medium in the

Table 3. Proliferation of Colon Cancer Cell HT29 and Breast Cancer Cell MCF-7 Treated with the Anthocyanin Fraction of the Fruit and Berry Extracts[a]

	HT29		MCF-7	
fruits/berries/ anthocyanin standards	proliferation, % of control	signif	proliferation, % of control	signif
rosehips	81.8 ± 6.1	***	104.4 ± 8.5	
blueberries	105.3 ± 5.3	*	74.9 ± 12.7	***
black currant	107.0 ± 12.1		55.5 ± 8.2	***
black chokeberries	62.8 ± 17.3	***	80.8 ± 21.2	**
apple (peel)	87.8 ± 8.9	***	106.1 ± 7.8	*
sea buckthorn	104.9 ± 8.2		99.2 ± 10.1	
plum	98.7 ± 5.8		99.5 ± 6.9	
lingonberries	85.1 ± 8.6	***	101.7 ± 7.1	
cherries	118.4 ± 13.9	***	105.9 ± 6.8	*
raspberries	92.1 ± 5.6	***	102.3 ± 5.1	
delphinidin-3-g, 200	13.5 ± 1.1	***	18.3 ± 0.9	***
cyanidin-3-g, 200	12.3 ± 2.2	***	15.0 ± 0.8	***
malvidin-3-g, 200	10.0 ± 0.7	***	15.7 ± 0.3	***
D + C + M, 200	73.0 ± 3.7	***	65.5 ± 3.5	***
delphinidin-3-g, 20	102.2 ± 10.1		94.8 ± 6.1	
cyanindin-3-g, 20	104.1 ± 11.0		103.9 ± 8.7	
malvidin-3-g, 20	109.7 ± 10.1	*	103.2 ± 11.2	
D + C + M, 20	106.0 ± 5.1	*	108.7 ± 8.4	*

[a] Results presented are from the highest concentration of the anthocyanin fraction of the extracts, or the two concentrations of anthocyanin standards used, according to **Table 2**. Significantly different from control: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Results are presented as means ± SD based on three independent replicate samples, and the proliferation tests were repeated on three different occasions.

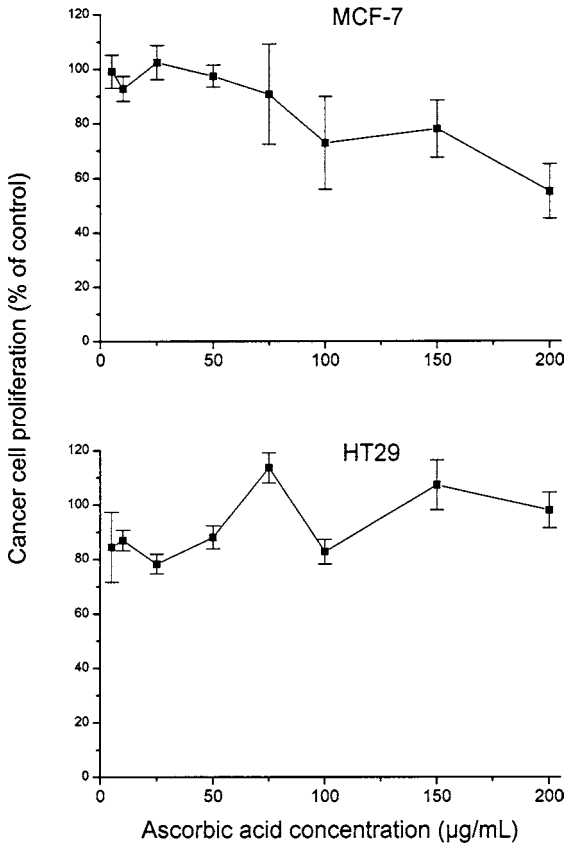

Figure 2. Effects of standard ascorbic acid on the proliferation of HT29 and MCF-7 cells. Results are presented as means ± SD based on three independent replicates for each concentration, and the proliferation tests were repeated on three different occasions.

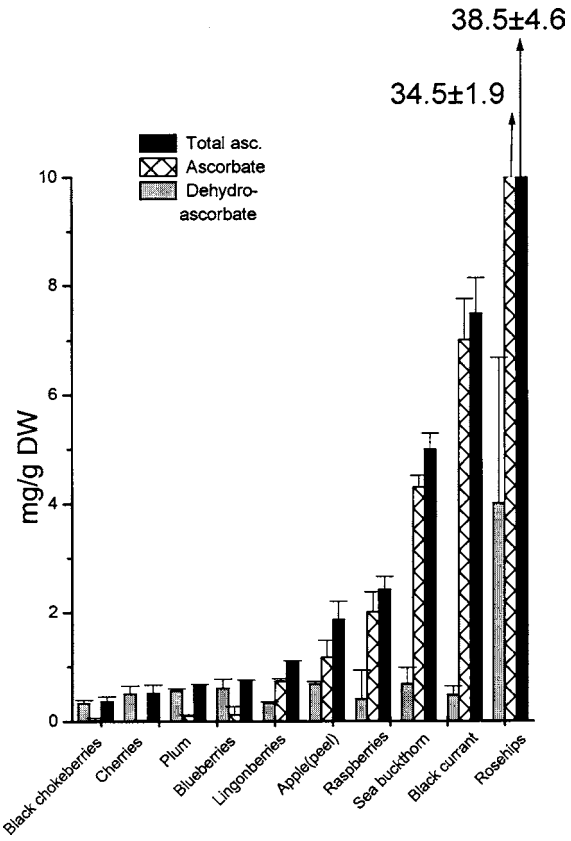

Figure 3. Content of vitamin C in 10 fruits and berries. Results are presented as means ± SD based on three to four independent replicate samples.

wells, whereas a mixture of these anthocyanins at a total concentration of 200 μg/mL did not decrease the cell proliferation to the same extent (**Table 3**). At a lower concentration of the anthocyanin standards, 20 μg/mL in the medium in the wells, no effects was found on the proliferation of HT29 and MCF-7 cells of the delphinidin or cyanidin standard, and a slight stimulating effect on cell proliferation was found for malvidin in HT29 cells and for the mixture of the three anthocyanin standards for both HT29 and MCF-7 cells.

Ascorbic acid standard inhibited proliferation of both HT29 and MCF-7 cells, although at different concentrations and to different extents (**Figure 2**). Proliferation of HT29 cells was significantly inhibited at lower concentrations, 5−50 μg/mL, and at 100 μg/mL, whereas a slight stimulating effect was found at 75 and 150 μg/mL. Proliferation of MCF-7 cells was significantly inhibited at higher concentrations, 100−200 μg/mL, and also at 10 μg/mL.

Content of Antioxidants in the Extracts of the 10 Fruits and Berries. Analyses of the extracts from the fruits and berries showed great differences in the content of vitamin C, carotenoids, flavonols, and other phenolic compounds. The level of the vitamin C (ascorbate and dehydroascorbate) content varied almost 100-fold, between 0.4 and 38.5 mg/g of dry weight (DW), whereas cherries contained the lowest and rosehips the highest contents (**Figure 3**). There were also differences in the ratio of dehydroascorbate/ascorbate, and plum, blueberries, cherries, and black chokeberries had a relatively high ratio (values shown only indirectly in **Figure 2**).

There were great differences in both the composition and the contents of the carotenoids among the investigated species. The content of total carotenoids varied almost 150-fold among the species, between 5 and 730 μg/g of DW, whereas cherries

Table 4. Content of Carotenoids (Micrograms per Gram of Dry Weight) in 10 Fruits and Berries Used in Cancer Cell Proliferation Experiments[a]

	lutein	lycopene	β-carotene	carotene ester 1	carotene ester 2	xanthophyll	cryptoxanthin	neolycopene
rosehips	19.7 ± 3.3	282.3 ± 16.3	145.2 ± 9.4	23.2 ± 1.0	191.0 ± 1.8	nd	49.0 ± 0.8	20.3 ± 1.6
blueberries	6.0 ± 0.3	nd[b]	3.3 ± 0.8	nd	nd	nd	nd	nd
black currant	16.8 ± 0.6	nd	15.1 ± 0.3	nd	nd	5.6 ± 0.3	nd	nd
black chokeberries	9.1 ± 1.1	nd	46.4 ± 2.2	nd	nd	2.7 ± 1.4	nd	nd
apple (peel)	10.6 ± 0.5	nd	8.5 ± 0.3	nd	nd	3.6 ± 0.2	nd	nd
sea buckthorn	15.6 ± 0.5	5.6 ± 0.4	41.7 ± 2.2	8.6 ± 1.0	41.4 ± 4.6	nd	nd	nd
plum	4.7 ± 1.3	nd	9.6 ± 0.6	nd	nd	nd	nd	nd
lingonberries	3.6 ± 0.1	nd	2.1 ± 0.3	nd	nd	nd	nd	nd
cherries	nd	nd	4.9 ± 1.0	nd	nd	nd	nd	nd
raspberries	5.5 ± 0.7	nd	nd	nd	nd	4.2 ± 0.4	nd	nd

[a] Results are presented as means ± SD based on three to four independent replicate samples. [b] Not detected.

Table 5. Content of Hydroxycinnamic Acids, Benzoic Acid, Ellagic Acid, and Flavonols (Micrograms per Gram of Dry Weight) in 10 Fruits and Berries Used in Cancer Cell Proliferation Experiments[a]

	HCA[b]	quercetin	quercetin-glycosides	other flavonols	benzoic acid	ellagic acid
rosehips	nd[c]	87 ± 17	88 ± 30	222 ± 33	nd	nd
blueberries	2565 ± 293	46 ± 9	nd	nd	nd	nd
black currant	113 ± 43	28 ± 9	99 ± 35	50 ± 34	nd	nd
black chokeberries	2333 ± 292	138 ± 14	nd	89 ± 4	nd	nd
apple (peel)	nd	534 ± 78	13 ± 7	nd	nd	nd
sea buckthorn	nd	21 ± 3	438 ± 77	215 ± 26	nd	nd
plum	300 ± 47	153 ± 41	nd	nd	nd	nd
lingonberries	nd	851 ± 79	181 ± 40	25 ± 3	2505 ± 28	nd
cherries	350 ± 34	9 ± 2	nd	nd	nd	nd
raspberries	nd	3 ± 1	nd	nd	nd	765 ± 142

[a] Results are presented as means ± SD based on three to four independent replicate samples. [b] Hydroxycinnamic acids. [c] Not detected.

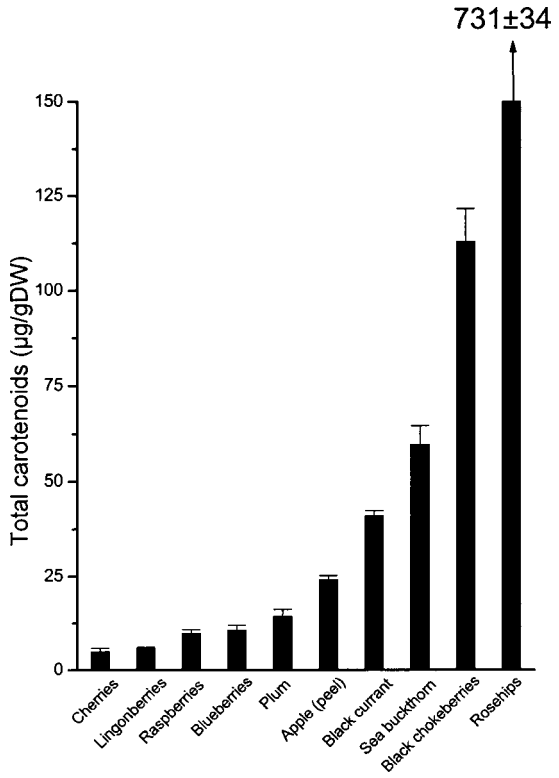

Figure 4. Content of total carotenoids in 10 fruits and berries. Results are presented as means ± SD based on three to four independent replicate samples.

contained the lowest and rosehips the highest contents (**Figure 4**). β-Carotene and lutein were the most common carotenoids and were detected in all investigated species except raspberries (β-carotene not detected) and cherries (lutein not detected) (**Table 4**).

Table 6. Content of Total Phenolics (Milligrams of Gallic Acid Equivalents per Gram of Dry Weight) and Total Anthocyanins (Milligrams per Gram of Dry Weight) in 10 Fruits and Berries Used in Cancer Cell Proliferation Experiments[a]

	total phenolics	total anthocyanins
rosehips	90.6 ± 2.9	0.4 ± 0.3
blueberries	26.7 ± 3.0	101.4 ± 17.9
black currant	28.8 ± 2.7	79.0 ± 11.5
black chokeberries	68.2 ± 3.6	67.7 ± 2.8
apple (peel)	20.8 ± 1.2	15.4 ± 0.5
sea buckthorn	11.9 ± 1.2	0.4 ± 0.3
plum	18.0 ± 1.2	25.5 ± 2.7
lingonberries	46.1 ± 1.6	19.6 ± 1.3
cherries	23.0 ± 1.6	84.0 ± 7.9
raspberries	30.5 ± 2.2	17.3 ± 1.0

[a] Results are presented as means ± SD based on three to four independent replicate samples.

Flavonols, hydroxycinnamic acids, ellagic acid, and benzoic acid were analyzed in the 10 fruits and berries, and also here large differences in the contents were found (**Table 5**). High levels of hydroxycinnamic acids were found in blueberries and black chokeberries, and relatively high levels were found in cherries and plum, whereas a high level of benzoic acid was found in lingonberries. Ellagic acid was found only in raspberries. The content of total antocyanins was highest in blueberries, black currant, cherries, and black chokeberries (**Table 6**). The level of total phenolics varied almost 8-fold among the species, and it was highest in rosehips, black chokeberries, and lingonberries (**Table 6**).

Correlation between the Content of the Different Antioxidants and Cancer Cell Proliferation. There was an inverse relationship between the content of vitamin C (ascorbate and dehydroascorbate) and cancer cell proliferation using a ranked correlation (Spearman correlation), so the extracts with the highest concentration of vitamin C inhibited cancer cell

proliferation to the highest extent, giving the lowest proliferation levels. This effect was significant for the highest concentration of the extracts (0.5%) for both HT29 ($r_s = -0.648$, $P = 0.043$) and MCF-7 cells ($r_s = -0.733$, $P = 0.016$), whereas a lower level of correlation was found for the second highest concentration (0.25%), with a lower significance level ($r_s = -0.564$, $P = 0.09$; and $r_s = -0.612$, $P = 0.06$). There was also a significant correlation between the concentration of ascorbate at the 0.25% concentration of the extracts and the inhibition of cell proliferation of MCF-7 ($r_s = -0.648$, $P = 0.043$).

There was also an inverse relationship found between the sum of the content of lutein and β-carotene and cancer cell proliferation of MCF-7 (Spearman correlation) for the second highest concentration (0.25%) of the extracts. The correlation coefficients were -0.67 ($P = 0.03$) for MCF-7 cells and -0.56 ($P = 0.09$) for HT29 cells. Calculated with Pearson correlation, the correlation coefficient for lutein at the same concentration of the extracts was -0.72 ($P = 0.018$) for MCF-7 cells. For the other investigated antioxidants, no significant correlations between the individual antioxidants in the total extracts and the inhibition of cell proliferation could be found (values not shown).

In addition, a significant inverse relationship could also be found, at the highest concentration according to **Table 2A**, between the content of anthocyanins in the fraction of the total extracts from the fruits and berries containing the anthocyanins and the proliferation of MCF-7. The correlation coefficients were -0.692 ($P = 0.027$) calculated with Spearman correlation and -0.774 ($P = 0.009$) calculated with Pearson correlation. No correlation was found between proliferation of HT29 cells and the concentration of anthocyanins in the anthocyanin fraction (values not shown).

DISCUSSION

Components in fruits and berries have been shown to inhibit cancer cell proliferation in vitro. It is, however, difficult to evaluate the relative importance of individual compounds for the anticancer effects of fruits and vegetables seen in vivo. The protective effect might be due to additive or synergistic actions of several compounds, but most investigations of the inhibitory effects on the proliferation of cancer cells in culture are focused on individual compounds or groups of compounds. Thus, the relative importance of the compounds in comparison to others is usually not simultaneously evaluated. Enhanced apoptosis and inhibition of cell proliferation was found in a human leukemia cell line, when cells were treated with quercetin and ellagic acid in combination, compared with the single compounds, proposed to be due to a synergistic rather than an additive effect (*22*).

The anticancer effects of fruits, berries, and vegetables may be exerted at several different levels. For example, bioactive compounds may be antimutagenic, inhibit cancer cell proliferation, or cause induction of apoptosis. In this work, the effects of 10 different extracts of fruits and berries on the proliferation of two different cancer cell lines were investigated. The fruits and berries were chosen to represent many different levels of compounds with potentially inhibitory effects on cancer cell proliferation, for example, vitamin C, carotenoids, and anthocyanins. The range of concentrations of the main antioxidants added to the growth medium of the cancer cells were at the levels that can be found in human tissues. The final concentration of vitamin C with the highest extract concentration added (0.5%) was between 2 μg/mL (cherry) and 190 μg/mL (rosehips) in the growth medium. This could be compared with a concentration of vitamin C found in human plasma in the range of 3.5−15.8 μg/mL (calculated from ref *23*). Higher concentrations have been reported in cerebrospinal fluid and gastric juice (*24*−*26*). The final concentrations of total carotenoids with the highest extract concentration added (0.5%) were in the range from 25 ng/mL (cherry) to 3.6 μg/mL (rosehips) in the growth medium, compared with human plasma concentrations of 0.5−0.6 μg/mL (*27*). The highest final concentration of β-carotene in the growth medium was 0.7 μg/mL (rosehips), compared with a plasma concentration of ~0.2 μg/mL (*27*).

There were large differences in the concentration of the analyzed antioxidants in the 10 investigated species, up to 100-fold for vitamin C and 150-fold for carotenoids. The extraction method used was chosen to give an acceptable yield of the various antioxidants. It was not optimized for each of the individual compounds, but the yields should nevertheless be similar in the 10 different species and therefore reflect the relative differences in the species of the individual antioxidants. Due to the large differences in some of the values of the concentrations of vitamin C and some of the carotenoids, the correlation calculations were also performed with ranked values (Spearman correlation) that do not postulate a Gaussian distribution. Considering that many compounds in plants have been reported to have inhibitory effects on cancer cell proliferation and are likely to contribute to the inhibition seen in this investigation, the correlation found between the content of vitamin C (ascorbate and dehydroascorbate) and inhibition of cell proliferation of both HT29 and MCF-7 cells was relatively strong and may indicate an important role of vitamin C.

However, the cancer chemopreventive mechanism of vitamin C is at present under discussion (*28*). Epidemiological investigations support a protective effect of vitamin C against several cancers (*10*, *29*). Ascorbic acid in plasma was shown to have the highest correlation with fruit and vegetable intake and was suggested to be an important component in the protective effect seen for fruits and vegetables (*30*). Lately the antioxidant role of ascorbic acid has been questioned due to reports of a pro-oxidant role in vivo. Markers of DNA damage mediated by oxygen radicals, 8-oxoguanine (8OHG) and 8-oxoadenine (8OHA), were investigated after 500 mg vitamin C supplementation of diets. Levels of 8OHG were found to decrease and of 8OHA to increase (*31*, *32*). The physiological effects of these contradictory changes are, however, not clearly understood (*33*). It has been suggested that the putative pro-oxidant effect of iron−ascorbate mixtures transiently accelerates DNA damage but that this leads to an up-regulation of repair systems, which lowers the levels of 8OHG (*34*).

In addition, although the epidemiological evidence supports an important protective role for vitamin C, results from in vitro tests with ascorbic acid have not found any clear effect. Ascorbic acid was found to arrest the growth of several investigated cancer cell lines, whereas other cancer cell lines were not affected (*35*). When tumor cells were treated with either ascorbic acid or a derivative, 6-*O*-palmitoyl-ascorbate-2-*O*-phosphate, inhibition of DNA synthesis and proliferation were found in the cells treated with the derivative, whereas no effects could be found in the cells treated with ascorbic acid (*36*). In this investigation, the same inhibition effect of proliferation seen in HT29 and MCF-7 cells treated with the extracts of the 10 fruits and berries could not be found when the cancer cells were treated with ascorbic acid standard alone, although there was an inhibition effect at the lower concentrations in HT29 cells and a higher inhibition effect in MCF-7 cells at higher concentrations. However, in another investigation, exposure of

a human squamous cell carcinoma cell line with a combination of a flavone and ascorbic acid resulted in inhibition of cell proliferation, whereas either of the single compounds had no effect (*37*), and ascorbic acid has also been found to improve the antineoplastic activity of several established cytostatics in human breast carcinoma cells in vitro (*38*). The correlation between the inhibition of cancer cell proliferation and the content of vitamin C found in this investigation may therefore be due to a synergistic effect of vitamin C and other substances. There was also a correlation found between the content of lutein and β-carotene and the inhibition of cancer cell proliferation. The importance of these carotenoids is supported by epidemiological evidence, which suggest a strong inverse relationship between the risk of cancer and dietary β-carotene intake or blood concentrations (*39*−*41*) and for blood serum concentrations of lutein (*40*). However, results from intervention trials involving β-carotene supplementation have been inconsistent and sometimes contrary to what was expected (*42*−*45*). Suggestions have been made about recommendations regarding intake in different groups of the population, antioxidant contra pro-oxidant properties of the antioxidants depending on concentration, and pro-cancer properties of oxidized carotenoids (*46*−*48*).

Even if in this investigation a correlation was found between the inhibition of cancer cell proliferation with vitamin C levels and also to a certain extent with levels of some carotenoids, other substances are also likely to contribute. The cell proliferation in HT29 as well as MCF-7 cells was strongly inhibited by the blueberry extract, although the levels of vitamin C and carotenoids were found to be relatively low. The concentrations of hydroxycinnamic acids as well as of total phenolics and total anthocyanins were high in blueberries. Compounds in these groups may thus account for the inhibitory effect of the blueberry extract. However, the fraction containing the anthocyanins of the extract of the blueberries only inhibited proliferation of MCF-7 cells to a lower extent than the total extract, so the inhibitory effect is probably only partly due to the anthocyanins. In HT29 cells proliferation was inhibited to a lower extent than in MCF-7 cells by the total blueberry extract, and the anthocyanin fraction of blueberries even showed a weak stimulation of the proliferation of HT29 cells, so in these cells other substances must account for the inhibitory effect or the tested concentrations might give another response. In support of these results with a lower response of HT29 cells are the findings in another investigation (*49*) that the sugar-conjugated anthocyanins were much less effective than the aglycon cyanidin in inhibiting the growth of HT29 cells and that HT29 cells were inhibited to a lower extent than the other colon cancer cells tested, HCT 116. In this investigation, no correlation was found between total anthocyanins or total phenolics in the total extract of the fruits and berries, in agreement with other investigations in which no correlation could be found between the inhibition of HepG3 cells and total phenolic or flavonoid content in cultivars of raspberries and strawberries (*49*, *50*). However, when a fraction containing the anthocyanins was separated from the rest of the total extract, a correlation was found between the content of anthocyanins in the fraction and the proliferation of MCF-7 cells, so the anthocyanins seem to contribute to the inhibition of proliferation in these cells.

In conclusion, the inhibition of cancer cell proliferation seen in these experiments correlated with vitamin C levels and also to certain extent with levels of some carotenoids, present at physiological levels. This might indicate an important role for these antioxidants, together with the anthocyanins, in controlling cancer cell proliferation also in vivo, possibly by synergistic effects. In the MCF-7 cells, the anthocyanins may contribute to the inhibition of proliferation.

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