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Berry Extracts Exert Different Antiproliferative Effects against Cervical and Colon Cancer Cells Grown in Vitro

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Polyphenol-rich berry extracts were screened for their antiproliferative effectiveness using human cervical cancer (HeLa) cells grown in microtiter plates. Rowan berry, raspberry, lingonberry, cloudberry, arctic bramble, and strawberry extracts were effective but blueberry, sea buckthorn, and pomegranate extracts were considerably less effective. The most effective extracts (strawberry > arctic bramble > cloudberry > lingonberry) gave EC_{50} values in the range of 25–40 μg/(mL of phenols). These extracts were also effective against human colon cancer (CaCo-2) cells, which were generally more sensitive at low concentrations but conversely less sensitive at higher concentrations. The strawberry, cloudberry, arctic bramble, and the raspberry extracts share common polyphenol constituents, especially the ellagitannins, which have been shown to be effective antiproliferative agents. However, the components underlying the effectiveness of the lingonberry extracts are not known. The lingonberry extracts were fractionated into anthocyanin-rich and tannin-rich fractions by chromatography on Sephadex LH-20. The anthocyanin-rich fraction was considerably less effective than the original extract, whereas the antiproliferative activity was retained in the tannin-rich fraction. The polyphenolic composition of the lingonberry extract was assessed by liquid chromatography–mass spectrometry and was similar to previous reports. The tannin-rich fraction was almost entirely composed of procyanidins of linkage type A and B. Therefore, the antiproliferative activity of lingonberry was caused predominantly by procyanidins.

KEYWORDS: Antiproliferative; anthocyanins; berries; cancer; ellagitannins; inhibition; lingonberry; polyphenols; proanthocyanidins; tannins

INTRODUCTION

Epidemiological studies have consistently shown an association between the consumption of fruit and vegetables and a reduced risk of human diseases, such as cardiovascular disease and cancer (*1–4*). Accordingly, there has been a focus on identifying components of fruit and vegetables responsible for anticancer effects (*5*). Fruit and vegetables are rich sources of antioxidants capable of scavenging oxygenated free radicals which can damage cellular components such as DNA, proteins, or membrane lipids (*6*). Control of this oxidative damage may influence the onset of carcinogenesis (*5, 7*).

Uncontrolled proliferation and suppressed apoptosis are important steps in the initiation and progression of cancers (*8*). Many studies have confirmed that berry extracts inhibit proliferation of cancer cells in vitro (*9–15*) and transformation into cancerous cells in vitro (*16*) and reduce tumor numbers in genetically predisposed mice (*17*) and the progression of experimentally induced tumors in animal models (*13, 18*). Intervention trials of black raspberries against human esophageal and colon cancer (*19*) are in progress.

Berries contain a complex mixture of antioxidants including vitamin C, carotenoids and xanthophylls, and polyphenols, which differ in a species-specific manner (*20, 21*). Individual polyphenols such as anthocyanins (*22*), ellagitannins (*15*), and ellagic acid (*23*) have been shown to inhibit cancer proliferation in vitro.Furthermore, other studies have suggested considerable potential for synergetic effects between different antioxidant components (i.e., vitamin C, carotenoids, and polyphenols (*12*)) and within polyphenol components (*15*).

This study ranks the antiproliferative effectiveness of a range of fruit extracts enriched in polyphenols, but devoid of vitamin C and carotenoids, using a screen based on the growth of human cervical carcinoma (HeLa) cells with comparative studies on human colon cancer (CaCo-2) cells. By comparing the phytochemical diversity of the berry extracts with their antiprolif-

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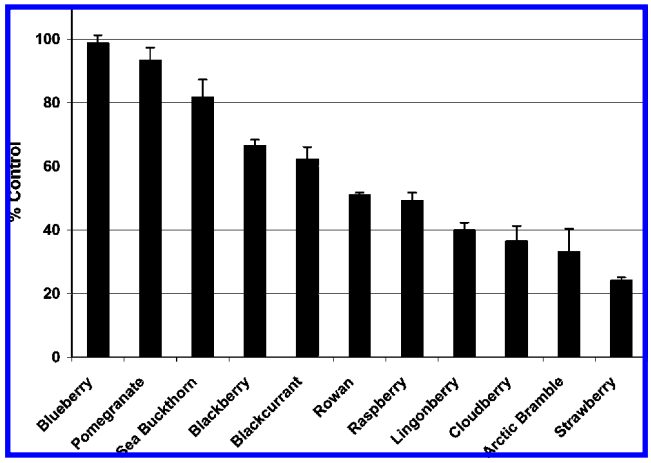

Figure 1. Viability of HeLa cells in the presence of berry extracts. All extracts applied at 50 μg of GAE/mL. Results are means of three replicate experiments ± standard error.

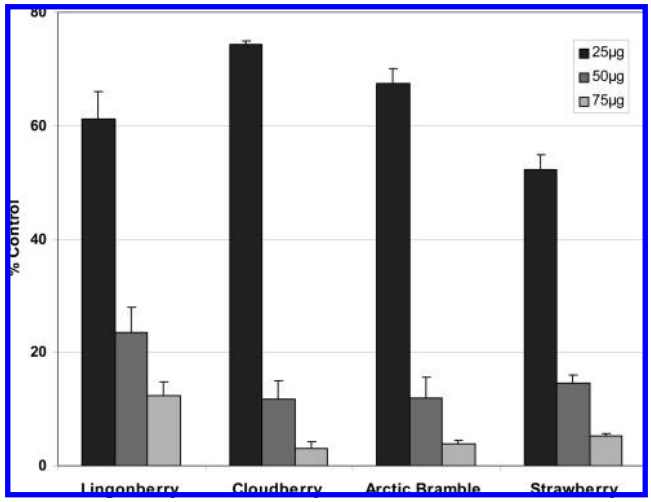

Figure 2. Dose effectiveness of berry extracts on viability of HeLa cells Three concentrations of extracts (25, 50, and 75 μg of GAE/mL) were applied. Results are means of three replicate experiments ± standard error.

erative effectiveness, key structural components relevant to the inhibition of cancer cell growth may be identified.

MATERIALS AND METHODS

Plant Material and Extraction. Blackcurrants were obtained from Bradenham Hall, Norfolk, United Kingdom and blueberries were grown at SCRI. Cloudberries, arctic bramble, lingonberries, sea buckthorn, and rowan berries were a gift from Dr. Harri Kokko, University of Kuopio, and were arranged through the European Union Northberry Project. Strawberries, blackberries, and pomegranates were purchased from a local supermarket. Raspberries were obtained from local farmers.

The fruits were extracted and polyphenol-rich fractions obtained by the methods described previously (*24*). Briefly, frozen fruit was homogenized in a Waring blender (6 × 20 s at full power) using an equal volume to weight of ice-cold 0.2% (v/v) acetic acid in water. The extract was filtered through a glass sinter and applied to C18 solid-phase extraction units (Strata C18-E, GIGA units, Phenomenex Ltd., U.K.) prewashed in 0.2% (v/v) acetic acid in acetonitrile and then preequilibrated in 0.2% (v/v) acetic acid in water. Unbound material, which contained the free sugars, organic acids, and vitamin C, was discarded. After extensive washes, the polyphenol-enriched bound

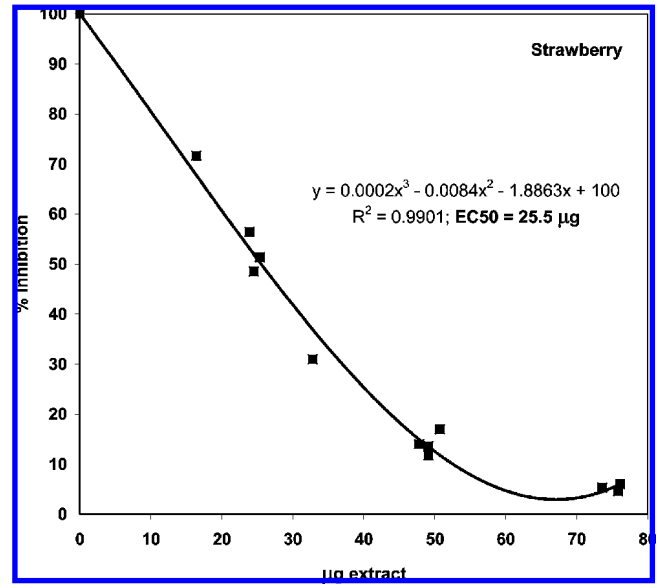

Figure 3. Dose effectiveness of strawberry extracts against HeLa cell viability The data are fitted according to the polynomial equation shown. The EC_{50} is calculated as the amount of extract required to reduce cell viability to 50% of control.

Table 1. EC_{50} values for CaCo-2 and HeLa cells

	EC_{50} values (μg/mL)	
berry extract	HeLa	CaCo-2
arctic bramble	26.4	24.4
cloudberry	31.6	29.4
lingonberry	28.7	38.3
strawberry	25.5	22.2

extracts were eluted with acetonitrile. The C18-bound extracts were evaporated to dryness in a Speed-Vac (Thermo Fisher, Basingstoke, U.K.).

Sorption to Sephadex LH-20 in aqueous ethanol and selective debinding with aqueous acetone is an established method for separating tannins from nontannin phenolics. The method was adapted from the *Tannins Handbook* (kindly made available at www.users.muohio.edu/hagermae/tannin.pdf). Briefly, a column of Sephadex LH-20 was washed in 80% (v/v) ethanol/water and then 50% (v/v) acetone/water before being equilibrated with three volumes of 80% ethanol. The lingonberry C18 extract was dissolved in 80% ethanol, applied to the column and the run-through plus a column volume of 80% ethanol collected as the unbound fraction. This material was bright red and obviously contained the bulk of the anthocyanins. The column was washed with three column volumes of 80% ethanol. The bound fraction was eluted with three volumes of 50% acetone. The unbound and bound fractions were evaporated to near dryness and then stored frozen.

Anthocyanin and Phenol Assays. The total anthocyanin concentration was estimated by a pH differential absorbance method (*25*). The absorbance value was related to anthocyanin content using the molar extinction coefficient calculated for cyanidin-3-*O*-glucoside (purchased from ExtraSynthese Ltd., Genay, France). Phenol content was measured using a modified Folin−Ciocalteau method (*25*). Phenol contents were estimated from a standard curve of gallic acid.

Cell Culture and Measurements of Cell Viability. Human cervical cancer (HeLa) cells were grown as described previously (*24*) and cultured at a cell density of 50000 cells/mL. Human colon cancer (CaCo-2) cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing D-glucose and L-glutamine (Bio-Whittaker product no. BE12-604F) and supplemented with 10% fetal calf serum (Gibco product no. 10500-056), 1% penicillin/streptomycin (Sigma product no. P-0781), 1% nonessential amino acids (ICN product

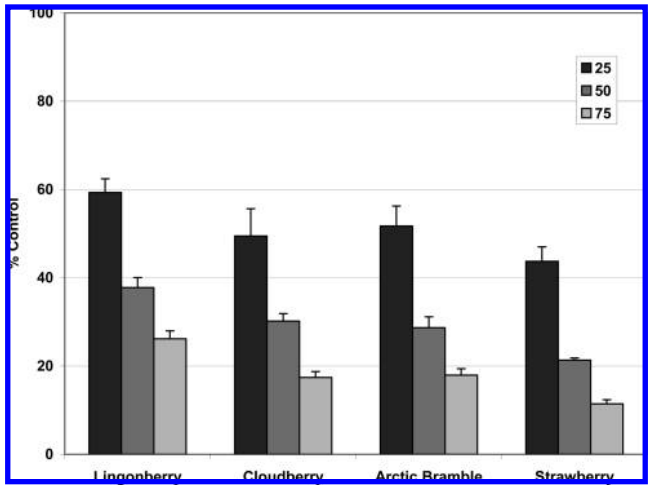

Figure 4. Effect of berry extracts on CaCo-2 cell viability. Three concentrations of fruit extracts (25, 50, and 75 μg of GAE/mL) were applied. Results are means of three replicate experiments ± standard error.

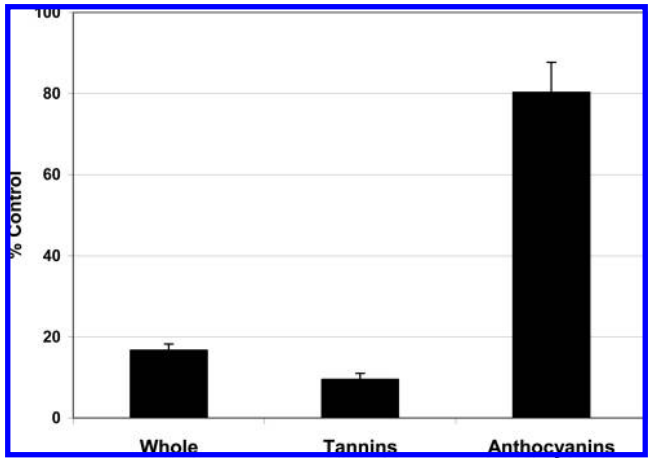

Figure 5. Effect of lingonberry fractions on HeLa cell viability. Extracts were applied at 40 μg of GAE/(mL of phenols). Results are means of three replicate experiments ± standard error.

no. 1681049), and 25 μg/mL gentamycin (Gibco product no. 15750). The cells were grown in 50 mL flasks at 37 °C in a constant humidified atmosphere of 5/95 CO_2/air. After trypsin detachment, as described previously (*26*), CaCo-2 cells were counted and subcultured at 50000 cells/mL.

The cell suspension (100 μL/well) was added to wells on a TC Microwell plate (Nunc product no. 167008). The plates were preincubated overnight. Using a template, each experimental treatment (fruit extract type and concentration) was replicated four times in randomly assigned positions on each of four 96-well plates. All extracts were filter sterilized in phosphate-buffered saline (PBS) prior to addition to plates, and all additions were in 10 μL volumes. Phenol contents were routinely checked to assess phenol losses due to filter sterilization. There were three controls: blanks which contained only PBS; controls with cells and PBS only; and treatment controls for each sample and concentration of extract that contained no cells, only PBS and extract. The plates were incubated for 72 h at 37 °C. Cell viability was assayed using the Dojindo CCK-8 kit following the manufacturer's instructions (NBS Biologicals, Cambridge, U.K.).

Liquid Chromatography–Mass Spectrometry (LC-MS). Samples (containing 20 μg of gallic acid equivalents by Folin assay) were analyzed on a LCQ-DECA system, comprising Surveyor autosampler, pump, photodiode array detector (PDAD), and a ThermoFinnigan mass spectrometer iontrap. The PDAD scanned three discrete channels at 280, 365, and 520 nm. Samples were eluted with a gradient of 5% acetonitrile (0.1% formic acid) to 30% acetonitrile (0.1% formic acid) on a C18 column (Synergi Hydro C18 with polar end capping, 4.6 mm × 150 mm, Phenomenex Ltd.) over 60 min at a rate of 400 μL/min. The LCQ-DECA liquid chromatography−mass spectrometer was fitted with an electrospray ionization interface, and the samples were analyzed in positive- and negative-ion mode. There were two scan events: full-scan analysis followed by data-dependent MS/MS of the most intense ions. The data-dependent MS/MS used collision energies (source voltage) of 45% in wide-band activation mode. The MS detector was tuned against cyanidin-3-*O*-glucoside (positive mode) and against ellagic acid (negative mode).

RESULTS

The screening method ranked the antiproliferative effects of the fruit extracts on the growth of the HeLa cells (**Figure 1**). Extracts of blueberry and pomegranate showed very little effect on the growth of the HeLa cells, whereas other samples were effective to some degree. Rowan berries, raspberry, lingonberry, cloudberry, arctic bramble, and strawberry were the most effective, all reducing viability to ≤50% of control at 50 μg/mL. Increasing amounts of the most effective extracts (strawberry > arctic bramble > lingonberry > cloudberry) caused increasing inhibition of HeLa cell growth (**Figure 2**). The median effective dose (concentration of extract that causes 50% inhibition, EC_{50}) was determined by plotting cell viability against the actual phenol concentration applied to the cultures (**Figure 3**). By plotting the slight variations in phenol content applied and fitting the viability data to polynomial functions, the EC_{50} values for arctic bramble, cloudberry, lingonberry, and strawberry were calculated as 26.4, 31.6, 28.7, and 25.5 μg, respectively (**Table 1**).

The viability of CaCo-2 colon cancer cells was also inhibited in a dose-dependent manner by these four berry extracts (**Figure 4**). In contrast to HeLa cells, CaCo-2 cells were more sensitive at the lowest concentration of extract (25 μg of GAE) but comparatively less sensitive at 50 and 75 μg of GAE. The EC_{50} values were calculated in a fashion similar to the HeLa cells. Arctic bramble, cloudberry, lingonberry, and strawberry gave EC_{50} values of 24.4, 29.4, 38.3, and 22.2 μg, respectively (**Table 1**).

The lingonberry extract was fractionated by chromatography on Sephadex LH-20 into an unbound fraction, which by eye obviously contained the bulk of the original anthocyanin content, and a bound fraction which should contain tannin-like compounds (*26*). The anthocyanin-rich unbound fraction was much less effective than the original lingonberry extract (**Figure 5**), and the antiproliferative activity was retained in the bound fraction.

The HPLC traces of the lingonberry whole extract (**Figure 6**) and the LH-20 bound tannin fraction (**Figure 7**) at 280 nm showed the recovery of components in these fractions. Twenty eight peaks were annotated in the original extract, and their putative identities based on their PDA spectra, mass spectra, and literature data are given in **Table 2**. The LH-20 unbound fraction gave traces very similar to the original fraction (results not shown). The tannin-rich fraction contained 38 identifiable peaks (**Figure 7**; **Table 3**).

Three main anthocyanins made up a major portion of the lingonberry extract and were identified as cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-arabinoside (*27, 28*) (**Table 2**). The recovery of these compounds in the LH-20 bound fraction was estimated using LC-MS at between

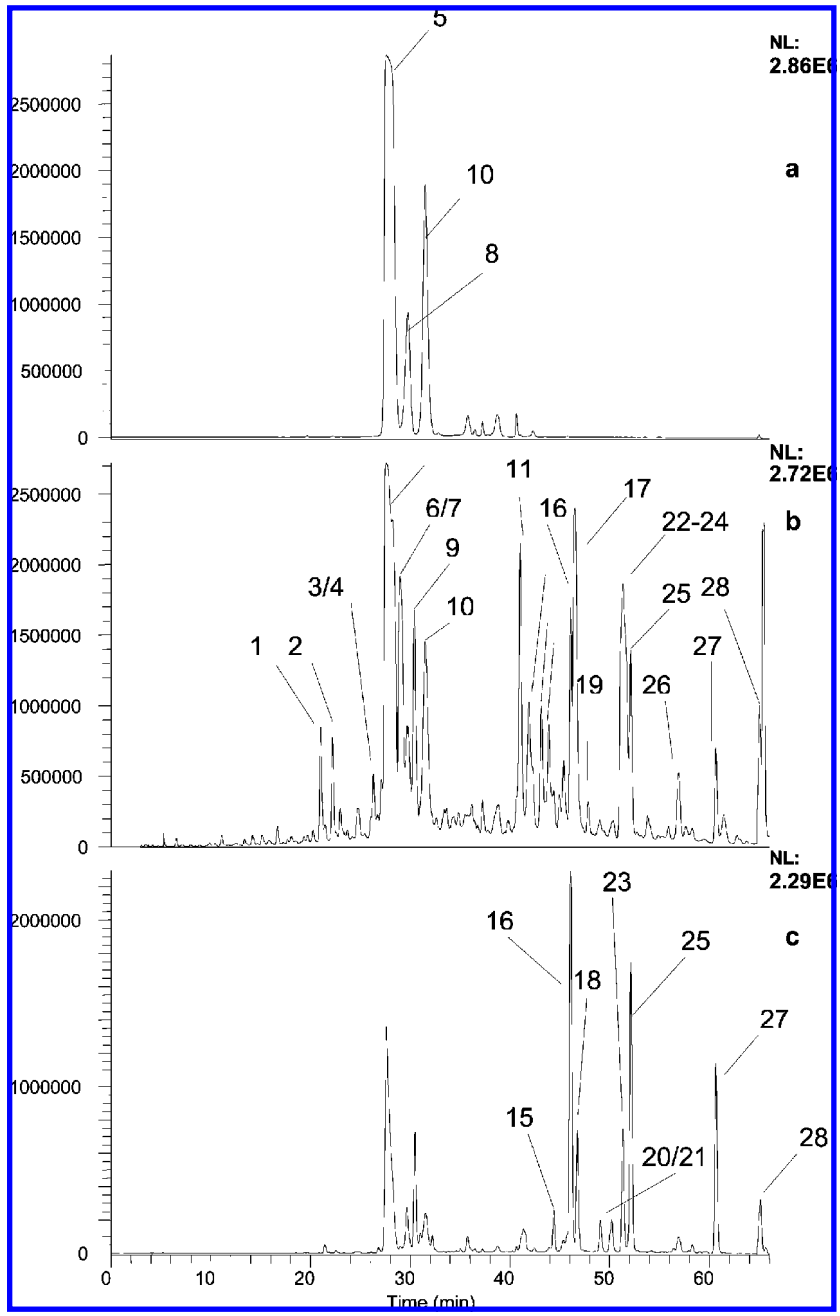

Figure 6. LC-MS traces of lingonberry extracts: Trace a, absorbance at 520 nm; trace b, absorbance at 280 nm; trace c, absorbance at 365 nm. The figure in the right-hand corner represents the full-scale deflection for each trace. The peaks are numbered as presented in **Table 2**.

0.1 and 0.3% of their content in the original extract (results not shown). All of the peaks noted in the tannin fraction (**Figure 7**) gave PDA spectra with maxima around 280 nm and mass spectra (**Table 3**) that identified them as proanthocyanidins composed of catechin units or procyanidins. Previous work has shown that the proanthocyanidins from lingonberry were procyanidins with no prodelphidins (*29*), and the assignments of putative identity are supported by previous work (*30*). Where two or more main *m*/*z* values were obtained, the compound was identified as the largest *m*/*z* signal as the other signals could occur because of in-source fragmentation. Not all components are completely separated, so further development of the LC-MS conditions are required to accurately describe the composition, but it is clear that this fraction is predominantly composed of procyanidins.

DISCUSSION

Berry extracts, particularly those from the *Rubus* family (raspberry, arctic bramble, and cloudberry) but also strawberry and lingonberry, were effective in preventing the proliferation of human cervical and colon cancer cells in vitro. This report extends previous reports on the antiproliferative effects of berry extracts (*9–15, 24*).

Many previous studies have focused on the antiproliferative effect of a single fruit. In studies that compared the effects of different fruits, there have been differences in the ranking of effectiveness. For example, extracts of rosehips, sea buckthorn, blueberries, blackcurrant, apple peel, plum, and lingonberry were effective inhibitors of colon cancer HT29 cells and breast cancer MCF-7 cells whereas cherry, black chokeberry, and raspberry extracts were less effective (*12*). However, these extracts were

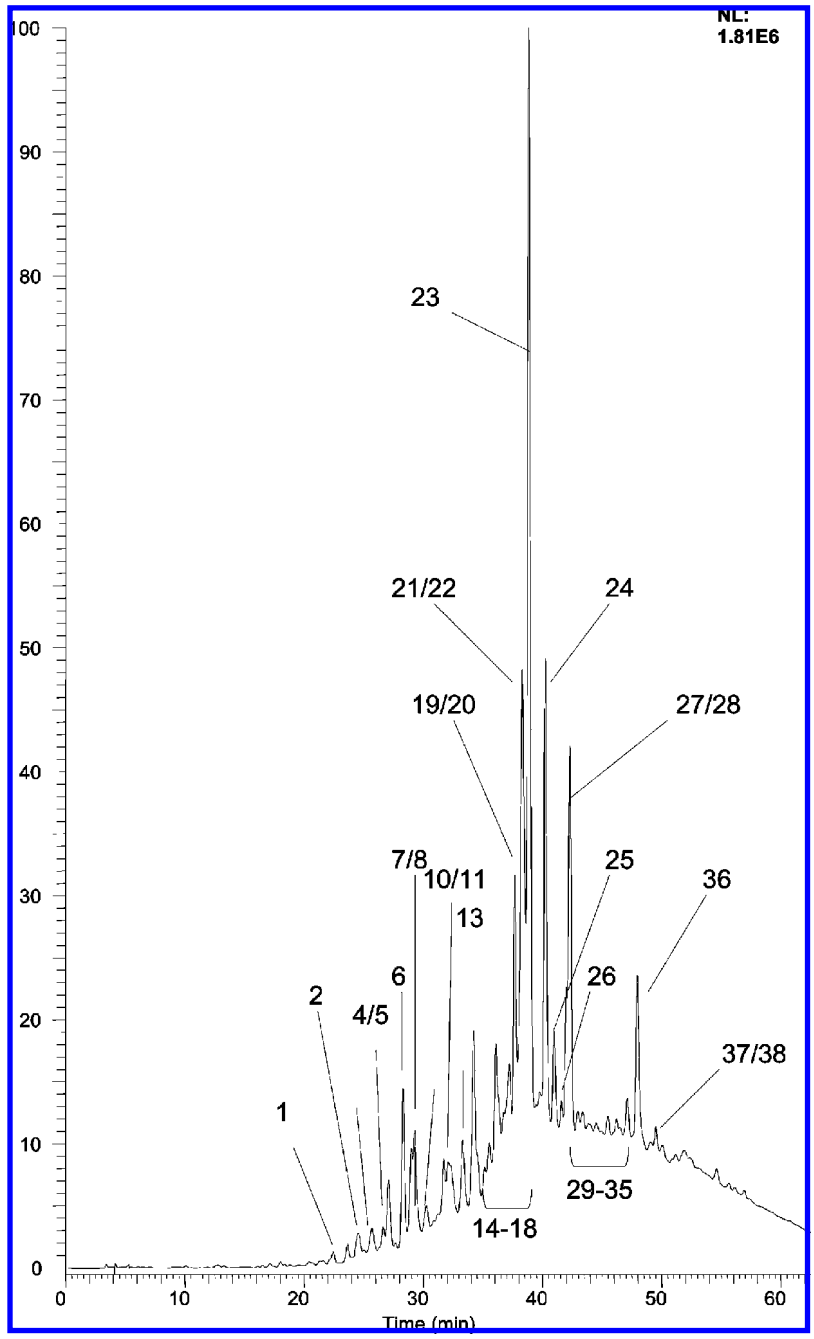

Figure 7. LC-MS trace of lingonberry tannin extract. The absorbance at 280 nm is shown. The figure in the right-hand corner represents the full-scale deflection for each trace. The peaks are numbered as presented in **Table 3**.

prepared in 50% ethanol/water and contained carotenoids, vitamin C, polyphenols, sugars, and organic acids. In fact, the antiproliferative effectiveness of these extracts was most closely correlated with vitamin C and carotenoid content. Other similar studies which ranked the antiproliferative effectiveness of methanolic berry extracts (*31, 32*) and berry juices (*33*) against a range of human cancer cell lines have found different orders of effectiveness.

All the extracts used in this study were enriched in polyphenols and depleted of sugars, organic acids, carotenoids, and vitamin C, and therefore the relative contribution of different polyphenol components can be observed. The lack of vitamin C and carotenoids may explain the difference in effectiveness of certain berries in this study and previous studies [e.g., sea buckthorn (*15*)].

Considering the recent papers extolling the effectiveness of pomegranate against prostate cancer in clinical trials (e.g., see ref *34*), the apparent antiproliferative ineffectiveness of the pomegranate extract in this study requires some explanation. In fact, previous studies with a pomegranate tannin extract, which, on initial LC-MS studies, appears to be similar to our extract (results not shown), showed that it was relatively ineffective against a range of colon cancer cell lines in vitro (*15*) and required doses of around 100 μg/mL to cause significant inhibition. This level of inhibition would not be picked up by our screen, which was carried out using 50 μg/mL GAE of extracts.

Published polyphenolic compositions (*20, 21, 28, 35*) for the most effective berry types strongly suggest that effectiveness of the *Rubus* family (raspberry, arctic bramble, and cloudberry)

Table 2. Major Polyphenol Peaks in Lingonberry Extract[a]

peak no.	RT (min)	PDA	*m/z* [M − H]	MS^2	putative identity[b]
1	21.03	295	371.1	249.0	ND
2	22.21	270	+501.1	322.1	ND
3	27.19	280	291.1	**139.1**, 165.0	epicatechin
4	27.53	325	**353.3**, 191.1	191.1	chlorogenic acid
5	27.64	515, 280	+**449.0**, 287.2	287.2	cyanidin-3-*O*-galactoside
6	29.00	315, 280	329.1	**185.0**, 121.1	hydroxycinnamic acid derivative
7	29.31	315, 280	379.1	249.1	hydroxycinnamic acid derivative
8	29.72	515, 280	+**449.0**, 287.2	287.2	cyanidin-3-*O*-glucoside
9	30.40	325, 280	461.1	415.0, multiple	hydroxycinnamic acid derivative
10	32.01	515, 280	+**419.1**, 287.2	287.2	cyanidin-3-*O*-arabinoside
11	41.02	320, 290	**355.1**, 193.0	193.0	ferulic acid hexoside
12	41.90	310			ND
13	43.16	285			ND
14	43.90	315	**355.1**, 193.0	193.0	ferulic acid hexoside
15	44.38	350	+**611.1** (*633.1*), 303.2	303.2	quercetin rutinoside
16	46.08	355	+**465.0**, 303.2	303.2	quercetin 3-*O*-galactoside
17	46.53	280	333.1	185.1, multiple	ND
18	46.75	355	+**465.0** (*487.2*), 303.2	303.2	quercetin 3-*O*-glucoside
19	47.85	280			ND
20	49.02	355	+**435.0** (*457.1*), 303.2	303.2	quercetin xyloside
21	50.22	355	+**435.0** (*457.1*), 303.2	303.2	quercetin xyloside
22	51.07	280	+579.0	365.0, 325.0, 277.1	PAC dimer
23	51.33	355	+**435.0** (*457.1*), 303.2	303.2	quercetin arabinoside
24	51.63	280	853.0	multiple	PAC
25	52.11	355	+**448.9** (*471.1*), 303.2	303.2	quercetin rhamnoside
26	56.91	325	**501.3**, 193.1	**307.0**, 193.0	hydroxycinnamic acid derivative
27	60.66	350	+**592.9** (*615.2*), 303.2	303.2	quercetin 4-HMG-rhamnoside
28	65.04	350	**575.2**, 285.1	285.1	kaempferol-4-HMG-rhamnoside

[a] All putative identifications are based on previous work (*27–30, 42, 53*). The main ion is shown in bold. + = positive ions. Figures in parentheses are the Na^+ (+22) adducts of the main ion, which are often seen in flavonols. [b] 4-HMG = 3-hydroxymethyl glutaroyl; PAC = proanthocyanidin; ND = not determined.

but also strawberry could be due to their high content of ellagitannins. Ellagitannins and ellagitannin-rich fruit extracts have previously been reported to inhibit cancer cell growth in vitro (e.g., see refs *24* and *36*), inhibit cancer progression in animal models (*17*), and be effective in human trials against prostate cancer (*34*). Their effectiveness may be due to the release of the potent antiproliferative compound, ellagic acid (*23*), under physiological conditions (*24, 37*).

There has been little evidence presented for the anticancer effects of lingonberry. Apart from the report discussed above (*12*) where lingonberry extracts were shown to inhibit cancer cell proliferation, they have also been shown to inhibit tumor progression in mice model systems (*17, 38*). As well as high levels of flavonoid components (anthocyanins, proanthocyanidins, and flavonols *28, 29*), lingonberry also contains a diverse range of other potentially bioactive components such as lignans (*39*), iridoid glycosides and hydrophilic carboxylic acids (*40*), stilbenes [including resveratrol (*41*)], and a range of other phenolic components not found in other berry species (*42*). Nevertheless, despite this phytochemical richness, the antiproliferative effect was retained in the lingonberry tannin-rich extract, which was composed almost entirely of proanthocyanidins, which indicates that these components are largely responsible for the antiproliferative effects. However, it is possible that other, as yet, unidentified components act synergetically or additively with the proanthocyanidins.

Previous work (*43*) indicated anticarcinogenic potential was concentrated in the proanthocyanidin-rich fraction of lingonberry, but this work focused on inhibition of ornithine decarboxylase, an indicator of anticarcinogenic activity. Conversely, anthocyanin-rich fractions from blueberry were more effective antiproliferative agents against certain human colon cancer cell lines than tannin- and flavonol-enriched fractions (*44*). The blueberry tannin fraction would contain mainly proanthocyanidins (*45*), but the difference in effectiveness between anthocyanin and tannin fractions was much less pronounced using CaCo-2 cells than was observed using HT-29 colon cancer cells. In any case, grape seed proanthocyanidins (*46*) have been demonstrated to be potent anticancer agents. Therefore, it is not surprising that lingonberry proanthocyanidins are also effective antiproliferative agents. Supporting evidence also comes from the structurally related gallotannins, which have long been known to be effective against chemically induced skin cancer (*47*).

The high antioxidant capacity of lingonberries (*48*) seems to be related to their high content of proanthocyanidins compared to other berries (*28*). Indeed, purified proanthocyanidins from lingonberry have been reported to have very high antioxidant activity in a range of assays (*49*). However, the mechanism whereby proanthocyanidins act as antiproliferative agents is as yet unknown.

It is clear that the serum bioavailability of many berry polyphenols is low (*50*) and probably orders of magnitude lower than the levels of berry extracts required for effective inhibition of HeLa cell proliferation in this study. Therefore it is very unlikely that cervical cancer cells in vivo would ever be exposed to these levels through normal diets. However, previous work (e.g., see ref *51*) suggested that a major portion of ingested polyphenols survive digestion in the upper gastrointestinal tract (GIT) and reach the colon, where they could be subject to fermentation by colonic microflora. This has been confirmed in human ileostomy patients (see ref *52*). Therefore, considering the high polyphenol content of berries (*20*), colonic epithelial cells (which are akin to the CaCo-2 cancer cells) and epithelial cells of the upper GIT could be exposed to these levels and we

Table 3. Major Polyphenol Peaks in Lingonberry Tannin Extract

peak no.	RT (min)	*m/z* [M − H]	MS[2a]	putative ID[b]
1	22.64	1153	865	EC4
2	24.55	1153	865	EC4
3	25.67	577	425	EC2
4	26.63	1153	865	EC4
5	27.08	865	713	EC3
6	28.29	865	713	EC3
7	28.98	1153	865	EC4
8	29.29	865	713	EC3
9	30.24	1441	multiple	EC5
10	31.71	1153	865	EC4
11	32.09	863	711	EC3A
12	32.27	865	713	EC3
13	33.31	1151	863	EC4A
14	34.22	863	711	EC3A
15	34.55	1153	865	EC4
16	35.14	1439	multiple	EC5A
17	36.08	865	713	EC3
18	36.35	863	711	EC3A
18	36.73	1153	865	EC4
19	37.20	1441	multiple	EC5
20	37.66	865	713	EC3
21	38.28	577	425	EC2
22	38.51	1153	865	EC4
23	38.84	1153	865	EC4
24	40.23	1153, 863	multiple	EC4, EC3A mix
25	40.96	863, 711, 575	711	EC3A
26	41.55	1151	863	EC4A
27	41.99	863	711	EC3A
28	42.29	575	425	EC2A
29	42.95	1151, 1439	multiple	EC5A
30	43.36	575, 863, 1151, 1439, 1727	multiple	EC6A
31	43.86	575, 863, 1151, 1439, 1727	multiple	EC6A
32	44.49	575, 863, 1151, 1439, 1727	multiple	EC6A
33	44.93	575, 863, 1151, 1439, 1727	multiple	EC6A
34	45.48	577, 865	713	EC3
35	47.10	1151, 575	863	EC4A
36	47.94	575	423, 285	EC2A
37	48.40	1727	multiple	EC6A
38	49.05	1151	863	EC4A
39	49.50	863	711	EC3A

[a] Only the most abundant MS^2 ion is denoted. [b] EC = epicatechin, EC2 = B-linked epicatechin dimer, EC2A = A-linked epicatechin dimer. The PDA spectrum of all components was centred on 280 nm.

conclude that the CaCo-2 screen is a physiologically relevant model for first-pass screening of components with anticancer effects.

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