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Blackberry, Black Raspberry, Blueberry, Cranberry, Red Raspberry, and Strawberry Extracts Inhibit Growth and Stimulate Apoptosis of Human Cancer Cells In Vitro

NAVINDRA P. SEERAM,* LYNN S. ADAMS, YANJUN ZHANG, RUPO LEE, DANIEL SAND, HENRY S. SCHEULLER, AND DAVID HEBER

Center for Human Nutrition, David Geffen School of Medicine, University of California, Los Angeles, California 90095

Berry fruits are widely consumed in our diet and have attracted much attention due to their potential human health benefits. Berries contain a diverse range of phytochemicals with biological properties such as antioxidant, anticancer, anti-neurodegerative, and anti-inflammatory activities. In the current study, extracts of six popularly consumed berries—blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry—were evaluated for their phenolic constituents using high performance liquid chromatography with ultraviolet (HPLC-UV) and electrospray ionization mass spectrometry (LC-ESI-MS) detection. The major classes of berry phenolics were anthocyanins, flavonols, flavanols, ellagitannins, gallotannins, proanthocyanidins, and phenolic acids. The berry extracts were evaluated for their ability to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cell lines at concentrations ranging from 25 to 200 μg/mL. With increasing concentration of berry extract, increasing inhibition of cell proliferation in all of the cell lines were observed, with different degrees of potency between cell lines. The berry extracts were also evaluated for their ability to stimulate apoptosis of the COX-2 expressing colon cancer cell line, HT-29. Black raspberry and strawberry extracts showed the most significant pro-apoptotic effects against this cell line. The data provided by the current study and from other laboratories warrants further investigation into the chemopreventive and chemotherapeutic effects of berries using in vivo models.

KEYWORDS: Berries; polyphenols; antiproliferative; apoptosis; cancer

INTRODUCTION

Epidemiological studies suggest that consumption of a phytochemical-rich diet, which includes fruits and vegetables, contribute toward reducing the risk of certain types of human cancers (*1*, *2*). Among colorful fruits, berries such as blackberry (*Rubus sp*.), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium macrocarpon*), red raspberry (*Rubus idaeus*) and strawberry (*Fragaria ananassa*) are popularly consumed in human diet in fresh and in processed forms such as beverages, yogurts, jellies, and jams. In addition, berry extracts are widely consumed in botanical dietary supplement forms for their potential human health benefits.

A wide number of laboratory and animal studies have shown that berries have anticancer properties (reviewed in ref *3*). The biological activities of berries are partially attributed to their high content of a diverse range of phytochemicals such as flavonoids (anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins, ellagitannins, and gallotannins), stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), and lignans (*3*). Berry bioactives impart anticancer effects through various complementary and overlapping mechanisms of action including the induction of metabolizing enzymes, modulation of gene expression and their effects on cell proliferation, apoptosis, and subcellular signaling pathways (reviewed in ref *4*). As part of our ongoing interest in phytochemical constituents and biological activity evaluation of berry fruits (*5*, *6*), we have focused our attention on six popularly consumed berries: blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry. The objectives of the current study were (i) to establish phenolic fingerprint profiles of the berry extracts using high performance liquid chromatography with ultraviolet (HPLC-UV) and electrospray ionization mass spectrometry (LC-ESI-MS) detection; (ii) to evaluate the berry extracts (at 25−200 μg/mL concentrations) for their ability to inhibit the proliferation of human oral (KB, CAL-27), prostate (LNCaP), breast (MCF-7), and colon (HT-29, HCT116) tumor cell lines, and (iii) to evaluate the berry

* To whom all correspondence should be addressed. Navindra Seeram, Center for Human Nutrition, 900 Veteran Avenue, Los Angeles, CA, USA. Tel: (310) 825-6150; fax: (310) 206-5264; e-mail: nseeram@mednet.ucla.edu.

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extracts (at 200 μg/mL concentrations) for their ability to stimulate apoptosis of the cyclooxygenase-2 (COX-2) enzyme expressing colon cancer cell line, HT-29.

The identification of phytochemical constituents of some edible berries has been reported (*5*, *7*−*13*), as has been their anticancer activity (*14*−*18*). However, this is the first evaluation of the antiproliferative and pro-apoptotic effects of blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry using extracts characterized by HPLC-UV and LC-MS against this panel of human tumor cell lines. The data provided by the current study and from others (*7*, *14*−*18*) warrant future studies to probe the chemopreventive and chemotherapeutic effects of berries using in vivo models.

MATERIALS AND METHODS

Reagents. All solvents were HPLC grade and purchased from Fisher Scientific Co. (Tustin, CA). Ellagic acid, catechin, epicatechin, and quercetin standards were purchased from Sigma Aldrich Co. (St. Louis, MO). Cyanidin, pelargonidin, peonidin, malvidin, and delphinidin aglycons and their respective 3-glucosides (anthocyanins) were purchased from Chromadex Inc. (Santa Ana, CA).

Berry Extracts. Fresh berry fruits: blackberry *(Rubus sp*.), black raspberry *(Rubus occidentalis*), blueberry var. Jersey (*Vaccinium corymbosu*m), cranberry var. Early Black (*Vaccinium macrocarpo*n), red raspberry (*Rubus idaeu*s), and strawberry (*Fragaria ananass*a) were purchased from local supermarkets (Westwood, Los Angeles, CA). The post-harvest dates for the berries were not provided on the label information of these packages. The edible parts of the berries were extracted in methanol containing 0.1% HCl by sonication for 20 min as previously reported (*5*). Briefly, a representative sample of fruits (from three packages weighing approximately 200 g) were separately blended (Waring Blender, New Hartford, CT) with methanol (100 mL containing 0.1% HCl) and then centrifuged. The supernatant liquids were concentrated in vacuo (Buchi Rotavap) at a temperature of 37 °C as reported for preparation of phenolic-rich extracts from berries (*6*, *7*). The berry extracts were dissolved to a concentration of 1 mg/mL by sonication in water/methanol containing 0.1% HCl (1:1, v/v) and injected directly on the HPLC for phenolic analyses. For the bioassays, the extracts were enriched in phenolics by removing sugars and acids using C-18 Sep-Pak cartridges as reported (*7*).

High Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) Detection and Electrospray Ionization Mass Spectrometry (ESI-MS) Methods. A Surveyor HPLC system equipped with a diode array absorbance detector (DAD), scanning from 250 to 600 nm, and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, USA) with a Symmetery C-18 column, 250 × 4.6 mm, i.d. 5 μm (Waters, MA) was used. The mobile phase consisted of a gradient system over 70 min of acetonitrile (ACN) and H_2O (1% formic acid) at a flow rate of 1 mL/min. The program was an isocratic elution with 10% ACN in H_2O (containing 1% formic acid) for 10 min followed by a linear gradient to 20% ACN in H_2O containing 1% formic acid at 70 min with a column regeneration time of 10 min between injections. The column was maintained at 25 °C. After the sample passed through the flow cell of the DAD, the column eluate was split and 0.2 mL/min was directed to a LCQ Advantage ion trap (Thermo Finnigan, San Jose, USA) mass spectrometer fitted with an electrospray (ESI) interface. Analyses utilized the positive ion mode (m/z M + H^+) for detection of anthocyanins and negative ion mode (m/z M − H^+) for all other compounds. Preliminary analyses were carried out using full scan, data dependent MS/MS scanning from m/z 250−2000. The capillary temperature was 275 °C, sheath gas and auxiliary gas were 45 and 0 units/min, respectively, and the source voltage was 4 kV. MS/MS fragmentation was carried out with 50% energy. Zoom scan analyses were carried out to determine the charge state of some of the ellagitannin-based compounds as reported (*5*). Identities of the compounds were obtained by matching their molecular ions (m/z) obtained by LC-ESI-MS and LC-ESI-MS/MS with literature data (*5*, *8*−*13*).

Cell Culture Materials. All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). KB oral cancer cells and MCF-7 breast cancer cells were grown in Minimum Essential Medium (MEM); CAL-27 oral cancer cells were grown in Dulbecco's Minimum Essential Medium (DMEM); LNCaP prostate cancer cells were grown in RPMI 1640; HT-29 and HCT116 colon cancer cells were grown in McCoy's 5A Medium, Modified. All media contained 10% fetal bovine serum (FBS) in the presence of 100 U/mL penicillin and 0.1 g/L streptomycin. Cells were incubated at 37 °C with 95% air and 5% CO_2. Cells used had 20 or fewer passages and were in their linear phase of growth during the experiment.

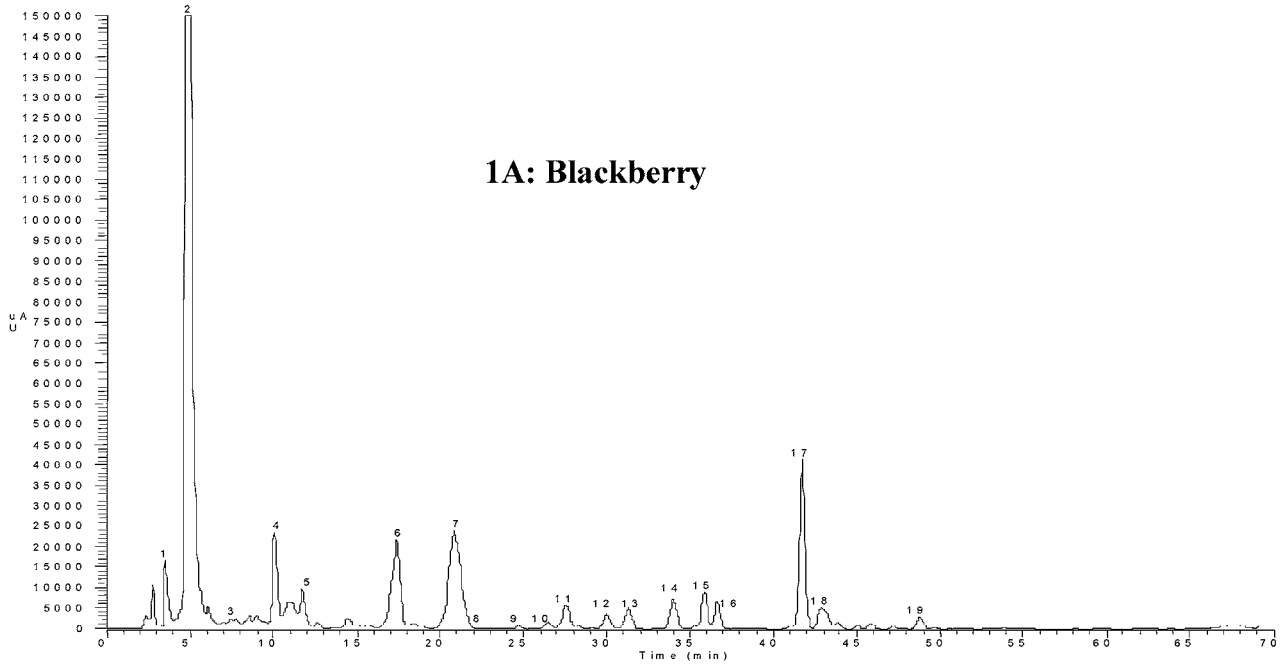
Cell Proliferation Assay. Proliferation was measured utilizing the CellTiter-Glo Luminescent Cell Viability assay (Technical Bulletin # 288, Promega Corp., Madison, WI). When added to cells, the assay reagent produces luminescence in the presence of ATP from viable cells. Cells were plated in 96-well plates at a density of 5000 cells/well and incubated for 24 h. Berry extracts were solubilized in deionized water by sonication, filter sterilized, and diluted to the desired treatment concentration with media specific to the cell type studied. Cells were treated with 100 μL of media or berry extracts and incubated for 48 h drug exposure duration as previously reported (*6*). Berry extracts were tested at 25, 50, 100, and 200 μg/mL concentrations. At the end of 48 h, plates were equilibrated at room temperature for 30 min, 100 μL of the cell viability assay reagent was added to each well, and cell lysis was induced on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescence signal, and results were read on an Orion Microplate luminometer (Bertholds Detection Systems, Pforzheim, Germany). All plates had control wells containing medium without cells to obtain a value for background luminescence, which was subtracted from the test sample readings. Data are expressed as the ratio of treated to untreated cells, mean ± SE for three replications.

Assessment of Apoptosis. Apoptosis was assessed utilizing the Cell Death Detection ELISA^{PLUS} assay (Boehringer Mannheim, Indianapolis, IN). This assay is a photometric enzyme-linked immunoassay that quantitatively measures the internucleosomal degradation of DNA, which occurs during apoptosis. The assay is a quantitative sandwich-enzyme-immunoassay utilizing monoclonal mouse antibodies directed against DNA and histones that detect specifically mono- and oligonucleosomes. Quantitative measurement of the amount of internucleosomal degradation is measured photometrically at 405 nm with ABTS as substrate (*20*). HT-29 cells (grown in McCoy's 5A Medium, Modified) were plated in 60-mm dishes (Falcon, BD Biosciences, San Jose, CA) at a density of 100 000 cells/dish and allowed to attach for 24 h. Cells were treated with media control or berry extracts at a concentration of 200 μg/mL for 48 h. Following treatments, nonadherent cells were collected and pelleted at 200*g* for 10 min. The supernatant was discarded; the cell pellet was washed with cold calcium magnesium free-phosphate buffered saline (CMF-PBS) containing 137 mmol/L sodium chloride, 1.5 mmol/L potassium phosphate, 7.2 mmol/L sodium phosphate, 2.7 mmol/L potassium chloride at pH 7.4 and recentrifuged. Adherent cells were washed with CMF-PBS, trypsinized, collected, and combined with nonadherent cells into a total of 1 mL of media. Both live and dead cells were then counted via Trypan Blue (Pierce, Rockford, IL) exclusion, and 10 000 cells were added to the microtiter plate for all treatment groups and the apoptosis assay was performed according to the manufacturer's instructions. Values were subtracted from background readings (media plus reagent, no cells) and expressed as absorbance of dye bound to antibodies bound to mono- and oligonucleosomes at 405 nm of each treated sample divided by media controls.

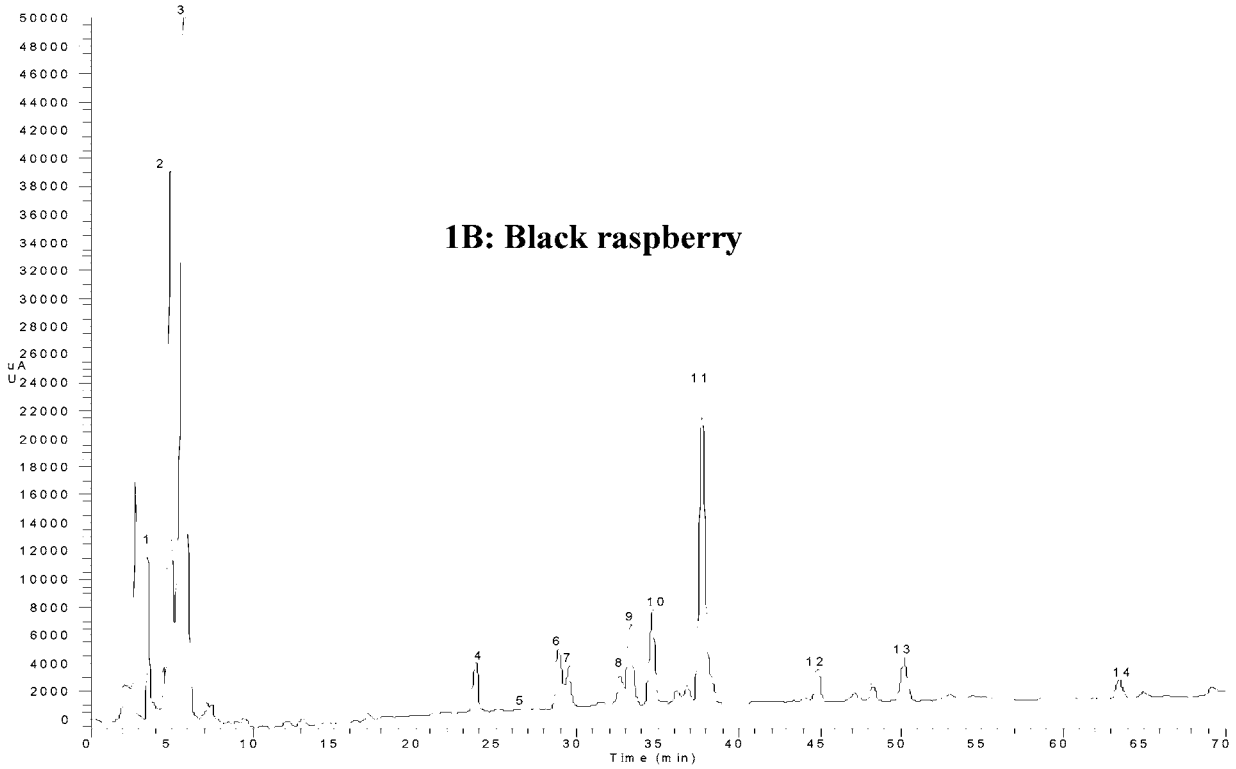
Statistical Analysis. Data were analyzed by either student's t-test, one-way ANOVA followed by Dunnett's Multiple Range test ($\alpha = 0.05$) with Graph Pad Prism 3.0 (Graph Pad Software Inc., San Diego, CA) as appropriate.

RESULTS AND DISCUSSION

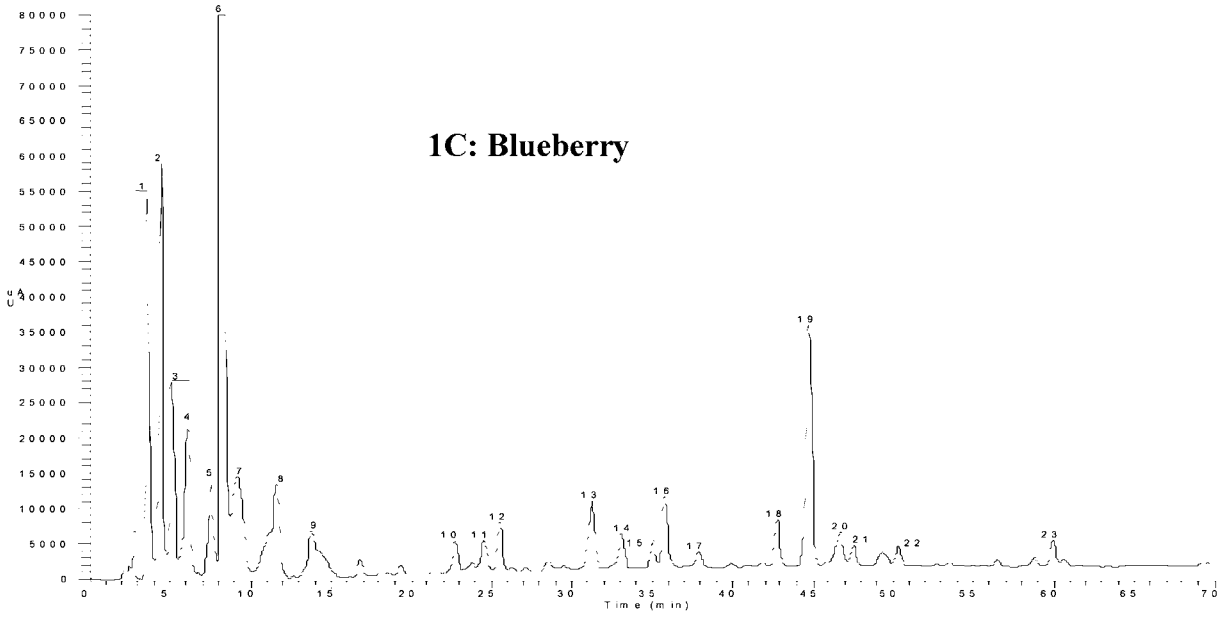
HPLC Profiling of Berry Extracts for Phenolics. Figure 1 shows the fingerprint phenolic profiles of blackberry (**Figure 1A**), black raspberry (**Figure 1B**), blueberry (**Figure 1C**), cranberry (**Figure 1D**), red raspberry (**Figure 1E**), and strawberry (**Figure 1F**) extracts. The individual phenolics from these



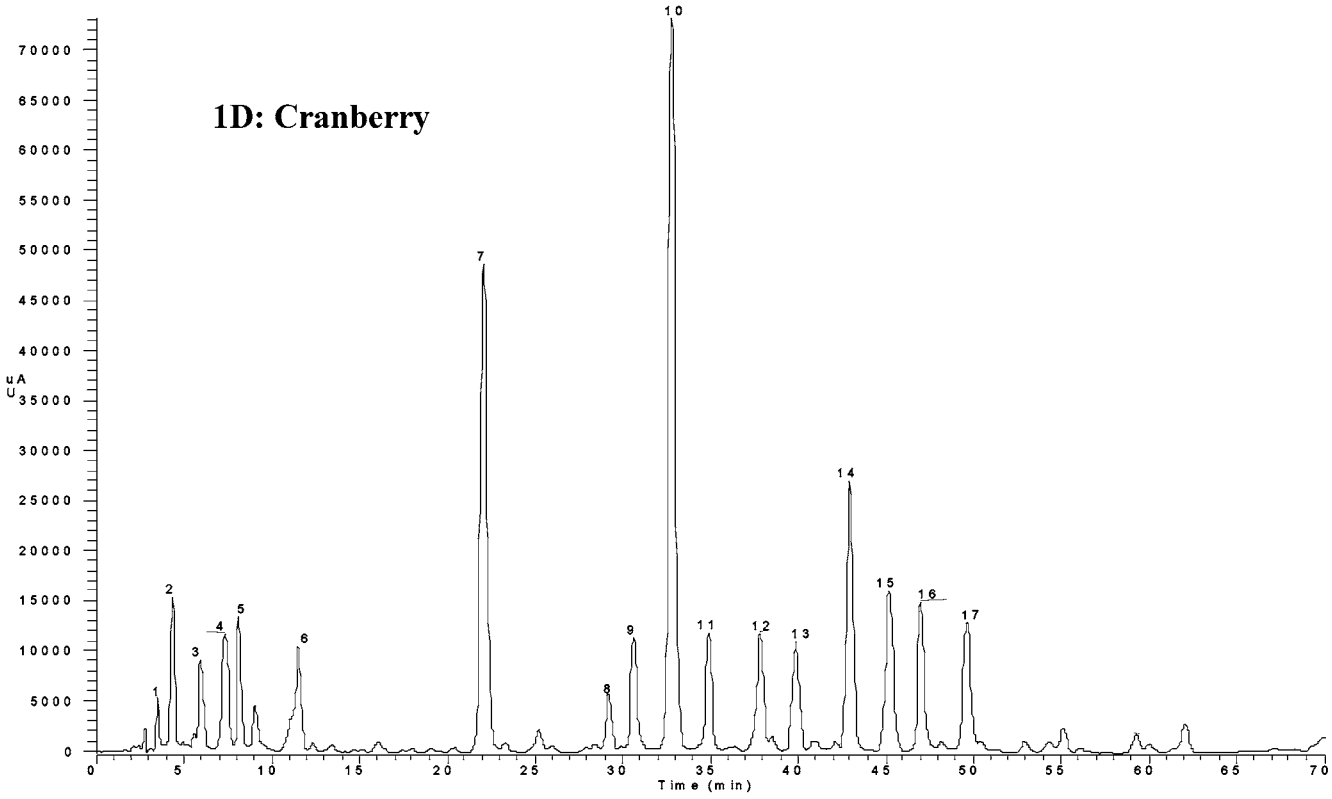
Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	3.52	280, 520	449	+	287	Cy-glucoside
2	4.72	280, 515	419	+	287	Cy-arabinoside
3	7.60	280, 510	433/449	+	271/287	Plg-glucoside/Cy-rutinoside
4	10.07	340, 520	463	-	301	Peo-glucoside
5	11.73	330, 520	419	+	287	Cy-xyloside
6	17.40	280, 330, 520	535	+	449, 287	Cy-(malonoyl)glucoside
7	20.83	280, 520	593,	-	, 287 /	Cy-dioxaloylglucoside
8	22.12	235, 280	1401	-	1251, 1235, 1099, 933,897, 633	unknown
9	24.84	235	1869	-	1567, 1235, 933, 897, 631	unknown
10	26.58	250, 330, 520	593	-	301	EA-derivative
11	27.57	250, 360, 515	433	-	301	EA-derivative
12	29.96	250, 360, 520	433	-	301	EA-derivative
13	31.28	250, 365	301	-	257, 229	EA
14	33.98	255, 350	463	-	301,179	Q-glucoside
15	35.87	255, 350	463	-	301	EA + Q-derivative
16	36.62	255, 350	477	-	301,179	Q-derivative
17	41.73	255, 355	607	-	463, 301	Q-derivative
18	42.88	255, 350	505	-	301,179	Q-derivative
19	48.78	255, 350	505	-	301,179	Q-derivative



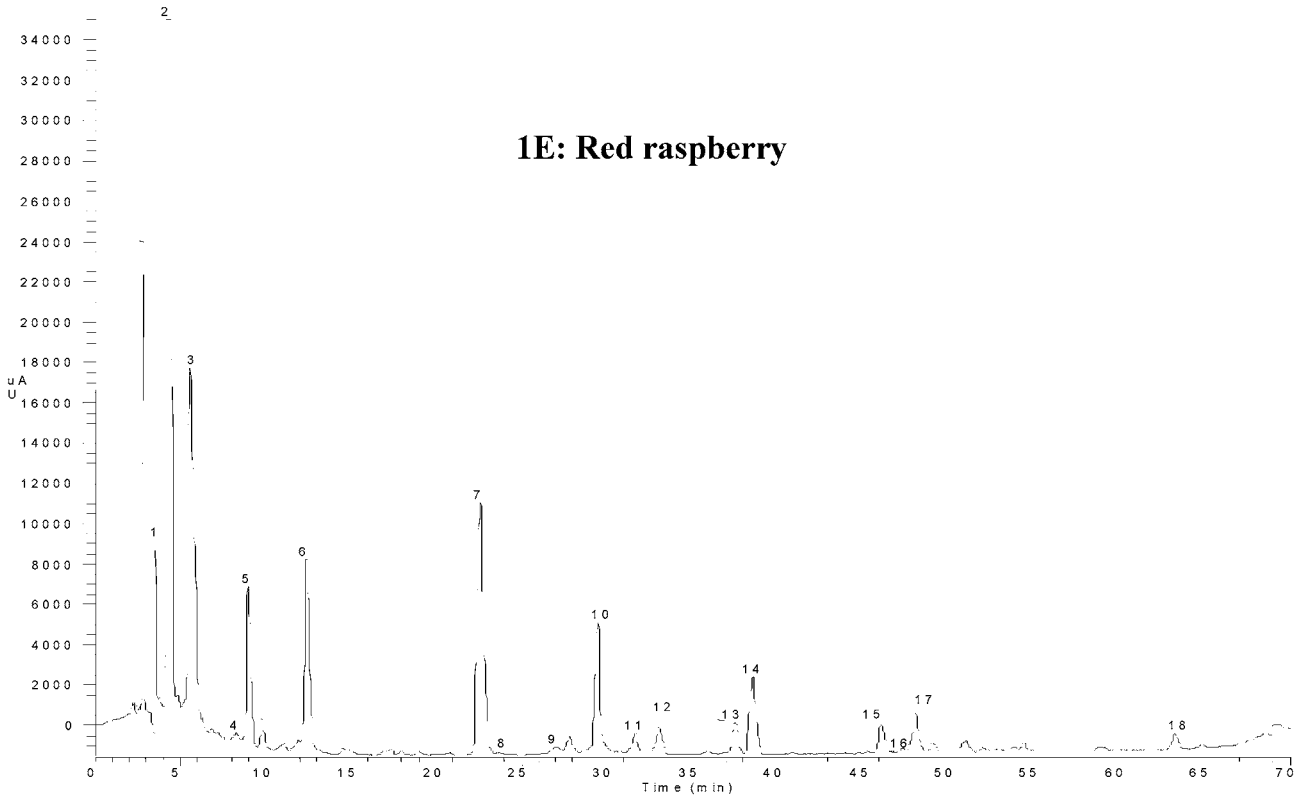
Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	3.55	235, 275, 525	757	+	611, 433, 287	Cy-sophoroside-rhamnoside
2	4.90	235, 280, 520	727	+	581, 433, 287	Cy-sambubioside-rhamnoside
3	5.77	235, 280, 520	595	+	449, 287	Cy-rutinoside
4	23.78	255, 370	741	-	301	EA-derivative
5	26.62	260, 345	1869	-	1567, 1235, 933, 897, 631	EA-derivative
6	28.88	245, 370	433	-	301	EA-derivative
7	29.52	250, 370	447	-	301	EA-derivative
8	32.72	255, 370	301	-	257, 229	EA
9	33.33	250, 360	609	-	301	EA-derivative
10	34.70	250, 360	609	-	301	EA-derivative
11	37.75	255, 355	477	-	301	Q-derivative
12	44.87	255, 370	447	-	315	unknown
13	50.22	255, 370	491	-	315, 301	unknown
14	63.52	255, 370	489	-	315	unknown



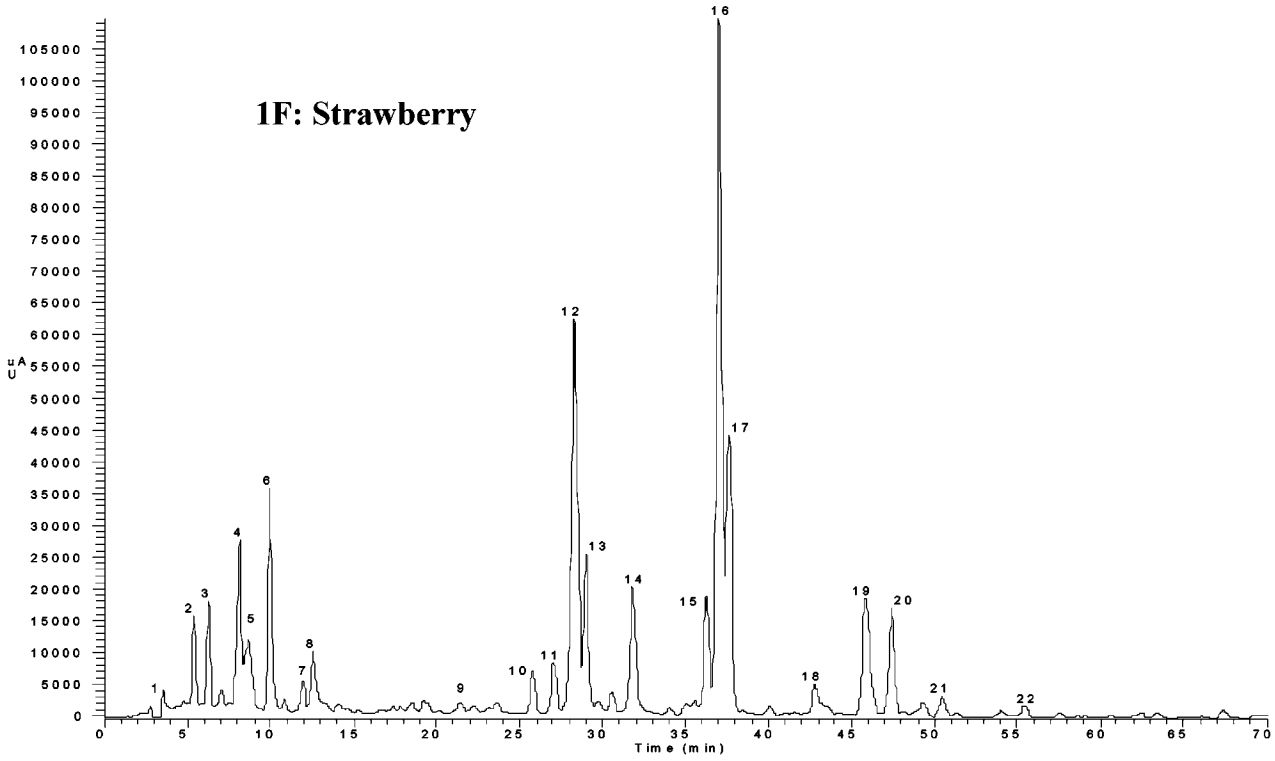
Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	3.57	235, 275, 525	465	+	303	Dp-galactoside
2	4.43	235, 280, 520	449/435	+	287/303	Cy-galactoside/Dp-arabinoside
3	5.12	235, 280, 520	479, 449	+	317, 287	Pt-galactoside
4	6.1	240, 280, 520	479, 419	+	317, 287	Pt-glucoside
5	7.53	235, 280, 520	463/449	+	301/317	Peo-galactoside/Pet-arabinoside
6	8.27	245, 325, 530	493	+	331	Mal-galactoside
7	9.18	235, 280, 525	493/	+	331	Mal-glucoside
8	11.63	235, 275, 525	463	-	331	Mal-arabinoside
9	13.8	240, 275, 530	463	-	331, 301	Mal/Peo-glycoside
10	22.78	235, 370	479	-	317	unknown
11	24.53	245, 370	493	-	317	unknown
12	25.53	245, 285, 325	367	-	285,179,161, 135	K-derivative
13	31.28	250, 370	463	-	316	unknown
14	33.1	250, 370	463	-	343, 301	unknown
15	35.07	250, 370	463	-	301,179	Q-derivative
16	35.78	250, 370	477	-	301,179	Q-derivative
17	37.92	250, 370	507, 433	-	507, 433,331, 301	unknown
18	42.8	250, 370	433	-	301	unknown
19	44.77	255, 345	447	-	301	Q-derivative
20	46.72	250, 370	477	-	331	unknown
21	47.55	250, 370	507	-	471, 344, 329, 315	unknown
22	50.33	255, 370	521	-	345	unknown
23	59.97	255, 370	491	-	345, 301	unknown



Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	3.5	240, 520	435	+	303	Dp-arabinoside
2	4.37	255, 510	449	+	287	Cy-glucoside
3	5.98	255, 510	419	+	287	Cy-arabinoside
4	7.37	260, 510	463	+	301	Peo-galactoside
5	8.15	310, 505	463	+	301	Peo-glucosoide
6	11.53	255, 510	433	+	301	Peo-arabinoside
7	22.1	265, 325	479	-	316	unknown
8	29.23	250	449	-	316	unknown
9	30.65	250, 340	449	-	316	unknown
10	32.82	245, 345	463	-	301, 179	Q-derivative
11	34.95	250	673, 575	-	575, 449, 423	unknown
12	37.87	245, 340	433	-	301	Q-derivative
13	39.9	245, 335	433	-	301	Q-derivative
14	42.98	245, 340	433	-	301	Q-derivative
15	45.22	245, 335	447	-	301	Q-derivative
16	47.02	245, 275, 345	507	-	344, 387, 301	unknown
17	49.67	245, 275, 360	n.d.	-	n.d	unknown



Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	3.51	235, 530	611	+	287	Cy-sophoroside
2	4.25	280, 520	611	+	287	Cy-sophoroside
3	5.58	280, 525	449	+	287	Cy-glucoside
4	8.11	235, 505	433	+	271	Plg-glucoside
5	8.98	235	n.d	-	463, 301	EA-derivative
6	12.4	235, 330	n.d	-	463,301	EA-derivative
7	22.57	235, 350	625	-	463, 301	EA-derivative
8	23.69	235	1401	-	1265, 933, 633, 1250	EA-derivative
9	27.01	235	1869	-	1567, 1265, 1235, 935	Sanguiin H-6
10	29.45	250, 360	433	-	301	EA-derivative
11	31.65	250	859	-	831.7, 795.7, 785.0, 765.4, 713.1,437.1, 301	EA-derivative
12	33.07	250	301	-	-257, 229	EA-derivative
13	37.53	250	463	-	301, 179	Q-derivative
14	38.55	250, 345	477	-	301	EA-derivative
15	46.1	245	447	-	315	unknown
16	47.74	245, 315	839	-	807.5, 779.3, 747.3, 638.4, 301	EA-derivative
17	48.12	250	475	-	301	EA-derivative
18	63.32	250	489	-	315	unknown



Peak #	HPLC-UV (min)	HPLC-UV (nm)	MS	ESI mode	MS/MS	Tentative ID
1	5.37	235, 350	783	-	481, 301	EA-derivative
2	6.16	275, 520	449	+	287	Cy-glucoside
3	8.13	235, 280, 350	639	-	463, 301	EA-derivative
4	8.68	275, 520	433	+	271	Plg-glucoside
5	10.13	310, 505	579	+	473,271	Plg-rutinoside
6	11.95	235, 310	325	-	265, 187, 145, 163	Coumaroyl-glucoside
7	12.55	235, 330	355	-	295, 217, 175, 193, 134	Coumaroyl-ester
8	21.3	235, 250	935	-	898, 633, 463, 301	Galloyl-HHDP-glucoside
9	25.8	240, 325	479	-	n.d	unknown
10	27.07	265, 350	609	-	301, 179, 151	Q-rutinoside
11	28.32	255, 355	595	-	300, 271, 179 1	unknown
12	29.02	250, 370	447	-	301, 257	Methyl-EA-pentose
13	31.8	250, 370	301	-	257, 229	EA
14	36.27	285	463	-	301, 179, 151	Q-glucoside
15	37.03	265, 355	477	-	301, 179, 151	Q-glucuronide
16	37.63	235, 260, 345	1869	-	1567, 1265, 1085, 935, 633	Sanguiin-H 6
17	42.8	250	593	-	285	K-coumaroyl-glucoside
18	45.85	260, 345	447	-	285, 151	Methyl-EA-pentose
19	47.43	265, 345	461	-	285	K-glucuronide
20	50.47	325	461	-	285, 281	K-glucuronide

Figure 1. HPLC-UV fingerprint profiles of berry extracts showing their diversity in phenolic constituents with accompanying tables showing peak LC-MS and LC-MS^n data. The HPLC-UV detection wavelength was set at 280 nm. Peak identification was aided by comparison with reference standards where available and from the literature (*5*, *8*–*13*). Peak numbers and retention times refer to the chromatograms as follows: 1A = blackberry; 1B = black raspberry; 1C = blueberry; 1D = cranberry; 1E = red raspberry; 1F = strawberry. LC-MS/MS methods were used to differentiate ellagic acid from quercetin (identical molecular weights of 302 amu) as reported (*5*). Cy, cyanidin; Dp, delphinidin; EA, ellagic acid; HHDP, hexahydroxydiphenoyl; K, kaempferol; Mal, malvidin; n.d. = not determined; Plg, pelargonidin; Peo, peonidin; Pt, petunidin; Q, quercetin.

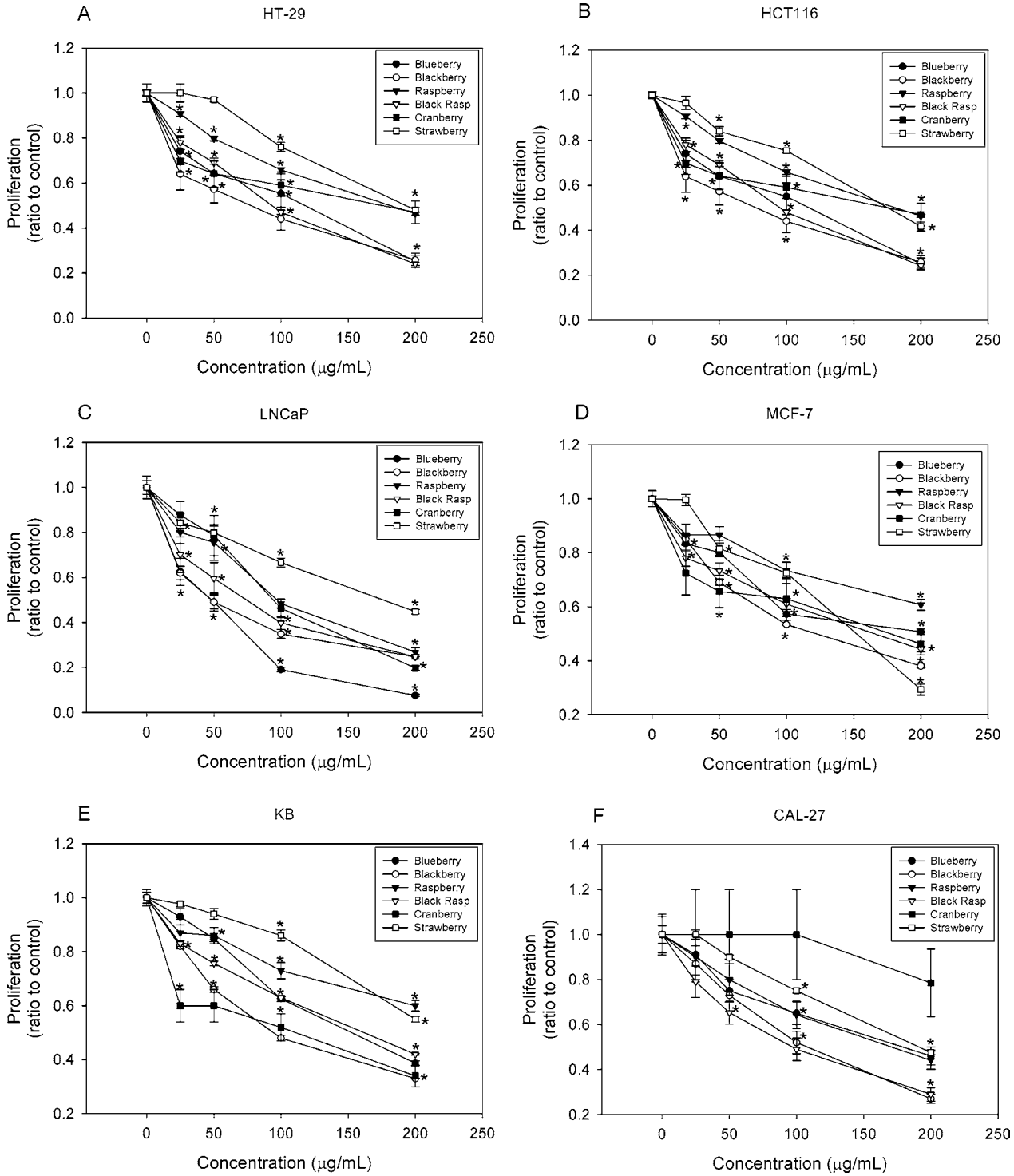


Figure 2. Inhibition of proliferation of human tumor cell lines. (A) = HT-29 (colon), (B) = HCT116 (colon), (C) = LNCaP (prostate), (D) = MCF-7 (breast), (E) = KB (oral), (F) = CAL-27 (oral). Cells were exposed to berry extracts at 25–200 μg/mL for 48 h. Proliferation was measured via the CellTiter-Glo Luminescent Cell Viability assay. Data are expressed as percentage of untreated cells, mean ± SE (n = 3). Single asterisk indicates a significant difference (p = 0.01) compared to untreated controls.

berries have been previously described (*5*, *6*, *8*−*13*) and are therefore not detailed in the current study. Nevertheless, accompanying tables with peak information and peak identification for the phenolics have been provided for the berry extracts. Whenever available, reference standards of phenolics were used to substantiate the identification of peaks in the berry extracts. Tentative identities of phenolics, which were not available as standard reference materials, were obtained by matching their molecular ions ($M + H^+$, for anthocyanins) or ($M - H^+$, for other phenolics) obtained by LC-ESI-MS and LC-MS/MS methods with the theoretical molecular weights from literature data (*5*, *8*−*13*). We utilized LC-MS/MS methods to distinguish between conjugates of quercetin and ellagic acid since their aglycons produce identical molecular ions on fragmentation ($M - H^+$, *m*/*z* 301) as previously reported (*5*). On MS/MS analyses, the quercetin *m*/*z* 301 ion further fragments to form characteristic *m*/*z* 179 and 151 ions, whereas the equivalent EA *m*/*z* 301 ion yields ions at *m*/*z* 257 and 229 (*5*).

The major groups of phenolic compounds present in berries are reported to be anthocyanins, flavonols, flavanols, ellagitannins, gallotannins, proanthocyanidins, and phenolic acids (*3*). The major anthocyanins identified in the berries (**Figure 1A−F**) correspond to the previous report (*10*) and are as follows. Blackberries contain cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-rutinoside, cyanidin-3-xyloside, cyanidin-3-malonylglucoside, cyanidin-3-dioxaloylglucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside. Black raspberries contain cyanidin-3-sophoroside rhamnoside, cyanidin-3-sambubioside rhamnoside, and cyanidin-3-rutinoside. Blueberries contain cyanidin-3-galactoside, delphinidin-3-galatoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, malvidin-3-galactoside, malvidin 3-glucoside, and malvidin-3-arabinoside. Cranberries contain cyanidin-3-glucoside, cyanidin-3-arabinoside, delphinidin-3-arabinoside, peonidin-3-galactodside, peonidin-3-glucoside, and peonidin-3-arabinoside. Red raspberries contain cyanidin-3-sophoroside, cyanidin-3-glucoside, and pelargonidin-3-glucoside. Strawberries contain cyanidin-3-glucoside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside.

With regard to the tannin composition, blackberry, black raspberry, red raspberry, and strawberry are known to contain predominantly hydrolyzable tannins (ellagitannins and gallotannins), whereas blueberries and cranberries are known to contain predominantly condensed tannins (proanthocyanidins) (*3*). It is noteworthy that proanthocyanidins do not appear as sharp peaks when monitored by HPLC-UV and appear as broad peaks at common HPLC-UV monitoring wavelengths for flavonoids and are therefore not visible in the chromatograms displayed in **Figure 1A−F** (shown at 280 nm). In addition, based on the extraction solvent (acidic methanol), which is suitable for the extraction of anthocyanins (*5*, *7*), it is unlikely that proanthocyanidins were present in the berry extracts used in our studies.

Antiproliferative Activities. Berry extracts were tested for their ability to inhibit the growth of human colon (HT-29, **Figure 2A**; HCT116, **Figure 2B**), prostate (LNCaP, **Figure 2C**), breast (MCF-7; **Figure 2D**) and oral (KB, **Figure 2E**; CAL27, **Figure 2F**) tumor cell lines. The IC50 values of the various berry extracts are shown in **Table 1**. With increasing concentration of berry extract, increasing inhibition of cell proliferation in all of the cell lines tested was observed, with different degrees of potency between cell lines. In other published in vitro studies (*6*, *14−16*), cell lines of differing origins have been shown to respond with varying degrees of sensitivity in growth toward berry extracts as seen in the current study.

Pro-Apoptotic Activities. The pro-apoptotic effects of each berry extract was evaluated on the HT-29 colon cancer cell line to ascertain whether the observed reduction in viable cell number was due to the induction of apoptosis (**Figure 3**). There is increasing evidence suggesting an association between cancer and COX-2 because prostaglandins are mediators of inflammation and chronic inflammation predisposes to carcinogenesis (*21*, *22*). Therefore, among the cell lines that we studied, we chose the HT-29 colon cancer cell that expresses the COX-2 enzyme for evaluation for pro-apoptotic activity. At the highest dose tested in the proliferation assays (200 μg/mL), two of the berry extracts exhibited significant pro-apoptotic activity ($p \leq 0.01$). Black raspberry extract induced apoptosis 3-fold over untreated controls, and strawberry extract induced apoptosis 2.8-fold over untreated controls ($p \leq 0.01$). Cells treated with the other berry extracts also showed increased levels of apoptosis compared to controls (blueberry: 1.8-fold, blackberry: 1.8-fold, and raspberry: 1.7-fold), whereas cranberry extract demonstrated no pro-apoptotic activity against this cell line.

Table 1. IC50 Values of Berry Extracts on the Proliferation of Human Tumor Cell Lines[a]

CAL-27	IC50	SE	KB	IC50	SE
blackberry	110.80	0.07	blackberry	98.04	0.02
blueberry	177.40	0.07	blueberry	171.30	0.03
raspberry	164.50	0.08	raspberry	95.40	0.02
black rasp	92.58	0.08	black rasp	80.80	0.11
cranberry	130.05	0.08	cranberry	180.61	0.06
strawberry	102.00	0.06	strawberry	81.51	0.03
HT-29	**IC50**	**SE**	**HCT116**	**IC50**	**SE**
blackberry	64.60	0.05	blackberry	65.00	0.05
blueberry	89.96	0.05	blueberry	90.00	0.05
raspberry	187.60	0.01	raspberry	188.00	0.01
black rasp	89.11	0.02	black rasp	89.00	0.02
cranberry	121.30	0.06	cranberry	121.00	0.06
strawberry	114.20	0.04	strawberry	62.00	0.02
MCF-7	**IC50**	**SE**	**LNCaP**	**IC50**	**SE**
blackberry	122.00	0.04	blackberry	49.61	0.04
blueberry	169.90	0.04	blueberry	36.45	0.05
raspberry	190.80	0.05	raspberry	100.00	0.06
black rasp	145.40	0.04	black rasp	66.75	0.06
cranberry	135.00	0.07	cranberry	100.00	0.07
strawberry	180.00	0.13	strawberry	178.00	0.07

. [a]IC50 values were calculated by nonlinear regression (curve fit) utilizing GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA).

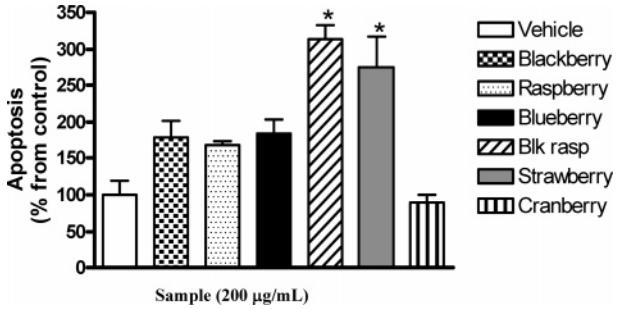


Figure 3. Pro-apoptotic activity of berry extracts against human HT-29 colon cancer cell line. Cells were exposed to berry extracts (200 μg/mL) for 48 h, harvested, and analyzed using the Cell Death Detection ELISA^{PLUS} assay. Values are means ± SE, $n = 3$. Data are expressed as absorbance at 405 nm of each sample over media controls. Single asterisk indicates a significant difference ($p \leq 0.01$) compared to untreated controls.

Apoptosis or programmed cell death is a major mechanism of cancer suppression. Generally, the growth rate of preneoplastic or neoplastic cells outpaces that of normal cells because of malfunctioning or dysregulation of their cell growth and cell death machineries. Therefore, induction of apoptosis or cell cycle arrest can be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, preinitiated, or neoplastic cells from the body. Berry extracts have been shown to have apoptotic effects in human cancer cells (*2*, *17*, *18*). Among berry phenolics, anthocyanins have been shown to be major contributors toward the induction of apoptosis. It is noteworthy that the test concentrations of the berry extracts used in these cell culture experiments far exceed levels of phenolics and/or their metabolites achievable physiologically, based on current knowledge of polyphenol bioavailability (*23*, *24*). Therefore, the limitations in this study are true of most cell culture studies. Factors such as (i) cell line specificity, (ii) test sample concentrations

(typically ranging up to 250 μg/mL for extracts, (iii) stability and/or potential transformation of phenolic compounds given the pH of cell culture media (*19*), (iv) duration of cell exposure to treatment samples, i.e., drug exposure time (typically 24, 48, or 72 h), (v) whether phenolic compounds are uptaken into cells and in what forms, (vi) generation of artifacts such as hydrogen peroxide by phenolic compounds in cell culture media (*25*), as well as other potential reactions should be considered when evaluating cell culture experimental results. Nevertheless, our studies provide preliminary data as to the ability of these compounds to inhibit the growth and induce apoptosis of different human cancer cell lines in vitro. Because extrapolations cannot be made between cell culture studies to humans, future animal and human studies should be designed to investigate the potential of berries for the prevention and treatment of chronic human diseases such as cancer.

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