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Effect of Black Raspberry (*Rubus occidentalis* L.) Extract Variation Conditioned by Cultivar, Production Site, and Fruit Maturity Stage on Colon Cancer Cell Proliferation

Jodee L. Johnson,[†,||] Joshua A. Bomser,[‡] Joseph C. Scheerens,[§] and M. Monica Giusti*[,†]

[†]Department of Food Science and Technology, 110 Parker Food Science and Technology Building, The Ohio State University, 2015 Fyffe Road, Columbus, Ohio 43210, United States

[‡]Department of Human Nutrition, 325 Campbell Hall, The Ohio State University, 1787 Neil Avenue, Columbus, Ohio 43210, United States

[§]Department of Horticulture and Crop Science, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, Ohio 44691, United States

ABSTRACT: Black raspberries have been shown to inhibit multiple stages of oral, esophageal, and colon cancer. The objective of this study was to evaluate how black raspberry extract variability conditioned by horticultural factors affected the antiproliferative activity of 75 black raspberry extracts using an in vitro colon cancer cell model. HT-29 cells grown in 96-well plates were treated with freeze-dried extracts at 0.6 and 1.2 mg of extract/mL of medium. Percent cell growth inhibition for each concentration of the extracts was determined using the sulforhodamine B assay. All extracts significantly inhibited the growth of HT-29 colon cancer cells in a dose-dependent manner. Cell proliferation was significantly influenced by cultivar, production site, and stage of maturity. The lack of correlation between growth inhibition and extract total phenolic and total monomeric anthocyanin assays suggested horticultural parameters influence bioactivity in a complex manner.

KEYWORDS: black raspberry, *Rubus occidentalis* L., anthoncyanins, phenolics, colon cancer

INTRODUCTION

Colon cancer is the third leading cause of cancer death among both men and women in the United States. In 2010 it was estimated that there were 102,900 new cases of colon cancer and 51,370 deaths from this disease (includes rectum cancer).[1] The main risk factors that contribute to a person developing colon cancer are heredity, age, diet, cigarette smoking, colorectal polyps, ulcerative colitis, and Crohn's disease.[2] Recently, food-based approaches for the prevention and/or treatment of cancer have been explored. Berry extracts rich in anthocyanins have been suggested as potential agents to reduce the risk of colon cancer by inhibiting the proliferation of human colon cancer cells in vitro, while having little effect on the growth of nontransformed colon epithelial cells.[3]

Anthocyanins are the flavonoid compounds responsible for the bright attractive red, orange, purple, and blue colors of the leaves, stems, roots, flowers, and fruits in plants. In plants, anthocyanins act as a defensive shield against UV rays, bacteria, viruses, fungi, and oxidative reactions.[4,5] In humans, anthocyanins have been shown to have a wide range of bioactivities including being potent antioxidants and possessing anticancer and anti-inflammatory properties.[6] Berries are one of the richest dietary sources of anthocyanins, with black raspberries having one of the highest anthocyanin contents among them.[7] Studies indicate that black raspberry phenolic antioxidants, especially the anthocyanins, may play an important chemoprotective role.[6,8,9] The source of fruit may affect its chemoprotective efficacy, as environmental and horticultural (production and postharvest) factors are known to affect the phenolic constituents and antioxidant properties of black raspberry fruits.[10]

This research is part of a large multidisciplinary effort to develop a high-field NMR- and bioassay-based investigative system to evaluate the interaction of plant-derived compounds as potential bioactive dietary intervention agents.[11] The objective for this study was to evaluate the polyphenolic variability in juices of 75 black raspberry samples and their solid phase extraction (SPE) extracts and to test the bioactivity of the extracts by measuring their inhibition of cell growth proliferation on a colon cancer in vitro cell system. Samples included berries from three cultivars, grown in seven locations and at three maturity stages. The hypothesis for this study was that changes in composition caused by the differences due to cultivar, location, and/or maturity would result in different bioactivities of the fruit. Substantial variability in fruit chemical composition and the concomitant variability in bioassay results are important elements of our investigative system, as they are essential for the robust regression analyses that identify important bioactive compounds and their interactions.

MATERIALS AND METHODS

Reagents and Solvents. Certified American Chemical Society (ACS) grade potassium chloride, sodium carbonate, and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Reagent grade gallic acid and Folin−Ciocalteau reagent were purchased from MP

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Table 1. Description of Black Raspberry Samples

production site location[a]	N[b]	cultivars			maturity stages[c]		
		Bristol	Jewel	MacBlack	underripe	ripe	overripe
1 northeast	18	X	X	X		X	X
2 west	9			X	X	X	X
3 northeast	12		X	X		X	X
4 northwest	6	X	X			X	
5 west	18		X	X	X	X	X
6 southwest	6		X			X	X
7 south	6		X	X	X		

[a] Production site locations were assigned with respect to Columbus, OH, the state's centrally located capital city (latitude N 40° 00′; longitude W 82° 53′). [b] N, sample number. Each of the 25 farm × cultivar × stage combinations is represented by three samples. [c] Maturity stages: underripe fruit was red-violet in color and 7−10 days from maturity; ripe fruit was dull black and could be easily removed from the torus with gentle force; overripe fruit was conditioned by holding ripe fruit at 20 °C for 48 h.

Biomedicals (Solon, OH) or from Sigma-Aldrich. Dulbecco's phosphate buffered saline solution and trypsin (2.5%) were purchased from Lonza Walkersville, Inc. (Walkersville, MD). Sulforhodamine B dye (75%), trichloroacetic acid (99%), and tris(hydroxymethyl) aminomethane (99%) were purchased from Sigma-Aldrich.

Plant Material. For this project, 75 samples (approximately 450 g) of ripe and/or underripe black raspberry fruits were hand-harvested into quart fiberboard containers from commercial farms throughout Ohio (Table 1). Fruit maturity was determined visually and by tactile means; underripe fruit was red-violet (burgundy) and adhered to the torus, whereas ripe fruit was dull black and could easily be removed from the torus with gentle finger force. Samples were transported on ice to the laboratory and examined for defects prior to processing. Intact underripe and ripe fruit samples were placed in polyethylene freezer bags and stored at −20 °C. In addition, samples of intact ripe berries were afterripened by placing them cavity-end down, individually (i.e., separated from one another), on polystyrene foam trays and storing them on the laboratory counter at 20−21 °C for 48 h. The resulting overripe fruits were re-examined for defects, and intact berries (edible) were frozen as described above.

To prepare juices for analysis and phenolic extraction, thawed black raspberries (200 g) were ground with 100 mL of water in a blender. The material was then filtered through cheesecloth followed by centrifugation to remove the residual pulp. The juice obtained was divided into aliquots and refrozen at −20 °C for subsequent analyses or extraction.

Extraction of Anthocyanins and Other Phenolics from Black Raspberry Juices. Freeze-dried black raspberry extracts were obtained with the following procedure. Phenolics were fractionated on a C-18 SPE cartridge (Phenomenex Strata C18-E, 5 g/20 mL Giga Tubes; Phenomenex, Torrance, CA) activated with acidic methanol (0.1% trifluoroacetic acid added to HPLC grade methanol), followed by 0.1% trifluoroacetic acid in deionized and distilled (DD) water. Samples (50 mL) were loaded onto the column. The cartridges were washed with 60−100 mL of DD water to remove sugars, organic acids, and other polar compounds. The phenolics/anthocyanins adhering to the column were eluted using approximately 30 mL of acidic methanol until eluates were colorless. Samples were taken to a final volume of 50 mL with methanol and partitioned into tared, screw-capped test tubes. The tubes were covered with aluminum foil to reduce light exposure, and the solvent was removed from each tube under a nitrogen stream in an N-Evap system (Organomation Associates, Inc., Berlin, MA; water bath at 35 °C). Solvent-free tubes were stored at −80 °C. Prior to obtaining final weights, extracts were freeze-dried using a Labconco freeze-dry system (condensing unit 7960046 and stoppering tray dryer 7948040, Labconco Inc., Kansas City, MO) to remove any residual moisture, placed in a desiccator, and then weighed to five decimal places. Weighed extracts were returned to −80 °C storage until they were subjected to chemical analyses and bioassay.

Analytical Procedures. The soluble solids (SS) content of juices were determined by refractometry, and juice titratable acidity (TA) levels were ascertained using the methodology of Perkins-Veazie et al.[12] Total phenolic (TP) contents were measured at 765 nm using the modified microscale protocol for Folin−Ciocalteau colorimetry[13] and quantified from spectral measurements as gallic acid equivalents (GAE) using a standard curve that ranged from 100 to 900 mg of gallic acid/L. Total monomeric anthocyanin (TMA) contents were determined at 520 and 700 nm using the pH differential method.[14] Pigment contents were expressed as cyanidin-3-glucoside equivalents (C3-GE), using a molar mass of 449.2 g mol^{-1} and an extinction coefficient of 26900 L cm^{-1} mol^{-1}. Absorbencies were measured for juices and extracts in a Beckman-Coulter DU 73 UV−visible spectrophotometer (Beckman-Coulter, Inc., Brea, CA) and a Shimadzu UV−visible spectrophotometer 2450 (Shimadzu Scientific Instruments, Inc., Columbia, MD), respectively.

Cell Culture. An HT-29 colon cancer cell line, derived from a colorectal adenocarcinoma, was purchased from American Type Culture Collection (Manassas, VA). The cells were grown in McCoy's 5A medium (Lonza Walkersville, Inc.) supplemented with 10% fetal bovine serum and incubated at 37 °C in a NuAire IR Autoflow CO_2 water-jacketed incubator at a modified atmosphere (5% CO_2, 95% O_2). All cell culture experiments were performed within the initial 20 passages.

Cell Growth Inhibition Assay. HT-29 colon cancer cells were seeded in 96-well plates at a concentration of 1.0×10^4 cells/well. After 24 h of incubation, the cells were treated with 0.6 and 1.2 mg of extract/mL of growth medium of each extract and incubated for an additional 48 h. These black raspberry treatment doses were based on results obtained in preliminary experiments.[15] There were three replications for each treatment concentration, and the control for the experiment was cells grown in media. Treatments were randomly assigned within the inner wells of a 96-well plate to avoid the possible effect of well position on cell growth. A sulforhodamine B assay was used to assess cell growth as described previously.[16] Briefly, this colorimetric assay was used to determine percent cell growth inhibition by measuring cellular protein content at 500 nm using a Synergy HT Multi-Detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The percent cell growth inhibition was calculated as follows:

$$\%\ \text{growth inhibition} = 100 - \left(\frac{T_{trt} - T_0}{T_{ctr} - T_0}\right) \times 100$$

T_0 = absorbance of cellular protein content after 24 h of incubation prior to black raspberry extract treatments, T_{trt} = absorbance of cellular protein content after 48 h of incubation with black raspberry extract treatments, and T_{ctr} = absorbance of cellular protein content without treatment of black raspberry extracts after the total incubation period of 72 h.

Statistical Analysis. All data were subjected to analysis of variance (ANOVA) with Tukey's post hoc comparisons (SAS 9.1 software). For all statistical calculations, a p value of <0.05 was considered to be statistically significant. The main effects of production site, cultivar, and maturity stage were analyzed independently. The dependent variables included juice SS, TA, TP, and TMA; extract TP and TMA; and HT-29 cell growth inhibition at 0.6 and 1.2 mg/mL. The interactive effects of independent variables on cell growth inhibition were tested using data subsets excluding unbalanced entries (i.e., those having a single entry for an interactive variable). Mean separation for interactive effects was accomplished by analyzing discrete subsets (e.g., maturity stages within cultivars or cultivars within production sites). Pearson correlation tests

Table 2. Main Effect Means and Probability Coefficients for Chemical Parameters of 75 Black Raspberry Juices and Corresponding SPE Extracts Statistically Analyzed by Production Site, Cultivar, or Maturity Stage

		juice (wet wt basis)					SPE[a] extracts (dry wt basis)		
source	N[b]	titratable acidity (%)	soluble solids (%)	TP (mg GAE/100 g)[c]	TMA (mg C3-GE/100 g)[d]	TMA/TP (%)	TP (mg GAE/g)[e]	TMA (mg C3-GE/g)[f]	TMA/TP (%)
production site									
1	18	1.19 ab[g]	10.1 b	440 b	281 b	64.1	259	124	47.9 ab
2	9	1.49 ab	9.6 b	415 b	298 b	71.9	283	131	45.1 ab
3	12	1.28 ab	9.6 b	422 b	279 b	67.1	288	142	49.8 ab
4	6	0.99 b	10.1 ab	388 b	260 b	67.2	270	134	48.8 ab
5	18	1.23 ab	10.7 ab	410 b	271 b	65.9	279	119	41.2 b
6	6	1.06 ab	12.9 a	639 a	494 a	78.8	304	169	56.5 a
7	6	1.67 a	9.8 ab	378 b	227 b	58.6	244	128	53.7 ab
p		0.0258	0.0294	0.0006	<0.0001	ns	ns	ns	0.0131
cultivar									
Bristol	9	1.23	10.0	449	290 ab	65.0	266	131	48.0
Jewel	33	1.18	10.6	453	322 a	70.7	277	134	47.8
MacBlack	33	1.35	10.1	412	260 b	63.8	275	129	46.9
p		ns	ns	ns	0.0305	ns	ns	ns	ns
maturity stage[h]									
underripe	15	1.80 a	9.3 b	315 c	199 c	62.9	248 b	109 b	42.7 b
ripe	33	1.20 b	9.4 b	411 b	289 b	70.1	277 ab	140 a	50.6 a
overripe	27	1.04 b	11.9 a	526 a	346 a	65.5	287 a	133 a	46.3 ab
p		<0.0001	<0.0001	<0.0001	<0.0001	ns	0.0425	0.0100	0.0281

[a] SPE, solid phase extraction. [b] N, sample number. [c] Total phenolic content expressed as mg gallic acid equivalents/100 g. [d] Total monomeric anthocyanin content expressed as mg cyanidin 3-glucoside equivalents/100 g. [e] Total phenolic content expressed as mg gallic acid equivalents/g. [f] Total monomeric anthocyanin content expressed as mg cyanidin 3-glucoside equivalents/g. [g] Means with similar letters are not significantly different as determined by Tukey's post hoc comparisons. [h] Maturity stages: underripe fruit was red-violet in color and 7−10 days from maturity; ripe fruit was dull black and could be easily removed from the torus with gentle force; overripe fruit was conditioned by holding ripe fruit at 20 °C for 48 h.

were then performed to ascertain relationships between bioactivity and analytical values.

RESULTS AND DISCUSSION

Analytical Evaluation of Black Raspberry Juices and Their SPE Extracts. Juice attribute levels (Table 2) were comparable to those reported for similar samples harvested from the same production sites in a previous season.[10] Fruit maturity was perhaps the most significant cause of variability in juice characteristics among samples. As is common in raspberries.[17] and most edible fruits,[18] TA levels decreased and SS, TP, and TMA constituents increased as berries ripened and began to senesce. The changes we report here were similar to patterns reported in black raspberry and other *Rubus* fruit.[19,20] In a detailed study of 'Jewel' and 'MacBlack' fruit of equivalent maturity stages, citric acid levels decreased by as much as 52%, whereas monosaccharides, TP, and TMA values increased as much as 37%, 67%, and 3.7-fold on a dry weight basis, respectively, as berries progressed from underripe to overripe.[19] Constituent patterns during ripening and senescence may be due in part to changes in dry matter; berries in this study averaged 18.9 ± 0.3, 16.8 ± 1.1, and 20.7 ± 1.6% dry weight, respectively, for underripe, ripe, and overripe fruit. However, phenolic, and specifically anthocyanin, synthesis is known to occur throughout the ripening and senescence processes in red raspberry[21] and other berry fruits.[22] Juice characteristics also varied significantly among production sites, in part due to differences in the maturity stages harvested at each location. Other factors of production or environment may also have conditioned site differences; phenolic data patterns among ripe samples from these locations were comparable with those of fruit obtained from these sites in a previous season [e.g., r = 0.71 for TP site means in both seasons].[10]

TP and TMA contents of the black raspberry SPE extracts were also highly variable (Table 2), with individual samples ranging from 102 to 410 mg GAE/g and from 20 to 216 mg C3-GE/g of eluate dry matter, respectively. Although the extraction procedure increased sample variability, it removed sugars, organic acids, and other compounds that interfere with NMR profiling, which is integral to our investigative system for evaluating bioactive component interactions. In addition, phenolic constituents and anthocyanins were concentrated in extracts by an average of (67 ± 3)- and (48 ± 2)-fold over those found in juice, respectively. The levels of TP and TMA in the black raspberry extracts used in this experiment were much higher than the reported literature values of 5938 mg GAE/100 g and 1770 mg C3-GE/100 g associated with freeze-dried whole black raspberries used in clinical studies.[23,24] These findings together suggest that the extraction procedures used in this study effectively concentrated the potentially chemoprotective compounds of interest, increasing sample suitability for bioassay.

Among production factors, stage of maturity had a significant effect on both the TP and TMA contents of black raspberry SPE extracts (Table 2). Similar to juices, underripe berry extracts

Table 3. Main Effect Means and Probability Coefficients for Percent Growth Inhibition in HT-29 Colon Cancer Cells Treated at Two Levels with 75 Black Raspberry Juice SPE Extracts Statistically Analyzed by Production Site, Cultivar, or Maturity Stage

source	N[a]	growth inhibition at 0.6 mg/mL (%)	growth inhibition at 1.2 mg/mL (%)
production site			
1	18	64 ab[b]	95 ab
2	9	61 ab	90 ab
3	12	65 ab	92 ab
4	6	64 ab	94 ab
5	18	62 ab	90 b
6	6	54 b	85 b
7	6	73 a	103 a
p		0.0019	0.0187
cultivar			
Bristol	9	67 a	98 a
Jewel	33	66 a	95 a
MacBlack	33	59 b	88 b
p		0.0017	0.0005
maturity stage[c]			
underripe	15	68 a	99 a
ripe	33	63 b	93 b
overripe	27	61 b	89 b
p		0.0226	0.0017

[a] *N*, sample number. [b] Means with similar letters are not significantly different as determined by Tukey's post hoc comparisons. [c] Maturity stages: underripe fruit was red-violet in color and 7−10 days from maturity; ripe fruit was dull black and could be easily removed from the torus with gentle force; overripe fruit was conditioned by holding ripe fruit at 20 °C for 48 h.

exhibited lower levels of phenolic constituents than either their ripe or overripe counterparts. The proportion of anthocyanins within the phenolic profile of samples varied among production locations, but was generally lowest in underripe fruit. Presumably, the level of phenolic compounds present within each sample affected extract antioxidant capacity. Moreover, because the primary black raspberry anthocyanins, cyanidin 3-rutinoside (C3-R) and cyanidin 3-xylosylrutinoside (C3-X), account for much of the fruit's antioxidant activity,[25] the proportion of anthocyanins present may also have affected an extract's phytonutrient value.

HT-29 Colon Cancer Cell Growth Inhibition. Percent cell growth inhibition for the black raspberry SPE extracts ranged from 33 to 82% at a concentration of 0.6 mg extract/mL medium and from 71 to 118% at a concentration of 1.2 mg extract/mL medium. Inhibitory responses within samples were dose-dependent and highly correlated (r = 0.86). A study conducted by Zhang et al.[26] demonstrated that a highly purified anthocyanin fraction from strawberries has the ability to inhibit HT-29 cells approximately 60% at a treatment concentration of 0.25 mg/mL. Other studies on the use of anthocyanin-enriched fractions from muscadine grapes and blueberries resulted in 50% inhibition of HT-29 cells at concentrations of 0.2 and 0.025−0.05 mg/mL, respectively.[27,28] These data suggest highly purified berry fractions to be antiproliferative at lower concentrations than the SPE-treated juices used in this study. In contrast, HT-29 cells grown in media containing 0.5−2% natural grape or cherry juice caused no cytotoxic effect; cell viability was approximately 96% when compared to controls (100%).[29] In their study, significant antiproliferative effects were achieved only after fruit juice concentrations reached 10% within the media.

Black raspberry extracts incorporated into the media at a high dosage (1.2 mg/mL) had a significantly more pronounced (p < 0.0001) antiproliferative effect on HT-29 colon cancer cells than their low-dose counterparts, with 24% of assay results exceeding 100% inhibition. Percent inhibition values of >100% indicated that these extracts contained bioactive compounds at levels that were cytotoxic to HT-29 colon cancer cells. Dai et al.[30] reported blackberry extracts from purees with relatively high levels of non-anthocyanin phenolics to be more cytotoxic to HT-29 cells than those isolated from freeze-dried material containing fewer phenolic constituents but similar levels of anthocyanins. In their study, the enhanced cytotoxicity from puree extracts was attributed to phenolic-induced production of reactive oxygen species (ROS) within cells. Although levels of oxidative stress in high-dose-treated HT-29 cells were not measured in our experiment, individual phenolic compounds may have been present at concentrations that promoted cell death in approximately one-fourth of the high-dose extracts. In contrast, Yi et al.[28] demonstrated that treatment with highly purified anthocyanin fractions from blueberries resulted in decreased proliferation and apoptosis in HT-29 cells.

Previous studies have shown berry cultivars to differentially affect extract composition and antiproliferative efficacy,[28,31,32] but to our knowledge, the influence of other production parameters on HT-29 cell bioassay results has yet to be reported. In this study, production site, cultivar, and stage of berry maturity each had a significant effect on the inhibition of extract-treated HT-29 colon cancer cell growth (Table 3). Among extracts from different cultivars, those of 'Jewel' and its maternal grandparent, 'Bristol', behaved similarly, whereas 'MacBlack' extracts were significantly less effective at inhibiting cell growth. Although extracts from these cultivars did not differ significantly in phenolic content (Table 2), the pattern of inhibition among cultivars seemed to follow that exhibited by juice TP and TMA values. Maturity stage also significantly influenced antiproliferative capability, with extracts from underripe berries outperforming their ripe and overripe counterparts. Patterns of growth inhibition with respect to berry maturity were significantly, but not strongly, correlated with measures of maturity in juices (r = 0.30 to 0.36 for TA and r = −0.34 to −0.36 for SS). Growth inhibition also appeared to be inversely related to those of juice and/or extract TP and TMA contents. Although cultivar × maturity stage interactions were not significant for either bioassay data set, individual analyses of maturity stages within cultivars revealed extracts of 'MacBlack' to be the predominant drivers of growth inhibition differences associated with the main effects of these two parameters (Figure 1). 'Bristol' and 'Jewel', both released by Cornell University's New York State Agricultural Experiment Station, were reported to be genetically similar,[33] whereas 'MacBlack', developed from wild germplasm of unknown parentage and privately released, was likely to differ substantively. Moreover, as 'Bristol' and 'Jewel' are early-season cultivars, they likely ripened under similar climate conditions, whereas the late-season cultivar, 'MacBlack', which ripens approximately 2 weeks later at each location, likely matured under different environmental influences.[10]

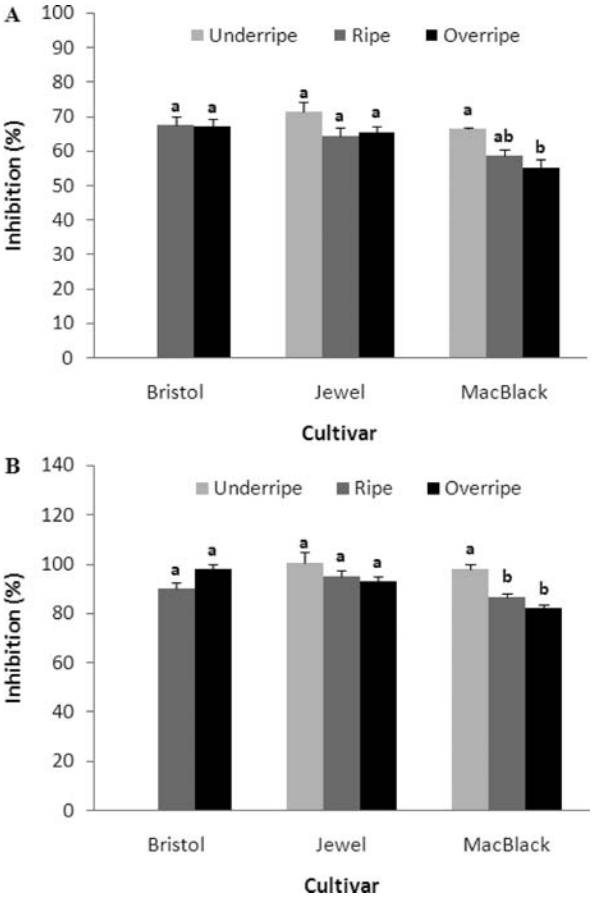

Figure 1. Comparison of the inhibition of HT-29 colon cancer cells by black raspberry extracts derived from three cultivars at three stages of maturity: (A) inhibition at a treatment concentration of 0.6 mg of extract/mL of medium; (B) inhibition at a treatment concentration of 1.2 mg of extract/mL of medium. Values represent means and standard errors of the mean for samples within each cultivar × maturity stage combination.

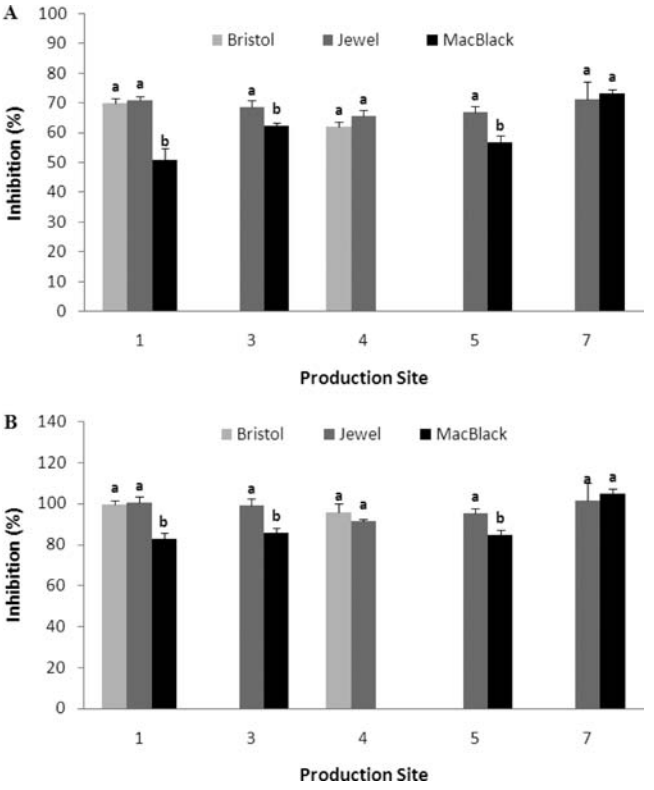

Figure 2. Comparison of the inhibition of HT-29 colon cancer cells by black raspberry extracts derived from three cultivars harvested at five production sites. (A) inhibition at a treatment concentration of 0.6 mg of extract/mL of medium; (B) inhibition at a treatment concentration of 1.2 mg of extract/mL of medium. Values represent means and standard errors of the mean for samples within each cultivar × production site combination.

Among harvest locations, extracts from production site 7, isolated solely from underripe fruit, were significantly more antiproliferative than those from production site 6 and from production site 5 when extracts were administered at the higher dose. Low- and high-dose extracts from production site 6 were the least bioactive against HT-29 cells, even though herein and in previous seasons, TP and TMA values of fruit harvested from this location were among the highest obtained from Ohio-grown fruit.[10] Production site × maturity stage interactions were not significant; however, production site × cultivar interactions were significant (p = 0.003 and 0.05, respectively) for both low and high doses of extract. Cultivar differences at each production site are depicted in Figure 2. Differences in inhibition levels associated with 'Bristol' and 'Jewel' extracts (production sites 1 and 4) were not significant. The inhibitory effects of 'MacBlack' differed significantly from the other two cultivars at production sites 1, 3, and 5, where samples representing multiple maturity stages were acquired, but not at location 7, where only underripe samples were obtained. In summary, our findings suggested that berry maturity, cultivar, and production site had a complex effect on black raspberry antiproliferative activity in an in vitro cell model.

Phenolic Constituents as Factors Influencing Bioactivity. Increased dietary intake of antioxidant-rich fruits and vegetables has long been advocated as a means to reduce the incidence, delay the onset, or lessen the severity of degenerative diseases and to lower the burden of these diseases on society's health care costs.[34−37] *Rubus* fruits, specifically those of black raspberry, are noted for their high antioxidant capacities.[20,38−40] In black raspberries, antioxidant capacity, TP, and TMA are highly correlated variables,[10] mainly due to the redox potency and relatively high concentration of cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside, the primary antioxidants in this fruit.[25] These relationships prompted us to examine correlations between extract TP, TMA, and TP/TMA and bioactivity measured in the HT-29 cell assay. Percent inhibition in our study was not correlated with any of the extract phenolic constituent variables. Other studies have also reported a lack of relationship between the antioxidant capacity of fruit juices and extracts and the level of growth inhibition in HT-29 and other cancer cell lines.[29,31]

However, in vitro studies have often confirmed strong antiproliferative effects to be associated with anthocyanin-rich,[28,41] phenolic-rich,[42] and tannin-rich[43] berry fractions or with individual berry compounds.[26] Anthocyanins were thought to be the most important chemopreventive compounds in black raspberries.[8,9] Aside from their direct effects as antioxidants, anthocyanins at relatively low doses have been found to induce synthesis of ROS-remediating enzymes, cause cell cycle arrest, and stimulate apoptosis, affecting the survival of cancer cells in vitro.[6,9] In vitro studies also found anthocyanins to stimulate cellular differentiation and reduce inflammation, angiogenesis,

and metastasis. The efficacy of anthocyanins against HT-29 cells was reported to be structure-specific, with monoglycosides exhibiting the most profound effects.[41,44] Regardless of structure, anthocyanins may be preferentially absorbed by cancer cells. Studies on a tumorigenic esophageal cell line indicated that the uptake of black raspberry cyanidin 3-glucoside (C3-G), C3-R, and C3-X to be 100-fold greater than in its low tumorigenic counterpart.[45] In addition, the cell growth medium that was used for our bioassay studies was at a pH close to neutral, which favors degradation of anthocyanins. Therefore, it is possible that the degradation products are playing an important role in the observed reduction of cell growth.

Other phenolic constituents of black raspberry including ellagic acid and its derivatives, flavonols (quercetin and kaempferol), and phenolic acids (ferulic acid, *p*-coumaric acid, and gallic acid) were also proposed as factors inhibiting the proliferation of cancer cells.[8,46] Ross et al.[43] demonstrated an ellagitannin-rich red raspberry extract administered to human cervical cancer cells to be more antiproliferative than an anthocyanin-rich fraction from the same source. These authors suggested that living cells can metabolize ellagitannins to free ellagic acid and proposed ellagic acid to be beneficial via the stimulation of apoptosis in cancer cells. Among individual phenolics isolated from strawberry, the flavonols, quercetin, kaempferol, and kaempferol-3-(6′-coumaroyl) glucoside, were most effective at inhibiting the proliferation of HT-29 cells.[26]

Although there is ample evidence for the bioactivity of individual berry compounds or compound classes, the chemopreventive or health-beneficial properties of these fruits were thought to be conditioned most likely by the additivity and/or synergy among multiple constituents.[30,42,44,47−49] In our study, cultivar, production site, and maturity stage may have substantially influenced the relative levels of the specific black raspberry constituents in fruit extracts acting in concert to inhibit HT-29 cell growth. Under this presumption, our inability to demonstrate a strong relationship between percent inhibition and chemical assays may have reflected the lack of specificity within the TP and TMA data rather than a lack of bioactivity among phenolic compounds. Evidence for constituent variability among our samples includes studies showing 'Jewel' and 'MacBlack' to contain identical anthocyanins, but they differ in their proportions.[15,19,50] Moreover, the relative concentration of individual anthocyanins, specifically C3-X and C3-R, are not consistent across locations[10,25] or harvest seasons. Throughout black raspberry maturation and senescence, the level of individual anthocyanins increases proportionally.[19,50] The influence of cultivar, maturity, location, or cultural conditions on berry anthocyanin, flavonol, proanthocyanin, ellagic acid, and/or phenolic acid profiles has also been described in red raspberry,[21] blackberry,[51] blueberry,[52,53] and strawberry.[53−57] In strawberry, 6 of 26 ripening-specific gene families were presumed to be involved in regulating the phenylpropanoid pathway,[58] and both enzymatic and gene activities controlling the formation of flavonoid classes were reported to fluctuate with ripening stage.[59,60]

In conclusion, we have demonstrated the proliferation of HT-29 colon cancer cells treated with phenolic-rich black raspberry extracts to be influenced by the horticultural parameters of the source material (i.e., cultivar, production site, and maturity) in a complex manner. In corroboration, our proliferation data were recently coupled with the metabolomic profiles of the same extracts developed by high-field NMR. Partial least-squares regression analyses of the data sets[11] indicated specific anthocyanins, flavonols, and other phenolic compounds to be important drivers of bioassay variability. The identification of these specific compounds and their interactions is in progress. Finally, the influence of production factors (genetic and environmental) on bioactivity suggested the importance of berry source for studies of chemoprevention, which validated the decision by the Ohio medical research team to single-source black raspberries for their laboratory and clinical research involving dietary intervention in aero-digestive cancers.[61]

AUTHOR INFORMATION

Corresponding Author

*Phone (614) 247-8016; fax (614) 292-0218; e-mail giusti.6@osu.edu.

Present Addresses

[||]Division of Nutritional Sciences, University of Illinois at Urbana−Champaign, 228 Edward R. Madigan Laboratory, 1201 West Gregory Drive, Urbana, Illinois 61801, United States.

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