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Inhibition of Estrogen-Mediated Mammary Tumorigenesis by Blueberry and Black Raspberry

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ABSTRACT: We previously demonstrated the protective effects of blueberry (BB) and black raspberry (BRB) supplemented at 2.5% dose in an ACI rat mammary tumor model. Here, we assessed a dose-related alteration in tumor indices with diet supplemented with 5% BB or BRB powder. The diet was well tolerated. Tumor palpation from 12 weeks revealed first tumor appearance by 84 days in the control group, that was delayed by 24 and 39 days with the BB and BRB diets, respectively (p = 0.04). Ellagic acid detected in the plasma of rats fed the BRB diet was in the range of 96.6−294.2 ng/mL. While the BB diet showed better efficacy in reducing mammary tissue proliferation and tumor burden, tumor latency was delayed efficiently by BRB. Furthermore, BB was effective in downregulating CYP1A1 expression, while BRB downregulated ERα expression effectively. Distinct anticarcinogenic effects of the two berries correspond to their distinct phytochemical signatures.

KEYWORDS: *blueberry, black raspberry, anthocyanidins, estrogen, ellagic acid, ACI rats, breast cancer*

■ INTRODUCTION

Prolonged exposure to estrogen is a major risk factor for breast cancer.[1] Estrogens are structurally related steroids that regulate cell proliferation and differentiation in reproductive and nonreproductive tissues.[2] Estrogen exerts its biological effects on the breast in two pathways: hormone receptor-mediated cell-proliferation, and metabolism and catechol estrogen-mediated DNA damage. The cell proliferative effect of estrogen leading to cancers is mediated by the estrogen receptors, ERα and ERβ. They are members of the nuclear steroid receptor superfamily and mediate their action by ligand-dependent binding as homo- or heterodimers, to the estrogen-response element of the target genes, resulting in their transcription regulation.[3,4] Estrogens influence breast cancer by inducing estrogen-regulated proteins that are autocrine, paracrine, and intracrine growth factors.[5] Prolonged exposure to estrogen results in an increased cell proliferation that causes an increase in spontaneous DNA replication errors, and a mutation in the target cell would enhance the replication of clones that carry the genetic errors.[6]

In addition, estrogens are metabolized to genotoxic metabolites which undergo redox cycling to generate reactive oxygen species that can damage DNA.[7,8] Estrogens are converted to metabolites and excreted from the system by the action of cytochrome P450s and phase II enzymes. Metabolism of estrogens mostly occurs in the liver, where estradiol is converted to the 2-hydroxy metabolite by CYP1A2 and the 16α-hydroxy metabolite by CYP3A4. Recent studies with human recombinant P450s have shown that CYP1A1 and 1A2 can also produce 4-hydroxy metabolites, although their highest activity is for 2-hydroxylation.[9,10] 4-Hydroxylation is the major pathway in the breast as the CYP1B1 expression is exceptionally high in this organ.[11] Both 2- and 4-hydroxyestradiol generate free radicals through redox cycling and causes DNA damage. However, due to its relatively ready conjugation, the 2-hydroxy metabolite is rapidly removed. Therefore, the presence of the 4-hydroxy metabolite at the target site is a critical player in carcinogenesis.[11]

There is a growing body of evidence indicating the benefits of berry fruit consumption for protection from several degenerative diseases including cancer. Berries are rich in anthocyanins, which give the characteristic blue and purple colors to the fruits.[12] Studies by Stoner et al.[13] have shown that black raspberry (BRB), black berry, and strawberry show a high anticarcinogenic potential against gastro-intestinal tumors. At a dose of only 5% in the diet, these berries show 30−60% reductions in all tumor indices studied.[13−15] Clinical studies on patients with Barett's esophagus consuming 30−45 g of lyophilized BRB per day show a significant reduction in the urinary biomarker for oxidative stress.[16] Ellagic acid, the key polyphenol present in BRB, is shown to modulate both phase I and phase II enzymes.[17] The anthocyanins present in berries are known to impart anticancer effects by induction of metabolizing enzymes, modulation of gene expression, modulation of cell proliferation, apoptosis, and their downstream signaling pathways.[18−20]

The chemical structure of anthocyanins and ellagic acid present in berries are depicted in Figure 1. These molecules have similarities in functional moiety suggesting a potential for SERM (selective estrogen receptor modulation)-like activity.[21] Both ellagic acid and berry anthocyanins are shown to have potent antiestrogenic activities.[22−24] Previous studies from our laboratory with 2.5% blueberry (BB) and BRB diets have shown protection against mammary tumors in rats induced by subcutaneous implantation of estrogen.[25,26] These studies provided insights into the modulation of some of the molecular events involved in estrogen metabolism by the berry diets. BRB

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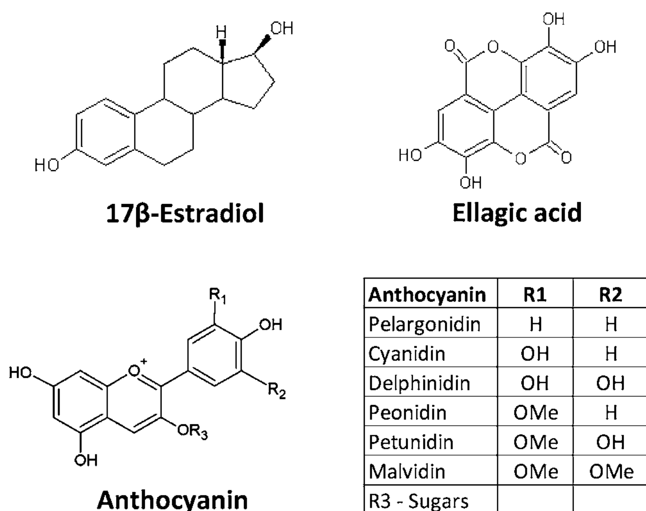


Anthocyanin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OMe	H
Petunidin	OMe	OH
Malvidin	OMe	OMe
R3 - Sugars		

Figure 1. Structures of 17β-estradiol, ellagic acid, and anthocyanins; the table indicates different functional groups of anthocyanin molecules.

in the diet up to a dose of 2.5% showed higher degree of protection against estrogen-mediated mammary carcinogenicity, compared to BB at the same dose. BB and BRB have distinct phytochemical profiles, and a short-term study using these berries at a 5% dose revealed better protection from BB compared to BRB against estrogen-induced ACI rat mammary tissue proliferation (unpublished data). In this study, we therefore explored the long-term effects of the berries supplemented in diets at 5% against estrogen-induced mammary tumorigenesis, in order to understand the synergistic effects of berry phytochemicals at a higher dose.

MATERIALS AND METHODS

Diet. BB and BRB were procured and processed as described previously.[26] Briefly, organic BB (Berkeley) were harvested, rinsed with deionized water, dehydrated using commercial dehydrators (at 35 °C−40 °C), powdered, lyophilized to remove residual moisture, vacuum packed, and stored at −20 °C until use. BRB was procured as freeze-dried powder from Van Drunen farms (Momence, IL). Control AIN-93 M diet and diets supplemented with berries were prepared in pellet form by Harlan-Teklad, Inc. (Madison, WI). AIN-93 M diet was supplemented with 5% BB or BRB powder, by replacing the corn starch and fiber content, to adjust for the calorie content.[26]

Animals and Treatment. All animal experiments were carried out in accordance to an approved protocol from the Institutional Animal Care and Use Committee. Five to 6 week-old female ACI rats were purchased from Harlan Sprague−Dawley, Inc. (Indianapolis, IN). The rats were housed in cages and fed food and water ad libitum. After acclimation for 7 days, the rats were divided into 6 groups (Table 1). Groups 1 and 4 received the AIN-93 M diet, groups 2 and 5 received a diet supplemented with BB powder (5%, w/w), and groups 3 and 6 received the AIN-93 M diet supplemented with BRB powder (5%, w/w). Body weights and diet consumption were assessed weekly for all groups until termination. Two weeks later, rats from groups 4, 5, and 6 were implanted with a 1.2 cm silastic implant containing 9 mg of 17β-estradiol (E_2), as described previously.[27] Seven animals from each group were euthanized after 3 weeks and 6 animals after 3 months of estrogen treatment. The remaining 26 animals from groups 4, 5, and 6 were palpated for mammary tumors from week 12. Rats were euthanized when the largest tumor reached 1.2 cm. When the palpable tumor incidence in group 4 was >85%, all animals were euthanized. Animals were euthanized by CO_2 asphyxiation, blood was drawn to isolate serum/plasma, and tumors were measured. Mammary tumors and adjacent tissues, and pituitary gland were weighed; a small piece of each tissue was transferred to 10% buffered formaldehyde for histopathological analysis and immunohistochemistry.

Table 1. Treatment Groups and Observation Time Points

		number of rats		
group #	group	3 weeks	3 months	7 months
1	untreated	7	6	6
2	5% BB	7	6	6
3	5% BRB	7	6	6
4	E_2	7	6	26
5	E_2 + 5% BB	7	6	26
6	E_2 + 5% BRB	7	6	26

Quantification of Serum Estrogen. Serum estrogen levels were measured using estradiol EIA kit (Cayman Chemical Co., Ann Harbor, MI) following the manufacturer's instructions. The limit of detection is 20 pg/mL.

Plasma Prolactin. Plasma prolactin levels were measured using the Rat Prolactin EIA kit (Alpco Diagnostics, Windham, NH).

Immunohistochemistry. Mammary tissue sections (5 μm) were stained for Proliferating Cell Nuclear Antigen (PCNA) using the Zymed Rat PCNA kit (Invitrogen Co., Carlsbad, CA) as described previously.[27] Slides were blind, 100 cells/field, and 5−6 fields/slide were stained for deeply stained epithelial nuclei by two independent scorers. The average of six scores by two cytopathologists were calculated and plotted as percentage of deeply stained cells.

RNA Isolation and qPCR. Total RNA was isolated from mammary tissues using Trizol (Invitrogen, Carisbad, CA). RNA concentrations were measured by Nanodrop ND-1000. qPCR was carried out with qScript One Step SYBR Green QRT-PCR kit (Quanta Biosciences, Gaithersburg, MD) using 7500 Fast-Real Time PCR system. The relative gene expressions were compared with the control using the $2^{\Delta\Delta Ct}$ method normalizing with 18S RNA. Oligonucleotide primers (Integrated DNA technologies, Coralville, Iowa) used were as follows: CYP1A1 (forward, 5′ TGGAGACCTTCCGACATTCAT 3′; reverse, 5′ GGGATATAGAAGCCATTCAGACTTG 3′); CYP1B1 (forward, 5′ AACCCAGAGGACTTTGATCCG 3′; reverse, 5′ CGTCGTTTGCCCACTGAAAA 3′); ERα (forward, 5′ GGCACATGAGTAACAAAGGCA3′; reverse, 5′ GGCATGAAGACGATGAGCAT3′); 18S (forward, 5′GGGAGGTAGTGACGAAAAATAACAAT3′; reverse, 5′TTGCCCTCCAATGGATCCT3′).

Cell Line and Treatment. T47D breast cancer cells (4,500 cells/well) were seeded in 96 well plates and grown with DMEM supplemented with 10% fetal bovine serum for 24 h. Cells were treated with 25, 50, 100, 200, or 400 μM delphinidin, malvidin, petunidin, peonidin, or cyanidin, or an equimolar mixture of all the 5 anthocyanidins at concentrations of 5, 10, 20, 40, or 80 μM each. Cells were treated for 48 h and cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyl tetrazoliumbromide (MTT) assay. After solubilization of formazan crystals, the color intensity was measured spectrophotometrically at 570 nm.

Phytochemical Profiles of Berry Extracts. Extraction, enrichment, and hydrolysis of the anthocyanins were carried out using the conditions described.[28] Briefly, BB and BRB powders were extracted with 75% aqueous ethanol containing 10 mM HCl and enriched by loading the concentrated extracts on a XAD-761 and diaion HP-20 (1:1) column. The polyphenolics, including anthocyanins and ellagitannins, were eluted with methanol under gravity. Pooled elutes were concentrated and hydrolyzed with 2 N HCl (~5 vol) at 120 °C for 2 h and purified using C18 column. Anthocyanidins and other polyphenolics were eluted with acidified (10 mM HCl) methanol. The enriched extracts were dried under reduced pressure using Savant Speed-Vac and stored at −20 °C until use.

UPLC (ultra-performance liquid chromatography) analyses were carried out on a Shimadzu UPLC system, equipped with an autosampler/injector, binary pump, fluorescent, and diode array detectors. BB and BRB extracts (5-μL injection: 1 mg/mL) were analyzed on a Shim-Pack XR-ODS II reverse phase column

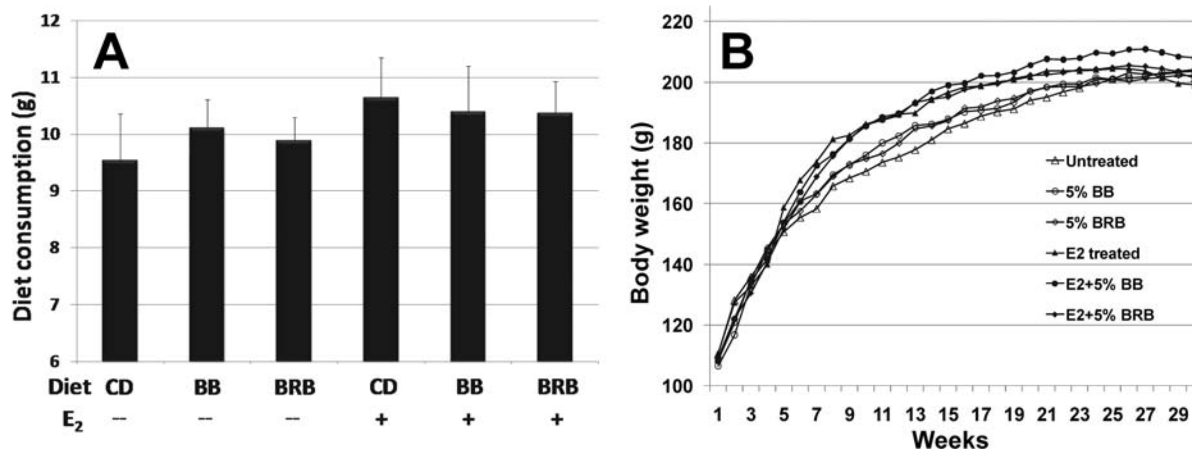


Figure 2. Average diet consumption and body weight gain: (A) diet consumption by the ACI rats fed 5% control diet, and diets supplemented with blueberry (BB) or black raspberry (BRB), with and without estrogen treatment during the course of the study; (B) body weights of rats receiving various treatments. SD (3.8–7.1%), not shown in graph to increase clarity.

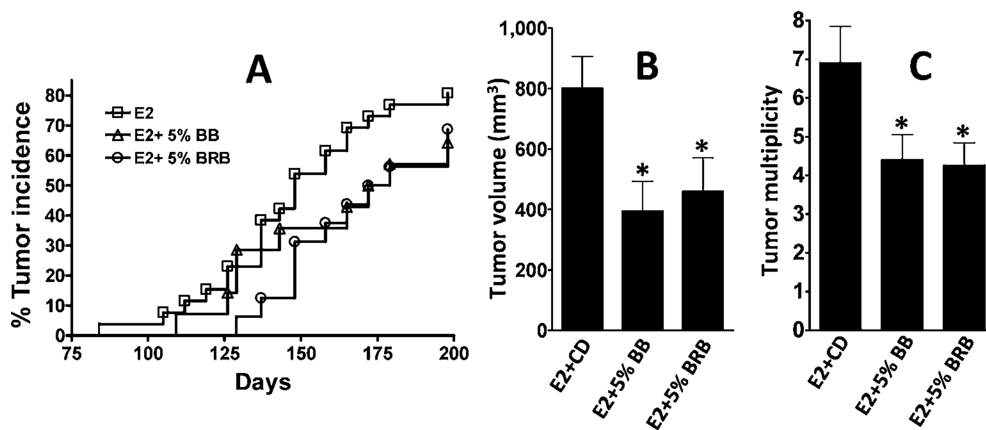


Figure 3. Effect of diets supplemented with blueberry (BB) and black raspberry (BRB) on (A) tumor incidence; (B) tumor volume; and (C) tumor multiplicity. Tumor incidence was calculated on the basis of palpation of the mammary tumors.

(Shimadzu; 150 × 3.0 mm i.d., 2.2 μm). Anthocyanidins and other ellagic acid/ellagitannins were separated in a linear gradient of 3.5% phosphoric acid (solvent A) and acetonitrile (solvent B) with a flow rate of 0.75 mL/min. In the gradient, initially solvent B was 15% for 2 min and increased to 20% after 3 min. Solvent B concentration was further increased to 60% from 3 to 10 min, held for 1 min, and again changed to 15% after 12 min. Anthocyanidins, ellagitannins, and other phenolics were monitored at 530, 366, and 280 nm, respectively.

Plasma Ellagic Acid. For detection of ellagic acid, 1 mL of rat plasma was acidified using 0.1 M potassium dihydrogen phosphate and phosphoric acid (final pH 2.5), extracted with acetonitrile, vacuum-dried, reconstituted in methanol,[29] and analyzed by UPLC. A Shim-pack XR-ODS-II column (3.0 × 150 mm; 2.2 μm) was used for separation at a flow rate of 0.7 mL/min under a binary linear gradient condition using mobile phases, solvent A (100 mM sodium phosphate with 10 mg/L SDS (sodium dodecyl sulfate), pH 2.5, with phosphoric acid) and solvent B (60% acetonitrile, 10% methanol, and 30% solvent A). The gradient condition was as follows: 0–1.77 min, 6% B; 1.77–5.47, 30% B; 5.47–7.29, 100% B; 7.29–9.12 min, 100% B; 9.12–12.0 min, 6% B. The PDA was set to scan from 200 to 600 nm. Ellagic acid standard was prepared in dimethyl sulfoxide (200 μg/mL) and serially diluted in methanol to 1000, 500, 250, 125, and 62.5 ng/mL. The ellagic acid peak from the plasma was confirmed by spectral purity (200–500 nm) and cochromatography with the reference to confirm identical retention time.

Statistical Analysis. Student's *t*-test was used for the comparison between the controls and among the E_2-treated groups. Comparisons of tumor incidence were carried out using the nonparametric log-rank test. Analysis of variance (ANOVA) was used to compare the effect of prolactin and pituitary weight among the E_2-treated groups. All statistical analyses were carried out using GraphPad Prism software (San Diego, CA), version 4.3. A *p*-value of <0.05 was considered significant.

RESULTS

Body Weight and Diet Consumption. No significant difference was observed with diet consumption among the various groups, suggesting no specific effect of the berry diets on food intake (Figure 2A). Subsequent to estrogen treatment, the body weights increased significantly ($P < 0.0001$) irrespective of their diets, compared to the respective control groups. However, by 22 weeks, there was no significant difference between the body weights of the control and E_2-treated groups (Figure 2B).

Tumor Incidence, Volume, and Multiplicity. Tumor latency was delayed by 24 days with BB and 39 days with BRB diet ($p = 0.04$) (Figure 3A). When 80% of rats in the control group developed palpable tumors, only 64% and 69% of rats from the BB and BRB diet groups, respectively, developed tumors. At termination, the tumor volume in the control group was 801.2 ± 105.3 mm^3, which was reduced to 395.1 ± 97.9 mm^3 (50.7% reduction) by BB diet ($p = 0.0116$) and to 461 ± 109.8 mm^3 (42.4% reduction) by the BRB diet ($p = 0.0407$).

Table 2. Effect of Berry Treatment on Organ Weights[a]

time point	organ	groups (mean ± SD)					
		untreated	BB	BRB	E_2	E_2 + 5%BB	E_2 + 5%BRB
3 wks	liver (g)	4.8 ± 0.2	5.0 ± 0.2	4.8 ± 0.5	6.9 ± 0.9	7.6 ± 0.7	6.6 ± 0.8
	mammary (g)	4.2 ± 0.3	2.4 ± 0.3	3.2 ± 0.4	5.3 ± 0.3	4.8 ± 0.7	4.3 ± 1.0
	pituitary (mg)	7.9 ± 1.3	9.0 ± 2.3	8.5 ± 1.2	19.4 ± 3.0	22.4 ± 2.0	18.7 ± 3.1
3 months	liver (g)	4.7 ± 0.3	5.3 ± 0.6	4.8 ± 0.1	7.9 ± 0.5	8.0 ± 0.8	7.4 ± 0.2
	mammary (g)	3.9 ± 0.5	3.7 ± 1.3	4.4 ± 0.8	6.8 ± 0.4	6.9 ± 0.8	6.7 ± 0.4
	pituitary (mg)	8.9 ± 0.6	10.5 ± 1.2	8.6 ± 1.2	39.4 ± 4.6	30.7[c] ± 3.7	42.8 ± 5.5
	p values					***0.0332***	
7 months	liver (g)	5.4 ± 0.4	6.1 ± 0.2	5.7 ± 0.4	6.9 ± 0.7	7.1 ± 0.8	6.5[c] ± 0.7
	p values						***0.0423***
	mammary (g)	2.8 ± 0.5	5.3[b] ± 0.7	5.1[b] ± 0.9	7.5 ± 0.7	6.7 ± 1.4	6.2[c] ± 1.2
	p values		***0.0005***	***0.0030***			***0.0423***
	pituitary (mg)	8.4 ± 0.8	12.1[b] ± 1.4	8.8 ± 2.5	66.5 ± 17.5	50.6[c] ± 11.3	49.5[c] ± 12.2
	p values		***0.0042***			***0.0035***	***0.0011***

[a]Values expressed are the mean ± SD. [b]Significantly higher than untreated rats. [c]Significantly lower than E_2 treated rats.

Tumor multiplicity was 6.9 ± 0.9 in the control group, which was reduced to 4.4 ± 0.6 by the BB diet ($p = 0.0241$) and 4.3 ± 0.6 by the BRB diet ($p = 0.0212$) (Figure 3B).

Liver, Mammary and Pituitary Gland Weights. At three weeks, the liver, mammary, and pituitary tissue weights were all significantly ($p < 0.0001$) elevated with estrogen treatment. However, there was no significant difference in the organ weights among the groups without E_2-treatment. Similarly, no differences were found in organ weights among the E_2-treated groups. At 12 weeks, while there was no difference in the liver and mammary weights of E_2-treated groups, the pituitary gland weight of rats fed the BB diet were significantly lower ($p < 0.05$), compared to the E_2-treated groups; however, BRB did not show this effect. At 7 months, the liver and mammary tissue weights of rats fed the BB diet did not show any significant difference from the E_2 alone-treated group. However, the pituitary gland weight was significantly reduced ($p = 0.0035$). Rats fed the BRB diet showed significant reduction in the liver ($p = 0.0423$), mammary ($p = 0.0002$), and pituitary ($p = 0.0011$) weights at 7 months (Table 2). One-way ANOVA showed a significant reduction in mammary weight among the BB-treated group ($p = 0.0237$) but not by BRB, while pituitary weight was significantly reduced by both BB ($p = 0.0338$)- and BRB-treated ($p = 0.0269$) groups over the three time-points analyzed.

Serum Estrogen. No significant modulation was found in the serum estrogen levels among the untreated and berry-fed rats, while a significant increase in the E_2-treated animals was observed ($p = 0.002$). This increase in the serum estrogen levels was significantly reduced by both the BB and BRB diets ($p = 0.019$ and 0.012, respectively) (Figure 4A).

Plasma Prolactin. No significant differences were found in the plasma prolactin levels between untreated rats and rats fed the 5% BB and 5% BRB diets. However, significant increase in the prolactin levels was observed at all time-points with E_2 treatment ($p < 0.0001$). At 3 weeks, the circulating prolactin levels were offset by berry diets by 1.4- to 1.5-fold ($p < 0.05$). At 3 months, the reduction in the prolactin levels by the two berries was more significant ($p < 0.001$). At 7 months, however, while BB still significantly reduced the prolactin levels, the reduction observed with BRB was not significant (Figure 4B). One-way ANOVA revealed the prolactin levels were better modulated by BRB ($p = 0.0067$) compared to BB ($p = 0.0232$).

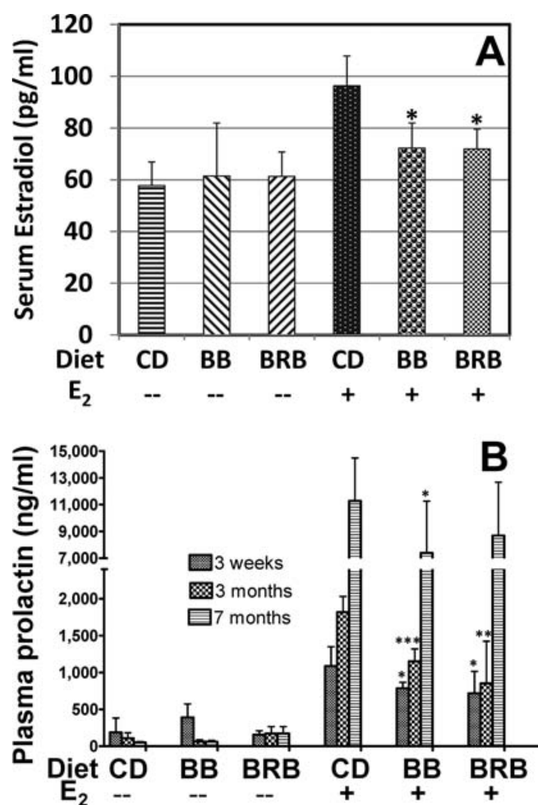


Figure 4. Circulating estradiol (A) and prolactin (B) levels measured at various time points in rats fed diets supplemented with blueberry (BB) and black raspberry (BRB).

Modulation of Estrogen-Related Gene Expression. The gene expression modulation was compared among the E_2-treated groups only. The CYP1A1 expression, which was increased by more than 3-fold in 3-weeks by E_2, was downregulated by the BB diet at 3 weeks and 3 months, while the BRB diet did not show any effect at the early time-point. BRB, however, downregulated CYP1A1 expression at 3 months (Figure 5A). CYP1B1 was not modulated either by E_2 or by the berry diets (data not shown). The estrogen receptor alpha was upregulated only at the 3-month and 7-month time points which was offset by both the berries, though BRB demonstrated better effect at both the time-points (Figure 5B).

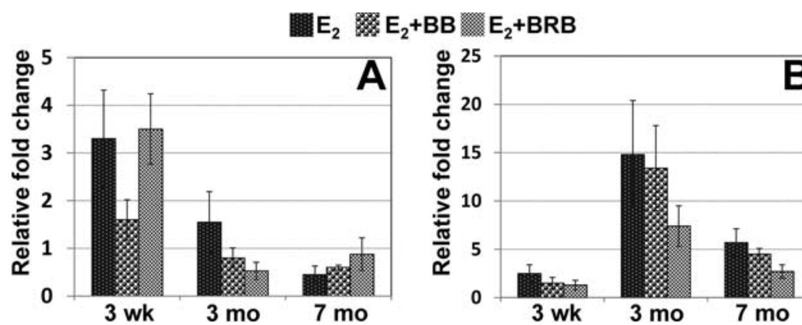


Figure 5. Effect of BB and BRB supplemented diets on E2 associated increase in mRNA levels of CYP1A1 (A) and ERα (B) by qPCR. The relative fold change was calculated using the $2^{\Delta\Delta Ct}$ method.

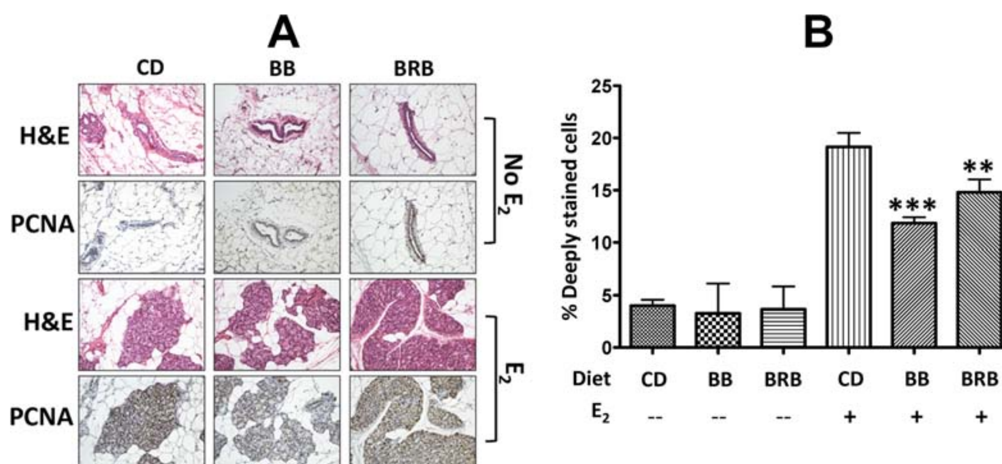


Figure 6. Antiproliferative effects of the control diet (CD) and diet supplemented with blueberry (BB) or black raspberry (BRB), with and without estrogen (E_2) treatment evaluated by (A) immunohistochemical staining for Proliferating Cell Nuclear Antigen (PCNA) at 7 months. Photomicrographs are 200× magnification of normal and hyperplastic mammary tissues; (B) corresponding graph representing the quantitation of deeply stained cells for PCNA.

Proliferation Index. Proliferation index assessed by staining for PCNA protein in mammary tissues revealed no difference among the untreated rats and rats fed BB or BRB diet alone. At 3 weeks, the proliferation increased in the E_2 alone-treated group by over 5-fold ($p < 0.001$). This increase in proliferation was offset by both BB ($p = 0.0276$) and BRB ($p = 0.0035$) by 23.6% and 30%, respectively (not shown). At 3 months, the percentage of intensely stained cells reduced from 35% to 22% in the E_2-treated rats (not shown). The proliferation rate was not significantly different in the berry diet groups. However, at 7 months, the rate of proliferation was reduced by 37.4% and 22.1% by the BB and BRB diets, respectively, which were significant ($p = 0.0002$ and 0.0237, respectively) (Figure 6).

Synergistic Activity of BB Anthocyanidins. BB anthocyanidins when tested for their antiproliferative activity in vitro using estrogen-positive human breast T47D cells revealed that over 50−120 μM of individual anthocyanidins were required to kill 50% of the cells (Figure 7). However, an equimolar mixture of the 5 anthocyanidins present in BB showed significantly higher efficacy, requiring less than 10 μM of each of them to achieve the same effect.

Phytochemical Profiles of Berry Extracts. HPLC analysis of BB extract revealed delphinidin (52.3%) as the predominant anthocyanidin. Other anthocyanidins detected include petunidin (14.2%), cyanidin (8.1%), and peonidin + malvidin (8.1%). However, cyanidin was essentially the only anthocyanidin present in black raspberry extract, besides ellagic acid and quercetin (Figure 8).

Phytochemical Profiles of Rat Plasma. Efficiency of ellagic acid extraction from plasma was determined by spiking

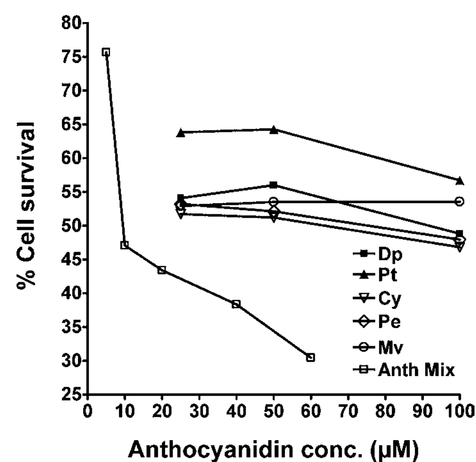


Figure 7. Antiproliferative effects of delphinidin (Dp), cyanidin (Cy), malvidin (Mv), peonidin (Pe), and petunidin (Pt) tested either individually or as an equimolar mixture against T47D breast cancer cells. Data represent the average of 3 experiments conducted at different times. SD (±3.2−6.8%) is not shown to improve clarity of the data.

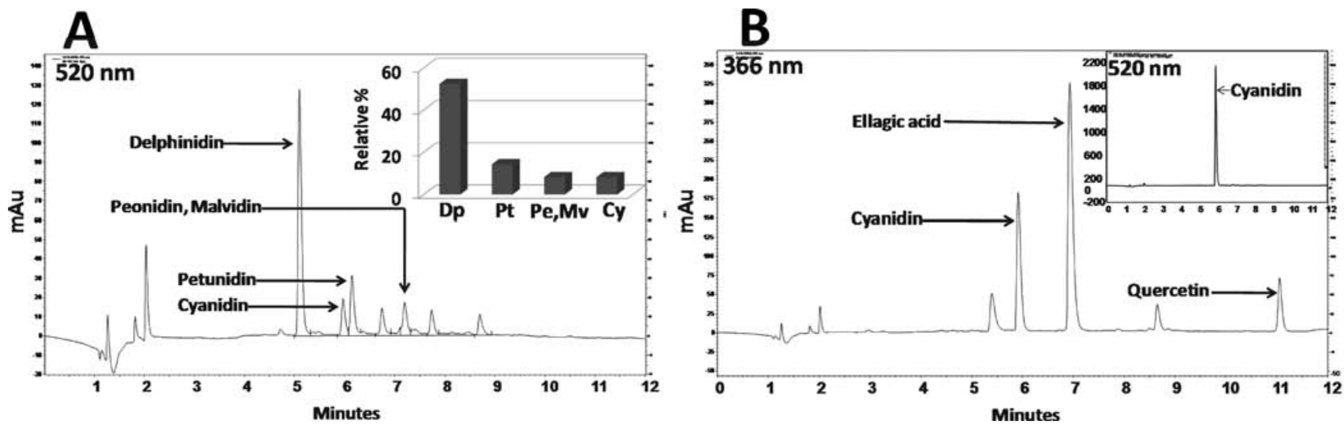
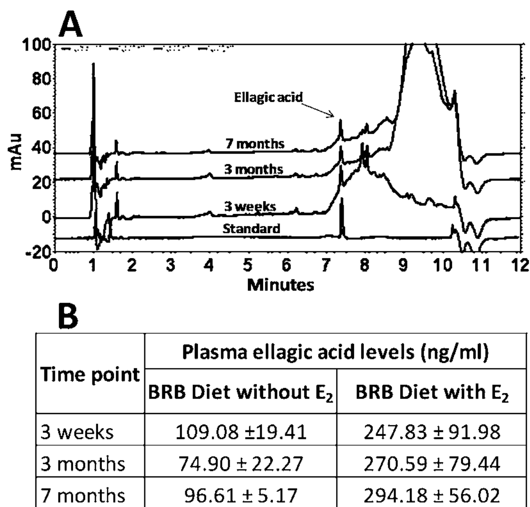


Figure 8. Ultra-performance liquid chromatography (UPLC) profiles of blueberry (BB) and black raspberry (BRB) phenolics: (A) detection and relative proportions of BB anthocyanidins; (B) detection of ellagic acid and cyanidin in BRB; mAu, milli absorbance units.

known amounts of ellagic acid to plasma from untreated animals, and a recovery of 85−90% was observed. The method had a detection limit of 500 pg of ellagic acid. The identification of ellagic acid was confirmed by spiking and spectral purity based on authentic standard (Figure 9). The plasma ellagic acid levels were determined at all the three time points studied. The plasma ellagic acid levels were found to be constant at all three time points, between 96.6 ± 5.17 and 109.1 ± 19.4 ng/mL, for rats fed the BRB diet alone. However, the levels were 2.5−3-fold higher ($p < 0.0004$), between 247.8 ± 92 and 294.2 ± 56 ng/mL, in rats fed the BRB diet and treated with E_2 implants.



Time point	Plasma ellagic acid levels (ng/ml)	
	BRB Diet without E_2	BRB Diet with E_2
3 weeks	109.08 ±19.41	247.83 ± 91.98
3 months	74.90 ± 22.27	270.59 ± 79.44
7 months	96.61 ± 5.17	294.18 ± 56.02

Figure 9. Detection (A) and quantitation (B) of ellagic acid from rat plasma at various time points by ultra-performance liquid chromatography (UPLC). Plasma was extracted from acidified plasma with acetonitrile and analyzed using C18 reverse phase column and UV detection (366 nm). Ellagic acid was confirmed by spectral purity and cochromatography with the standard.

■ DISCUSSION

This study was conducted to assess the protection offered by BB and BRB against mammary tumorigenesis when administered at 5% in the diet. The objective of this study was to (1) compare and contrast the distinct effects of the two berries, (2) determine their relative antiproliferative activity in mammary tissue, and (3) correlate the distinct phytochemical signature of the two berries with disease prevention. The findings were also compared with the results of our earlier studies with lower doses of berries and ellagic acid. Rats fed the diets containing 5% berries showed no change in body weight and diet consumption, suggesting that these doses are well tolerated.

It is interesting to note that the BRB diet delayed the first tumor appearance better than BB at the dose tested by nearly 15 days. BB was relatively more effective in reducing the rate of proliferation and tumor volume compared to BRB. Similarly, BB was more effective in downregulating CYP1A1 expression compared to BRB. However, BRB was more effective in downregulating the ERα gene expression more effectively than BB. Yet both the diets showed a similar effect in reducing the circulating estrogen levels. This highlights the distinct phytochemical profiles of the two berries. BB has 4-fold lower levels of total phenolics and 8-fold lower levels of anthocyanin content compared to BRB in our preparations. However, what make these two berries ideal candidates are their distinct phytochemical signatures: BB contains a diversity of anthocyanins, glycones of delphinidin, malvidin, cyanidin, peonidin, and petunidin, while BRB contains almost exclusively cyanidin glycones (Figure 10) along with an abundant amount of ellagic acid. Thus, it appears that the anthocyanin mixture in BB is capable of regulating the proliferation rate of the

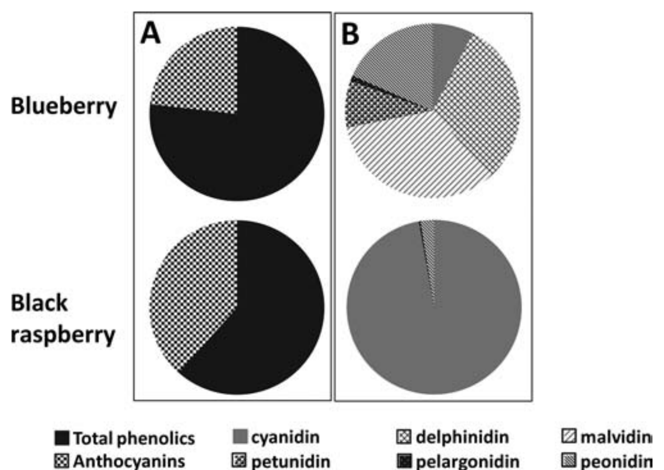


Figure 10. Phytochemical profiles of blueberry (BB) and black raspberry (BRB): (A) total phenolics and anthocyanidin content. (B) Relative abundance of the various anthocyanins.[21]

mammary cells more effectively, while the ellagic acid–cyanidin combination of black raspberry exerts its effect by delaying the neoplastic transformation. It remains to be studied if BB might be more effective if the phenolic contents of both the berries are adjusted to the same levels. Alternately, other cultivars of blueberry such as Rubel, which have higher total anthocyanins and phenolics, can be tested instead of the Berkeley used in this study, which has relatively lower amounts of phenolics. Considering that the tumor volume was not further reduced by increasing the berry dose from 2.5% to 5%, it appears that these berries exert their major effects during the preinitiation/ preneoplastic stages of mammary tumorigenesis by increasing the latency from 18 to 24 for BB and 20 to 39 for BRB and may have a lesser effect once the cells have become malignant.

Previous studies with 1% and 2.5% BB and BRB showed BRB as having a better antiproliferative effect.[25] However, with an increase in the dose to 5%, BB appears to be more effective. In addition, the tumor volume was more effectively reduced by BB compared to BRB in this study. Assessment of the inhibition of cell proliferation in vitro by either the individual anthocyanidins or a mixture of all the 5 anthocyanidins present in BB revealed that while 50–120 μM of each of them was required individually to kill 50% of the T47D cells, when in mixture, less than 10 μM each was sufficient to achieve the same effect. It is evident from these studies that the anthocyanidins of BB act synergistically and therefore supersede the effect of BRB at a higher dose in limiting the rate of proliferation. The anthocyanidins have a structural similarity in the functional moiety to estradiol and are therefore expected to have SERM-like properties. In vitro studies with anthocyanidins have shown a weak estrogenic activity on breast cancer cell lines and an antiestrogenic activity in the presence of estrogen, which was related to the number of hydroxyl groups present.[24] This explains the slight increase in proliferation in the mammary tissue of rats fed the berry diets alone, which was reversed in the presence of estrogen, at all time points in our studies.

While no significant difference was observed in the liver and mammary tissue weights of rats fed the BB diet at all the time points studied, it is noteworthy that the BRB diet significantly reduced the weight of the liver, mammary and pituitary tissues at termination. One-way ANOVA revealed that BB was better in reducing the mammary weight significantly over time compared to BRB. However, the weight of the pituitary gland was most offset in rats fed the BB diet at both the 3-month and 7-month time-points, while BRB only significantly modulated at the tumor time-point. Similarly, prolactin was better modulated by BB at all the time points than by BRB. This also correlates with the profiles of circulating prolactin levels. As discussed previously,[27] prolactin secreted by the pituitary gland has a vital role in mammary tumorigenesis in this model, and therefore, reduction in pituitary weight and the corresponding prolactin levels at various time points suggest that berries are effective against this axis of tumor formation. It is not clear, however, whether the prolactin levels are directly modulated by berries or whether the production of prolactin is reduced due to modulation in circulating estrogen levels facilitated by the berry diets.

Ellagic acid bioavailability was confirmed by its presence in plasma. The BRB used in this study contains 0.3 mg ellagic acid/g.[30] Therefore, at 5% in the diet, an average of 0.15 mg of ellagic acid/ellagitannins was administered to these rats based on a 10 g diet intake per day (15 ppm). On the basis of 8.12 mL of total plasma for rats weighing 200 g,[31] detection of about 100 ng of ellagic acid/mL plasma in the group receiving the BRB diet alone suggests that ≈0.6% of ellagic acid was bioavailable in the plasma. The plasma ellagic acid bioavailability in the group receiving both estrogen and BRB treatments was significantly higher (about 294 ng/mL translating to 1.8% bioavailability). This suggests that the metabolism of ellagic acid may be altered in the presence of high amounts of estradiol due to the modulation or competitive inhibition by estrogen of the enzymes involved in the metabolism of ellagic acid, like GST, UGT, and ST.[32] It is noteworthy that when ACI rats were treated with estrogen implants and received ellagic acid via the diet at 400 ppm, the plasma ellagic acid levels were in the same vicinity (298 ng/mL; unpublished data) as found in the present study in which ellagic acid in the BRB diet was present at only 15 ppm. This suggests that the ellagic acid available in the plasma in this study is the cumulative amounts obtained as pure ellagic acid as well as from the breakdown of several ellagitannins present in black raspberry, implying that ellagic acid present as ellagitannins in BRB may have better bioavailability.

Chemoprevention studies with BRB were shown mostly effective in only skin[33] and gastrointestinal tract tumors.[34] The lack of efficacy of ellagic acid in preventing dimethylbenz[*a*]-anthracene-induced mammary tumors even at a dose of 8,000 ppm[35] was mostly attributed to the lack of bioavailability at target site. However, it appears that the efficacy is also dependent on the animal model used. Chemopreventive agents have traditionally been tested against bolus doses of chemical carcinogens that are irrelevant to environmental exposure. In our study, we utilized a low-dose estrogen exposure model to induce mammary tumors in rats. The circulating estrogen levels in this model reach ≈200 pg/mL, which is also relevant in humans. It is also well-known that the pharmacokinetics of agents is different at higher doses when compared to lower doses. Apart from ellagic acid, we were unable to measure the plasma levels of other phytochemicals, particularly anthocyanins that are present in significant amounts. Lower total phytochemicals but diverse anthocyanin content of BB showed a better protective effect compared to BRB suggesting synergistic activity. Additionally, the efficacy we observed with the two berries could also be the additive effects from a multitude of agents present in them than from a single agent. When seven different types of berries with varying levels of anthocyanin and ellagitannin content were compared for their potency in reducing *N*-nitrosomethylbenzylamine-induced rat esophageal tumors, it was observed that they all showed similar effects, suggesting that the effect may be coming from some other components of these berries, such as carotenoinds, zeaxanthin, anthraquinones, etc.[36]

In summary, this study has shown protective effects of berries against mammary tumors and has several important highlights: the rat model used circumvents most, if not all, of the disadvantages of traditional carcinogen models which rely on bolus doses of carcinogens; therefore, the translatability is enhanced; the doses at which berries were administered were safe and produced no toxicity; while reduction in tumor burden was not enhanced with 5% berry diets compared to 2.5%, the protective effect from BB seemed to have superseded that of the black raspberry despite 8- and 4-fold lower levels of anthocyanins and total phenolics, respectively; BB was more effective in regulating the rate of proliferation of the mammary tissue at a higher dose, while BRB was efficient in delaying the first tumor appearance, suggesting distinct molecular targets of

the two berries in accordance to their distinct phytochemical profiles. During the process of disease progression, the numbers of biomolecules that are abnormally expressed are limited. However, these abnormalities increase with disease progression. Therefore, targeting just one biomarker with one molecule often results in ineffectiveness or negative outcome.[37] On the basis of our allometric scaling calculations,[21] 1 cup of fresh or frozen berries a day is sufficient to provide the necessary protection from the carcinogenic effects of circulating estrogen.

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ABBREVIATIONS USED

BB, blueberry; BRB, black raspberry; E_2, 17β-estradiol

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