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Berry Fruits Modulated Endothelial Cell Migration and Angiogenesis via Phosphoinositide-3 Kinase/Protein Kinase B Pathway in Vitro in Endothelial Cells

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ABSTRACT: Polyphenolic-rich berry fruits are known to activate redox-sensitive cellular signaling molecules such as phosphatidylinositol-3-kinase (PI3 kinase)/kinase B (Akt), resulting in a cascade of downstream signaling pathways. This study investigated the ability of strawberry (SB), wild blueberry (WBB), and cranberry (CB) extracts to induce the activation of PI3 kinase/Akt signaling in vitro in human umbilical endothelial cells (HUVECs) and whether this activation would enhance cell migration and angiogenesis. Anthocyanin profiles of the extracts were characterized using HPLC-ESI/MS, and Akt activation was investigated using the Alpha Screen SureFire assay. The total anthocyanin contents of SB, WBB, and CB extracts were 81.7, 82.5, and 83.0 mg/100 g fresh weight, respectively. SB, WBB, and CB extracts activated Akt in a dose-dependent manner via PI3 kinase and induced cell migration and angiogenesis in vitro in HUVECs. The results from this study suggest that polyphenolics in berry fruits may play a role in promoting vascular health.

KEYWORDS: *wild blueberry, cranberry, strawberry, polyphenolic compounds, anthocyanins, p-Akt, cell migration, tube formation*

■ INTRODUCTION

Consuming a diet rich in polyphenolic compounds derived from fruits and vegetables has been reported to lower the risk for developing chronic diseases including cardiovascular diseases and cancers.[1−3] These effects have been attributed to the antioxidant properties of these compounds. However, the biological effects of polyphenols may extend well beyond the direct modulation of oxidative stress. Beneficial effects of polyphenolic compounds in the cardiovascular system are mainly associated with maintaining a healthy endothelium. The endothelium, the layer of cells that line the cavities of the heart, the blood, and lymph vessels, plays a significant role in maintaining vascular integrity and cardiovascular function.[4] In pathologic conditions, especially under oxidative stress and inflammation and particularly with the presence of cardiometabolic risk factors, the endothelium undergoes structural and functional alterations, thus losing its protective role and becoming a proatherosclerotic structure.[5] In the earliest stages, changes in the endothelium are predominantly functional and termed accordingly "endothelial dysfunction". Endothelial nitric oxide synthase (eNOS) plays a central role in endothelial function. It has been found that impaired activation/expression of eNOS results in reduced vasodilation, endothelial cell migration, and angiogenesis in vitro.[6,7]

Berry fruits such as strawberry (SB), wild blueberry (WBB), and cranberry (CB) are known to be rich sources of polyphenolic compounds and have been studied extensively in the past decade for their health-promoting properties when consumed by humans.[8,9] Polyphenolic compounds present in berry fruits vary widely in chemical structure, from simple phenolic acids to complex flavonoid polymers. Flavonoids are the largest subcategory of polyphenols and have been studied for their antioxidant activity in vitro[10] and in vivo.[8] Polyphenolic compounds are known to activate redox-sensitive cellular signaling molecules such as phosphatidylinositol-3-kinase (PI3 kinase), resulting in a cascade of downstream signaling paths.[10−12] We and others have shown that extracts of strawberry, grape seed, tea, and cocoa that are rich in polyphenols induce endothelial dependent relaxation (EDR) by activating eNOS via the PI3 kinase/protein kinase B (Akt) pathway.[10−12] Activation of the PI3 kinase/Akt/eNOS signaling cascade is also purported to enhance cell migration and angiogenesis.[7] Because polyphenols are known to activate the kinase/Akt/eNOS signaling cascade, we hypothesize that polyphenol-rich extracts of berries would enhance cell migration and angiogenesis in vitro in endothelial cells.

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Although the biological activities of various polyphenols have been studied, the present study was conducted to understand the association/relevance between polyphenolic profile of fruit extracts and their biological effects as evaluated in an in vitro model system. Polyphenolic/anthocyanin-rich berry fruit extracts (SB, WBB, and CB) were used to investigate the activation of the redox-sensitive PI3 kinase/Akt pathway in vitro in human umbilical endothelial cells (HUVECs). We also investigated the effects of berry fruit extracts on endothelial cell migration and formation of capillary-like tubes upon the activation of downstream signaling cascades.

■ MATERIALS AND METHODS

Berry Materials and Reagents. Lowbush "wild" blueberries (*Vaccinium angustifolium* Ait.) (WBB) were provided by the Wild Blueberry Association of North America (WBANA, Orono, ME). Cranberries (*Vaccinium macrocarpon* Ait. cv. 'Stevens') were obtained from a grower in Wisconsin. Freeze-dried strawberry powder was supplied by the California Strawberry Commission (CSC, Watsonville, CA, USA). Standards such as cyanidin-3-*O*-glucoside chloride (Cy-3-glc), cyanidin-3-*O*-arabinoside chloride (Cy-3-ara), cyanidin-3-*O*-galactoside chloride (Cy-3-gal), peonidin-3-*O*-glucoside chloride (Pn-3-glc), peonidin-3-*O*-galactoside chloride (Pn-3-gal), peonidin-3-*O*-arabinoside chloride (Pn-3-ara), delphinidin-3-*O*-glucoside chloride (Dp-3-glc), malvidin-3-*O*-glucoside chloride (Mv-3-glc), and malvidin-3-*O*-galactoside chloride (Mv-3-gal) were obtained from ChromaDex, Inc. (Irvine, CA, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade water, methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Ethyl acetate was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Phosphate-buffered saline (PBS) solution was purchased from Fisher BioReagents (Fair Lawn, NJ, USA).

Sample Preparation and Extraction of Anthocyanin Compounds. WBB and CB were lyophilized using a Millrock Bench-Top Freeze-Dryer (Kingston, NY, USA). The resulting powder was milled with a coffee grinder and then stored at −20 °C. During extraction following the modified method of Asami et al.,[13] 2.5 g each of freeze-dried SB, WBB, and CB powder was added to 50 mL of mixed solvent (acetone/water/acetic acid, 70:29.5:0.5, v/v/v). After 1 h of vigorous shaking in the dark, the mixture was centrifuged at 10000*g* for 20 min at 4 °C and then filtered with Whatman no. 2 filter paper. The supernatant was freeze-dried following removal of the solvent mixture using a N_2 gas stream. Each freeze-dried berry extract (SB, WBB, and CB) was weighed, resuspended in PBS solution (pH 7.4) at the desired concentration, and stored at −20 °C prior to analyses. The extracts were dissolved in PBS (pH 7.4, 100 mg of extracted powder in 1 mL of PBS) and used for cell culture, oxygen radical absorbance capacity (ORAC), and anthocyanin analyses.

HR-MS (Exactive Orbitrap) Identification of Anthocyanin Compounds. An Accela HPLC system composed of an HPLC pump, a photodiode array (PDA) detector, and an Exactive Orbitrap mass spectrometer fitted with an electrospray interface (ESI) probe (Thermo Fisher Scientific) was used to identify the anthocyanins in WBB and CB extracts following the procedure described previously.[14] Samples were filtered through a 0.45 μm filter (Whatman), and 5 μL of extract was injected onto the HPLC. Anthocyanins were separated on a Synergi RP-Max 250 × 2.0 mm (4 μm i.d.) reversed-phase column (Phenomenex, Torrance, CA, USA). The solvents used were 1% formic acid in water (solvent A) and 1% formic acid in acetonitrile (solvent B). Gradient elution was 5−50% acetonitrile (1% formic acid) in 1% aqueous formic acid for a total run time of 30 min. The flow rate was 400 μL/min, and the oven temperature was 40 °C. After passing through the flow cell of the PDA detector, the column eluate was directed to an Exactive Orbitrap trap mass spectrometer fitted with an ESI probe. The mass spectrometer was set up in positive ionization to scan from m/z 150 to 2000 at a resolution of 100 000, at m/z 200 with a scan cycle time of 1 s. The capillary temperature was 275 °C, and the source voltage was 4 kV.

HPLC Quantitative Analysis of Anthocyanin Compounds. A Waters (Milford, MA, USA) Alliance 2795 HPLC system equipped with a model 996 PDA detector, model 2475 fluorescence detector, Quattro Micromass triple-quadrupole mass spectrometer, and MassLynx (v. 4.1) software was used to separate and quantify anthocyanins in WBB and CB extracts. Separations were performed on a Synergi Max-RP column (150 × 3.0 mm, 4 μM; Phenomenex). The mobile phase consisted of 1% formic acid in methanol (solvent A) and 1% formic acid in acetonitrile (solvent B). The gradient elution program was set as follows: 2% B from 0 to 4 min, 2−20% B linear from 4 to 20 min, 20−80% B from 20 to 24 min, 80% B from 24 to 30 min, and then returning to initial concentration of 2% from 30 to 35 min to re-equilibrate the system. The flow rate was 0.4 mL/min, and the injection volume was set at 20 μL. The column temperature was maintained at 40 °C, and the autosampler was cooled at 15 °C. The PDA was set at 520 nm to monitor the UV−visible absorption of anthocyanins, and the UV−visible spectra were recorded from 200 to 600 nm with a resolution of 1.2 nm and acquisition of 1 spectra/s. After passing through the flow cell of the PDA, the column eluate was split, and 0.2 mL/min was diverted to a mass spectrometer fitted with an ESI interface. The positive ionization mode (ESI+) of the mass spectrometer was utilized for the detection of anthocyanins. The mass spectrometer conditions were set up as follows: source temperature, 100 °C; desolvation temperature, 300 °C; nitrogen desolvation flow, 800 L/h; capillary voltage, 3000 V; cone voltage, 30 V; and MS-MS collision energy, 15 V. Quantification of anthocyanins was based on peak areas determined at 520 nm and calibration curves (0, 5, 10, 25, 50, 75, and 100 μg/mL) using mixed reference standards (Dp-3-glc, Cy-3-gal, Cy-3-glc, Cy-3-ara, Pn-3-gal, Pn-3-glc, Pn-3ara, Mv-3-gal, and Mv-3-glc). Mv-3glc was used to quantify peaks when standards were not available commercially. The calibration curves for the all of the reference standards were linear with $R^2 \geq 0.998$.

Total Phenolic and Ascorbic Acid Assays. The total phenolic content was estimated colorimetrically using the Folin−Ciocalteu phenol reagent according to the method of Slinkard and Singleton.[15] The ascorbic acid concentration in the freeze-dried extracts of SB, WBB, and CB was measured using HPLC as described by Sanchez-Mata et al.[16]

Oxygen Radical Absorbance Capacity Assay. The ORAC assay was performed on fruit extracts according to the method described by Prior et al.[17] with minor modifications. Fruit extracts were diluted at ambient temperature. A Perkin-Elmer Envision plate reader (Waltham, MA, USA) was used to measure the fluorescent decay of fluorescein (485 nm excitation/528 nm emission) for 60 min (1 min interval) after the addition of peroxyl radicals (AAPH) to the fruit extract. ORAC values were determined using standard Trolox concentration. A dilution series (1:2) of standards in the concentration range of 1.5625−25.0 μM was prepared. ORAC values were expressed as micromole Trolox equivalents (TE) per gram of dried extract. A standard curve was prepared by plotting Trolox concentrations versus the area under the fluorescence decay of fluorescein curve obtained for each Trolox concentration. ORAC values for the fruit extracts were calculated from the standard curve.

In Vitro Cell Culture Assays. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Washington, DC, USA) and grown in endothelial cell growth medium (EGM, Lonza) with 4% fetal bovine serum (FBS). In response to different concentration/time factors, cell viability was investigated using the Trypan blue exclusion test,[11] and apoptosis was investigated using the Caspase 9 Colorimetric Activity Assay Kit (Millipore, Waltham, MA, USA). Experiments were conducted with cells having a passage number of 2. Cells were preincubated in 2% media for 12 h to investigate the effect of berry extracts on the activation of Akt. However, preincubation time was reduced to 1 h in the experiments that investigated the effect of berry extracts on the cell migration and angiogenesis due to prolong experimental periods.

Table 1. Anthocyanins Detected from Wild Blueberry and Cranberry Extracts in Phosphate-Buffered Saline Using Reversed-Phase HPLC-MS[a]

peak	t_R (min)	anthocyanin	chemical formula	exact mass (m/z)[b]	actual mass (m/z)[c]	wild blueberry[d] (mg/100 g FW)	cranberry (mg/100 g FW)
1	16.96	delphinidin-3-*O*-galactoside	$C_{21}H_{21}O_{12}$	465.103305	465.10	6.5 ± 2.8*	nd[e]
2	18.50	delphinidin-3-*O*-glucoside	$C_{21}H_{21}O_{12}$	465.103305	465.10	4.0 ± 2.7	nd
3	19.73	cyanidin-3-*O*-galactoside	$C_{21}H_{21}O_{11}$	449.108390	449.11	9.5 ± 4.6	14.8 ± 3.0
4	21.43	cyanidin-3-*O*-glucoside	$C_{21}H_{21}O_{11}$	449.108390	449.11	5.8 ± 2.3	tr
5	22.51	petunidin-3-*O*-galactoside	$C_{22}H_{23}O_{12}$	479.118955	479.11	1.1 ± 0.8*	nd
6	22.51	cyanidin-3-*O*-arabinoside	$C_{20}H_{19}O_{10}$	419.097825	419.10	2.7 ± 0.7	23.4 ± 6.3
7	23.65	petunidin-3-*O*-glucoside	$C_{22}H_{23}O_{12}$	479.118955	479.11	4.9 ± 2.2*	nd
8	24.83	peonidin-3-*O*-galactoside	$C_{22}H_{23}O_{11}$	463.124040	463.12	7.6 ± 3.3	29.9 ± 6.4
9	26.55	peonidin-3-*O*-glucoside	$C_{22}H_{23}O_{11}$	463.124040	463.12	2.3 ± 5.6	0.8 ± 0.3
9	26.55	malvidin-3-*O*-galactoside	$C_{23}H_{25}O_{12}$	493.134605	493.13	8.9 ± 2.4	nd
10	28.10	peonidin-3-*O*-arabinoside	$C_{21}H_{21}O_{10}$	433.113475	433.11	nd	7.6 ± 1.6
10	28.10	malvidin-3-*O*-glucoside	$C_{23}H_{25}O_{12}$	493.134605	493.13	13.1 ± 2.8	6.5 ± 1.5
11	29.43	malvidin-3-*O*-arabinoside	$C_{20}H_{19}O_{11}$	463.124040	463.12	5.2 ± 2.4*	nd
12	31.10	delphinidin-3-(6″-acetoyl) glucoside	$C_{22}H_{23}O_{13}$	507.113870	507.11	1.4 ± 0.5*	nd
13	32.49	petunidin-3-(6″-acetoyl) galactoside	$C_{24}H_{25}O_{13}$	521.128967	521.13	tr	nd
14	34.46	cyanidin-3-(6″-acetoyl)glucoside	$C_{23}H_{23}O_{12}$	491.118955	491.12	1.1 ± 0.5*	nd
15	35.90	petunidin-3-(6″-acetoyl) glucoside	$C_{24}H_{25}O_{13}$	521.128967	521.13	0.8 ± 0.2*	nd
16	36.85	malvidin-3-(6″-acetoyl) galactoside	$C_{24}H_{27}O_{13}$	535.144617	535.14	tr	nd
17	39.15	peonidin-3-(6″-acetoyl)glucoside	$C_{24}H_{25}O_{12}$	505.134605	505.13	tr	nd
18	40.25	malvidin-3-(6″-acetoyl)glucoside	$C_{25}H_{27}O_{13}$	535.144617	535.14	7.6 ± 3.5*	nd
		total anthocyanins				82.5	83.0

[a]Identification and quantification using HPLC and Quattro-Micro MS coupled with PDA at 520 nm. [b]Identified using Exactive Orbitrap high-resolution MS. [c]Actual mass from Quattro Micro MS. [d]The asterisk (*) indicates expression as malvidin-3-*O*-glucoside equivalents. tr, trace. [e]nd, not detected.

Effects of Berry Fruit Extracts on Phosphorylated Akt (Ser-473) Levels in Vitro in HUVECs. The effect of SB, WBB, and CB extracts on the activation of Akt to p-Akt was investigated using HUVECs. Confluent HUVECs were starved for 12 h in a media containing 2% serum followed by treatment with berry extracts. To identify the optimum concentration effects of SB, WBB, and CB extracts on the activation of Akt, HUVECs were treated with different dilutions of berry extracts (0.1, 0.2, 0.5, and 1 mg freeze-dried extract/mL diluted extract in cell culture medium). Because it is known that polyphenolic compounds activate Akt via the redox-sensitive PI3 kinase pathway,[7] wortmannin (Ascent Scientific LLC, Princeton, NJ, USA), a PI3 kinase inhibitor, was used to confirm the mechanism of action. A negative control group of HUVECs was treated with 30 nmol/L of wortmannin, a dose that does not induce cytoxicity in HUVECs, for 30 min before exposure to berry extracts and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 to confirm PI3 kinase pathway involvement. Our previous work has indicated that polyphenolic compounds induced the maximum phosphorylation of Akt at 10 min in vitro in HUVECs.[10−12] Therefore, cells were incubated with different concentrations of berry extracts for 10 min. At the end of 10 min, the reaction was stopped by adding ice-cold PBS solution, cells were washed twice with the cold PBS buffer, and then cell lysates were prepared in cell lysis buffer (PerkinElmer, Waltham, MA, USA) containing protease inhibitors (Calbiochem, EMD Serono Inc., Rockland, MA, USA). Cell lysates obtained from the above experiments were analyzed for p-Akt (Ser-473) and total Akt protein using the Alpha Screen SureFire Assay and Perkin-Elmer Envision 2103 Multi label Reader as per the manufacturer's instructions (PerkinElmer). The phosphorylation levels of Akt (p-Akt) were expressed as p-Akt/Total Akt in response to different treatments and compared with PBS-treated controls.

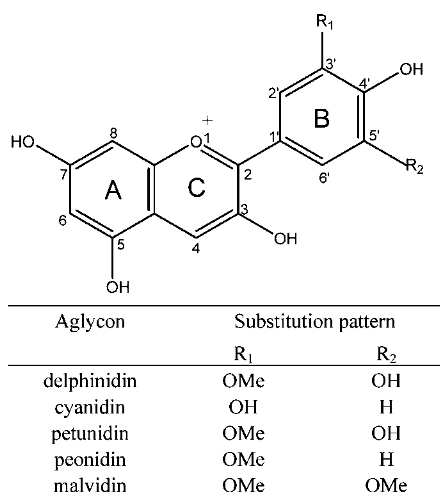
Effects of Berry Fruit Extracts on Endothelial Cell Migration. The cell migration assay (scratch assay) was performed to investigate the effect of SB, WBB, and CB extracts (0.05, 0.1, and 0.2 mg/mL) on endothelial cell migration.[7] HUVECs were grown (>90% confluent) in 6-well plates and starved for 1 h in a medium containing 2% serum. The bottom of each well containing a HUVEC monolayer was scraped (straight line) with a 200 μL sterile pipet tip. Cell debris was removed by washing the plate with PBS solution. Cells were treated with different concentrations of berry extracts in a medium containing 2% serum and then incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Half of the plates were treated with wortmannin (30 nmol/L) for 30 min before the addition of fruit extracts to confirm the involvement of the PI3/Akt signaling pathway. Parallel control experiments were carried out with cells treated with PBS solution. The number of cells that migrated beyond the scratched lines at 0, 5, 12, and 24 h were counted under 40× magnification level using an Olympus CKX41 microscope (Center Valley, PA, USA) by a single investigator in a blinded manner.

Effects of Berry Fruit Extracts on Angiogenesis (Capillary-like Tube Formation) in Vitro in HUVECs. In vitro experiments using HUVECs evaluated the effects of SB, WBB, and CB extracts (0.05, 0.1, and 0.2 mg/mL) on the formation of capillary-like tubes that mimic new blood vessel formation (angiogenesis).[7] HUVECs were starved for 1 h in endothelial growth medium containing 2% serum. The cells were washed with PBS solution and then plated in 48-well plates that had been precoated with 100 μL of growth factor-reduced Matrigel Matrix (BD Bioscience, Franklin Lakes, NJ, USA). The cells were incubated for 24 h with fruit extracts at 37 °C in a humidified incubator containing 5% CO_2. Some cell plates were treated with wortmannin and berry extracts to confirm involvement of the PI3/Akt signaling pathway in capillary-like tube formation. Capillary-like tubes were photographed after 0, 5, 12, and 24 h under 40× magnification level using an Olympus CKX41 microscope. All completed side branches were counted by a single investigator in a blinded manner.

Statistical Analysis. The Sigma Plot 11 statistical program (Systat Software, Inc., Chicago, IL, USA) was used to analyze the data. Normality and equal variance tests were performed followed by one-way ANOVA for multiple-comparison tests and to compare with the control. Treatments were considered to be significantly different at $P < 0.05$. Results are given as the mean ± SE of at least three experimental replications.

■ RESULTS

Characterization of Anthocyanin Compounds in Berry Fruit Extracts. The anthocyanin composition of WBB and CB extracts was characterized using reversed-phase HPLC in this study. The characterization of the anthocyanin profile of SB extract was reported earlier by Edirisinghe et al.[8] Identification and peak assignment of anthocyanins in the WBB and CB extracts were initially conducted on the basis of accurate mass data obtained from a high-resolution Exactive Orbitrap mass spectrometer (see Table 1, footnote *b*). Identification was also conducted by comparing the retention times of the peaks obtained during HPLC separations with those from reference standards and published data. Hence, the anthocyanin peaks found during HPLC separations for WBB and CB extracts were assigned as delphinidin-3-*O*-galactoside (**1**), Dp-3-glc (**2**), Cy-3-gal (**3**), Cy-3-glc (**4**), petunidin-3-*O*-galactoside (**5**), Cy-3-ara (**6**), petunidin-3-*O*-glucoside (**7**), Pn-3-gal (**8**), Pn-3-glc/Mv-3-gal (**9**), Pn-3-ara/Mv-3-glc (**10**), malvidin-3-*O*-arabinoside (**11**), delphinidin-3-(6″-acetoyl)glucoside (**12**), petunidin-3-(6″-acetoyl)galactoside (**13**), cyanidin-3-(6″-acetoyl)glucoside (**14**), petunidin-3-(6″-acetoyl)glucoside (**15**), malvidin-3-(6″-acetoyl)galactoside (**16**), peonidin-3-(6″-acetoyl)glucoside (**17**), and malvidin-3-(6″-acetoyl)glucoside (**18**) (Table 1). The structures of these anthocyanins inherent in WBB and CB are shown in Figure 1.



Aglycon	Substitution pattern	
	R_1	R_2
delphinidin	OMe	OH
cyanidin	OH	H
petunidin	OMe	OH
peonidin	OMe	H
malvidin	OMe	OMe

Figure 1. Basic structure of the naturally occurring aglycons and glycosides in wild blueberry (WBB) and cranberry (CB). The chemical structure indicates the two aromatic rings (A and B) and the R_1 and R_2 substitution sites.

In CB extracts, five major compounds (**3**, **6**, **8**, **9**, and **10**) and a trace compound (**4**) were detected (Figure 2). In contrast, 15 major anthocyanin compounds (**1**−**12**, **14**, **15**, and **18**) and 3 trace compounds (**13**, **16**, and **17**) were detected in WBB extracts. Quantification of the anthocyanins in both WBB and CB was based on the retention times and standard curves obtained from the mixture of nine reference standards (Dp-3-glc, Cy-3-gal, Cy-3-glc, Cy-3-ara, Pn-3-gal, Pn-3-glc, Pn-3-ara, Mv-3-gal, and Mv-3-glc). In addition, the reference standard, Mv-3-glc, was used to quantify the remaining anthocyanin peaks detected in WBB. Mv-3-glc (13.1 ± 2.8 mg/100 g wet basis) was the most abundant anthocyanin in WBB, followed by Cy-3-gal (9.5 ± 4.6 mg/100 g wet basis), Mv-3-gal (8.9 ± 2.4 mg/100 g wet basis), Pn-3-gal and malvidin-3-(6″-acetoyl)-glucoside (7.6 ± 3.3 and 7.6 ± 3.5 mg/100 g fresh weight, respectively), and Dp-3-gal (6.5 ± 2.8 mg/100 g fresh weight). In contrast, the three most dominant compounds in CB were Pn-3-gal, Cy-3-ara, and Cy-3-gal (29.9 ± 6.4, 23.4 ± 6.3, and 14.8 ± 3.0 mg/100 g based on fresh weight of berries, respectively). The two minor anthocyanins in CB samples were Pn-3-ara and Mv-3-glc. Edirisinghe et al.[8] previously reported that one major and two minor anthocyanins were detected in extracts of freeze-dried strawberry powder. Pelargonidin-3-*O*-glucoside (Pg-3-glc) (11.5 mg/100 g fresh weight) was the most abundant anthocyanin, whereas pelargonidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside were the minor anthocyanin components of the strawberry powder extract.[8]

Total anthocyanin content was calculated as the sum of identified and quantified anthocyanins in each fruit. The total anthocyanin contents for WBB, CB, and SB were 82.5, 83.0, and 81.7 ± 5.9 mg/100 g fresh weight, respectively.

Total Phenolic Content, Ascorbic Acid Content, and Antioxidant Capacity of Berry Fruit Extracts. The total phenolic contents for SB, WBB, and CB extracts used in the cell culture experiments were 26.15 ± 0.49, 25.56 ± 1.64, and 28.02 ± 1.84 μg gallic acid equivalents (GAE)/mL, respectively. The ascorbic acid contents in the freeze-dried SB, WBB, and CB extracts used in the cell culture experiments were 2.85 ± 0.06, 0.102 ± 0.003, and 1.32 ± 0.01 mg/g, respectively. The ORAC assay was used to quantify the antioxidant capacity of berry fruit extracts. The ORAC value of SB (669 ± 30 μmol TE/g) was significantly higher than those of WBB and CB (244 ± 12 and 298 ± 16 μmol TE/g, respectively, $P < 0.01$).

Effects of Fruit Extracts on the Activation of Akt in Vitro in HUVECs. Fruit extracts were tested across a range of 0.01−10 mg/mL, and the optimum activation was observed within 10 min.[10−12] At berry extract concentrations >1 mg/mL, the pH of the cell culture media was altered beyond the physiologically relevant level (pH <7.0). Therefore, concentrations above 1 mg/mL were not used in these experiments. Cell viability and apoptosis of HUVEC cells were not significantly affected at the concentrations tested (0.01−1.0 mg/mL for 10 min) as measured by Trypan blue exclusion test (99 ± 1.0% in all treatments) and Caspase 9 activity assay [PBS, 36.4 ± 1.6; SB, 37.4 ± 2.1; WBB, 35.5 ± 1.0; and CB, 35.8 ± 2.0 μmol/L chromophore *p*-nitroaniline (*p*NA)], respectively, compared to PBS-treated controls ($P > 0.05$).

SB, WBB, and CB extract treatments resulted in significantly increased phosphorylation of Akt in a dose-dependent manner in vitro in HUVECs ($P < 0.05$, Figure 3). The maximum p-Akt threshold was observed at 1 mg/mL for SB and CB extracts and at 0.5 mg/mL for the WBB extract. Furthermore, the maximum phosphorylated Akt levels in response to SB (at 1 mg/mL) and WBB (0.5 mg/mL) were 2- and 3-fold higher, respectively, compared to that of CB (at 1 mg/mL, $P < 0.05$).

To investigate the involvement of PI3 kinase in berry-induced activation of Akt, cells were pretreated with wortmannin, a PI3 kinase inhibitor. As shown in Figure 3, phosphorylation of Akt was significantly decreased in the presence of wortmannin compared to the cells treated with

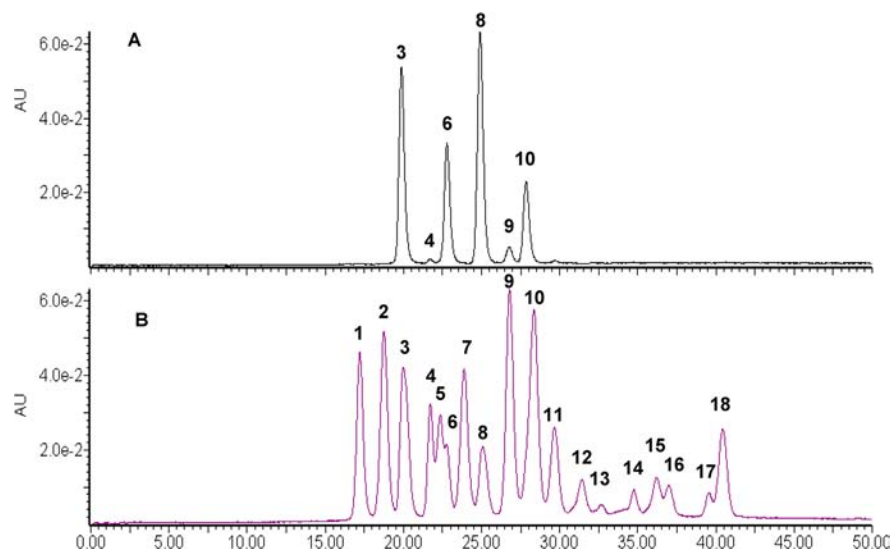


Figure 2. Anthocyanin profiles of (A) cranberry (CB) and (B) wild blueberry (WBB) extracts in phosphate-buffered saline detected at 520 nm. Peak identities and quantities are summarized in Table 1.

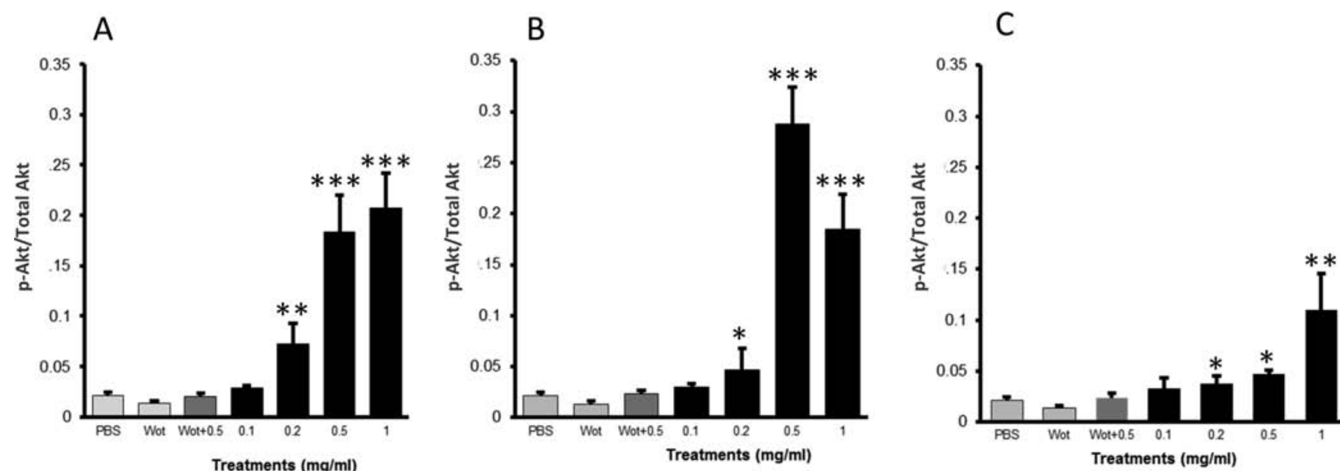


Figure 3. Dose effect of various berry extracts on the activation of Akt in vitro in HUVECs. HUVECs were starved for 12 h followed by treatment with 0.1, 0.2, 0.5, and 1 mg/mL of berry extracts [(A) strawberry (SB); (B) wild blueberry (WBB); (C) cranberry (CB)] and then incubated for 24 h at 37 °C in a humidified incubator containing 5% CO_2. The ordinates are the relative ratios of the phosphorylated (Ser-473) and nonphosphorylated form of Akt (p-Akt/Total Akt). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, significant compared to respective PBS controls ($n = 3$). Wot, wortmannin.

berry extracts alone (without wortmannin). These data support the involvement of PI3 kinase in the events of activating Akt in HUVECs in response to exposure to SB, WBB, and CB extracts.

Effects of Berry Extract on Cell Migration and Angiogenesis (Capillary-like Tube Formation) in Vitro in HUVECs. In cell migration and angiogenesis experiments, HUVEC viability and apoptosis, as assessed by the Trypan blue exclusion test and Caspase 9 activity, respectively, were significantly attenuated in response to SB, WBB, and CB extracts at concentrations of 0.5 mg/mL and above (≥0.5 mg/mL) 5 h post-treatment ($P < 0.05$). However, cell viability and apoptosis up to 24 h post-treatment were not significantly altered in response to exposure of HUVECs to berry extracts at concentrations of 0.2 mg and below (≤0.2 mg/mL, $P > 0.05$, data not shown). Therefore, all the cell migration and capillary-like tube formation assays were performed at extract concentrations of 0.05, 0.1, and 0.2 mg/mL. In cell migration and angiogenesis experiments, scratch marks and capillary-like tubes, respectively in the surface of HUVEC-coated plates were observed at four different times in a 24 h time interval. After 12 and 24 h, in control and fruit extract treatments, HUVECs migrated sufficiently to eradicate evidence of scratch marks in the HUVEC monolayers even though the control group shows many empty spaces due to cell death. This is probably due to the depletion of nutrients in the media (no medium change). Therefore, 12 and 24 h time points were not considered in the analysis. After optimization of cell migration and angiogenesis assays, significant changes were observed at the 5 h time point for 0.2 mg/mL concentration. Therefore, we have reported data at 5 h with 0.2 mg/mL berry concentrations (Figures 4 and 5).

At the end of 5 h, cell migration was significantly increased in response to all three berry extracts compared to their respective PBS controls ($P < 0.01$, Figure 4). However, no significant differences were seen between the different berry extracts ($P > 0.05$). Cell migration in response to exposure to berry extracts in the presence of wortmannin is shown in Figure 4. Cell migration was significantly reduced in HUVECs treated with

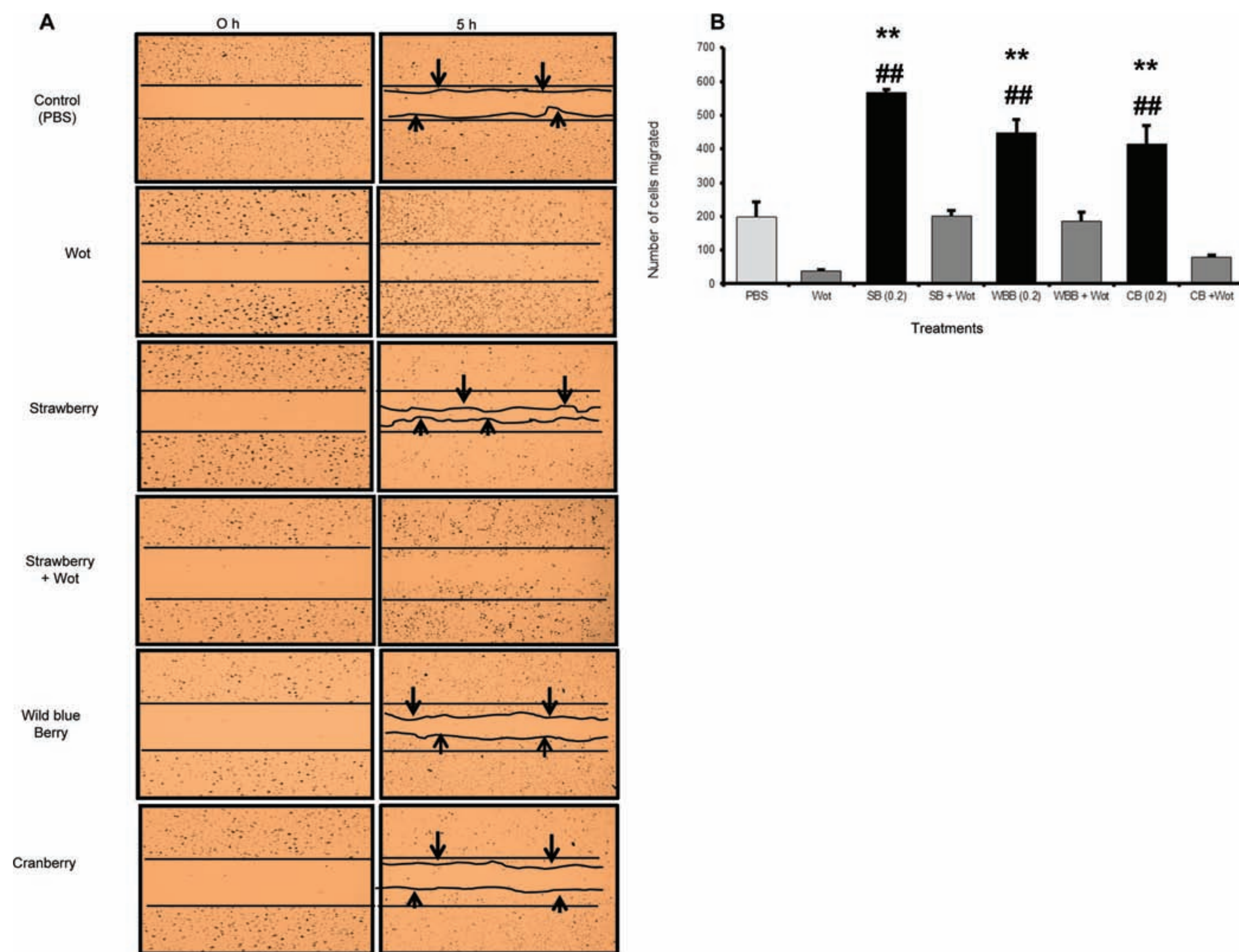


Figure 4. Effect of different berry extracts on cell migration in vitro in HUVECs. HUVECs were starved for 1 h followed by treatment with 0.2 mg/mL of berry extracts [strawberry (SB); wild blueberry (WBB); cranberry (CB)] and then incubated for 24 h at 37 °C in a humidified incubator containing 5% CO_2. (A) Representative photographs of cells (40×) were taken at the beginning and after 5 h. The number of cells migrated beyond the scratch lines after 5 h was counted under the microscope by a single investigator in a blinded manner. (B) Histograms represent mean ± SD cell migration beyond the scratch lines (n = 3). **, P < 0.01, vs PBS-treated control groups. ##, P < 0.01, vs respective wortmannin-treated groups. No significant differences were found among the different berry extracts (P > 0.05). Wot, wortmannin.

wortmannin and berry extracts compared to cells treated with berry extracts alone (P < 0.01). However, in the presence of wortmannin, cell viability at the end of 5 h decreased, and cells experienced morphological changes as observed under the microscope.

Capillary-like tube formation was significantly increased in response to all three berry extracts compared to their respective PBS controls after 5 h (P < 0.01, Figure 5). Similar to the results found in cell migration experiments, no significant differences (P > 0.05) in tube formation were found between the different berry extracts (0.2 mg/mL). As expected, capillary-like tube formation was significantly abolished when cells were treated with berry extracts and wortmannin at the end of 5 h (P < 0.001, Figure 5). Wortmannin may have some other side effects and may inhibit other related molecular signaling events in cell migration and angiogenesis. This is apparent in the control experiments (cell migration and angiogenesis were significantly lower compared to PBS).

DISCUSSION

The vascular endothelium is a vital homeostatic cell layer responsible for a variety of functions, such as thromboresistance, control of vascular tone, and vascular growth.[18] In the present study, extracts of SB, WBB, and CB dose-dependently activated Akt, a key intermediate in endothelial function. Furthermore, we demonstrated that activation of Akt by the berry extracts was mediated by PI3 kinases. Endothelial cell migration and angiogenesis require activation of several signaling pathways that converge on cytoskeletal remodeling following a series of events in which the endothelial cells extend and contract their cytoskeleton to form new cells. Several factors/signaling events are involved in the regulation of endothelial cell migration and angiogenesis including vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR), PI3/Akt, Rho kinase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase cascades. However, the main focus of this study was to investigate the effect of berry extracts on the modulation of endothelial cell migration and angiogenesis and not to ascertain

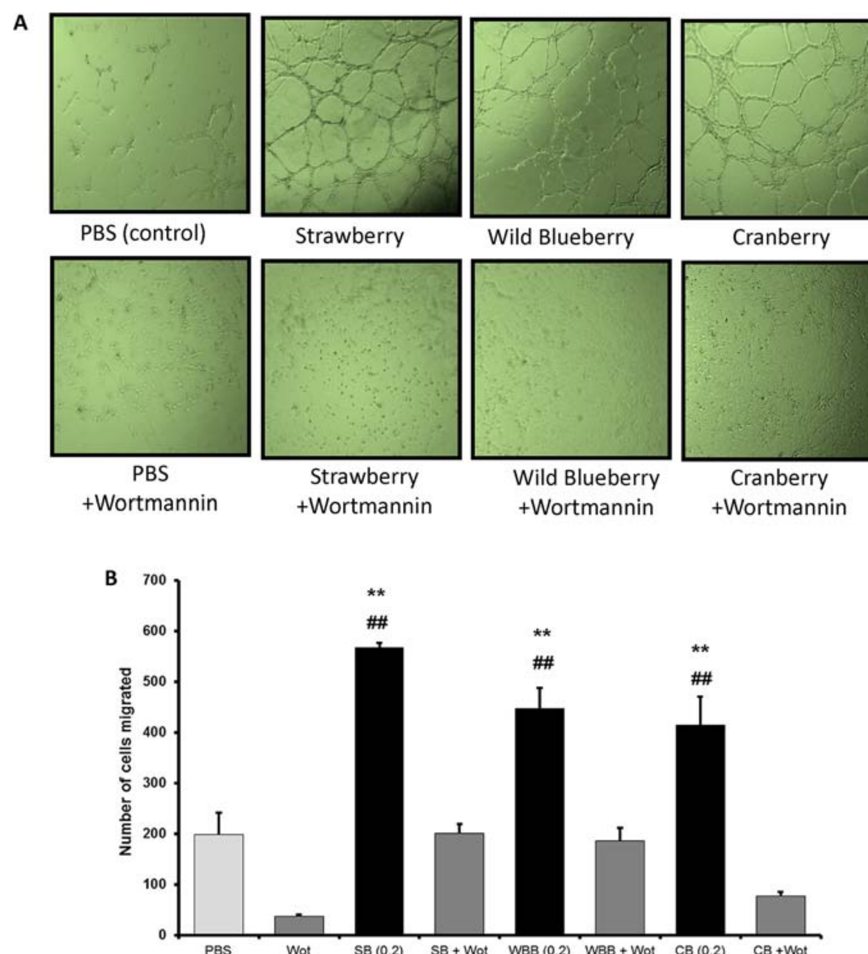


Figure 5. Effect of different berry extracts on capillary-like tube formation in vitro in HUVECs. HUVECs were starved for 30 min followed by treatment with 0.2 mg/mL of berry extracts [strawberry (SB); wild blueberry (WBB); cranberry (CB)] and then incubated for 24 h at 37 °C in a humidified incubator containing 5% CO_2. (A) Representative photographs of cells (40×) were taken at the beginning and after 5 h. The number of capillary-like tube branches was counted under the microscope by a single investigator in a blinded manner. Effect of strawberry extracts in the presence of wortmannin was given as a representative slide to indicate the involvement of PI3 kinase/Akt signaling in the berry extract-induced capillary-like tube formation. (B) Histograms represent mean ± SD number of completed capillary-like tube branches ($n = 3$). **, $P < 0.01$, vs PBS-treated control groups. ##, $P < 0.01$, vs respective wortmannin-treated groups. No significant differences were found among the different berry extracts ($P > 0.05$). Wot, wortmannin.

the detailed molecular mechanism(s) of endothelial cell migration and angiogenesis induced by berry extracts. eNOS, which is activated by PI3/Akt through the production of NO, is known to play a major role in endothelial cell migration and angiogenesis.[19] We have previously reported that eNOS is activated by polyphenolic compounds via the PI3 kinase/Akt signaling pathway.[9−11] Hence, we consider that the PI3/Akt/eNOS-mediated mechanism is the main focus for this study. However, we are not excluding the possibility that this activation may also be induced by other cell signaling cascades such as VEGF/VEGFR and NADPH oxidase.

The berry fruits investigated in this study contain significant quantities of anthocyanins. Although we observed diversity and distinct differences in the composition of individual anthocyanins in WBB and CB in this study, and SB in our previous paper,[8] the total anthocyanin contents of these fruits were similar. For WBB fruits, the total anthocyanin content was 82.5 mg/100 g wet basis, whereas that of CB was 83.0 mg/100 g fresh weight. The total anthocyanin content of strawberry is 81.65 ± 5.9 mg/100 g fresh weight.[8] These values are almost within the range reported previously for lowbush blueberries (95−255 mg/100 g)[20] and cranberries (13.6−171 mg/100 g),[21−23] respectively. Furthermore, we measured the total antioxidant capacity of berry fruits using the ORAC assay, which indicated that SB had significantly higher antioxidant capacity compared to WBB and CB. This may be due to higher content of several other undetected polyphenolic compounds and antioxidant vitamins such vitamin C in strawberry. The vitamin C content analyzed in the freeze-dried SB extract used in the cell culture experiments was 2.85 ± 0.06 mg/g, which is in agreement with a previous paper.[10] In contrast, the ascorbic acid contents in WBB and CB extracts were only 0.102 ± 0.003 and 1.32 ± 0.01 mg/g, respectively, which may partially account for the differences observed in ORAC values.

PI3 kinase is a redox-sensitive kinase, and therefore we speculated that berry-derived polyphenolic compounds via their antioxidant properties may change the redox status of endothelial cells, resulting in the activation of PI3 kinase. Thus, berry fruits with higher antioxidant capacity as estimated by the ORAC assay would be speculated to cause greater redox changes and higher levels of activated Akt in endothelial cells compared to berries with lower ORAC values. However, we did

not observe this to be the case in the present study. The WBB extract at a concentration of 0.5 mg/mL caused the highest activated Akt levels ($P < 0.05$) compared to SB (1 mg/mL) and CB (1 mg/mL) extracts. It would have been ideal if we were able to measure the changes in the redox status of the endothelial cells. However, changes in the redox status of cells occur within milliseconds, making this determination difficult to measure. Several other factors such as the redox status of the endothelial media, intracellular redox status, and incubator conditions maybe responsible for the changes in the redox status in cell culture experiments. Although the total anthocyanin contents of SB, WBB, and CB extracts used in the cell culture experiments were very similar (3.40, 3.44, and 3.47 μg/mL for SB, WBB, and CB extracts, respectively, at 0.2 mg/mL), the effects of individual anthocyanin compounds present in different berry extracts on the activation of Akt are not known. In addition, there were no remarkable differences in the total phenolic content for SB, WBB, and CB extracts used in the cell culture experiments (26.15 ± 0.49, 25.56 ± 1.64, and 28.02 ± 1.84 μg of GAE/mL for SB, WBB, and CB, respectively, at 0.2 mg/mL).

Although the activation of PI3/Akt/eNOS induced by polyphenolic compounds has been demonstrated previously, the functional aspects of this event, such as endothelial cell migration and angiogenesis, at the concentration found in human plasma after ingestion of berry fruits (physiologically relevant concentrations) have not been reported. In the present study, we found that berry extracts not only activated PI3/Akt but also enhanced cell migration and angiogenesis as assessed by scratch and capillary-like tube formation assays, respectively. However, the cellular events observed in cell migration and angiogenesis in this study could also be attributed to several other factors, and not limited only to the activation of PI3 kinase. We are not discounting the possibility that enhanced migration and angiogenesis by the berry extracts may have also occurred through nonspecific growth effects in the cell culture system due to other nutrients present in the berry.

Both angiogenesis and cell migration are important in neovascularization. Angiogenesis is driven by endothelial cells, which, on activation by angiogenic growth factors, migrate into the interstitial matrix, proliferate, and form new capillary-like structures.[24] Several in vivo studies have reported improvement of endothelial function with exposure to polyphenolic compounds.[25] However, the functional studies in humans are mostly related to vasodilation. A study reported by Lekakis et al.[26] found that polyphenolic compounds from red grapes acutely improve endothelial function in patients with coronary heart disease as assessed by flow-mediated vasodilation. Chronic cranberry juice consumption reduced carotid femoral pulse wave velocity, a clinically relevant measure of arterial stiffness.[27] Dal-Ros et al.[28] reported that intake of grape polyphenols protects against aging-induced endothelial dysfunction and decline in physical performance in human subjects. Although the endothelial function is a broad term that implies regulation of endothelial cell functions, migratory as well as tube formation properties (neovascularization) are important components in repairing vasculature, especially in chronic metabolic disease conditions and aging.[29] Therefore, our data support the available evidence suggesting that polyphenolic-rich fruits have favorable effects on endothelial function and, moreover, extend findings to include other functional indicators of endothelial health. Future studies in vivo will be required to verify the relevance of these in vitro findings.

Bioavailability is a key factor in determining the physiological effects of food-derived polyphenols in humans. However, the role of polyphenols in the etiology and treatment of chronic disease is hardly definitive, and some have challenged the findings in studies that defined the mechanisms of action of polyphenols and their bioavailability within the body. The total anthocyanin contents of berry extracts (0.2 mg/mL) used in the cell migration and angiogenesis assays were 3.40, 3.44, and 3.47 μg/mL for SB, WBB, and CB, respectively. Pg-3-glc, Mv-3-glc, and Pn-3-gal are the major anthocyanins in SB, WBB, and CB, respectively, and their concentrations in the cell culture media were 1108, 1107, and 2690 nmol/L. Edirisinghe et al.[8] reported that the concentration maxima (C_{max}) of pelargonidin sulfate and Pg-3-glc were 139 ± 7 and 14 ± 1 nmol/L, respectively, after ingestion of a strawberry beverage containing 10 g of freeze-dried strawberry powder (~100 g of fresh strawberry). The physiological relevance of high doses is questionable, because these doses are not feasible to obtain in in vivo systems. One must consider dose as a key factor involved in understanding how these polyphenolic compounds might benefit human health. However, not all studies regarding the use of polyphenols address this factor. For example, Ohga et al.[30] have demonstrated that the polyphenol epigallocatechin-3-gallate (EGCG) in green tea suppresses tumor growth by direct action on tumor cells and by inhibition of angiogenesis. However, the dose of EGCG used in that study was 25−50 μmol/L. The corresponding anthocyanin content of berry extracts at concentrations >0.5 mg/mL exceeds ~8.5 mg/mL.

Seeram et al.[31] demonstrated that blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro at concentrations ranging from 25 to 200 μg/mL. Kim et al.[32] have also demonstrated that proanthocyanidin-rich isolate from cranberry (0−100 μg/mL) blocked the activation of the Akt in vitro in HUVECs. However, in the studies done by Seeram et al.[31] and Kim at al.,[32] quantitative polyphenolic analyses of the extracts were not done. Furthermore, the extracts were relatively pure and concentrated compared to the extracts used in the present study. The present investigation also observed similar effects such as apoptosis, antiangiogenic, and antiproliferative activities at doses of >0.5 mg/mL of berry extracts. The study done by Chen et al.[33] reported increased angiogenesis and cell migration in HUVECs treated with the serum taken from human subjects after treatment with polyphenol extracts obtained from purple sweet potato leaves. However, they observed that direct treatment with polyphenol extracts of 0.2−0.6 mM GAE inhibited proliferation, migration, and tube formation in vascular endothelial growth factor-treated HUVECs.

The individual anthocyanin (i.e., major anthocyanin in SB, Pg-3-glc) concentrations in the berry extract (0.2 mg/mL) investigated in cell migration and angiogenesis assays in the present study are greater than those reported in human blood after the consumption of appreciable amounts of berry fruits.[8] However, they are within 1 order of magnitude of the levels found in human blood after the consumption of berry fruits. We also observed changes at the concentrations of individual anthocyanin closely matched with in vivo concentration (i.e., at 0.05 mg/mL). However, the changes observed with tube formation and cell migrations at low concentrations were

statistically not significant. The active compounds in vivo may not be the native anthocyanins found in berry extracts, which were evaluated in our in vitro studies. The active compounds are more likely to be anthocyanin metabolites.[34] Anthocyanins are extensively conjugated in the body; upon ingestion, they are rapidly absorbed in the gastrointestinal tract. Then, they are quickly metabolized and excreted in the bile and urine either as nonconjugated or methylated and glucuronidated forms. For example, Kay et al.[35] demonstrated the metabolic conversion of cyanidin 3-glycosides into glucuronide conjugates as well as methylated and oxidized derivatives of cyanidin 3-galactoside and cyanidin glucuronide. Also, Felgines et al.[36] have shown that the strawberry anthocyanin, Pg-3-glc, was transformed into glucuronated and sulfonated derivatives in humans. Furthermore, a recent study has shown extensive modification of anthocyanins by colonic microflora in the gut, which resulted in the deglycosylation and demethylation of the aglycones.[37] The nonconjugated forms quantified in the berry extracts represent a minor fraction of the circulating metabolites in vivo,[8] which is indeed the limitation of this study. Very little is currently known regarding the biological activities of these conjugated metabolites in both in vitro and in vivo systems.[8] This is clearly an area for future work.

In summary, the present study demonstrated that SB, WBB, and CB extracts induced cell migration and angiogenesis via the PI3 kinase/Akt pathway in vitro in endothelial cells. To maintain proper perspective on the potential benefits of berry fruits on human health, there must be continued recognition of the true implication of in vitro versus in vivo studies.

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Author Contributions

A.Z.T., Jr., and C.C. equally contributed as first authors. A.Z.T., Jr., I.E., B.B.-F., and L.S.J. designed the research. A.Z.T., Jr., C.C., I.E., K.D.W., J.E.J., K.B., A.K., and R.K.T. conducted the research. A.Z.T., Jr., C.C., and I.E. analyzed the data and compiled the manuscript. All authors read and approved the final submitted manuscript.

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Notes

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