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Characterization of Blackberry Extract and Its Antiproliferative and Anti-Inflammatory Properties

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ABSTRACT Blackberries are rich in polyphenols, including anthocyanins. Polyphenols are hypothesized to have biological activities that may impact positively on human health. In these studies, an anthocyanin-rich extract from Hull blackberries grown in Kentucky was obtained and fully characterized in terms of total anthocyanin and phenolic content, polymeric color, anthocyanin composition, and total antioxidant capacity. *In vitro* cell culture studies showed that the blackberry extract inhibited HT-29 colon tumor cell growth in a concentration-dependent manner with 49.2 μg of total anthocyanins/mL inhibiting HT-29 cell growth up to 66% at 72 hours. Likewise, in a concentration-dependent manner, total anthocyanin concentrations in the range of 0–40 μg/mL suppressed both high-dose (10 μg/mL) and low-dose (0.1 μg/mL) lipid A-induced interleukin-12 release from mouse bone marrow-derived dendritic cells. These results suggest that Hull blackberry extract (HBE) has potent antioxidant, antiproliferative, and anti-inflammatory activities and that HBE-formulated products may have the potential for the treatment and/or prevention of cancer and/or other inflammatory diseases.

KEY WORDS: • ***anthocyanin*** • ***antioxidation*** • ***colon cancer*** • ***phenolics***

INTRODUCTION

Fruits and vegetables are rich sources of naturally occurring antioxidants, including vitamins C and E, β-carotene, and phenolics. Among the phenolic compounds, anthocyanins have drawn increasing attention since they possess potent antioxidant activity. A comparison of the antioxidative properties of anthocyanins with other widely known antioxidants showed that the anthocyanins had higher activity than vitamin E, ascorbic acid, and β-carotene and were comparable to butylated hydroxytoluene and butylated hydroxyanisole.[1–4] Moreover, several studies have shown that the content and antioxidant activities of total anthocyanins and total phenolics in various fruits are highly correlated.[5–10] However, studies using extracts from different fruits and vegetables have suggested that there may be synergic or additive biological effects due to unique combinations of anthocyanins and phenolics.[11,12]

Anthocyanins are water-soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium. Anthocyanins exist at low pH as a flavylium cation, which is their naturally occurring form. The flavylium cation is highly electron deficient, which leads to their potent activity toward free radicals and oxygen reactive species. The flavylium cation is intensely colored (red or orange at low pH), while the ring-opened chalcone is uncolored at a pH of about 4.5 and above. The pH-dependent color difference of the anthocyanins is unique from polymerized anthocyanin-tannin or other pigments and serves as the basis of several quantitation methods for monomeric anthocyanins.

Because antioxidants function as potential inhibitors of numerous degenerative diseases, including cancer, inflammation, and heart diseases, various anthocyanin-containing extracts or anthocyanin-rich fractions from fruits and vegetables have been investigated for their therapeutic potential. For example, several groups have investigated the antitumor effects of anthocyanins on human cancer cell lines[12–17] and in animal models.[18,19] Wang *et al.*[4] reported that anthocyanin-rich extracts inhibited the enzyme activity of cyclooxygenase-2 and acted as modulators of the immune response in activated macrophages via inducing tumor necrosis factor-α production.[20] The expression of cyclooxygenase-2 in lipopolysaccharide-stimulated macrophages was also suppressed by anthocyanins at both the protein and RNA levels.[21] Studies in animal models also demonstrated the anti-inflammatory properties of anthocyanin-containing extracts.[22–25]

Blackberries (belonging to the Rosaceae family) are among the earliest fruits used medically. As early as the 16th Century, blackberry juice was used in Europe to treat infections of the mouth and eyes. Recent research has revealed that blackberries have among the highest antioxidant capacity of any fruit and vegetables.[26] However, studies to elu-

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cidate the potential therapeutic properties of blackberries have been limited. Since blackberries are currently grown in large scale in Kentucky, there was an interest in both characterizing and developing anthocyanin-rich blackberry extract products for potential nutritional and medical applications. Thus, the objectives of these studies were (1) to characterize blackberry extracts for total anthocyanin and phenolic content, polymeric color, anthocyanin composition, and total antioxidant capacity (TAC) by the Trolox equivalent (TE) antioxidant capacity (TEAC) method and (2) to evaluate the bioactivities of blackberry extract in *in vitro* models of colon tumor cell proliferation and lipid A-induced inflammation.

MATERIALS AND METHODS

Materials

Blackberry puree was from Hull blackberries grown and kindly provided by WindStone Farms (Paris, KY). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), potassium persulfate, formic acid (ACS grade, >96%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin-Ciocalteau phenol reagent, and gallic acid (98% purity) were purchased from Sigma (St. Louis, MO). Hydrogen chloride (~1.25 mol/L in ethanol) was purchased from Fluka (St. Gallen, Switzerland). USP-grade ethanol (absolute; 200 proof) was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY). High performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of Hull blackberry extract (HBE)

The Hull blackberries were harvested on July 26, 2005 at WindStone Farms. The seeds and skin of the berries were removed using a Langsenkamp (Indianapolis, IN) type 161 Colossal Pulper having two agitator arms with brushes with a stainless steel chamber and a stainless steel catch pan with two outlets (one threaded and one with a sanitary fitting) with a 10-horsepower, three-phase, 60-cycle, 230/460 voltmeter. Whole blackberries were passed through the Lagsenkamp pulper at a rate of 50–75 gallons/minute to produce a homogeneous blackberry puree free of skin and seeds. The blackberry puree was stored frozen at −20°C until processed for the present studies.

The frozen blackberry puree was lyophilized in a VirTis (Gardiner, NY) model AD2 lyophilizer and ground into a powder. An aliquot of blackberry powder (2.5 g) was treated under sonication for 30 minutes with 60 mL of ethanol containing 0.01% HCl. The supernatants were collected after filtration and dried by rotary evaporation at 40°C. The dried extract was dissolved in 10 mL of deionized water, filtered through a nylon syringe filter (pore size 1.0 μm), and then stored frozen at −20°C.

Monomeric anthocyanins and polymeric color measurement

Monomeric anthocyanin content was determined by the pH-differential method of Giusti and Wrolstad.[27] Briefly, samples were diluted 1:100 (vol/vol) in a 25 mmol/L potassium chloride buffer (pH 1.0) and a 0.4 mol/L sodium acetate buffer (pH 4.5), and then the absorbance was read against a blank at 510 nm and 700 nm with 1-cm-pathlength disposable cuvettes. Total anthocyanin content was calculated using an extinction coefficient of 26,900 L cm^{-1} mg^{-1} and a molecular weight of 449.2 g/mol of cyanidin-3-glucoside. Color density and polymeric color were calculated using absorption at 420, 510, and 700 nm with and without bisulfite treatment. The percentage of polymeric color was determined by the ratio of polymerized color to color density.

Total phenolic measurement

Total phenolic content in HBE was estimated based on the method of Singleton and Rossi[28] using gallic acid as a standard. Twenty microliters of diluted samples was added to 1.58 mL of distilled water along with 100 μL of a 2 *N* Folin-Ciocalteu phenol reagent. All solutions were mixed thoroughly and then allowed to sit at room temperature for 1 minute. Three hundred microliters of saturated sodium carbonate solution (200 g/L) was then added to each sample. The absorbance was measured at 765 nm with a Beckman (Fullerton, CA) ultraviolet (UV)-visible spectrophotometer after incubation for 2 hours at room temperature. Total phenolics were determined based on the standard curve generated with 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of gallic acid.

TEAC assay

The TEAC assay for HBE was carried out using a Beckman DU640B spectrophotometer following procedures described by Re *et al.*[29] $ABTS^{\bullet+}$ was produced by reacting 7 mmol/L ABTS with 2.5 mmol/L potassium persulfate for 16 hours in the dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Twenty microliters of HBE samples was added to 980 μL of diluted $ABTS^{\bullet+}$ solution, such that each final sample produced between 20% and 80% inhibition of the blank absorbance. The absorbance readings were taken continuously every 6 seconds for 6 minutes at 734 nm at 30°C. Trolox standards in ethanol with final concentration ranging from 0 to 16.8 μmol/L were prepared and assayed under the same conditions. The TAC of HBE was calculated and expressed as μmol of TE/g of dried blackberry extract (DBE).

HPLC-UV-mass spectrometry (MS) analysis of anthocyanins

The HPLC-UV-MS analysis was performed using an X-Bridge™ C18 column (250 mm × 4.6 mm, particle size 5 μm) (Waters, Milford, MA) equipped with an X-Bridge™

Table 1. Composition and Characterization of DBE

	Level (mg/g of DBE)			
	Total anthocyanins[a]	*Total phenolics*[b]	*Polymeric color (%)*	*TAC (μmol of TE/g of DBE)*
Blackberry extract ($n = 3$)	6.80 ± 0.31	17.32 ± 0.74	2.2 ± 1.0	66.98 ± 1.27

[a]Total anthocyanins were expressed as cyanidin-3-glucoside equivalent.
[b]Total phenolics were expressed as gallic acid equivalent.

C18 guard column with a Waters model 2690 separation module equipped with a model 996 photodiode array detector, and coupled on-line with a Waters Micromass ZMD 4000 mass spectrometer. The mobile phase consisted of 10% formic acid (A) and 100% acetonitrile (B). The elution conditions were as follows: 0–45 minutes, linear gradient from 1% to 16% B (vol/vol); 46–50 minutes, linear gradient from 16% to 100% B; and 51–60 minutes, 100% B; post-time 5 minutes with 1% B; flow rate 1 mL/minute. The UV-visible detection wavelength was 524 nm, and the injection volume was 50 μL of HBE. The MS instrument was operated at the following settings: ESP+ mode; capillary voltage, 3.0 kV; cone voltage, 35 V; desolvation temperature, 300°C; source temperature, 100°C; scan range, 100–1,000 *m/z*.

Cell culture

HT-29 human colorectal cancer cells (HTB38; ATCC, Baltimore, MD) were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 10 μg/mL streptomycin and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Bone marrow cells were obtained by flushing the femurs of BALB/c mice (Harlan Sprague-Dawley Laboratories, Indianapolis, IN) with 1× Hanks' balanced salt solution. Cells were cultured in 100-mm-diameter bacteriological Petri dishes at 2×10^5 cells/mL in 10 mL of complete RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 1 mmol/L HEPES, 2 μmol/L L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, and 50 μmol/L 2-mercaptoethanol) containing 20–25 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) at 37°C in 7% CO_2. The cells were supplemented with an additional 10 mL of complete RPMI 1640 with 20–25 ng/mL GM-CSF on day 3. On day 6, 10 mL of supernatant was removed from each plate and spun down. The cells were resuspended in fresh 10 mL of complete RPMI 1640 with 20–25 ng/mL GM-CSF and added back to the Petri dishes. Nonadherent to lightly adherent cells were harvested on day 7 as dendritic cells (DCs) and used for the *in vitro* studies.

Cell proliferation assay

HT-29 cells were seeded at a density of 1.2×10^4 cells per well in 96-well plates and incubated under normal growth conditions overnight to allow cells to attach and proliferate. HBE was added in final concentrations ranging from 13.6 to 49.2 μg of monomeric anthocyanins/mL of medium to each well. Vehicle controls were the normal media with the corresponding pH adjusted using 2.5 *N* HCl solution of each treated group. At the 0.5-, 2-, and 4-hour time points, the medium was replaced with fresh medium, and the cells were cultured for a total of 48 or 72 hours. Cell growth was measured using the MTT assay as previously described.[30] Briefly, an MTT stock solution (5 mg/mL) was prepared by dissolving MTT in phosphate-buffered saline, pH 7.4. The stock solution was added at 1:10 (vol/vol) to the medium in each well, and plates were incubated in the dark at 37°C for 4 hours. Next, supernatant was removed, and 200 μL of 0.04 *N* HCl in isopropanol was added to each well. After being kept in the dark at room temperature for 1 hour, plates were read at 570 nm using an enzyme-linked immunosorbent assay plate reader. Cell viability was calculated as follows:

$$\% \text{ inhibition} = (Abs_{\text{Ctrl}} - Abs_{\text{t}})/Abs_{\text{Ctrl}} \times 100\% \quad (1)$$

where Abs_{t} is the absorbance of cells treated with HBE and Abs_{Ctrl} is the absorbance of the corresponding vehicle control.

Interleukin-12 (IL-12) release assay

Day 7 harvested bone marrow-derived DCs (BMDDCs) were plated in 200 μL of complete RPMI 1640 containing 20–25 ng/mL GM-CSF at 4×10^5 cells per well in 48-well tissue culture plates (Costar, Corning, NY) at 37°C in 7% CO_2 overnight. The medium was removed and replaced with fresh complete RPMI 1640. HBE was added in concentrations ranging from 5.1 to 37.3 μg of monomeric anthocyanins/mL of medium, and plates were incubated for 30 minutes. High-dose (10 μg/mL) or low-dose (0.1 μg/mL) lipid A from *Salmonella minnesota* R595 (Re) (List Biological Laboratories, Campbell, CA) was then added to each well with or without HBE treatment. After 24 hours, supernatant in each well was collected and stored at −80°C until IL-12 measurement. Total IL-12 concentration in supernatant was measured using a murine total IL-12 enzyme-linked immunosorbent assay kit (Pierce, Rockford, IL) according to the instructions from the manufacturer.

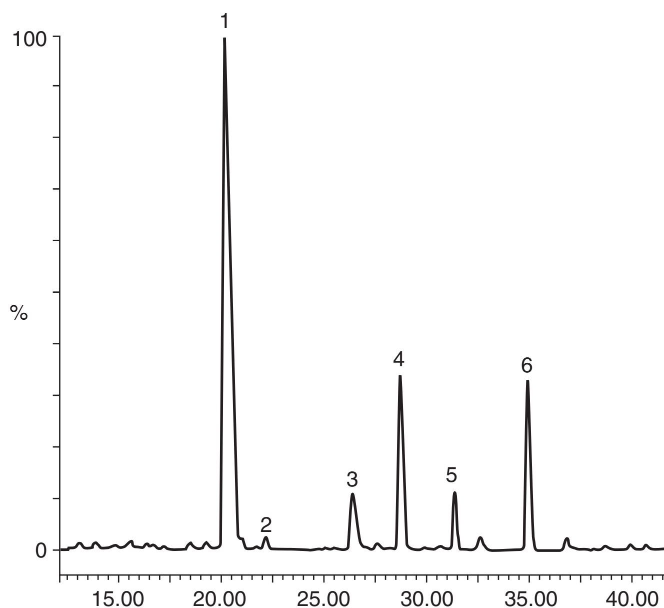

FIG. 1. HPLC chromatogram of anthocyanins in HBE identified by liquid chromatography-electrospray/MS as (peak 1) cyanidin-3-glucoside, (peak 2) cyanidin-3-arabinoside, (peak 3), delphinidin-3-xyloside (tentative), (peak 4) cyanidin-3-xyloside, (peak 5) cyanidin-3-malonylglucoside, and (peak 6) cyanidin-3-dioxalylglucoside.

Statistical analysis

All values of each assay other than cell culture studies were based on independent triplicate samples of HBE and calculated as the mean ±SD. Statistical analysis was performed using Student's *t* test. A value of $P \leq .05$ was considered significant.

RESULTS

Characterization of HBE

In these studies, a relatively simple extraction procedure with mildly acidified ethanol treatment was utilized to obtain a water-soluble HBE with a pH of 1.9. The average yield of DBE was 470.44 ± 9.27 mg/g of blackberry powder. Total anthocyanin content in the HBE was 6.80 ± 0.31 mg of cyanidin-3-glucoside equivalent/g of DBE. Total phenolic content in the HBE was found to be 17.32 ± 0.74 mg of gallic acid equivalent/g of DBE. The total polymeric color, which is the color contributed by polymerized material in HBE, was calculated to be 2.2 ± 1% (Table 1).

The TAC of HBE was determined using an improved TEAC assay,[29] which assessed the capacity of a compound or sample to scavenge $ABTS^{\bullet +}$ in terms of TE. The average TAC value of HBE was 66.98 ± 1.27 μmol of TE/g of DBE (Table 1).

The HPLC profile of HBE showed six major peaks as identified as 1–6 (Fig. 1). Peak identification was carried out based on the molecular weight and structural information obtained from their MS spectra, in addition to their retention times from HPLC-UV-visible spectra (Table 2). It is notable that other small peaks as shown in the chromatogram in Figure 1 were not identifiable by MS, and their identities remain unknown. Cyanidin-3-glucoside (peak 1) was the main component (71.0%) in HBE with the respective parent and daughter ion pairs (*m/z* 449/287). The other three major peaks (peak 4, 12.4%; peak 5, 3.5%; peak 6, 11.6%) had *m/z* values of 419/287, 535/287, and 593/287, which were identified as cyanidin-3-xyloside, cyanidin-3-malonylglucoside, and cyanidin-3-dioxalylglucoside, respectively, in accordance with data previously reported by Stintzing *et al.*[31] Cyanidin-3-arabinoside (peak 2) was also identified, and was in agreement of the initial report by Dugo *et al.*[32] using blackberry extracts. Another small peak (peak 3), with the respective parent and daughter ion pairs (*m/z* 435/303), was detected in the blackberry extract and is being reported for the first time. Based on its retention time, this compound has been tentatively identified as delphinidin-3-xyloside.

Effect of HBE on growth inhibition of colon tumor cells

In this study, HT-29 cells were exposed to HBE for 2 hours and then incubated with fresh media for 48 or 72 hours (Fig. 2). The growth of HT-29 cells was inhibited by 24–53% ($P < .001$) at concentrations ranging from 13.6 to 49.2 μg of monomeric anthocyanins/mL of medium at 48 hours. At the 72-hour time point, although the HT-29 cell growth inhibition was not significant ($P = .2$) at the con-

TABLE 2. PEAK ASSIGNMENTS, RETENTION TIME, AND MASS SPECTRAL DATA OF BLACKBERRY ANTHOCYANINS DETECTED BY HPLC AND ELECTROSPRAY IONIZATION-MS

Peak	*Retention time (minutes)*	*[M+]*	*Aglycon*	*Sugar moiety*	*Peak identification*
1	20.1	449/287	Cyanidin	Hexose	Cyanidin-3-glucoside
2	21.5	419/287	Cyanidin	Pentose	Cyanidin-3-arabinoside
3	26.4	435/303	Delphinidin	Pentose	Delphinidin-3-xyloside
4	28.7	419/287	Cyanidin	Pentose	Cyanidin-3-xyloside
5	31.3	535/287	Cyanidin	Malonyl-hexose	Cyanidin-3-malonylglucoside
6	34.9	593/287	Cyanidin	Dioxalyl-hexose	Cyanidin-3-dioxalylglucoside

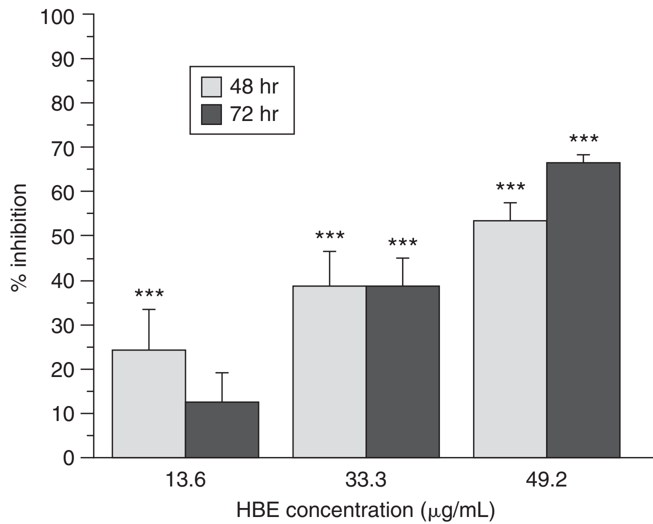

FIG. 2. Effect of HBE on growth of HT29 cells. HT29 cells were exposed to HBE for 2 hours, and cell viability was measured after 48 and 72 hours. Growth inhibition was observed in a concentration-dependent manner for both time points. Results are presented as mean ± SD values (n = 8–10) for each concentration. HBE concentration is expressed as μg of monomeric anthocyanins/mL of medium. ***$P \leq$ .001 compared to corresponding vehicle controls.

centration of 13.6 μg of monomeric anthocyanins/mL of medium, significant inhibition of 39% and 66% ($P < .001$) was observed at higher concentrations of 32.8 and 49.2 μg of monomeric anthocyanins/mL of medium, respectively. Interestingly, there were no significant differences in the growth inhibition rates and patterns at 48 and 72 hours after exposing the HT-29 cells to HBE in the dose range of 13.6 to 49.2 μg of monomeric anthocyanins/mL of medium for 0.5, 4 h, 24 h, and 48 hours (data not shown). These data indicate that the active components (including anthocyanins) that lead to inhibition by the HBE may be rapidly taken up by the cells. In addition, HBE in doses ranging from 0.66 to 2.53 μg of monomeric anthocyanins/mL of medium was added to the HT-29 cells and allowed to remain in the well for 48 hours. The results showed no significant differences in inhibition at 48 hours versus control cells, indicating that lower doses of extract in contact for cells for longer periods of time were not effective (data not shown).

In another experiment, instead of adding HBE directly to the cells, medium containing HBE was added to the HT-29 cells after incubation for 0.5 hours at 37°C. The results showed an inhibition rate at all concentrations similar to when HBE at a concentration of 13.6 μg of monomeric anthocyanins/mL of medium was added directly to cells. Since anthocyanins are known to be less stable at neutral pH than low pH, this result may suggest that there are other active components in the HBE. However, this aspect was beyond the scope of the present studies and remains an active area of investigation.

Effect of HBE on inhibition of lipid A-induced IL-12 release from DCs

To investigate whether HBE inhibits IL-12 release from DCs, DCs were exposed to HBE with or without lipid A induction of IL-12. As shown in Figure 3A, baseline release of IL-12 from nonstimulated DCs was low, with an IL-12 concentration of only 1.46 ng/mL. However, for all con-

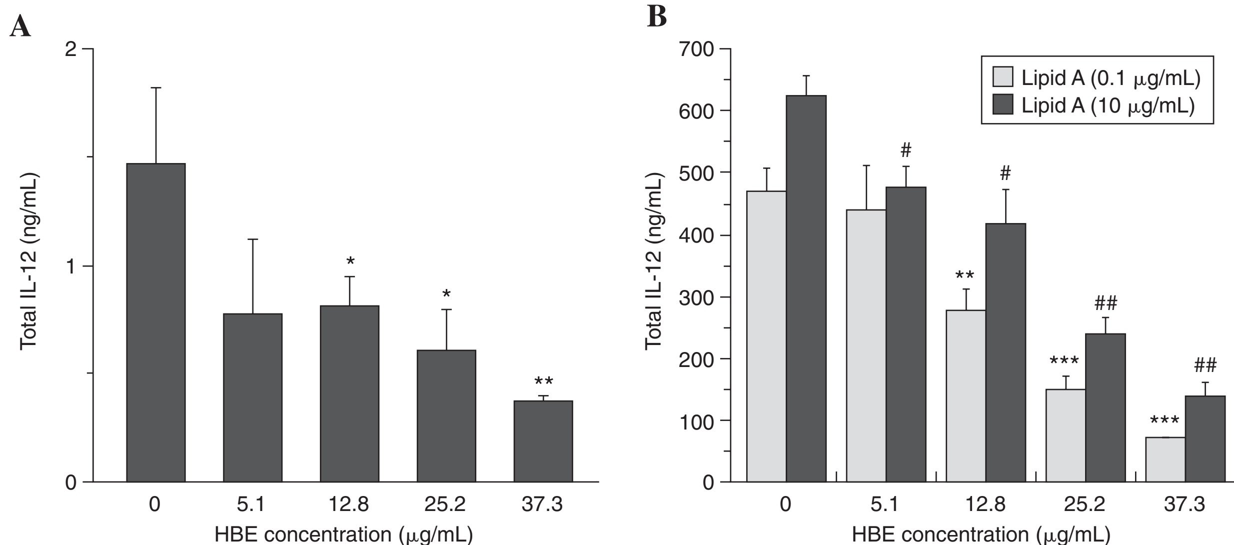

FIG. 3. Effect of HBE on lipid A-induced IL-12 release from murine DCs. DCs were exposed to HBE without (**A**) or with (**B**) lipid A for 24 hours. Inhibition of IL-12 release was observed in all cases. Results are presented as mean ± SD values (n = 3) for each concentration. HBE concentration is expressed as μg of monomeric anthocyanins/mL of medium. (A) *$P \leq .05$, **$P \leq .01$ compared to the vehicle control without HBE and lipid A treatment. (B) **$P \leq .01$, ***$P \leq .001$ compared to the vehicle control with low-dose lipid A treatment; $^{\#}P \leq .01$, $^{\#\#}P \leq .001$ compared to the vehicle control with high-dose lipid A treatment.

centrations of HBE added, the IL-12 release was reduced in a concentration-dependent manner with only 0.37 ng of IL-12/mL secreted using a concentration of 37.3 μg of monomeric anthocyanins/mL of medium. As expected, both high-dose (10 μg/mL) and low-dose (0.1 μg/mL) lipid A resulted in very high release of IL-12 from DCs of 624 ng/mL and 468 ng/mL, respectively (Fig. 3B). In the low-dose lipid A treatment group, the concentration of IL-12 in cell culture supernatant was decreased in a concentration-dependent manner from 468 to 72 ng/mL when HBE was added in the range from 5.1 to 37.3 μg of monomeric anthocyanins/mL. A similar pattern was observed in the high-dose lipid A treatment group, with the reduction of IL-12 release ranging from 474 to 138 ng/mL when HBE was added in the range from 5.1 to 37.3 μg of monomeric anthocyanins/mL. Thus, HBE significantly inhibited the release of IL-12 from murine BMDDCs with or without lipid A treatment. These results suggest that the pigmented, anthocyanin-containing extract from blackberries may have significant anti-inflammatory properties.

DISCUSSION

In this research, a water-soluble extract of Hull blackberry, rich in anthocyanins, showed significant antioxidant, antiproliferative, and anti-inflammatory properties. HPLC-MS results showed that the anthocyanins in HBE are mainly cyanidin-based. There are numerous reports on the growth inhibition of cancer cells *in vitro* by various anthocyanin-containing extracts or purified anthocyanin fractions from various kinds of fruits and vegetables.[12–14,33–36] Among them, Olsson *et al*.[37] showed an average inhibition of 53% on HT-29 cells at the highest concentration of strawberry extracts. Yi *et al*.[34] found that the 50% inhibitory concentration for blueberry extracts with HT-29 cells ranged from 1,000 to 3,000 μg/mL. Commercially prepared grape, bilberry, and chokeberry anthocyanin-rich extracts were investigated by Zhao *et al*.,[12] and it was demonstrated that all of the three extracts inhibited HT-29 cell growth, with chokeberry anthocyanin-rich extract being the most potent inhibitor. In addition, Parry *et al*.[38] reported similar results with black raspberry, cranberry, and chardonnay grape seed flour extracts on the antiproliferative effects on HT-29 cells. However, for all of these studies mentioned above, the HT-29 cells were treated with extracts for at least 24 hours. In addition, to our knowledge, the present study is the first to investigate the growth inhibition effect of an aqueous extract from blackberries on the proliferation of HT-29 cells. Moreover, in the present studies, cells were treated with a reduced contact time of 0.5–4 hours instead of the typical 24 hours. It was found that HBE inhibited the growth of HT-29 cells in a concentration-dependent manner. In addition, the inhibition rates of HT-29 cells versus anthocyanin concentration were comparable in these studies to extracts derived from other fruits. The studies by Malik *et al*.[15] using anthocyanin-rich berry extracts suggested that the increase in inhibition with exposure time (24, 48, and 72 hours) was mainly due to growth of control cells over time as there was little or no change in the cell number exposed to the berry extracts. However, these authors exposed the cells to fresh extract every 24 hours up to 72 hours and compared the cell number to that of control. In contrast, in the present studies, cells were exposed to berry extract for a fixed period of time (*i.e.*, 2 hours) and then removed and replaced with fresh medium without extract for additional incubation up to 48 and 72 hours. As a consequence, a trend of increasing inhibition over time was not observed in the present studies. For example, the percentage inhibitions of 38.6% and 38.99% were not statistically different at the 48- and 72-hour time point after a 2-hour exposure to a concentration of 32.8 μg of monomeric anthocyanins/mL of medium, respectively. There are a few studies that have been performed to elucidate the mechanism behind the chemoprevention effects of anthocyanins or anthocyanin-rich extracts on cancer cells. Hibiscus,[39] lingonberry,[40] and bilberry[17] anthocyanin-rich extracts were found to inhibit the growth of HL-60 (human leukemia cells) through the induction of apoptosis. Hou *et al*.[41] showed anthocyanins inhibit tumorigenesis induced by tetradecanoylphorbol 13-acetate on mouse JB6 ($^+$) cells by blocking activation of the mitogen-activated protein kinase pathway. Several groups have reported that anthocyanins could suppress the cyclooxygenase activity, which may also play a key role on carcinogenesis.[4,21,42] Hakimuddin *et al*.[43] found that the inhibition of MCF-7 cell proliferation by a flavonoid fraction from a red wine was related to its inhibition of calcium/calmodulin-promoted phosphodiesterase activity. The molecular mechanism by which the HBE inhibits the growth of various cancer cells is currently being investigated in our laboratories.

Previous studies by Pergola *et al.*[44] demonstrated that part of the anti-inflammatory activity of a specific blackberry extract was due to the suppression of nitric oxide production in J774 cells by cyanidin-3-*O*-glucoside. Rossi *et al*.[45] showed that the anthocyanin fraction from blackberry extract exerted multiple protective effects in carrageenan-induced pleurisy in rats. Nevertheless, most of the *in vitro* studies utilizing anthocyanin-containing extracts from other fruits or vegetables have focused on the effect of the extracts on nitric oxide synthesis and tumor necrosis factor-α levels *in vitro* using activated macrophages.[20,21,46] Other studies have assessed the effects of extracts on the inflammation induced by hydrogen peroxide and tumor necrosis factor-α in human microvascular endothelial cells.[24] To our knowledge, there have been few or no studies assessing the anti-inflammatory effects of anthocyanin-containing extracts on DCs. DCs are potent antigen-presenting cells and function as initiators and modulators of the immune response. Lipid A is known to induce maturation of DCs, resulting in synthesis of high levels of pro-inflammatory IL-12 that enhances both innate (natural killer cell) and acquired (B and T cells) immunity. Our results showed that HBE inhibited IL-12 release from DCs in both background and stimulated levels, which further demonstrated the anti-in-

flammatory properties of the anthocyanin-rich extract of blackberry.

It has been shown that the bioavailability of anthocyanins by oral consumption is very low. For example, as reported by Milbury *et al.*,[47] the average maximum plasma concentration of anthocyanins in the blood of humans was about 43.75 ng/mL after the consumption of 720 mg of anthocyanins. These concentrations are about 1,000 times less concentrated that those used in these studies (*i.e.*, 13.6–49.2 μg/mL). However, to achieve the therapeutic levels of anthocyanins used in these present *in vitro* studies, our laboratories are developing formulations containing HBE to better target anthocyanins to tumors. For example, an oral capsule containing HBE may be targeted to the colon to release a high local concentration of anthocyanins at a tumor or pre-tumor site.

In conclusion, the present studies demonstrated that HBE has significant antioxidant, antiproliferative, and anti-inflammatory actions that may be exploited for the treatment and/or prevention of colon cancer and/or inflammatory diseases.

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