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ARTICLE

Antioxidant and Anti-inflammatory Activities of Polyphenolics from Southeastern U.S. Range Blackberry Cultivars

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The antioxidant and topical anti-inflammatory activities of low and high molecular weight phenolic fractions (LMPF and HMPF, respectively) isolated from three blackberry cultivars (i.e., Navaho, Kiowa, and Ouachita), bred to tolerate the warm and humid climatic conditions of the southeastern United States, were investigated by the in vitro ferric reducing antioxidant power (FRAP) assay and an in vivo mouse ear edema model. Seventy percent (v/v) acidified acetone was employed to extract phenolics from the Georgia-grown blackberry cultivars, which were subsequently cleaned up on an Amberlite XAD-16 column and then further fractionated with Sephadex LH-20 to LMPF and HMPF. The anti-inflammatory response from topical application of solutions of the LMPF and HMPF as well as indomethacin, a potent nonsteroidal anti-inflammatory drug, was assessed in the TPA mouse ear model. All treatments significantly ($P < 0.05$) reduced TPA-induced irritation injury. Furthermore, mouse ear myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte infiltration, was assessed and found to be significantly ($P < 0.05$) reduced after topical application of indomethacin and all blackberry preparations. Correlation coefficients of 0.925 and 0.923 ($P < 0.01$) were determined when the anti-inflammatory activities of the blackberry fractions were compared to their total phenolics contents and antioxidant activities (i.e., FRAP values), respectively.

KEYWORDS: Blackberries; polyphenolics; anti-inflammatory activity; antioxidant activity; edema; myeloperoxidase activity; polymorphonuclear leukocyte infiltration

INTRODUCTION

Epidemiological studies have shown that the consumption of fruits and vegetables confers health benefits to man and reduces the risk of developing chronic and neurodegenerative diseases such as cardiovascular disease, cancer, and Alzheimer's disease (*1*, *2*). In vitro and in vivo studies suggest that the antioxidant, anti-inflammatory, and anticarcinogenic properties of fruits and vegetables are attributed, at least partly, to their polyphenolic constituents such as flavonoids (*3*). Although the exact mechanisms by which polyphenolics affect health are currently being elucidated, research to date continues to support daily consumption of fruits and vegetables. Berries are among 50 products that are ranked highest in polyphenolic antioxidant levels (*4*) and, therefore, highly recommended for the human diet.

Our understanding of the biochemical role of antioxidants in the body from both endogenous and exogenous sources at retarding inflammation is still in its infancy. In vitro studies have shown anti-inflammatory activities arising from polyphenolics. For instance, a muscadine skin extract rich in polyphenols inhibited the release of both the superoxide anion radical from phorbol myristate acetate-activated neutrophils and cytokines [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β)] from lipopolysaccharide (LPS)-activated peripheral blood mononuclear cells (*5*). Anthocyanidins contributed to the inhibition of tumorigenesis by blocking the activation of the mitogen-activated protein kinase (MAPK) pathway in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor mouse JB6 cells (*6*). Additionally, anthocyanin-rich berry extracts showed considerable inhibitory effects on NO production in LPS/interferon-γ (IFN-γ) activated RAW 264.7 macrophages (*7*): NO is known to be an important mediator of acute and chronic inflammation. Quercetin, a flavonol found in berries, suppresses TNF-α-induced expression of interleukin-8 (IL-8) and the monocyte chemoattractant protein (MCP-1) due to its capacity to inhibit the activation of nuclear factor-κB (NF-κB). NF-κB is a protein complex that controls the transcription of DNA and is a cell-signaling molecule for inflammation. It can regulate genes [e.g., the cyclooxygenase-2 (COX-2)-encoding gene] inducing the expression of proinflammatory prostaglandins, chemokines

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(i.e., chemotactic cytokines that attract inflammatory cells to the sites of inflammation), enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules, which play a key role in the initial recruitment of neutrophils to the sites of inflammation. The flavonoids found in berries are capable of reducing redox activation of NF-κB. Therefore, diets rich in phenolic antioxidants from small fruits (e.g., blackberries) may assist in keeping NF-κB at bay.

In vivo studies using animals have shown that the consumption of polyphenols can alter the progression of experimental disease states. Antitumor activities have been reported for resveratrol, a stilbene found in grape skins (*8*), and (−)-epigallocatechin-3-gallate, from green tea (*9*). Investigations have demonstrated that the anthocyanins from tart cherries have the ability to suppress inflammation-induced pain indices in rats (*10*). Greenspan et al. (*5*) reported that rats fed a diet of 5% (w/w) muscadine grape skins exhibited anti-inflammatory activity with a 50% reduction in hind paw edema in carrageenan-treated animals. Topical application of anthocyanins and a hydrolyzable tannin-rich pomegranate fruit extract on mouse skin significantly inhibited phosphorylation by MAPKs, activation of NF-κB, and activity of COXs, the latter being important enzymes involved in mediating the inflammatory process (*11*).

Neutrophil granulocytes or polymorphonuclear (PMN) leukocytes are the first inflammatory cell type to migrate to the site of inflammation due to chemical attractant signals such as IL-8 and INF-γ (a process known as chemotaxis). Myeloperoxidase (MPO) is a heme-containing peroxidase of PMN leukocytes that functions in body-defense mechanisms against a broad range of organisms (e.g., a bacterial infection). Activation of PMN leukocytes by inflammatory stimuli results in the release of lysosomal enzymes and MPO. MPO catalyzes H_2O_2 to produce HOCl, a strong oxidant generated in the presence of chloride ions. Hypochlorous acid, an antimicrobial oxidant, is a harmful agent that participates in an increasing number of inflammatory-mediated disorders (*12*). For example, Baldus et al. (*13*) demonstrated that there is a significant correlation between serum MPO levels and cardiovascular diseases.

Activation of PMN leukocytes by inflammatory stimuli causes the release of MPO and generates oxidants such as HOCl, which plays a significant role in the pathophysiology of vascular and other inflammatory diseases. Kato et al. (*14*) reported that commercially available phenolic antioxidants such as epigallocatechin gallate, epicatechin gallate, and curcumin function as strong inhibitors of MPO activity. A significant reduction in MPO activity by a muscadine extract of skins and seeds was reported in the TPA-induced inflammation mouse ear model (*15*). Limited knowledge is available on the effectiveness of blackberries at curbing inflammation. Duthie (*16*) reported that black raspberry extracts altered the expression of genes associated with inflammation and carcinogenesis. The author noted that COX-2 gene expression and subsequent prostaglandin production as well as NO synthase activity were inhibited in premalignant rat esophageal cells following feeding with the berry extract.

Blackberries (*Rubus* spp.) are an important small fruit crop in the United States and are commercially produced over a wide geographic range. The blackberry is a rich source of anthocyanins and other polyphenolic antioxidants including ellagic acid and its derivatives. In particular, cyanidin-based compounds have been found to be the major anthocyanins in blackberries (*17*). With regard to the tannin composition, blackberries predominantly contain hydrolyzable tannins, that is, ellagitannins and gallotannins (*18*). The fruit, therefore, possesses a considerable potential for extraction of biologically active compounds that could, when consumed at appropriate levels, potentially alleviate or attenuate symptoms of inflammatory diseases.

The aim of this study was to investigate the antioxidant and topical anti-inflammatory capacities of blackberry phenolics from three cultivars (i.e., Navaho, Kiowa, and Ouachita) bred for the hot, humid climatic conditions of the southeastern U.S. using the in vitro ferric reducing antioxidant power (FRAP) assay and an in vivo mouse ear edema model, respectively.

MATERIALS AND METHODS

Chemicals. Phenolic compounds including (+)-catechin, (−)-epicatechin, myricetin, quercetin, and a series of phenolic acids comprising gallic, ellagic, vanillic, caffeic, *p*-coumaric, *trans*-cinnamic, protocatechuic, syringic, chlorogenic, and *p*-hydroxybenzoic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A pure standard of kuromanin chloride (i.e., cyanidin-3-*O*-glucoside chloride) was acquired from Indofine Chemical Co. (Hillsborough, NJ). Chemicals for analytical and anti-inflammatory assays included 12-*O*-tetradecanoylphorbol-13-acetate (TPA), hexadecyltrimethylammonium bromide, indomethacin, 3,3′,5,5′-tetramethylbenzidine dihydrochloride, *N*,*N*-dimethylformamide, hydrogen peroxide solution (30% w/v), Folin−Ciocalteu's phenol reagent, sodium carbonate, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulfate heptahydrate, sodium acetate, acetic acid, triethanolamine, sodium dodecyl sulfate (SDS), monobasic potassium phosphate, and dibasic potassium phosphate; all were obtained from Sigma-Aldrich. The HPLC-grade solvents acetonitrile, water, and methanol were purchased from Fisher Scientific Co. (Suwanee, GA) as were ACS-grade acetone, 95% ethanol (v/v), glacial acetic acid, and hydrochloric acid.

Collection of Samples. Mature blackberries (*Rubus* spp.) were harvested from Jacob W. Paulk Farms, Inc. (Wray, GA), industrial operation in May 2006 and 2007. The three blackberry cultivars collected were Navaho, an erect thornless variety; Kiowa, an erect thorny variety; and Ouachita, a very erect thornless variety. All three cultivars, which grow particularly well in the hot, humid conditions of the southeastern United States, were patented by and released from the University of Arkansas. These cultivars are different from those in the dominant blackberry production areas of the northwestern United States. Hand-picked blackberries were transported to the Department of Food Science and Technology, The University of Georgia (UGA), in Athens, GA. Representative samples from each cultivar were lyophilized using a UNITOP 600 L VirTis freeze-dryer (The VirTis Co., Inc., Gardiner, NY), transferred to polyethylene pouches, and then stored at −40 °C until analyzed.

Preparation of Crude Blackberry Extracts (CBEs). Freeze-dried blackberry samples (i.e., containing fruit receptacles, skins, and seeds) from each cultivar were ground in a commercial coffee mill (KitchenAid Portable Appliances, St. Joseph, MI). Fifteen grams of blackberry powder was mixed with 150 mL of 70% (v/v) acidified acetone [containing 0.1% (v/v) HCl] and blended using a PT-3100 Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at 15000 rpm for 10 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). The extraction process was repeated two times as described above. All filtrates were pooled, and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corp., New Castle, DE) at 40 °C. Sample extractions of each cultivar were performed in triplicate.

Preparation of Polyphenolic Extracts (PPEs). Ten milliliters of each CBE (containing 13% solids) was applied to the top of a chromatographic column (30 mm i.d. × 340 mm e.l., Kontes, Vineland, NJ) packed with Amberlite XAD-16 [(bead size = 20−60 mesh), Sigma-Aldrich] and washed with ~300 mL of deionized water to remove sugars and organic acids. After the first 100 mL, the pH of the eluent was checked with pH paper test strips every 20 mL until a neutral pH was reached. The PPE was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor at 40 °C. The PPE was lyophilized using a FreeZone 2.5 L benchtop freeze-dryer (Labconco Corp., Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles in a 4 °C refrigerator.

Preparation of Low and High Molecular Weight Phenolic Fractions (LMPF and HMPF, Respectively). For each cultivar, 200 mg of

lyophilized PPE was dissolved in 10 mL of 95% (v/v) ethanol, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30 mm i.d. × 360 mm e.l., Kontes) packed with Sephadex LH-20 [(bead size = 25−100 μm), Sigma-Aldrich]. Five hundred milliliters of ethanol ([95% (v/v)] was used as the mobile phase to elute the LMPF. The system was changed over to 50% (v/v) acetone, and ∼300 mL was required to elute the HMPF from the Sephadex LH-20 column. The bovine serum albumin (BSA) assay (a detailed procedure for this assay is described below) was performed to check qualitatively for the presence of hydrolyzable and condensed tannins. For the LMPF and HMPF, ethanol and acetone were evaporated with the Büchi rotavapor at 40 °C, respectively, and each residue was lyophilized. Freeze-dried fractions were stored in amber-glass bottles at 4 °C until analyzed.

HPLC Analysis. An Agilent 1200 series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV−vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was employed for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) was used. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 10 min using 100% A, giving a total run time of 60 min. The injection volume of each fraction (0.5 mg/mL methanol) was 20 μL. Detection wavelengths were 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids), 360 nm (flavonols), and 520 nm (anthocyanins). Tentative identification of separated components was made by matching UV−vis spectra and retention time mapping with authentic standards.

Bovine Serum Albumin Precipitation Assay. The effect of blackberry tannins resulting in the formation of an insoluble tannin−protein complex was measured qualitatively by the BSA precipitation assay of Hagerman and Butler (*19*). Briefly, 1 mL of a methanolic solution (1.0 mg/mL) of each fraction (i.e., LMPF and HMPF) was pipetted into a 15 mL Falcon centrifuge tube. To each was added 2.0 mL of a standard protein solution [i.e., 1.0 mg of BSA/mL of 0.2 M acetate buffer (pH 5.0, containing 0.17 M NaCl)]. The solutions were vortexed, allowed to stand at room temperature for ∼15 min, and then centrifuged using a Centrific centrifuge (model 1228, Fisher Scientific, Pittsburgh, PA) for ∼15 min at 5000*g*. The supernate was discarded. The surface of the residual pellet and the walls of the tube were carefully rinsed with 1 mL of 0.2 M acetate buffer (pH 4.0) without disturbing the pellet. The pellet was then dissolved in 4 mL of SDS−triethanolamine solution [1% (w/v) SDS and 5% (v/v) triethanolamine in deionized water]. One milliliter of $FeCl_3$ reagent (0.01 M $FeCl_3 \cdot 6H_2O$ in 0.01 N HCl) was added, and the solution was vortexed. Approximately 20 min after the addition of the iron(III) reagent, sample absorbance readings were measured at λ = 510 nm with an Agilent 8453 photodiode array spectrophotometer (Agilent Technologies, Wilmington, DE). The average A_{510} of triplicate samples of the SDS−triethanolamine solution plus $FeCl_3$ reagent was subtracted from the A_{510} of each sample to correct for background absorbance.

Total Phenolics Content (TPC) Assay. The TPCs of the LMPF and HMPF were determined colorimetrically by the classical Folin−Ciocalteu assay (*20*). Briefly, 0.5 mL of a methanolic solution of each fraction (5 μg/mL) was pipetted into a test tube followed by the addition of 8.0 mL of deionized water, 0.5 mL of 2 N Folin−Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na_2CO_3 solution. The contents were vortexed for 15 s followed by a 60 min resting period at room temperature to allow for optimal color development. Absorbance readings of samples were taken at λ = 750 nm with the Agilent spectrophotometer. Quantification was based on a standard curve generated with gallic acid. Sample TPCs were expressed as milligrams of gallic acid equivalents (GAE) per 100 mg of the respective fraction.

Ferric Reducing Antioxidant Power (FRAP) Assay. The antioxidant capacity of each fraction was determined according to the method of Benzie and Strain (*21*). Briefly, the FRAP reagent was prepared fresh each day by adding 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of acetate buffer (300 mM, pH 3.6). The reagent was kept warm at 37 °C in a Precision model 182 water bath (Precision Scientific Inc., Chicago, IL) until further use. Three hundred microliters of the FRAP reagent and 10 μL of each sample solution [i.e., 50 μg/mL of 50% (v/v) ethanol] were pipetted into a borosilicate glass cuvette and mixed well by pumping the mixture through the pipet tip. After a 6 min quiescent period, 340 μL of deionized water was added. Absorbance readings were measured at λ = 593 nm using a UV−vis spectrophotometer (Beckman DU-650, Beckman Instruments, Inc., Fullerton, CA). Aqueous solutions of known Fe(II) concentrations in the range of 0.15−1.5 mM were employed to construct a calibration curve. FRAP values were calculated from a standard curve of $FeSO_4 \cdot 7H_2O$ and expressed as millimoles of Fe(II) equivalents per 100 mg of the respective fraction.

Animal Model Studies. *Animals.* All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UGA and conducted according to IACUC guidelines. The sample size (n) of eight animals for each test group was justified on the basis of preliminary experiments showing that sample standard deviation for determinations of ear edema was ∼5% of the measured value and the average expected difference (d) between TPA-treated ears and PPE-treated ears was about 0.2 mm. Assuming that $\alpha = 0.05$ and $1 - \beta = 0.9$, the formula employed was $n = 1 + 21 \times (s/d)^2$ (*22*). The formula gave 6.25, which was increased to eight in case of unexpected experimental problems.

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN), weighing between 22 and 25 g, were housed in groups of four in large shoebox cages. All groups were fed a standard rodent diet (TestDiet 570B, Purina Mills, St. Louis, MO) ad libitum with free access to water. Throughout the experiment, animals were in a fed condition. Photoperiods equaled 12 h of daylight and 12 h of darkness daily, with the environmental temperature maintained at 21 °C. Outside preliminary trials, three separate experiments with animals were performed over the course of this study.

12-O-Tetradecanoylphorbol-13-acetate-Induced Mouse Ear Edema. The mice were divided into 9 groups with each group containing 8 animals. Group 1 was designated the control in which 10 μL of acetone was applied to both the inner and outer ear surfaces. Thirty minutes after application of the acetone, 10 μL of ethanol (50%, v/v) was applied to each side of each ear without producing any inflammation; this treatment was a control to test for a possible effect arising from ethanol in blackberry sample extracts. For groups 2−9, edema was induced by topical application of 10 μL of an acetonic TPA solution (0.1 mg/mL) to each side of each ear. Group 2 was the vehicle control group, also called the TPA-positive control. Thirty minutes after the TPA application, 10 μL of 50% (v/v) ethanol was applied to each side of each ear without any anti-inflammatory treatment. Group 3 was the treatment-positive control group; that is, 30 min after the TPA application, 10 μL of a nonsteroidal anti-inflammatory drug (indomethacin, 25 mg/mL acetone) was applied to each side of each ear; this quantity represents a potent dose of indomethacin. For treatment groups 4−9, 10 μL of LMPF or HMPF solutions [i.e., 50 mg from Kiowa, Navaho, or Ouachita cultivars/mL of 50% (v/v) ethanol] were applied to each side of each ear, respectively, 30 min after the application of TPA. A summary of the experimental design is given in **Table 1**.

Edema was expressed as the increase in ear thickness resulting from inflammation. Ear thickness was measured using a micrometer (Mitutoyo series IP65, Mitutoyo America, Aurora, IL) at 0, 4, and 24 h after the TPA application. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges, and the thickness was recorded in millimeters. At 24 h, each animal was sacrificed with CO_2 inhalation according to the IACUC-approved protocol. Ear punch biopsies (i.e., 6 mm diameter hole punches) were taken immediately, weighed, and stored in an ultralow-temperature freezer at −80 °C. A single investigator performed all ear measurements and biopsies to standardize the procedure and reduce experimental error. The percent inhibition of edema was calculated using the following formula:

$$\begin{aligned} \%\ \text{inhibition} \\ = [1 - &(\text{difference in thickness (mm) of inflamed treated ear} \\ &- \text{difference in thickness (mm) of control ear}) \\ &/(\text{difference in thickness (mm) of inflamed ear} \\ &- \text{difference in thickness (mm) of control ear})] \times 100 \end{aligned}$$

Results represent the mean ± standard deviation of three separate experiments.

Table 1. Experimental Design for the TPA-Induced Mouse Ear Edema Study[a]

animal group	treatment	topical application of solution on both the inner and outer mouse ear surfaces at	
		t = 0 min	t = 30 min
1	control group	acetone	ethanol (50%, v/v)
2	TPA-positive control group	TPA solution	ethanol (50%, v/v)
3	treatment-control group	TPA solution	indomethacin solution[b]
4	treatment group	TPA solution	N/LMPF solution[c]
5	treatment group	TPA solution	N/HMPF solution[d]
6	treatment group	TPA solution	K/LMPF solution
7	treatment group	TPA solution	K/HMPF solution
8	treatment group	TPA solution	O/LMPF solution
9	treatment group	TPA solution	O/HMPF solution

[a] Each group comprised eight mice. Ten microliters of a 0.1 mg/mL TPA solution was applied to both the inner and outer ears of mice in groups 2−9. Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; *t*, time; N/LMPF and N/HMPF, Navaho low molecular weight phenolic fraction and high molecular weight phenolic fraction, respectively; K/LMPF and K/HMPF, Kiowa low molecular weight phenolic fraction and high molecular weight phenolic fraction, respectively; O/LMPF and O/HMPF, Ouachita low molecular weight phenolic fraction and high molecular weight phenolic fraction. [b] Ten microliters of a 25 mg/mL indomethacin solution in acetone was applied to both the inner and outer ears of mice. [c] Ten microliters of 50 mg/mL LMPF in ethanol (50%, v/v) from each cultivar was applied to both the inner and outer ears of mice. [d] Ten microliters of 50 mg/mL HMPF in ethanol (50%, v/v) from each cultivar was applied to both the inner and outer ears of mice.

Myeloperoxidase (*MPO*) *Assay*. Tissue MPO (MPO, EC 1.11.1.7) activity was measured in biopsies taken from both ears 24 h after TPA administration using the method of Suzuki et al. (*23*) as modified by De Young et al. (*24*). This assay is based on the oxidation of 3,3′,5,5′-tetramethylbenzidine·dihydrochloride (TMB) in the presence of H_2O_2 catalyzed by the MPO enzyme. When oxidized, TMB produces a chromophore giving a blue color. Because the extent of TMB oxidation is dependent on the MPO concentration, the formation of oxidized product is related to MPO activity via an increase in absorbance. Each mouse ear biopsy (∼8−18 mg) was placed in a 5 mL test tube to which 0.75 mL of a HTAB solution [80 mM phosphate-buffered saline (PBS), pH 5.4, containing 0.5% (v/v) hexadecyltrimethylammonium bromide (HTAB)] was added. Each sample was homogenized for 45 s at 4 °C using a tissue homogenizer (Tekmar Tissumizer, model SDT-1810, Tekmar Co., Cincinnati, OH). The homogenate was transferred quantitatively to a microcentrifuge tube with rinsing using an additional 0.75 mL of the HTAB solution. The 1.5 mL sample was centrifuged in an Eppendorf model 5417R refrigerated microcentrifuge (Eppendorf AG, Hamburg, Germany) at 12000*g* for 15 min, maintained at 4 °C. Triplicate 30 μL aliquots of the resulting supernate were added to a COSTAR 96-well microtiter plate (Corning Inc., Corning, NY). For the MPO assay, 200 μL of a mixture containing 100 μL of 80 mM PBS (pH 5.4), 85 μL of 0.22 M PBS (pH 5.4), and 15 μL of 0.017% (v/v) H_2O_2 was added to each well. To initiate the reaction, 20 μL of 18.4 mM TMB in 8% (v/v) aqueous *N*,*N*-dimethylformamide was added. Microtiter plates were incubated in an Isotemp Plus incubator (Fisher Scientific) at 37 °C for 3 min, after which they were immediately placed on ice. The reaction was then stopped by the addition of 30 μL of 1.46 M sodium acetate, pH 3.0. MPO activity was measured colorimetrically using an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) at λ = 630 nm by top scanning. The percent inhibition of MPO activity was determined from optical density (OD) readings at 630 nm/biopsy; the mean ± standard deviation is reported from three separate experiments.

$$\begin{aligned}\%\ \text{inhibition} = [1 - (&\text{Abs at 630 nm/biopsy inflamed treated ear} \\ &- \text{Abs at 630 nm/biopsy control ear}) \\ &/(\text{Abs at 630 nm/biopsy inflamed ear} \\ &- \text{Abs at 630 nm/biopsy control ear})] \times 100\end{aligned}$$

Statistical Analysis. System software (SAS, version 9.1) was used for statistical analysis of data. All observations were expressed as the mean ± standard deviation (n = 8). Statistical evaluations used *t* tests and one-way analysis of variance (ANOVA) with post hoc tests for significance of differences by Duncan's multiple-comparison test. Statistical significance was considered at $P < 0.05$. The SAS software was also used to determine correlation coefficients between different TPCs and percent inhibition of edema or MPO activity.

Table 2. Relative Yield (Percent, w/w) of CBE, PPE, LMPF, and HMPF from Lyophilized Georgia-Grown Blackberry Cultivars (Navaho, Kiowa, and Ouachita)[a]

blackberry cultivar	CBE	PPE	LMPF	HMPF
Navaho	1.85 ± 0.18	1.27 ± 0.03	0.75 ± 0.06	0.38 ± 0.01
Kiowa	1.25 ± 0.09	0.80 ± 0.11	0.44 ± 0.07	0.24 ± 0.04
Ouachita	1.36 ± 0.26	0.90 ± 0.30	0.52 ± 0.14	0.25 ± 0.10

[a] Values are means of triplicate determinations ± standard deviation. Abbreviations: CBE, crude blackberry extract; PPE, polyphenolic extract; LMPF, low molecular weight phenolic fraction; HMPF, high molecular weight phenolic fraction.

RESULTS AND DISCUSSION

Fractionation of Blackberry Phenolics. The CBE was obtained using a two-stage extraction with 70% (v/v) acidified acetone [containing 0.1% (v/v) HCl]. Seventy percent acetone is known to assist with the extraction of the important antioxidant tannin constituents. The CBE was partially purified using Amberlite XAD-16 particles to remove sugars and organic acids. Although some low molecular weight polar phenolics might have been removed in the aqueous washing step, the bulk of the polyphenolic constituents was retained on the column until eluted with anhydrous methanol; the resultant product was a crude PPE. The PPE for each cultivar was further fractionated into a LMPF and a HMPF via Sephadex LH-20 column chromatography, as has been described under Materials and Methods. The extraction and chromatography relative yields for CBE, PPE, LMPF, and HMPF are listed in **Table 2**.

Partial Characterization of Blackberry Polyphenolics. Phenolic compounds were quantified using response factors of the representative standard near their characteristic wavelength of maximum absorption (i.e., hydroxybenzoic acids and flavan-3-ol at 280 nm; hydroxycinnamic acids at 320 nm; flavonol and flavonol glycosides at 360 nm; and anthocyanins at 520 nm). The LMPF contained a dominant peak eluting at a retention time of 11.4 min. The DAD spectrum for this peak was identical to that of the cyanidin-3-*O*-glucoside standard, and the compound was later confirmed by ESI-TOF-MS (details not given in this paper). Our results are in agreement with other studies indicating that cyanidin-3-*O*-glucoside is the primary anthocyanin in blackberry (*17*, *25*). Its content in the cultivars ranged from 229 ± 0.1 to 309 ± 0.2 μg/mg LMPF. This is significant, as cyanidin-3-*O*-glucoside has been reported to have the highest antioxidant capacity of 14 different anthocyanins tested (*26*). The content of cyanidin-3-*O*-glucoside in the LMPF was present in the following order: Kiowa > Navaho > Ouachita. Gallic acid and *p*-coumaric acid were also detected in the LMPF of the Navaho cultivar and tentatively identified by comparison of their UV spectra and retention times with those of commercially available standards. Traces of flavan-3-ols [i.e., (+)-catechin and (−)-epicatechin, the monomers of proanthocyanidins (PACs)] and flavonols (i.e., quercetin) were also found in the LMPF. The HMPF for each cultivar was characterized by the presence of ellagic acid, its derivatives, and ellagitannins. The total amount of ellagic acid and derivatives ranged from 164 ± 1.5 to 552 ± 0.4 μg/mg HMPF. The greatest content of these was found in the Navaho cultivar followed by Kiowa and then Ouachita. A minor peak was also present in the chromatograms that could not be identified on the basis of spectral characteristics of the available standards.

The BSA precipitation assay was employed as a qualitative tool to detect/confirm the presence of tannins in each fraction,

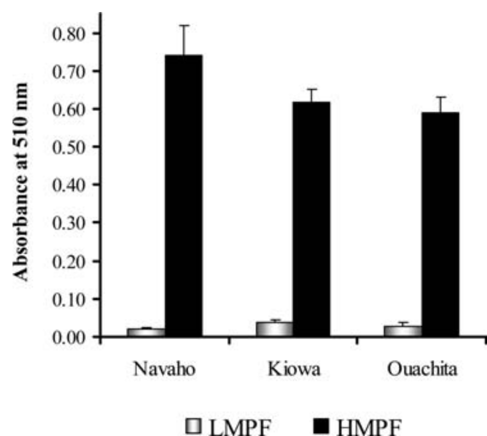

Figure 1. Relative tannin contents in the LMPF and HMPF of three blackberry cultivars. Abbreviations: N/LMPF and N/HMPF, Navaho low and high molecular weight phenolic fractions, respectively; K/LMPF and K/HMPF, Kiowa low and high molecular weight phenolic fractions, respectively; O/LMPF and O/HMPF, Ouachita low and high molecular weight phenolic fractions, respectively. Results are triplicate determinations ± standard deviation.

Table 3. Total Phenolics Content and Antioxidant Activity of the LMPF and HMPF from Georgia-Grown Blackberry Cultivars (Navaho, Kiowa, and Ouachita)[a]

blackberry cultivar	TPC (mg of GAE/100 mg of respective fraction)	FRAP (mmol of Fe^{2+} equiv/100 mg of respective fraction)
Navaho (LMPF)	72.6 ± 2.1	2.34 ± 0.15
Navaho (HMPF)	89.9 ± 1.9	3.92 ± 0.24
Kiowa (LMPF)	77.5 ± 2.1	2.70 ± 0.03
Kiowa (HMPF)	83.8 ± 1.0	3.45 ± 0.42
Ouachita (LMPF)	68.0 ± 1.9	2.03 ± 0.02
Ouachita (HMPF)	76.9 ± 1.2	2.21 ± 0.03

[a] Values are means of triplicate determinations ± standard deviation. Abbreviations: TPC, total phenolics content; GAE, gallic acid equivalents; FRAP, ferric reducing antioxidant power; LMPF, low molecular weight phenolic fraction; HMPF, high molecular weight phenolic fraction.

as this assay is applicable for both hydrolyzable and condensed tannins (*19*). The precipitation assay is based on the capacity of tannins to interact with protein in a manner that results in precipitation. It is important to note, however, that not all tannin−protein complexes will precipitate from solution. Absorbance readings at $\lambda = 510$ nm of the tannin−protein precipitate, dissolved in SDS−ethanolamine to which the ferric chloride reagent was added, for the LMPF and HMPF of all three cultivars are given in **Figure 1**. As expected, the high optical densities for the HMPFs indicated the presence of tannins, whereas the LMPFs were low in both hydrolyzable and condensed tannins.

TPCs and Antioxidant Activities. **Table 3** gives the TPCs of each fraction from the Navaho, Kiowa, and Ouachita cultivars. The results are reported as milligrams of GAE per 100 mg of respective fraction as opposed to (+)-catechin or (−)-epicatechin equivalents, because blackberries contain more hydrolyzable than condensed tannins (*27*); the TPCs ranged from 68.0 ± 1.9 to 89.9 ± 1.9 mg of GAE/100 mg of respective fraction. Of the three cultivars investigated, the Navaho HMPF possessed the highest TPC, but it was not significantly ($P > 0.05$) different from that of its Kiowa counterpart. It is somewhat difficult to compare our results with those reported in the literature, because most researchers have not removed sugars, organic acids, and water-soluble compounds (such as vitamin C), which can interfere with the reaction involving Folin−Ciocalteu's phenol reagent (*28*). When factoring in moisture contents as well as extraction and chromatography yields from the various mass balances, the blackberries for the three cultivars investigated in this study contain ~545 mg of GAE/100 g of fresh berries: our results are ~2 times those reported by Kao et al. (*29*) for the Navaho variety grown in Alabama (232.6 mg of GAE/100 g of fresh berries), and are comparable to the TPC reported by Sellappan et al. (*30*) (417.8 mg/100 g of fresh berries) in Kiowa. Variations may be due to differences in extraction methodologies employed for the phenolics or the presence of nonphenolic compounds such as ascorbic acid, monosaccharides/disaccharides, and organic acids, as well as differences in blackberry cultivar, stage of maturity, and environmental factors, such as light, temperature, agronomic practices, and geographical area.

Antioxidant activity was measured for the LMPF and HMPF from the blackberry cultivars by the classical FRAP assay. FRAP values ranged from 2.03 ± 0.02 to 3.92 ± 0.24 mmol of Fe^{2+} equiv/100 mg of respective fraction. The Navaho HMPF showed the highest FRAP value as compared to HMPFs from Ouachita and Kiowa. Pellegrini et al. (*31*) reported that berries, as a general category (i.e., black currant, strawberry, raspberry, and blueberry), possessed marked antioxidant capacity and that the blackberry was most effective at 5.15 mmol of Fe^{2+} equiv/100 g of fresh weight).

Anti-inflammatory Activity of Blackberry Polyphenolics. TPA can act as an inducer of epidermal hyperplasia, a tumor promoter, and an activator of various biological systems. TPA-induced ear edema is an in vivo model of acute inflammation; it offers a simple and useful assay for screening the efficacy of topical anti-inflammatory capacities of plant extracts, in this case blackberry preparations. The exact mechanism by which topical application of the phorbol ester induces an inflammatory response is not completely understood; however, the activation of protein kinase C with subsequent cytosolic phospholipase A_2 stimulation, arachidonic acid (AA) mobilization, and biosynthesis of prostaglandins and leukotrienes is involved (*32*). TPA applied to the mouse ear initiates a cascade of events leading to inflammatory processes such as increased vascular permeability, edema, and mast cell infiltration. One of the early hallmarks of skin irritation and local inflammation is the thickening/swelling of the dermal layer of skin. The TPA-induced irritation injury was very obvious when the mouse ears were examined during the course of this study; TPA-treated ears appeared visibly red and swollen. According to Carlson et al. (*33*), edema formation will begin <1 h post-TPA administration and reach its peak within 4−6 h. In our study, topical application of LMPF and HMPF from all three blackberry cultivars markedly attenuated the TPA-induced inflammation in the ears of treated mice, that is, in terms of both edema and the migration of PMN leukocytes to the site of inflammation, which was assessed by measuring MPO activity.

The experiment showed that the nonsteroidal anti-inflammatory drug (i.e., indomethacin) and treatments of the LMPF and HMPF from all blackberry cultivars significantly ($P < 0.05$) reduced the TPA-induced skin inflammatory response; **Figure 2** depicts the changes in ear thickness at both the 4 and 24 h test periods. Although one concentration is reported here, preliminary experiments with mice showed that the efficacy of topical application of indomethacin, the LMPF, and the HMPF at reducing edema after TPA-induced injury was concentration-dependent. At 4 h, the LMPF from Navaho, Kiowa, and Ouachita blackberries had reduced acute edema in the mouse ears by 46, 48, and 32%, respectively, as compared to the TPA-positive control group (see **Figure 2**). A similar finding was noted for treatment of the HMPF from Navaho, Kiowa, and Ouachita blackberries; that is, the TPA-induced skin inflammatory

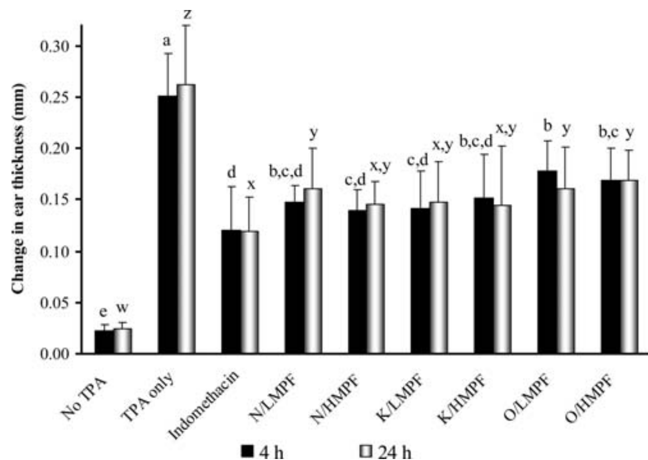

Figure 2. Change in ear thickness (mm) of mice after 4 and 24 h of TPA-induced injury. Ear thickness was measured with a digital micrometer. Values are means of triplicate experiments ± standard deviation. When one-way ANOVA was significant, differences in ear thickness for either the 4 or 24 h group were determined using Duncan's multiple-comparison test ($P < 0.05$). Means with the same letter at 4 h (a−e) and 24 h (w−z) are not significantly ($P > 0.05$) different.

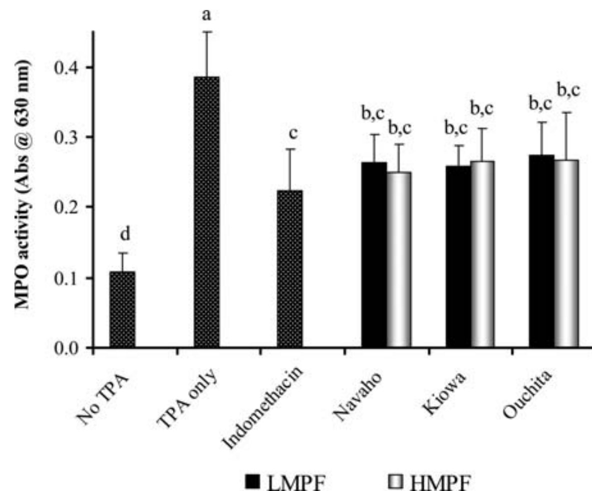

Figure 3. Colorimetric assay determination of myeloperoxidase (MPO) activity for biopsies of mouse ears after 24 h of TPA-induced injury. Values are means of triplicate experiments ± standard deviation. When one-way ANOVA was significant, differences in MPO activity for different groups were determined using Duncan's multiple-comparison test ($P < 0.05$). Means with the same letter are not significantly ($P > 0.05$) different.

response was reduced in the mouse ears by 49, 44, and 36%, respectively. Even though the HMPF possesses a greater content of tannins (particularly hydrolyzable tannins with numerous gallic acid residues) relative to the LMPF (see results of the BSA assay in **Figure 1**), no marked differences in inhibiting the acute edema were evident between these two fractions. Indomethacin (10 μL of 25 mg/mL acetone to each ear), as expected, effectively and significantly ($P < 0.05$) reduced edema in TPA-treated mouse ears by 57 and 60% after 4 and 24 h, respectively. Blackberries in the groups treated with HMPF from Navaho, Kiowa, and Ouachita reduced edema significantly by 49, 50, and 39% respectively. These values were similar to those observed at 4 h. Mice treated with the LMPF from Navaho, Kiowa, and Ouachita blackberries reduced the edema at 24 h by 43, 48, and 43%, respectively. The reduction in edema is not unexpected, as phytochemical-based phospholipase A_2 inhibitors have been shown to be effective at reducing leukocyte infiltration and edema in the TPA model of ear inflammation by preventing the release of AA from membrane bilayer. Phenolic acids have also been reported to inhibit COX and LOX enzyme activities, resulting in a reduction in TPA-induced injury (*34*), but the efficacy of the phenolics depends directly upon their chemical structures.

When the masses of ear punches taken at 24 h were examined, reductions in the TPA-induced injury by 53% from indomethacin and 37, 43, and 35% from the LMPF of Navaho, Kiowa, and Ouachita, respectively, as well as 48, 35, and 41% from the HMPF of Navaho, Kiowa, and Ouachita, respectively, were observed (data not shown). In all groups, the inhibition percentage was compared against the ear punch mass in the TPA-positive control group.

Neutrophil infiltration to the dermis is generally at its maximum within 24 h following TPA-induced injury. Assaying the MPO enzyme serves as a good index of granulocyte infiltration. The percent inhibition of MPO activity relative to a TPA-positive control sample is indicative of anti-inflammatory action (*35*). This neutrophil enzyme plays an essential part in the innate immune system by catalyzing the production of HOCl from H_2O_2. Flavonoid polyphenolics with high antioxidant activities have been reported to potently reduce inflammation and the production of H_2O_2 in this animal model (*36*). In our study, MPO activity was measured in the mouse ear biopsies taken 24 h after TPA administration (see **Figure 3**), as an index of neutrophil infiltration. On the basis of the observed absorbance readings and the equation defined under Materials and Methods, application of indomethacin significantly ($P < 0.05$) inhibited MPO activity by ~59%, application of the LMPF from Navaho, Kiowa, and Ouachita by 44, 46, and 41%, respectively, and application of the HMPF from Navaho, Kiowa, and Ouachita by 49, 43, and 43%, respectively. Huang et al. (*37*) reported that the potential degree of anti-inflammatory activity attributed to polyphenolics is proportional to the number of hydroxy groups residing in the bioactives. In our study, no statistically significant ($P > 0.05$) differences were observed in ear edema or MPO activity among the groups treated with the LMPF and HMPF from the three cultivars. This indicates that a wide range of molecular weight polyphenolics possess topical anti-inflammatory activity. Less effective transdermal penetration of tannin constituents in the HMPF due to their larger molecular weights relative to phenolic compounds in the LMPF might decrease the level of the phenolics within the ear tissue for HMPF treatments. Yet, Rocha et al. (*38*) did confirm the penetration of proanthocyanidin B_2 ($C_{30}H_{26}O_{12}$, FW = 578.5 g/mol) through the skin to the dermis layer.

Correlations between Observed Antioxidant and Anti-inflammatory Activities of Blackberry Polyphenolic Fractions. Correlations between the anti-inflammatory response from the blackberry LMPFs/HMPFs to the TPA-induced injury (as measured by the percent inhibition in edema) with the colorimetrically determined TPCs and antioxidant activities (as assessed by the FRAP assay) were established. **Figure 4** illustrates the positive linear correlations found. Correlation coefficients, r, of 0.925 and 0.923 ($P < 0.01$) were determined for the anti-inflammatory activity of the blackberry samples with the TPCs and FRAP values, respectively. These results suggest that the anti-inflammatory effects observed from blackberries are directly related to the level of total polyphenolics present. Interestingly, blackberries are highly recommended foods because they contain the highest antioxidant content per serving (i.e., 5.746 mmol/serving) of all berries (*4*). An r of 0.995 was determined when the TPCs of blackberry fractions were compared to their respective FRAP values.

Dietary Significance of Consuming Blackberries. Why investigate the anti-inflammatory activity of blackberry phenolics?

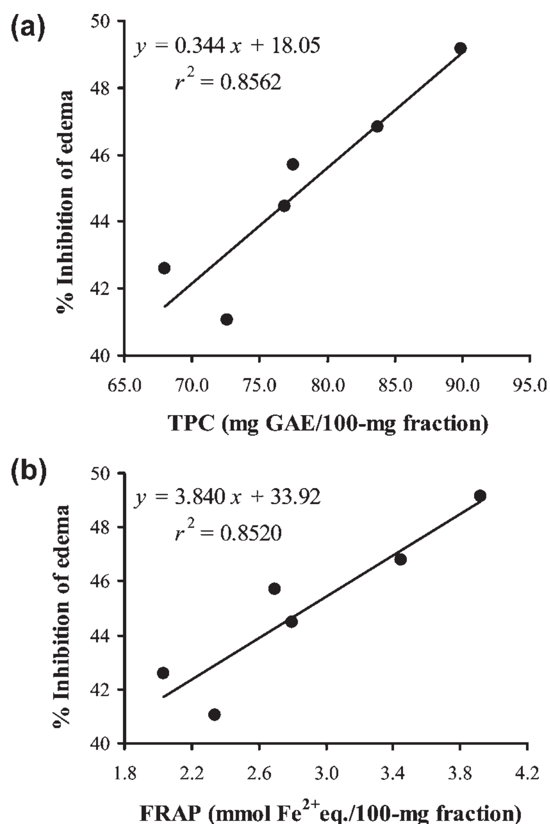

Figure 4. Correlations established between the percent inhibition of edema (i.e., ear thickness) and (**a**) the total phenolics contents (TPCs) or (**b**) ferric reducing antioxidant power (FRAP) values of the LMPFs and HMPFs from blackberries.

Due to undesirable side effects from many common anti-inflammatory drugs, dietary intervention strategies for both the prevention and treatment of chronic inflammatory states are gaining interest, with particular emphasis on the former. Polyphenols endogenous to fruits, vegetables, herbs, and spices have been shown to impart an inhibitory effect on inflammatory responses, and flavonoids specifically have been found to inhibit histamine release from human mast cells (*39*). Chung et al. (*36*) reported that edema formation may also be regulated by H_2O_2 generation, as evident from the anti-inflammatory activity of several phenolic antioxidant compounds against TPA-induced inflammation. For instance, peonidin, an anthocyanin rich in raw cranberries, has been reported to inhibit TPA-induced COX-2 expression (*40*). Theaflavins and their derivatives exhibited a strong anti-inflammatory activity due to their capacity to retard AA metabolism and inhibit overexpression of proinflammatory cytokines such as IL-1β and IL-6. Serraino et al. (*41*) demonstrated that cyanidin-3-*O*-glucoside, which represents $>85\%$ of the total anthocyanins in blackberry juice, prevents vascular hyporeactivity and endothelial dysfunction by scavenging peroxynitrite ($ONOO^-$); the reaction product between NO and the superoxide anion radical, is known to cause cellular and tissue injury in a variety of inflammatory states.

The precise mechanisms by which the LMPF and HMPF from blackberries exert anti-inflammation have not been determined. Possibly the phenolics within these fractions can interact with COX-2 and NF-κB or inhibit the release of pro-inflammatory cytokine interleukins. Pro-inflammatory cytokines can trigger the up-regulation of other pro-inflammatory cytokines and chemokines, as well as increase the expression of many cellular adhesion molecules (CAMS), selectins, integrins, and immunoglobulins (*42*). Perhaps the polyphenolics of blackberries interrupt this cell signaling pathway. Cells in injured skin, such as dermal dendritic cells, epidermal Langerhans cells, melanocytes, fibroblasts, and migrating leukocytes, are known as the source and target of interleukins. Moreover, the IL-1β pathway is involved in the induced transcription of a series of genes, including adhesion molecules, chemokines, secondary cytokines, NO synthase, and COX, all relevant to skin inflammation, and plays an important role in the modulation of inflammation (*43*).

The present study demonstrates that topical application of the LMPF (cyanidin-3-*O*-glucoside was the predominant anthocyanin) and the HMPF (ellagic acid and its derivatives were the main components) from Georgia-grown blackberry cultivars can cause a significant reduction in TPA-induced ear edema of mice. Even though different classes of phenolic compounds were present with various molecular weights, transdermal penetration of these compounds into the ear tissue appears to have occurred; that is, inflammatory markers were reduced in all treatment groups. Wang et al. (*44*) reported that the polyphenolics (particularly the anthocyanins) in rat diets containing freeze-dried black raspberries were effective in reducing *N*-nitrosamine tumorigenesis in the esophagus of the rats, indicating that the polyphenolics were being absorbed and have chemopreventive potential. Furthermore, the black raspberry fortified diets inhibited inflammation. Extending these findings to the present study, it is predicted that blackberry polyphenolics, which have been shown to impart topical anti-inflammatory activity, will be absorbed to some extent and impart anti-inflammatory activity in vivo.

The blackberry fractions also possessed marked antioxidant capacities, which exhibited a strong positive correlation with the observed anti-inflammatory activities. Although this research involved topical application, a portion of fruit polyphenolics will be absorbed in the gastrointestinal tract. Shukitt-Hale et al. (*45*) demonstrated that the polyphenolics in a 2% blackberry-supplemented diet, when fed to rats from 19 to 21 months of age, retarded and even reversed age-related decrements in motor and cognitive performance. These in vivo effects were postulated as being the result of the blackberry polyphenols increasing antioxidant and/or anti-inflammatory levels in the rats or their direct effects on signaling in the brain. We believe that the potent antioxidant and topical anti-inflammatory properties observed in our study add to the other known benefits of blackberries and can significantly enhance this fruit's market potential as a constituent in functional food/nutraceutical beverage formulations.

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