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ARTICLE

Inhibition of Pro-inflammatory Responses and Antioxidant Capacity of Mexican Blackberry (*Rubus* spp.) Extracts

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Total polyphenolic and anthocyanin- and proanthocyanidin-rich fractions from wild blackberry genotypes (WB-3, WB-7, WB-10, and WB-11), a domesticated noncommercial breeding line (UM-601), and a commercial cultivar (Tupy) were evaluated for inhibition of pro-inflammatory responses [nitric oxide (NO) production, inducible nitric oxide synthase (iNOS) expression, cyclooxygenase-2 (COX-2) expression, and prostaglandin E_2 (PGE_2)] in RAW 264.7 macrophages stimulated by lipopolysaccharide (LPS). At 50 μM [cyanidin-3-*O*-glucoside (C3G) or catechin equivalent], most fractions significantly ($P < 0.05$) inhibited all markers. The anthocyanin-rich fraction from WB-10 and the proanthocyanidin-rich fraction from UM-601 exhibited the highest NO inhibitory activities (IC_{50} = 16.1 and 15.1 μM, respectively). Proanthocyanidin-rich fractions from the wild WB-10 showed the highest inhibition of iNOS expression (IC_{50} = 8.3 μM). Polyphenolic-rich fractions from WB-7 and UM-601 were potent inhibitors of COX-2 expression (IC_{50} = 19.1 and 19.3 μM C3G equivalent, respectively). For most of the extracts, antioxidant capacity was significantly correlated with NO inhibition. Wild genotypes of Mexican blackberries, as rich sources of polyphenolics that have both antioxidant and anti-inflammatory properties, showed particular promise for inclusion in plant improvement programs designed to develop new varieties with nutraceutical potential.

KEYWORDS: Inflammation; wild blackberries; polyphenolic-rich fractions (PAE); antioxidant capacity; anthocyanins (ANC); proanthocyanidins (PAC); *Rubus*

INTRODUCTION

Chronic inflammation is associated with several human pathologies and causes the up-regulation of several pro-inflammatory proteins in affected tissues. Among the numerous pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) produce nitric oxide (NO) and prostaglandin E_2 (PGE_2) in lipopolysaccharide (LPS)-activated macrophages and in other stimulated cells (*1*, *2*). These enzymes are involved in initiating obesity, cardiovascular disease, neurodegenerative disease, diabetes, and cancer (*1*, *3*, *4*). Cancers may arise from chronic irritation and inflammation or, conversely, an oncogenic change can induce an inflammatory microenvironment that promotes the development of tumors (*1*).

Natural bioactive compounds including polyphenolic phytochemicals from fruits, vegetables, grains, legumes, tea, wine, and other plant-derived products may protect against cancer, degenerative diseases, and chronic and acute inflammation (*5*). Polyphenols are non-nutritive constituents produced by secondary metabolism in plants. These include several classes of phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (anthocyanins, flavanols, and flavonols), condensed tannins (proanthocyanins), stilbenoids, and hydrolyzable tannins (ellagitannins and gallotannins) (*6*, *7*). Polyphenols are scavengers of a wide variety of reactive species such as superoxide, hydroxyl radical, peroxyl radical, hypochlorous acid, and peroxynitrous acid, resulting in less reactive radicals (*8*, *9*). Polyphenols in fruits such as blueberry, gooseberry, lingonberry, and blackberries can act effectively as free radical inhibitors (*10*). Scavenging of free radicals and inhibition of inflammation may contribute to the prevention of chronic human diseases (*11*).

Currently, a variety of nonsteroidal anti-inflammatory drugs are used to treat chronic inflammatory diseases; however, these drugs have notable side effects. Thus, phytochemicals that can be used as natural preventive agents, for example, those found in

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fruits and vegetables, are an attractive alternative (*12*, *13*). Both the polyphenolic content and antioxidant activity of berries (*9*, *14*−*18*) are conditioned by the genotype of fruit (species and variety), environmental conditions, and production techniques (*13*, *19*). However, little is known about the anti-inflammatory activity of different blackberry genotypes (*20*). Thus, the objectives of this study were to evaluate the inhibition of pro-inflammatory responses in RAW 264.7 macrophages stimulated by LPS after treatment with extracts rich in polyphenols, anthocyanins, and proanthocyanidins from wild and cultivated Mexican blackberries and to compare their antioxidant capacities.

MATERIALS AND METHODS

Plant Materials. Four Mexican wild blackberries (*Rubus* spp.), designated WB-3, WB-7, WB-10, and WB-11, were collected in the spring of 2008 in Uruapan Michoacán, Mexico. These wild genotypes (commonly called frutillas or moras) are popular sources of food and pigments and are commonly wildcrafted in the region; leaves are typically used in teas to alleviate stomach pain. WB-3 and WB-10 were selections of the wild species *Rubus adenotrichus*, WB-7 was from *Rubus corifolius*, and WB-11 was from *Rubus glaucus*. A commercial blackberry (Tupy cultivar) and a domesticated noncommercial breeding line (UM-601) were collected in Los Reyes, Michoacan, Mexico, by the Agrobiologic Laboratory of Universidad Michoacana de San Nicolás de Hidalgo, Uruapan, Michoacán, Mexico, in the winter of 2008. Immediately after harvest, all fruits were washed and frozen at −80 °C. The samples were then lyophilized and stored at −20 °C until use in bioassays.

Chemicals. Sodium pyruvate solution (100 mM), penicillin (1000 units/mL), streptomycin (1000 units/mL), sodium nitrite, sulfanilamide, *N*-1-(naphthyl)ethylenediamine dihydrochloride, and LPS from *Escherichia coli* O55:B5 were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and macrophage RAW 264.7 cell line were purchased from American Type Culture Collection (Manassas, VA), and fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). COX-2 and inducible nitric oxide synthase (iNOS) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-mouse IgG horseradish peroxidase secondary antibody was purchased from GE Healthcare (Buckinghamshire, U.K.). All other chemicals were purchased from Sigma unless otherwise specified.

Polyphenolic (PAE), Anthocyanin (ANC), and Proanthocyanidin (PAC) Extracts. Freeze-dried blackberries (100 g) were blended in a flask with 500 mL of acidified 80% methanol (0.3% trifluoric acid, TFA) (1:5 w/v) and filtered through cotton three times to separate purple pigment from pulp, using a protocol recently described by Cuevas-Rodríguez et al. (*21*). Briefly, the extract was partitioned with ethyl acetate (EtOAc) (1:5) to remove lipophilic material, and after solvent removal, the aqueous portion (120 mL) was loaded on an Amberlite XAD-7 column (30 × 10 cm) and washed thoroughly with acidified water (0.3% TFA) to remove free sugars, pectins, and other impurities. One liter of methanol (0.3% TFA) was added to the column to elute the pigmented polyphenolic mixture. Methanol was evaporated, and the polyphenolic mixture was lyophilized to yield ∼4 g of dry powder, rich in total polyphenolics, which was designated post-Amberlite extract (PAE). Subsequently, 2.0 g of the PAE was dissolved in 5 mL of MeOH and applied to a column packed with Sephadex LH-20 (30 × 3 cm) preconditioned with H_2O/MeOH 80:20 (0.3% TFA). Twelve fractions (50 mL each) were collected starting when the colored material began to elute from the column. Using two consecutive solvents (20% MeOH and 70% acetone), anthocyanin-rich fractions (fractions 1−9) and proanthocyanidin-rich fractions (fractions 10−12) were eluted from the Sephadex LH-20 column. Solvents were evaporated (≤40 °C), and then the fractions were immediately frozen at −80 °C and freeze-dried. On average, across all genotypes, Sephadex LH-20 fractions 2 and 11 had the most abundant dry mass and were assigned as fractions rich in anthocyanins (ANC) and proanthocyanidins (PAC), respectively, for comparative bioactivity analysis along with the parent PAE and crude extracts (CE).

Total Polyphenol Content (TPC). The TPC of each of the blackberry extracts (CE, PAE, ANC, and PAC) was measured according to a modified Folin−Ciocalteu method (*22*). Briefly, 0.5 mL of 1 N Folin−Ciocalteu reagent was added to 0.5 mL of diluted blackberry extract (50 μg/mL), and this mixture was allowed to stand for 2−5 min before the addition of 1 mL of 20% Na_2CO_3. The solution was then allowed to stand for an additional 10 min before reading at 765 nm in a SpectraMax Plus spectrophotometer (Sunnyvale, CA). The polyphenol content was estimated using a standard curve of gallic acid and was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight extract (DW).

Antioxidant Capacity. Total antioxidant capacity was estimated using the oxygen radical absorbance (ORAC) assay (*23*, *24*) by measuring the protection by the extracts from free radicals generated by β-phycoerythrin (b-PE) in the presence of 2,2′-azobis(2-methylpropanimidamide) (AAPH). The assay was carried out in black-walled 96-well microplates (Fisher Scientific, Hanover Park, IL). Each well had a final volume of 200 μL. The following reactants were added in this order: 20 μL of 75 mM phosphate buffer, pH 7; 20 μL of either Trolox standard (1 mM final concentration) or sample (1.0−3.0 μg equiv C3G or (+)-catechin/mL); 120 μL of b-PE (70 nM final concentration); and 60 μL of AAPH (12 mM final concentration). As a blank, 25 μL of 75 mM phosphate buffer, pH 7, was added instead of Trolox or samples. Immediately after the addition of AAPH, plates were placed in an FL ×800 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT), set with excitation filter 485 nm and emission 582 nm and then read every 2 min for 3 h until 95% loss of fluorescence was reached. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated on the basis of the differences in the area under the fluorescence decay curve between the blank, standard, and a sample and expressed as micromoles of Trolox equivalents (TE) per gram of DW. Trolox (4−153 μM) was used as a standard ($y = 0.20x + 0.81$, $R^2 = 0.99$).

Cell Culture, Treatment, and Cell Viability. The macrophage cell line RAW 264.7 was cultured in growth medium containing DMEM, 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO_2/95% air. Cell treatment was conducted by seeding 2×10^5 cells in a six-well plate, and the cells were allowed to grow for 48 h at 37 °C in 5% CO_2/95% air. The cells were treated with blackberry PAE, ANC, and PAC extracts (0.5, 5.0, and 50 μM equivalents of cyanidin-3-*O*-glucoside for PAE and ANC and catechin for PAC) and 1 μg/μL LPS, added together at the same time, for 24 h. Cell viability was determined using the CellTiter 96 AQueous One Solution proliferation assay using the novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tretrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corp., Madison, WI). Briefly, 5×10^4 cells were seeded in a 96-well plate, and the total volume was adjusted to 200 μL with growth medium. The cells were allowed to grow for 24 h at 37 °C in 5% CO_2/95% air. After treatment, the growth medium was replaced by 100 μL of fresh growth medium, and 20 μL of MTS/PES ([(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inert salt, and an electron coupling reagent, phenazine ethosulfate (PES)] (Promega Corp.) was added to each well. The plate was incubated for 2 h at 37 °C, and the absorbance was read at 515 nm. The percentage of viable cells was calculated with respect to cells treated with dimethyl sulfoxide. Concentrations that allowed >90% cell viability were used to determine markers of inflammation.

Nitrite Measurement. NO production was measured after 24 h of cell treatment and LPS induction. A 100 μL aliquot of the culture supernatant was plated in a 96-well plate, and an equal amount of Griess reagent (1% sulfanilamide and 0.1% *N*-1-(naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4) was added. The plate was then incubated for 5 min at room temperature and the absorbance measured at 550 nm. The amount of NO was calculated using a sodium nitrite standard curve ($y = 0.13x + 0.12$, $R^2 = 0.99$).

Prostaglandin E_2 Measurement. After 24 h of cell treatment and LPS induction, the culture supernatant was collected. PGE_2 was measured using a PGE_2 EIA monoclonal kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Briefly, 50 μL of diluted cell supernatant was plated in a 96-well goat anti-mouse IgG coated plate and incubated for 18 h at 4 °C. After incubation, the plate was washed using the provided wash buffer, and the color was developed by adding 200 μL of Ellman's reagent and shaking the plate for 60−90 min in the dark.

Table 1. Total Polyphenolic Content (TPC) in Various Extracts from Wild, Commercial, and Domesticated Breeding Lines of Mexican Blackberries[a]

	TPC (mg of GAE/g of DW)			
	CE	PAE	ANC	PAC
WB-3	25.5 ± 1.1a	95.7 ± 3.0e	237.8 ± 6.2c	598.0 ± 10.5a
WB-7	18.4 ± 1.1c	187.2 ± 10.9a	211.4 ± 17.1d	565.6 ± 16.6b
WB-10	19.2 ± 0.7c	134.3 ± 2.7b	318.1 ± 18.8a	495.4 ± 32.1cd
WB-11	23.6 ± 1.1ab	92.67 ± 1.8e	250.7 ± 5.0b	454.9 ± 27.9d
Tupy	14.2 ± 0.4d	111.8 ± 4.5c	221.5 ± 11.7cd	521.7 ± 4.1c
UM-601	22.4 ± 0.2b	103.6 ± 1.7d	294.6 ± 11.6a	526.3 ± 19.4c

[a] Values are expressed as mean ± SD, $n = 6$. Different letters within columns show significant differences at $P < 0.05$ using LSD multiple-range test. TPC, total polyphenolic content; DW, dry weight; GAE, gallic acid equivalent; CE, crude extract; PAE, extract rich in polyphenols (post-Amberlite extract); ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; Tupy, commercial cultivar; UM-601, domesticated noncommercial blackberry breeding line; WB, wild blackberry species.

The amount of PGE_2 was calculated using the PGE_2 standard curve ($y = -37.29x + 108.6$, $R^2 = 0.98$).

Measurement of iNOS and COX-2 Protein Expression. iNOS and COX-2 enzyme expressions were determined in the cell lysates using the Western blot technique. Briefly, treated cells were washed with ice-cold DMEM and ice-cold phosphate-buffered saline before treatment with 200 μL of Lamemli buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β-mercaptoethanol as the lysing buffer. The cell lysates were boiled for 5 min, and approximately 25 μg of protein was loaded in 4−20% Tris-HCl ready gels (Bio-Rad Laboratories) for protein separation. Separated proteins were transferred to a PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. The membranes were washed with 0.1% TBST (five times, 5 min each) and incubated with either COX-2 or iNOS mouse monoclonal antibody (1:200) at 4 °C overnight. The membrane was washed again and incubated with anti-mouse IgG horseradish peroxidase conjugate secondary antibody for 3−4 h at room temperature. After incubation, the expression of COX-2 and iNOS was visualized using chemiluminescent reagent (GE Healthcare, Buckinghamshire, U.K.) following the manufacturer's instructions. The membrane was imaged with a Kodak Image Station 440 CF (Eastman Kodak Co., New Haven, CT).

Statistical Analysis. Experiments were repeated at least three times for consistency. Unless otherwise stated, the data are expressed as mean ± SD. The data obtained were analyzed using two-way ANOVA, SAS Institute (*25*), to compare experimental treatments with control values. The comparisons between treatments were performed using LSD mean separation, and differences were considered to be significant at $P < 0.05$. The concentration to inhibit 50% (IC_{50}) of NO and PGE_2 production and COX_2 and iNOS expression was determined by nonlinear regression (curve fit) using GraphPad Prism software version 4.00 (Graph Pad Software, San Diego, CA).

RESULTS AND DISCUSSION

Total Polyphenol Content. Phenolics are compounds with one or more aromatic rings and one or more hydroxyl groups (*7*). They are of particular importance because their consumption may ameliorate inflammation (*17*). The TPC of the blackberry fractions from all six genotypes is presented in **Table 1**. The TPC for blackberry crude extracts ranged from 14.2 ± 0.4 to 25.5 ± 1.1 mg of GAE/g of DW, where WB-3 and WB-11 had the highest TPC and were significantly higher than the commercial cultivar (Tupy) ($P < 0.05$). Polyphenolic content in a berry can be conditioned not only by genotype but by exposure of the plant to abiotic and biotic stresses such as temperature, irradiation, herbivore, and pathogenic infection (*14*, *16*). The TPCs reported in this study were within the ranges of those reported for other *Rubus* spp. (*9*, *10*, *26*−*28*). After a cleanup procedure using Amberlite XAD-7 resin, the average TPC across all genotypes was increased from an average of 20.5 mg of GAE/g in CE to 120.9, 255.7, and 527.0 mg of GAE/g of DW in the PAE, ANC, and PAC blackberry extracts, respectively. The ANC contained mainly cyanidin 3-*O*-glucoside, in addition to cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-arabinoside, and cyanidin 3-*O*-(6-*O*-malonyl)-glucoside, whereas PAC contained monomeric and polymeric forms of proanthocyanidins as well as ellagitannins such as pedunculagin, sanguiins H-6 and H-10, nobotanin A, malabathrin B, and lambertianin A (*21*). Ellagitannins have previously been reported in blackberry and raspberry species and were associated with antimicrobial bioactivities (*6*, *29*, *30*).

Table 2. Antioxidant Capacity (ORAC) of Various Extracts from Wild, Commercial, and Domesticated Breeding Lines of Mexican Blackberries[a]

	ORAC of extracts (μmol of TE/g of DW)			
	CE	PAE	ANC	PAC
WB-3	361.9 ± 76.5a	2534 ± 130.2c	3430.8 ± 225.1c	3926.1 ± 18.8d
WB-7	240.8 ± 122.4d	2407.4 ± 130.2c	3562.3 ± 264.3c	3512.4 ± 50.6e
WB-10	272.0 ± 122.4d	4038.5 ± 71.4a	5761.3 ± 154.6a	4201.4 ± 2.4c
WB-11	261.5 ± 120.6c	2524.8 ± 65.2c	2381.5 ± 346.2d	4395.7 ± 9.5c
Tupy	169.9 ± 71.2e	3367.7 ± 122.6b	5205.6 ± 282.1b	4686.3 ± 173.2b
UM-601	287.5 ± 140.6b	4158.7 ± 92.6a	3519.3 ± 189.8c	5161.9 ± 6.6a

[a] Different letters within columns indicate treatments were statistically different ($P \leq 0.05$), $n = 4$. ORAC, oxygen radical absorbance capacity; TE, Trolox equivalent; DW, dry weight; CE, crude extract; PAE, extract rich in polyphenols (post-Amberlite extract); ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; Tupy, commercial cultivar; UM-601, domesticated noncommercial breeding line of blackberry; WB, wild blackberry genotypes.

Antioxidant Capacity. Among the most commonly employed methods to evaluate a total antioxidant capacity is the ORAC method, which employs fluorescein as the target molecule (*31*). The most prevalent compounds in berries are anthocyanins, proanthocyanidins, and ellagitannins, which collectively are responsible for much of their antioxidant capacity (*7*). The antioxidant capacities of CE, PAE, ANC, and PAC extracts are shown in **Table 2**. The antioxidant capacities for crude extracts for all genotypes ranged from 169.9 to 361.9 μmol of TE equiv/g of DW. The CE antioxidant capacity for WB-3 was significantly higher than for all other genotypes, whereas the commercial Tupy cultivar had the lowest antioxidant capacity ($P < 0.05$). These ORAC value ranges were similar to those previously reported for blackberries cultivated in different regions of the United States and Mexico (*14*). The antioxidant capacities of all semipurified blackberry extracts (PAE, ANC, and PAC) in all genotypes increased significantly ($P < 0.05$) compared to the corresponding crude extracts (**Table 2**). The cleanup procedure that yielded fractions with much higher polyphenolic concentrations also resulted in significant increases in the antioxidant capacity across all fractions. PAE blackberry extracts exhibited ORAC values 8.3−19.8 times higher than those found with the crude extracts. The antioxidant capacity for PAE extracts ranged from 2407.4 to 4158.7 μmol of TE equiv/g of DW. PAE fractions from both UM-601 and WB-10 blackberries had the highest ORAC values ($P < 0.05$). The antioxidant capacity for blackberry extracts rich in anthocyanins (ANC) was 9.1−30.61 times higher than those for crude extracts. WB-10 exhibited significantly greater ($P < 0.05$) antioxidant activity than all other genotypes. The same trend was observed with PAC, which showed antioxidant capacities 10.8−27.6 times higher than those of the corresponding crude extracts. The range of antioxidant activity for blackberry PAC extracts was from 3512.4 to 5161.9 μmol of TE equiv/g of DW. The domesticated UM-601 blackberries had the highest antioxidant activity among the PAC fractions of all of the blackberries tested ($P < 0.05$). It is of interest that the concentrations of total polyphenols in the PAE, ANC, and PAC extracts were approximately 6.2, 12.8, and 26.6 times greater than that of the crude

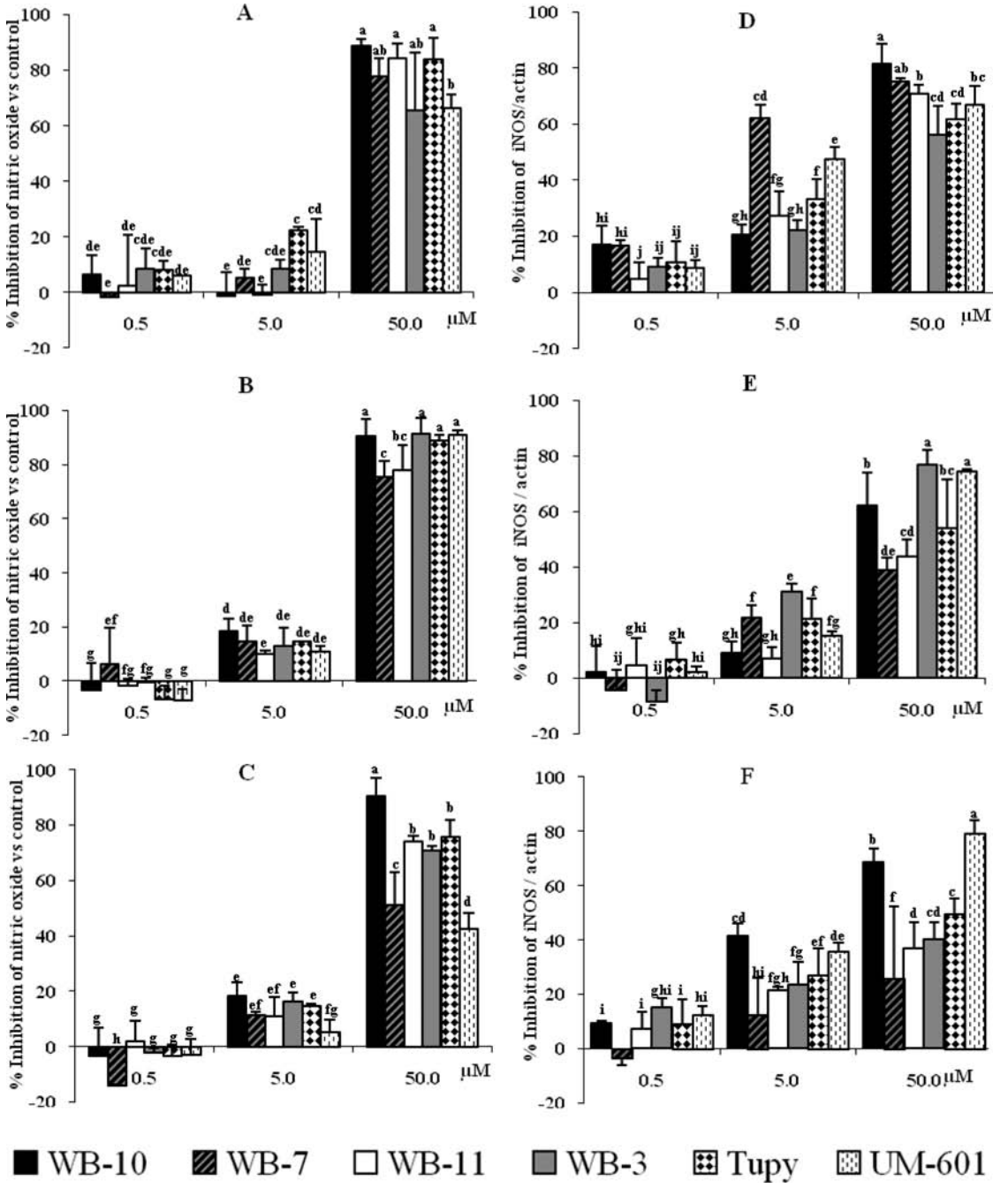

Figure 1. Inhibition of NO production and expression of iNOS by RAW 264.7 macrophages induced by LPS after treatment with extracts (0.5, 5.0, and 50 μM equivalents of cyanidin 3-*O*-glucoside) from wild, commercial, and domesticated breeding lines of Mexican blackberries: inhibition (%) of NO with PAE (**A**), ANC (**B**), and PAC (**C**); inhibition (%) of iNOS expression with PAE (**D**), ANC (**E**), and PAC (**F**). PAE, extract rich in polyphenolics; ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; WB, wild blackberry species; UM-601, domesticated breeding line of blackberry; Tupy, commercial cultivar. Inhibition was calculated relative to macrophages treated with 1 μg/mL LPS alone. Data represent the mean ± SD from two independent experiments and at least triplicate analysis, at $P < 0.05$.

extract, respectively. The antioxidant capacities (ORAC values) of ANC, PAC, and PAE of most genotypes were comparable. This may be due to the presence of other phenolic compounds in PAE, which are active antioxidants, that may have additive or synergistic antioxidant activity with anthocyanins and proanthocyanidins (*32*).

Effect of Extracts on NO Production and iNOS Expression. Some flavonoids and other polyphenolic compounds have been shown to exhibit inhibitory effects on NO production and iNOS expression (*33*). Therefore, we tested the effects of blackberry extracts on NO production and iNOS protein expression in LPS-stimulated macrophages. Blackberry extracts did not affect the viability of RAW 264.7 cell up to 50 μM (data not shown); thus, the inhibitory effects were not attributable to cytotoxic effects. Several of the PAE, ANC, and PAC extracts (**Figure 1A−C**) inhibited NO production up to 22% at 0.5 and 5 μM concentrations. At 50 μM concentration, the inhibition of these pro-inflammatory markers by all of the PAE, ANC, and PAC extracts was increased significantly ($P < 0.05$) compared with untreated LPS-stimulated macrophages. The ANC extract for the wild WB-10 genotype exhibited the highest activity against LPS-induced NO production with an IC_{50} value of 16.1 μM, followed by the

Table 3. Inhibitory Concentration of Pro-inflammatory Responses (IC_{50}) for Various Extracts from Wild, Commercial, and Domesticated Breeding Lines of Mexican Blackberries[a]

	IC_{50} [μM C3G equivalent (PAE and ANC) or catechin equivalent (PAC)]								
	PAE			ANC			PAC		
	NO	iNOS	COX-2	NO	iNOS	COX-2	NO	iNOS	COX-2
WB-3	39.9	28.3	35.0	22.3	>50	>50	21.4	>50	>50
WB-7	28.2	24.5	19.1	27.5	38.9	>50	33.1	>50	>50
WB-10	28.8	21.8	25.0	16.1	36.3	32.3	19.0	8.3	45.7
WB-11	30.9	28.2	22.0	24.7	>50	>50	29.5	>50	>50
Tupy	17.5	19.0	20.0	17.8	10.6	22.5	20.9	48.9	28.9
UM-601	26.7	15.9	19.3	19.8	24.5	28.6	15.1	25.1	34.6

[a] IC_{50}, the concentration (μM) that resulted in 50% reduction of production/expression of pro-inflammatory responses, $n = 4$. PAE, extract rich in polyphenols (post-Amberlite extract); ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; Tupy, commercial cultivar; UM-601, domesticated blackberry breeding line; WB, wild blackberry species. Because the concentration of polyphenolics in the crude extracts (CE) was very low and no effect was observed in this assay, it was not included in this table.

commercial blackberry Tupy (IC_{50} = 17.8 μM) and the domesticated breeding line UM-601 (IC_{50} = 19.8 μM), whereas other genotypes possessed moderate activity (**Table 3**). In PAC extracts, UM-601 and WB-10 blackberries displayed the most potent effect against NO production with IC_{50} = 15.1 μM and 19.0 μM, respectively, followed by Tupy (IC_{50} = 20.9 μM) and WB-3 (IC_{50} = 21.4 μM), whereas other genotypes possessed moderate activity (**Table 3**).

The iNOS expression in macrophages exposed to different blackberry extracts are shown in **Figure 1D−F** and followed the same trend as with NO inhibition. The potency of each of the blackberry extracts tested for the expression of iNOS is shown in **Table 3**. With PAE extracts, UM-601 showed the highest inhibition of iNOS expression (IC_{50} = 15.9 μM), followed by Tupy (IC_{50} = 19.0 μM). For ANC extracts, Tupy displayed more potent iNOS expression in the RAW 264.7 macrophage cells induced by LPS than ANC from any other genotype, with an IC_{50} value of 10.6 μM. However, the PAC for WB-10 showed the highest inhibition of iNOS expression (IC_{50} = 8.3 μM) as compared to the PAC for all other blackberry genotypes. It was reported that iNOS can produce >1000-fold of NO, a signaling molecule that plays a key role in the pathogenesis of inflammation and is considered to be a pro-inflammatory mediator, when overproduced in abnormal situations (*3*, *4*, *34*). Aberrant activation of cyclooxygenase/prostaglandin signaling is widespread in human neoplasia. NO has also been shown to cause DNA damage as well as mutations in vivo (*35*). The formation of carcinogenic *N*-nitrosamines, resulting from elevated NO formation, has been demonstrated in cell cultures and in vivo (*35*, *36*).

Antioxidant capacity and inhibition of NO production were significantly correlated in the PAE extracts for most of the genotypes (**Table 4**), indicating that various classes of phenolics in the PAE extracts had the ability to scavenge peroxyl and superoxide anion radicals. The ORAC values in ANC extracts were highly correlated with NO production, for example, WB-11 (R^2 = 0.918), cultivar Tupy (R^2 = 0.918), and UM-601 (R^2 = 0.960). For PAC extracts high correlations were observed for only three of the genotypes: WB-3 (R^2 = 0.966), Tupy (R^2 = 0.865), and UM-601 (R^2 = 0.966). When flavonoids are used as antioxidants, ROS are scavenged and therefore they can no longer react with nitric oxide, resulting in less cellular damage. Also, NO can be viewed as a radical itself, and it has been reported that nitric oxide molecules are directly scavenged by flavonoids (*20*). Wang and Mazza (*37*) reported that inhibitory effects on NO

Table 4. Correlation Coefficient (R^2) Analysis of Antioxidant Capacity and Inhibition of Nitric Oxide for Various Extracts from Wild, Commercial, and Domesticated Breeding Lines of Mexican Blackberries at 50 μM Concentration[a]

	antioxidant capacity/NO inhibition (%) of extracts		
	PAE	ANC	PAC
WB-3	0.823*	0.410	0.966*
WB-7	0.812**	0.848	0.466
WB-10	0.826*	0.658	0.287
WB-11	0.578	0.918*	0.778
Tupy	0.996**	0.918*	0.865*
UM-601	0.941**	0.960**	0.966*

[a] Within a column, R^2 values with * and ** are significant at $P < 0.05$ and $P < 0.01$, respectively. PAE, fraction rich in polyphenols (post-Amberlite extract); ANC, fraction rich in anthocyanins; PAC, fraction rich in proanthocyanidins. Tupy, commercial cultivar; UM-601, domesticated noncommercial breeding line of blackberry; WB, wild blackberry genotypes.

production significantly correlated with the content of individual categories of phenolic compounds present in berries.

The inhibition of NO production in macrophages could be explained by C3G interfering with the signal pathway for inflammation, due to the ROS scavenging capacity of C3G (*38*). Pergola et al. (*20*) reported that at least some part of the anti-inflammatory activity of the anthocyanin fraction of blackberry was due to suppression of NO production by C3G, which was the main polyphenol present in the blackberry extracts (*21*). Inflammation is a critical factor in tumor progression. Cells undergoing inflammation produce various pro-inflammatory responses that can damage DNA, leading to tumor initiation and promotion (*1*, *3*, *34*). Several studies have shown that the induction of iNOS produces large amounts of NO during endotoxemia and under inflammatory conditions. Therefore, compounds that inhibit iNOS expression and/or enzyme activity may be beneficial in averting inflammatory diseases caused by an overproduction of NO.

Effect of Extracts on PGE_2 Production and COX-2 Expression. The effects of different concentrations of blackberry extracts on COX-2 expression in RAW 264.7 macrophages induced by LPS are shown in **Figure 2**. For blackberry PAE extracts (0.5 and 5 μM), inhibition of COX-2 protein expression ranged from 4.3 to 50.9%. Cells treated with PAE at 50 μM showed significantly ($P < 0.05$) increased COX-2 inhibition. For blackberry PAE extracts at 50 μM concentrations (**Figure 2A**), inhibition of COX-2 ranged from 50.9 to 76.6%. WB-7 exhibited the highest activity against COX-2 with an IC_{50} value of 19.1 μM, followed by the UM-601 blackberries (IC_{50} = 19.3 μM), whereas other genotypes possessed moderate activity (**Table 3**). For ANC extracts at the 50 μM concentrations, the inhibition of COX-2 was 33.1−60.8% (**Figure 2B**). The PAC extracts at 50 μM concentrations inhibited COX-2 expression by 44.9−75.6% (**Figure 2C**).

The effects of blackberry extracts (50 μM) on the inhibition of PGE_2 production in RAW 264.7 macrophages induced by LPS are presented in **Figure 3**. For PAE extracts, inhibition ranged from 28.0 to 51.6%. The ANC extracts from UM-601, Tupy, and WB-10 blackberries provided higher inhibition of PGE_2 production than any of the PAE or PAC extracts (**Figure 3**). The results in this study show the capability of different polyphenol extracts to inhibit pro-inflammatory responses in macrophages (RAW 264.7) induced by LPS. During inflammation, iNOS and COX-2 are overexpressed, and some studies have indicated that there might be cross-talking between the COX-2 and iNOS genes in macrophages and that such relationships are complex and cell-type specific (*38*). Because iNOS is an enzyme responsible for the overproduction of NO during inflammation (*3*, *34*, *37*, *39*), compounds that are able to reduce NO production without affecting

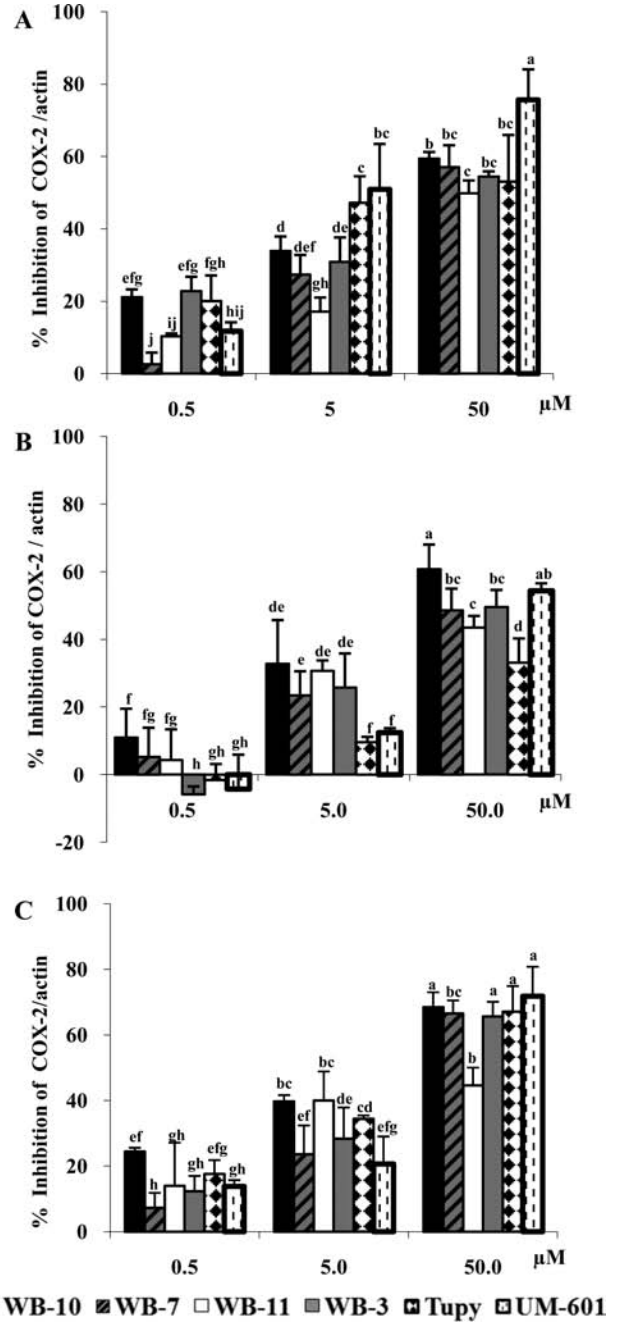

Figure 2. Inhibition of COX-2 expression in RAW 264.7 macrophages induced by LPS after treatment with extracts (0.5, 5.0, and 50 μM equivalents of cyanidin 3-*O*-glucoside) from wild, commercial, and domesticated breeding line of Mexican blackberry extracts: inhibition (%) of COX-2 expression with PAE (**A**), ANC (**B**), and PAC (**C**) extracts. PAE, extract rich in polyphenolics; ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; WB, wild blackberry species; UM-601, domesticated breeding line of blackberry; Tupy, commercial cultivar. Inhibition was calculated relative to macrophages treated with 1 μg/mL LPS alone. Data represent the mean ± SD from two independent experiments and at least a duplicate analysis, at $P < 0.05$.

eNOS or nNOS (isoforms of NOS) may be desirable anti-inflammatory agents (*39*). In this context, it has been reported that the high affinity of polyphenols for proteins and a possible subsequent conformational change of the enzyme might be associated with the inhibitory effect on iNOS enzymatic activity (*40*). On the other hand, elevated prostaglandin levels are exhibited in many human cancers due to the up-regulation of

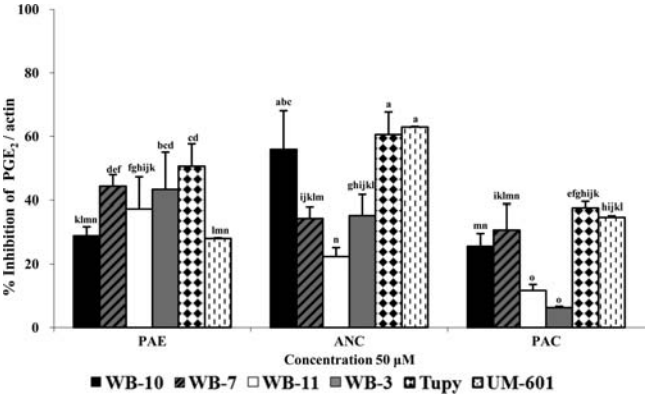

Figure 3. Inhibition of prostaglandins (PGE_2) production in RAW 264.7 macrophages induced by LPS after treatment with extracts (50 μM equivalents of cyanidin 3-*O*-glucoside for PAE and ANC, and 50 μM equivalents of catechin for PAC) from wild, commercial, and domesticated breeding line of Mexican blackberry extracts: inhibition (%) of prostaglandins with PAE, ANC, and PAC extracts. PAE, extract rich in polyphenolics; ANC, extract rich in anthocyanins; PAC, extract rich in proanthocyanidins; WB, wild blackberry species; UM-601, domesticated breeding line of blackberry; Tupy, commercial cultivar. Inhibition was calculated relative to macrophages treated with 1 μg/mL LPS alone. Data represent the mean ± SD from two independent experiments and at least duplicate analysis, at $P < 0.05$.

COX-2. Aberrant activation of cyclooxygenase/prostaglandin signaling is widespread in human neoplasia, and increased expression of COX-2 was also demonstrated in animal models of colitis and in human inflammatory bowel diseases (*1*, *41*). These findings demonstrated a marked increase in COX-2 during the inflammatory process and led to the notion that COX-2 alone is involved in inflammation (*34*, *42*). Polyphenolic-rich PAE fractions from the wild WB-7 and the domesticated breeding line UM-601 were the most potent inhibitors of COX-2 expression.

The combined results from this study suggest that dietary consumption of blackberries may reduce the oxidative stress generated by NO and the expression of pro-inflammatory proteins such as COX-2 and iNOS, thus increasing the body's protection against oxidation−inflammation-related diseases and, further, that polyphenolic-rich fractions from blackberry may be attractive targets for use as nutraceutical agents. Given the particularly strong bioactivities demonstrated by the wild Mexican blackberries, including the UM-601 breeding line derived from wild parents, we conclude that these genotypes may be useful in plant-breeding programs designed to obtain new varieties with enhanced nutraceutical potential.

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