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Antioxidant and Anti-inflammatory *in Vitro* Activities of Phenolic Compounds from Tropical Highland Blackberry (*Rubus adenotrichos*)

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ABSTRACT: This study evaluates the antioxidant and anti-inflammatory activities in a polyphenol extract from blackberries. The antioxidant activity measured via oxygen radical absorbance capacity (ORAC) was higher for the blackberry extract (4339 ± 144 μM TE/g) than for quercetin and ellagic acid. The blackberry phenolic compounds protected liposomes and liver homogenates against lipid peroxidation; in both models, the antioxidant activity (IC_{50} = 7.0 ± 0.5 and 20.3 ± 4.2 μg/mL, respectively) was greater than that found with Trolox. The extract inhibited superoxide production by NADPH oxidase in THP-1 cells and nitrite production in J774A.1 cells stimulated with LPS+IFNγ, with nitrite production decreasing after 4 h of incubation with the extract, mainly through a strong scavenging activity. However, 24 h of treatment reduced the amount of nitrites (IC_{50} = 45.6 ± 1.2 μg/mL) because of a down-regulation of iNOS protein expression, as demonstrated by Western blotting. The inhibitory activities found in blackberry phenols suggest a potential beneficial effect against oxidative stress and inflammatory processes.

KEYWORDS: *blackberry, Rubus adenotrichos, antioxidant activity, lipid peroxidation, anion superoxide, iNOS, macrophages cell lines*

INTRODUCTION

Functional foods have received attention from consumers due to increasing evidence regarding the relationship between diet and health.[1,2] Berries have been proposed as fruits that can potentially improve human health due to their high phenolic compound content. These molecules strengthen the antioxidant defenses of the body and reduce reactive oxygen species, molecules that are associated with such pathological processes as cardiovascular diseases, inflammation, and diabetes.[3,4]

Different biological properties have been reported for the phenolic compounds in berries, and their antioxidant capacities and radical-scavenging activities have been widely demonstrated using *in vitro* techniques and *in vivo* models.[5−7] The antiproliferative and anticancer activities of these polyphenols have been documented in human oral, breast, colon, cervical, and prostate cancer cell lines.[8−10] Polyphenols have also been reported to attenuate some inflammation intermediates, including nitric oxide, NF-*k*B, and TNFα.[11,12]

Moreover, some clinical trials have demonstrated that berry consumption could mitigate the oxidative stressors involved in atherogenesis and cardiovascular diseases.[13,14] For example, berry consumption has been associated with decreases in LDL oxidation, lipid peroxidation, serum glucose, and total cholesterol levels and an increase in HDL cholesterol. The mechanisms suggested include the upregulation of endothelial nitric oxide synthase, a decrease in oxidative stress and in the activity of carbohydrate digestive enzymes, and the inhibition of inflammatory gene expression and foam cell formation.[15]

The major biologically active compounds in berry fruits include such polyphenols as anthocyanins, phenolic acids, tannins (gallo- and ellagitannins), flavonols, flavanols, carotenoids, and vitamin C.[16,17] The main polyphenols in blackberry *Rubus adenotrichos* are cyanidin 3-glucoside, cyanidin-3-malonyl glucoside, lambertianin C, and sanguiin H-6.[18]

The *Rubus* genus is cultivated worldwide. However, the genotype, species, environment, maturity stage, and cultivation conditions influence the content of anthocyanins and hydrolyzable tannin compounds in the berries.[19,20] For this reason, the objective of this research was to characterize the health-

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promoting properties of the major blackberry variety consumed in Costa Rica: *Rubus adenotrichos* cv. 'vino'.

MATERIALS AND METHODS

Chemicals. All the solvents for HPLC and polyphenol purification were obtained from JT Baker (Griesheim, Germany). Amberlite XAD-7, 2,2-difenyl-1-picrylhydrazyl (DPPH), 2,2-azobis-2-methyl-propionamidine-dihydrochloride (AAPH), *tert*-butyl hydroperoxide (TBHP), thiobarbituric acid (TBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were acquired from Sigma Aldrich (St. Louis, MO, USA). The standards ellagic acid and quercetin were obtained from Fluka (Buchs, Switzerland), and cyanidin-3-glucoside was obtained from Extrasynthese (Lyon, France). For cell culturing, RPMI 1640 with glutamax, fetal bovine serum, streptomycin, penicillin, and human IFNγ were purchased from Life Technologies (St. Aubin, France). LPS (*Escherichia coli*, serotype 055:B5), retinoic acid, 1,25-dihydroxycholecalciferol, phorbol 12-myristate 13-acetate (PMA), and lucigenin were purchased from Sigma Aldrich (Lyon, France). Recombinant murine IFNγ was purchased from Genzyme (Le Perray en Yvelines, France). For the Griess reagent, sodium nitroprusside (SNP), sulfanilamide, naphthyletylenediamine dihydrochloride, and sodium nitrite were acquired from Merck (Darmstadt, Germany).

Blackberry Sample. This study used fully ripe blackberries (*Rubus adenotrichos* cv. 'vino') harvested in Costa Rica (Cartago). The blackberries were freeze-dried, packaged in metallic bags, and stored at −20 °C until used for extract preparation. The polyphenol purification was according to Mertz et al.[18] Briefly, 35 g of blackberry powder was extracted twice for 15 min with acetone/water/formic acid (70:30:2). The extract was filtered and concentrated under vacuum to remove the acetone (40 °C). The aqueous layer was mixed with ethyl acetate (2:1), separated, and loaded onto an Amberlite XAD-7 column (150 mm × 20 mm) packed in water. The column was washed with water to remove the sugars, and the phenolic compounds were eluted with methanol/water (80:20). The polyphenols were concentrated under vacuum and freeze-dried. The sample was stored at −20 °C for further analysis.

HPLC Analysis. The polyphenol extract was analyzed by HPLC for anthocyanins and ellagitannins following the protocols described by Mertz et al.[18] and Acosta-Montoya et al.[20] Briefly, the HPLC quantitative analysis was performed with a Dionex liquid chromatograph system equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an end-capped reverse-phase Lichrospher ODS-2 column (250 mm × 4.6 mm i.d., 5 μm) (Interchim, Montluçon, France). Mertz et al.[18] previously identified sanguiin H6, lambertianin C, and cyanidin-malonyl-glucoside following the same HPLC procedure using an additional hyphenation of the diode array detector (DAD) to an electrospray ion trap mass spectrometry detector (ESI-TRAP-MS/MS). As the same method was followed in this paper, a tentative identification of the phenolic compounds was performed based on the work of Mertz et al.[18] Quantification of the polyphenols in the extract was performed using calibration curves established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins.

Antioxidant Assays. *DPPH Radical-Scavenging Activity.* The radical-scavenging activity of the blackberry extract was evaluated by assessing the direct DPPH-scavenging activity in the extract.[21] DPPH (0.25 mM) was prepared in methanol, and 0.5 mL of this solution was incubated with 1 mL of various sample dilutions. The mixtures were incubated at room temperature in the dark for 30 min, and the absorbance of DPPH was measured at 517 nm. Sample blanks were prepared for each dilution. The percentage of the radical-scavenging activity of the extract was plotted against the sample concentration to calculate the IC_{50}, which is the amount of extract necessary to reach the 50% radical-scavenging activity. The samples were analyzed in triplicate. Commercial quercetin and ellagic acid were used as reference standards.

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was performed according to Ou et al.[22] Fluorescein was used as a fluorescent probe, and oxidation was induced with AAPH. The assays were performed using spectrofluorimeter equipment (Biotek Instruments, Winooski, VT, USA), and the ORAC values were expressed as micromoles of Trolox equivalents (μmol TE/g of extract). Commercial quercetin and ellagic acid were used as reference standards.

Nitric Oxide-Scavenging Activity. Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and rapidly converted into the stable product nitrite. The nitrite concentration was measured by the Griess reaction.[23]

SNP (5 mM) was mixed with different concentrations of blackberry extract and incubated for 60 min under direct light to enhance NO production. The Griess reagent (1% sulfanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4) was added, and the absorbance was measured at 540 nm in reference to the absorbance of standard solutions of sodium nitrite. Sample blanks were prepared in each experiment.

NO-scavenging activity was calculated as the amount of extract necessary to reduce 50% of the NO generated by SNP (IC_{50}). The sample was analyzed in triplicate. Commercial quercetin and ellagic acid were used as reference standards.

Inhibition of Lipid Peroxidation in Liposomes. Liposomes were prepared according to Pérez et al.[24] Briefly, 25 mg of commercial lecithin was dissolved in 2.15 mL of chloroform, and then 350 μL of methanol was added. This mixture was dried under a nitrogen atmosphere. The lecithin was resuspended in 4.5 mL of warm PBS and sonicated for 1 h at 4 °C to form liposomes.

To test the capacity of the extract to protect lipid peroxidation, oxidative stress was induced with AAPH. Various dilutions of blackberry extract (50 μL) were mixed with 0.45 mL of liposomes and 0.2 mL of AAPH (final concentration of 10 mM), and these solutions were incubated in the dark for 2 h at 37 °C. To determine the malondialdehyde (MDA) concentration, 0.2 mL of 5% TCA (trichloroacetic acid) and 1 mL of 0.75% TBA were added, and the samples were heated at 96 °C for 30 min. After cooling, 0.25 mL of 3% SDS was added, and the mixture was centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. The thiobarbituric acid reactive substances (TBARS) concentration was assessed using the molar absorption coefficient for MDA: 1.56×10^5 $cm^{-1} \cdot M^{-1}$. The MDA concentrations were plotted against the sample concentration, and the results were expressed as the amount of extract that inhibits 50% of lipid peroxidation (IC_{50}). The extract was tested in triplicate. Commercial Trolox was used as a reference standard.

Inhibition of Lipid Peroxidation in Liver Homogenates. This procedure was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 19-06). Sprague−Dawley rats (220 g ± 20 g), obtained from LEBi (Laboratorio de ensayos biológicos, Universidad de Costa Rica), were anesthetized with CO_2 and sacrificed by decapitation. The liver tissue of each rat was obtained and homogenized in PBS using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 rpm for 15 min to reduce the amount of suspended solids. Different concentrations of blackberry extract (75 μL) were added to 0.75 mL of liver supernatant and incubated for 30 min at 37 °C. Oxidative stress was then induced with TBHP (*tert*-butyl hydroperoxide) at a final concentration of 1.7 mM and incubated for 1 h at 37 °C, and the TBARS were measured as the end product of lipid peroxidation.

TBARS were assayed according to Mihara and Uchiyama.[25] Briefly, 0.25 mL of liver homogenate, 0.25 mL of 35% TCA, and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added, and the mixture was heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, and the sample was mixed and centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated as described previously, and the results were expressed as nmol MDA/g liver tissue. The MDA concentrations were plotted against the sample concentration to calculate the IC_{50}.

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The assay was performed using liver tissue from five rats. To establish the basal level of lipid peroxidation, the MDA levels without TBPH were assessed in each liver homogenate. Sample blanks were prepared in each experiment, and the extract was tested in triplicate. Commercial Trolox was used as a reference standard.

Inhibition of Superoxide Anion Production. The human promonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Cell No. TIB-202, Rockville, MD, USA). The cells were grown in RPMI 1640-glutamax medium red phenol containing 10% fetal calf serum, 10^5 U/L penicillin/streptomycin, and 10^{-4} g/L fungizon. To differentiate promonocytes, 3.5×10^5 cells/mL were incubated with retinoic acid (10^{-6} M), 1,25-dihydroxycholecalciferol (10^{-7} M), and IFN-γ (10 U/mL). The differentiated monocytes became adherent cells after 72 h.

In the first step, the cells were treated with different concentrations of the extract for 24 h to calculate the IC_{50} of the blackberry phenols. The medium and extract were then removed, and the cells were washed and collected by scraping into RPMI 1640 medium without red phenol. A cell suspension of 1×10^6 cells/mL was prepared and incubated with lucigenin (10^{-4} M) for 30 min, and the monocyte cell suspension was stimulated with PMA (10^{-7} M) to induce O_2^- production. The O_2^- reduced the lucigenin bioluminescence probe, and the luminescence generated was immediately recorded for 1 h at 37 °C using a Victor Wallac Luminometer (WALLAC Co, Turku, Finland). After the luminescence assay, the cells were dissolved in 1 M NaOH to determine the protein concentration (Dc Protein Assay, Bio-Rad, Hercules, CA, USA). The luminescence intensity was normalized to the amount of protein present. The response of PMA-stimulated cells without blackberry extract was used as a control and was considered equal to 100% of the superoxide anion production.

In the second step, to determine the kinetics of O_2^- inhibition activity in the blackberry extract, the same protocol was performed using a unique concentration of 80 μg/mL (defined as the IC_{50}) that was incubated for 15 min, 30 min, 1 h, 4 h, 8 h, and 24 h. Each experiment was performed in triplicate.

Anti-inflammatory Assays. *Cell Viability.* The murine macrophage cell line J774A.1 was obtained from ATCC. The cells were grown in RPMI 1640-glutamax medium containing 5% inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (20 μg/mL).

To test the cytotoxicity, 10^5 cells/well were seeded in a 96-well plate and incubated for 20 h with different concentrations of the blackberry extract. The cells were washed, and each well was filled with 200 μL of medium and 20 μL of a tetrazolium salt, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), mixed with an electron-coupling reagent, PMS (phenazine methosulfate), diluted in PBS. The plate was incubated for 4 h, and the absorbance was measured at 550 nm. The percentage of viable cells was calculated using the absorbance of the control cells without extract as 100%. The extract was tested in triplicate.

Measurement of Nitric Oxide. J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and were pretreated with different concentrations of the blackberry extract for 4 or 24 h. After the treatment, the cells were stimulated for 24 h with a mixture of LPS (10 ng/mL) and murine IFNγ (10 U/mL). The supernatants were collected, and the concentration of nitrite, which reflects the intracellular NO synthase activity, was determined using the Griess reagent, as described above. In some experiments, the extract was eliminated ("Wash") before stimulation with LPS+IFNγ, and in others, it was not ("No-wash"). Each experiment was performed in triplicate.

Western Blotting Analysis. To study the effect of the blackberry extract on iNOS expression, J774A.1 macrophages were pretreated for 24 h with the blackberry extract; the cells were washed with medium and stimulated with LPS (10 ng/mL) and murine IFNγ (10 U/mL) for 10 h. The cells were then washed and incubated with a lysis buffer (1X PBS, 1% Triton X-100, and protease inhibitor cocktail, Roche Applied Science, Mannheim, Germany) for 30 min on ice. The lysed cells were collected by scraping and then centrifuged at 10,000 rpm for 2 min. The supernatant was collected, and the proteins were used for electrophoresis.

A 30-μg sample of protein was mixed with loading buffer (125 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.5% bromophenol blue) and boiled for 10 min. The samples were loaded onto an 8% SDS-polyacrylamide gel and separated at 0.04 A. The proteins were transferred to a nitrocellulose membrane at 0.07 A overnight. The membrane with the proteins was blocked (5 mM Tris, 0.12 mM NaCl, 0.05% Tween, and 5% nonfat dry milk) for 1 h, washed (0.05% TBS-Tween), and incubated with a rabbit polyclonal anti-iNOS (M-19) antibody diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After the incubation, the membrane was washed and incubated with a secondary antibody, antirabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 1 h at room temperature. Lastly, the membrane was washed, and the proteins were detected using a chemiluminescence system (ECL, Amersham, Little Chalfont, Buckinghamshire, U.K.). The band analysis was performed with Image Lab software (Bio-Rad, Hercules, CA, USA). The densitometric analysis of the iNOS bands was normalized to β-actin.

IL-6 Assay. J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well. The cells were pretreated with different concentrations of blackberry extract for 24 h. After the treatment, the cells were washed, and each well was filled with fresh medium and a mixture of LPS (10 ng/mL) and murine IFNγ (10 U/mL). After a 24 h incubation with LPS+IFNγ, the supernatants were collected, and the IL-6 concentration was measured using a murine IL-6 EIA kit (eBioscience, Montrouge, France) following the manufacturer's instructions.

Statistical Analysis. The results from each experiment represent the means ± standard error. An analysis of variance (ANOVA) followed by a post hoc test was used to compare the differences between the antioxidant capacities of blackberry extract, quercetin, and ellagic acid. In addition, an ANOVA was used to compare between the controls and treatments of the blackberry extract in the lipid peroxidation and anti-inflammatory methods. A p-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The HPLC chromatogram of the blackberry extract shown in Figure 1 was similar to that published previously by Mertz et al.[18] These authors performed a tentative identification by HPLC-DAD/ESI-TRAP-MS of the same fruit variety used in this study, and the blackberry samples used in both studies were collected at the same location. Moreover, the present study followed an HPLC protocol that was identical to that of Mertz

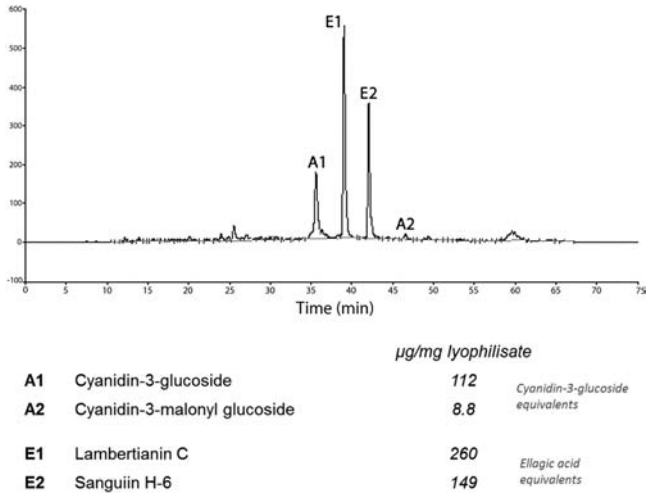

Figure 1. HPLC chromatogram of the blackberry polyphenol extract. (Corresponding putative identification of peaks A1, A2, E1, E2 previously published by Mertz et al.[18])

et al., and the retention times coincide. Correspondingly, our tentative phenolic profile for the blackberry extract includes the anthocyanins cyanidin 3-glucoside and cyanidin-3-malonyl glucoside and the ellagitannins lambertianin C and sanguiin H-6 as the main components. According to the literature, this polyphenol profile is common because cyanidin 3-glucoside is the most widespread anthocyanin in nature and is commonly found in colored fruits;[26] sanguiin H-6 is also the most common ellagitannin found in berries.[27] These types of polyphenols have been described in the literature as key contributors to antioxidant capacity. Anthocyanidins, such as cyanidin-3-glucoside, are known antioxidants due to their hydroxyl substituent in the structure of the B ring.[28] Ellagitannins, due to their high molecular weight structures, exhibit an important ability by donating a hydrogen atom and scavenging unpaired electrons.[27]

Table 1 shows the free radical-scavenging capacity of blackberry phenols assessed by different *in vitro* methods; the activities were compared to a reference radical scavenger flavonoid, quercetin, and to the ellagitannins subunit, ellagic acid. The scavenging activity in the extract against the free radical DPPH was significantly lower than that of quercetin and ellagic acid. The NO-scavenging activity of the extract was significantly higher than that of ellagic acid and lower than that of quercetin; however, in the case of peroxyl radicals measured by ORAC, the antioxidant activity in the extract was significantly higher than those of quercetin and ellagic acid. The differences in the antioxidant capacity evaluated by these assays can be explained by the fact that each method evaluates the scavenging activity against diverse radicals and by different mechanisms.[29−31] One of the factors that determines the radical-scavenging capacity is the chemical structure of the free radical used in each antioxidant assay method: the antioxidant performance will differ because the kinetic and stoichiometric factors related to each free radical are different.[30,31] The DPPH and NO assays use nitrogen radicals, whereas ORAC uses a peroxyl radical. Additionally, the mechanisms are different: DPPH and NO-scavenging involve direct methods, whereas ORAC uses a competition method that involves a probe in addition to the free radical and antioxidant. In addition, the ORAC method uses a peroxyl radical, which is more appropriate to evaluate antioxidants because it has a key role as a chain-carrying radical in lipid peroxidation.[31]

Table 1. Free Radical-Scavenging Capacity of Blackberry Phenol Extract, Quercetin, and Ellagic Acid[a]

	DPPH IC_{50} (μg/mL)	ORAC mM TE/g	NO-scavenging IC_{50} (μg/mL)
blackberry extract	2.57 ± 0.11 a	4.34 ± 0.14 a	24.5 ± 1.3 a
quercetin	2.17 ± 0.08 b	2.07 ± 0.08 b	6.6 ± 0.8 b
ellagic acid	2.22 ± 0.07 b	0.53 ± 0.02 c	298 ± 14 c

[a]Each value is the mean ± SE of three replicate experiments. The means in columns followed by different letters differed significantly ($p < 0.05$).

Despite the fact that lipid peroxides exert physiological beneficial effects, there is ample evidence demonstrating that these molecules also provoke membrane damage and tissue injury and are associated with the progression of many diseases. As discussed by Niki,[32] cellular enzymatic oxidation is a specific process, as opposed to the random oxidation of lipids by free radicals. Accordingly, lipid peroxidation is one of the most extensively researched processes induced by reactive oxygen species (ROS); indeed, it is important to evaluate the role of antioxidants.[33] In this study, the inhibition of lipid peroxidation was first assessed using artificial lecithin liposomes and then using rat liver homogenates. AAPH (in the liposome model) and TBHP (in the liver homogenate model) were used because of their ability to be metabolized into free radical intermediates that cross cellular membranes, leading to the production of highly reactive hydroxyl radicals that initiate lipid peroxidation.[30,34] Figure 2A shows that the blackberry phenols decreased the levels of lipid peroxidation in the liposomes in a dose-dependent manner; the concentration of phenols that was necessary to decrease 50% of the MDA concentration in the control liposomes (IC_{50}) was 7.0 ± 0.5 μg/mL. As shown in Figure 2B, a similar inhibitory effect was observed for the rat liver homogenates, with an IC_{50} of 20.3 ± 4.2 μg/mL. The IC_{50} values in both assays were significantly ($p < 0.05$) better than that of Trolox, a hydrosoluble artificial analog of vitamin E. The Trolox IC_{50} value in the liposomes was 13.3 ± 0.9 μg/mL, and the IC_{50} value was 43.1 ± 0.3 μg/mL in the liver homogenates. Similar *in vitro* results were reported for other polyphenol-rich extracts, and some *in vivo* models also demonstrated that polyphenol intake could prevent lipid peroxidation.[28,34−36] Hwang et al.[34] suggested that protection against lipid peroxidation is a consequence of free radical quenching.

In addition to mediating damage from outside the cell, such as in lipid peroxidation, ROS also modify the redox status

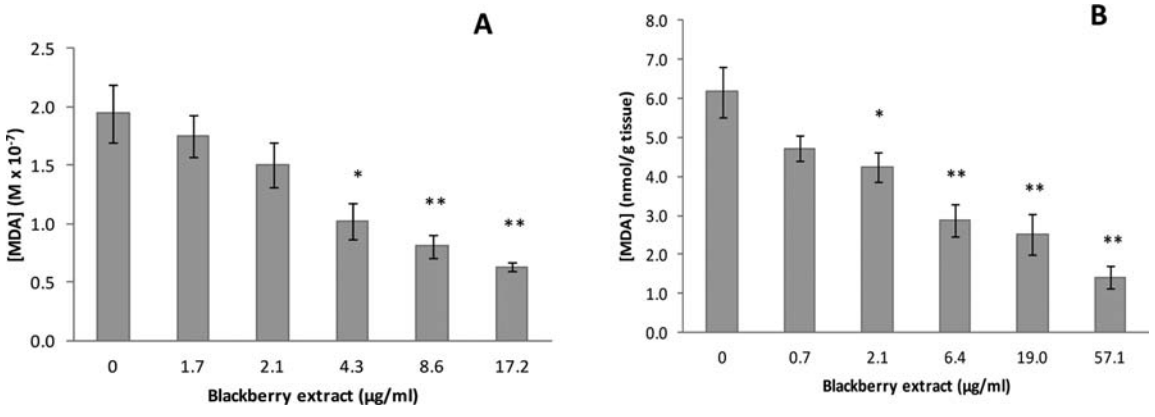

Figure 2. Inhibitory capacity of the blackberry extract against lipid peroxidation in liposomes (A) and in liver homogenates (B). Each value is the mean ± SE (three independent experiments). *$p < 0.05$, **$p < 0.01$ compared to controls without blackberry treatment.

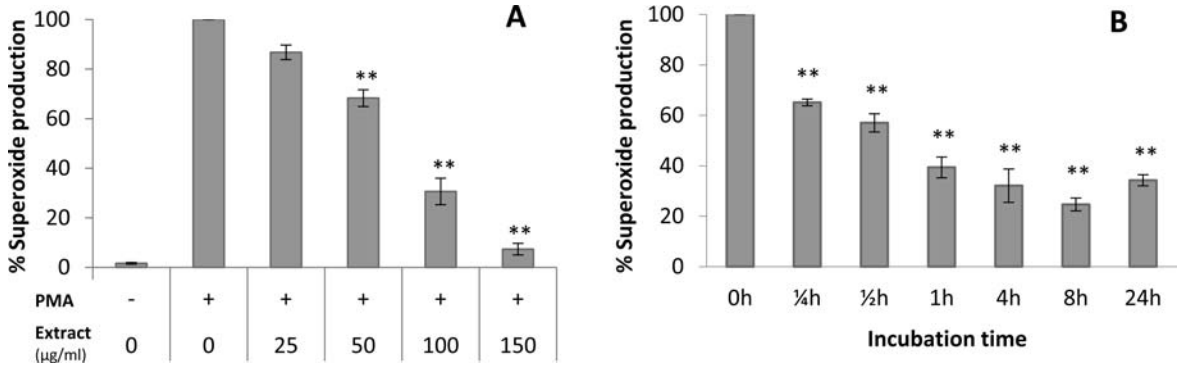

Figure 3. Inhibitory effect of the blackberry extract against the superoxide production induced in THP-1 cells. Dose−effect assay (A) and kinetic assay (B). Each value is the mean ± SE (four independent experiments). *$p < 0.05$, **$p < 0.01$ compared to controls without blackberry treatment.

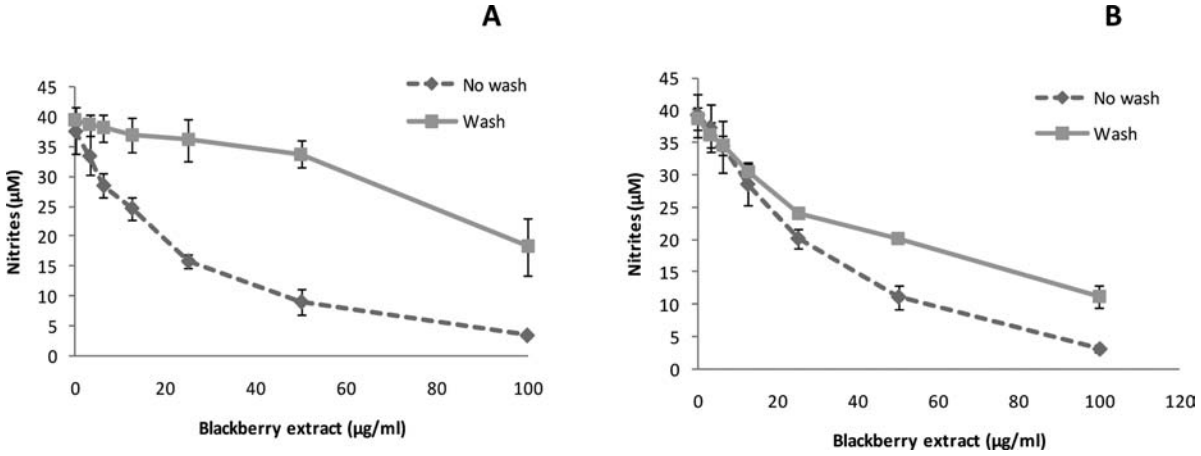

Figure 4. Effect of blackberry extract on LPS+IFNγ-induced nitrite production. Blackberry extract, 4 h pretreatment (A), and blackberry extract, 24 h pretreatment (B). The data are expressed as the means ± SEM from three separate experiments performed in triplicate.

within cells, along with such intracellular ROS as nitric oxide and superoxides, and play important roles in the modulation of inflammation and in immune regulation.[37] Figure 3A shows that the blackberry phenols significantly inhibited the superoxide anion production by NADPH oxidase ($p < 0.05$), from 50 μg/mL up to 150 μg/mL of extract, with an IC_{50} value at 80.3 ± 4.1 μg/mL. Figure 3B shows the kinetics of the superoxide inhibition activity in blackberry extract performed at the IC_{50} value (80 μg/mL). These results clearly show a significant inhibition ($p < 0.05$) of 35%, even after a short incubation time of 15 min. The maximum inhibition (76%) was observed after 8 h of incubation. Similar effects were reported in animal models supplemented with raspberry juice or with pure polyphenols.[38,39]

In addition to the inhibitory effect on superoxide production, the blackberry phenols inhibited nitrite production when used to pretreat macrophage cells later exposed to LPS+IFNγ. Figure 4A shows that 4 h of pretreatment with blackberry phenols reduced NO production in a dose-dependent manner only when LPS+IFNγ was coincubated with the extract (no-wash). If the extract was eliminated prior to the addition of LPS+IFNγ (wash), the inhibition of NO production was observed only at the highest concentration tested. The IC_{50} values after 4 h of incubation were 24.4 ± 3.2 μg/mL and 99.1 ± 7.3 μg/mL under the no-wash and wash conditions, respectively. The different results suggest that the extract only exerts scavenging activity with a 4 h incubation, as was previously confirmed in the NO-scavenging assay.

Figure 4B shows that a 24 h preincubation of the blackberry phenol with macrophage cells later stimulated with LPS+IFNγ decreased NO production both when the extract was incubated simultaneously with LPS+IFNγ and when it was eliminated before the addition of LPS+IFNγ. The IC_{50} values after 24 h of incubation were 30.4 ± 3.7 μg/mL and 45.6 ± 1.2 μg/mL under the no-wash and wash conditions, respectively, suggesting that the extract regulates iNOS expression in cells because the inhibition was found even when the extract was removed. This regulation was confirmed by Western blotting (Figure 5), which showed a concentration-dependent decrease

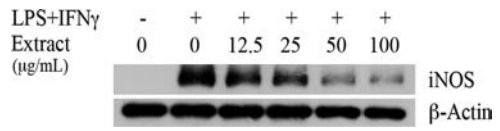

Figure 5. Western blot analysis of the effect of blackberry extract on LPS+IFNγ-induced iNOS expression. Lysed cells were subjected to Western blot using an antibody specific for iNOS. β-actin was used as a quality control.

in iNOS when the cells were incubated with the blackberry extract; the Western blot results show reductions of 29, 40, 74, and 79% at 12.5, 25, 50, and 100 μg/mL extract, respectively. Similar results have been shown in the extracts of other berries and with purified polyphenols.[10,40−42] The blackberry extract alone did not influence nitrite production and iNOS protein

expression in the J774A.1 cells relative to the control cells (data not shown), and blackberry extract concentrations under 100 μg/mL did not result in a loss of J774A.1 cell viability over 20 h of incubation, as assessed by an MTS/PMS assay (data not shown).

The inhibitory effect of blackberry phenols on the production of superoxides and nitric oxide manifests in a decrease in the oxidative stress in the cell, thereby modifying the redox status of the signaling molecules and transcription factors involved in the progression of inflammation. Specifically, the transcriptional regulatory factor NF-*k*B, which is involved in iNOS gene expression, has been reported to be sensitive to ROS.[37,43,44] We suggest that the blackberry radical-scavenging activity demonstrated in this study could decrease the concentrations of different types of ROS; this would prevent, at least in part, the activation of NF-*k*B, thus provoking a decrease in iNOS expression, as was observed by Western blotting and as has been described for other types of phenolic compounds.[45]

Inflammation is a complex process that involves other cytokines in addition to NO, superoxides, and iNOS. For this reason, the capacity of blackberry extract to inhibit IL-6 production was assessed, and the results are shown in Figure 6.

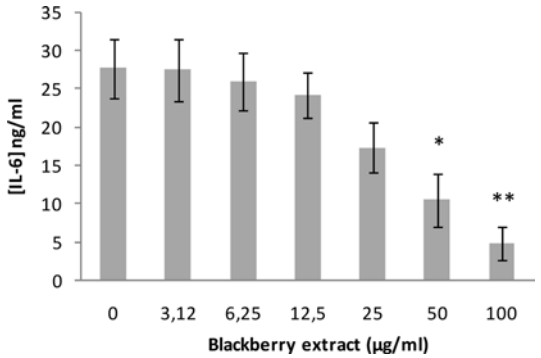

Figure 6. Effect of blackberry extract on LPS+IFNγ-induced IL-6 production. The data are expressed as the means ± SEM from three separate experiments performed in triplicate. *p < 0.05, **p < 0.01 compared to controls without blackberry treatment.

J774A.1 cells treated with the blackberry extract and then exposed to LPS+IFNγ demonstrated a capacity to inhibit IL-6 production. The treatment reduced IL-6 production in a concentration-dependent manner, with differences that were significant (p < 0.05) from the value obtained with the 50 μg/mL concentration. Cho et al.[46] reported similar results for the flavonoid quercetin, demonstrating that quercetin blocks the activation of NF-*k*B and other mitogen-activated protein kinases that regulate cytokine secretion. Further studies must be performed to evaluate the effect of the blackberry extract on other cytokines.

This study demonstrated the potential influence of blackberry phenols on inflammatory processes through their inhibitory properties against superoxides, nitric oxide, and IL-6. However, this influence can be widespread because these molecules also mediate cytotoxic effects, such as DNA damage, LDL oxidation, tyrosine nitration, and the inhibition of mitochondrial respiration.[37,43]

More research is required to consider both the antioxidant capacity of the blackberry polyphenols and also some alternative mechanisms of action.[47] Furthermore, such aspects as polyphenol bioavailability, metabolism, tissue distribution, and deconjugation by gut microbiota, as have been performed for other types of berries, need to be well characterized for blackberries.[47,48] Nonetheless, the blackberry extract evaluated in this paper clearly exhibits a potential benefit with regard to its capacity to protect against oxidative stress and inflammatory processes. This protection is mainly due to the very potent antioxidant activities of the major molecules present in the extract. Some authors suggest that ROS might be upstream targets of polyphenols during pathological processes.[49] Therefore, the inclusion of this fruit in the daily diet would be beneficial, and the fruit could be promoted as a functional food.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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