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ARTICLE

Antioxidant Capacity and in Vitro Inhibition of Adipogenesis and Inflammation by Phenolic Extracts of *Vaccinium floribundum* and *Aristotelia chilensis*

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Interest in berries from South America has increased due to their potential health benefits. The objective of this study was to characterize the anthocyanins and proanthocyanidins of *Vaccinium floribundum* and *Aristotelia chilensis*, total phenolics, and antioxidant capacity and to evaluate, in vitro, the ability of their phenolic extracts to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes. The anti-inflammatory property of these extracts on RAW 264.7 macrophages was also investigated. Antioxidant capacity, measured as oxygen radical scavenging capacity and expressed as Trolox equivalents, was higher in the berries of *A. chilensis*. Phenolic extracts inhibited lipid accumulation by 4.0−10.8% when adipocytes were treated at maturity and by 5.9−37.9% when treated throughout differentiation. Furthermore, a proanthocyanidin-enriched fraction from *V. floribundum* significantly increased Pref-1 expression in preadipocytes. Phenolic extracts decreased the production of nitric oxide (3.7−25.5%) and prostaglandin E_2 (9.1−89.1%) and the expression of inducible nitric oxide synthase (9.8−61.8%) and cycloxygenase-2 (16.6−62.0%) in lipopolysaccharide-stimulated RAW 264.7 macrophages. *V. floribundum* and *A. chilensis* phytochemicals limit adipogenesis and inflammatory pathways in vitro, warranting further in vivo studies.

KEYWORDS: *Aristotelia chilensis*; *Vaccinium floribundum*; adipogenesis; anthocyanins; inflammation; proanthocyanidins; obesity

INTRODUCTION

Berries and their derived products have shown a positive impact on metabolic syndrome related conditions, including cardiovascular diseases, diabetes, and inflammation (*1*, *2*). Their biological properties have been largely attributed to high levels of various phenolic compounds, as well as to the interactive synergies between their natural phytochemical components (*3*). Interest in berries from South America has increased in recent years mainly due to their potential health benefits and growing consumer interest in novel exotic fruit selections in the marketplace (*4*).

Aristotelia chilensis is a fruit-bearing shrub that thrives in the temperate forests of central to southern Chile and western Argentina. It belongs to the Elaeocarpaceae family and is commonly known as "maqui". *A. chilensis* yields a small edible purple/black berry averaging 5 mm in diameter with typically 3−4 seeds. The leaves and fruits of *A. chilensis* have been used in folk medicine to treat a variety of ailments including sore throat, kidney pains, ulcers, fever, inflammation, and diarrhea (*5*, *6*). Studies on the phytochemical composition of the berry of *A. chilensis* have indicated the presence of phenolic acids, proanthocyanidins, and anthocyanins as well as other flavonoids (*7*, *8*). The berry of *A. chilensis* has been shown to inhibit low-density lipoprotein (LDL) oxidation and to protect against intracellular oxidative stress in human endothelial cells and against acute isochemia/reperfusion in vivo in rat hearts (*9*, *10*).

Vaccinium floribundum, commonly known as "mortiño", is a deciduous, spreading shrub that belongs to the family Ericaceae. It bears a round blue to nearly black edible berry of about 8 mm in diameter. This berry is found mostly in northern South America, where it grows at elevations from 1800 to 3800 m (*11*). In Ecuador, local communities have used this plant to treat various medical conditions such as diabetes and inflammation (*12*). The chemical composition and the phenolic profile of *V. floribundum* have been reported, revealing predominantly quercetin, hydroxycinnamic acids, and cyanidin-3-glucosides (*13*). There are no papers concerning the biological properties of this berry.

Within recent decades, the incidence of obesity has increased drastically (*14*) and has become a worldwide health concern due to its association with an increased risk of morbidity and mortality (*15*, *16*). Obesity is a complex metabolic disorder that results from an imbalanced energy intake and energy expenditure, leading to an increase in adipocyte size and number (*17*). The amount of adipose tissue can be reduced by inhibiting adipogenesis and fat deposition (*18*). Adipogenesis is the cellular transition through which a fibroblastic cell first develops into a preadipocyte and finally into a mature adipocyte (*19*). Obesity is

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also associated with a chronic inflammatory state characterized by abnormal production of cytokines and the activation of inflammatory signaling pathways (*20*). Furthermore, chronic inflammation plays a crucial role in the development of metabolic disorders linked to obesity, including insulin resistance and atherosclerosis (*21*, *22*). Two important enzymes involved in activating the inflammatory response are inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). iNOS and COX-2 can catalyze the synthesis of nitric oxide (NO) and prostaglandin E_2 (PGE_2), respectively, which in turn cause sepsis, sepsis shock, and systemic inflammatory response syndrome (*23*). Therefore, inhibition of the expression of these enzymes or of their products can help reduce inflammation and related conditions.

In this study, we investigated the in vitro antioxidant capacity (AC) of *V. floribundum* and *A. chilensis* berry extracts, characterized their anthocyanin and proanthocyanidin constituents, and evaluated the ability of their phenolic fractions to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes. In addition, the anti-inflammatory properties were evaluated in vitro using lipopolysaccharide-stimulated (LPS) RAW 264.7 macrophages by investigating the NO and PGE_2 production as well as iNOS and COX-2 expression. A commercial powder of *V. floribundum* was also evaluated and compared to the freeze-dried berry extracts.

MATERIALS AND METHODS

Materials. Ripe *A. chilensis* berries were collected in January 2009 from the Entrelagos region in Chile (S 40° 40′ 48.5″, W 72° 33′ 43.3″). Ripe *V. floribundum* berries were collected during late November 2008 in the grasslands of Simiatug, Ecuador. The berries were cleaned by removing leaves, stems, and damaged berries. The whole berries were freeze-dried, sealed in plastic bags, and shipped to our laboratory. In addition to the collected berries, a *V. floribundum* commercial powder (Mortiño, a natural product produced by Simiatug Samai) was purchased from a local market in Quito, Ecuador. This powder was prepared by dehydrating fresh berry at low temperatures (< 45 °C) and high ventilation. The berries and the commercial powder were stored at −80 °C until usage.

Swiss albino mouse 3T3-L1 fibroblasts, macrophage RAW 264.7 cell line, and Dulbecco's modified Eagle's medium (DMEM) were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). Isobutylmethylxanthine (IBMX), dexamethasone (DEX), insulin, sodium pyruvate solution, penicillin (1000 U/mL), streptomycin (1000 U/mL), sodium nitrite, sulfanilamide, *N*-1-(naphthyl)ethylenediamine-diHCl, LPS from *Escherichia coli* O55:B5, Trolox, Folin−Ciocalteu's phenol reagent, 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH), Amberlite XAD-7 resin, Oil Red O, trans-4-carboxy-5-octyl-3-methylene-butyrolactone (C75, > 98% purity), and epigallocatechin gallate (EGCG, ≥95% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 was purchased from GE Life Sciences (Buckinghamshire, U.K.). Actin mouse mAb epitope mapping at the C-terminus of actin of human origin, COX-2 mouse mAb against amino acids 580−598 of human COX-2 and inducible iNOS mouse mAb epitope mapping at the C-terminus of mouse iNOS, and DLK goat pAb epitope mapping at the C-terminus of human Pref-1/DLK-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG horseradish peroxidase conjugate secondary antibody was purchased from GE Healthcare (Buckinghamshire, U.K.). All solvents for high-performance liquid chromatography (HPLC) were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA).

Preparation of the Phenolic-Rich Extract. Berry extraction and fractionation were performed on the basis of procedures developed by Grace et al. (*24*). The freeze-dried berries and the commercial powder were blended with 80% ethanol acidified with 0.3% trifluoroacetic acid (TFA). The extract was then filtered through cotton, followed by Whatman no. 4 and then no. 1 filter papers with the aid of suction. The collected hydroalcoholic extract was evaporated using a rotary evaporator at a temperature not exceeding 40 °C. The aqueous concentrate was partitioned with ethyl acetate (4 × 500 mL) to remove lipophilic material.

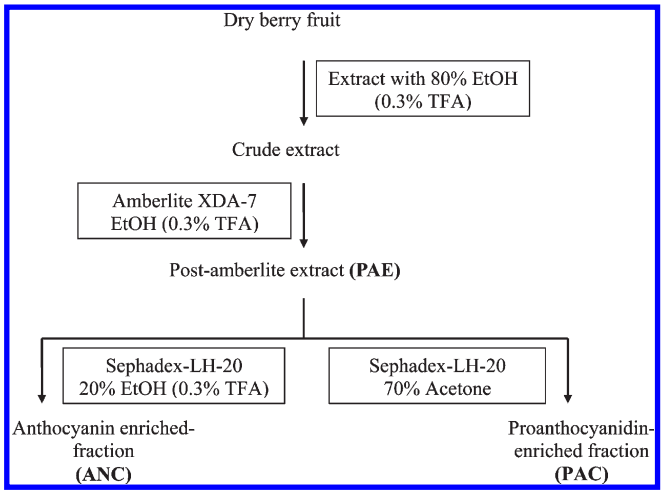

Figure 1. Flowchart showing extraction and anthocyanin and proanthocyanin enrichment process. Abbreviations: EtOH, ethanol; TFA, trifluoroacetic acid; PAE, post-Amberlite extract; ANC, anthocyanin-enriched fraction; PAC, proanthocyanidin-enriched fraction. Adapted from Grace et al. (*24*).

The aqueous layer was retained and loaded onto an Amberlite XAD-7 column (30 × 10 cm) preconditioned with acidified water (0.3% TFA). The resin was washed thoroughly with acidified water (0.3% TFA, ∼3 L) to remove free sugars, pectins, and phenolic acids. The polyphenolic mixture was then eluted with acidified ethanol (0.3% TFA), evaporated, and freeze-dried to yield post-Amberlite extract (PAE).

Preparation of Anthocyanin- and Proanthocyanin-Enriched Fractions. The process used to prepare anthocyanin- and proanthocyanidin-enriched fractions is illustrated in **Figure 1**. The enriched fractions were prepared by placing 2 g of the PAE on a Sephadex LH-20 column (30 × 3 cm). Anthocyanins were obtained from an isocratic elution of 20% aqueous ethanol acidified with 0.3% TFA. The column was then washed with 70% aqueous acetone to elute polymeric proanthocyanidins. All fractions were concentrated and freeze-dried to yield the anthocyanin-enriched (ANC) or proanthocyanidin-enriched (PAC) fraction.

Anthocyanin and Proanthocyanidin Analysis. Anthocyanin separation was conducted on an 1100 HPLC (Agilent Technologies, Santa Clara, CA) using a Supelcosil-LC 18 column (250 × 4.6 mm, 5 μm) (Supelco, Bellefonte, PA). Samples were prepared by dissolving 5 mg of extract or enriched fractions in 1 mL of methanol and filtering through 0.45 μm nylon filters (Fisher Scientific) before injection. The mobile phase consisted of 5% formic acid in H_2O (A) and 100% methanol (B). The flow rate was held constant during the sample analyses at 1 mL/min with a step gradient of 10, 15, 20, 25, 30, 60, 10, and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47, and 60 min, respectively. The same instrumentation was used to separate proanthocyanidins. Mobile phase consisted of 94.9% H_2O, 5% acetonitrile, and 0.1% formic acid (A) and of 94.9% acetonitrile, 5% H_2O, and 0.1% formic acid (B). The flow rate was held constant at 1 mL/min with a step gradient of 0, 5, 15, 30, 60, 90, 0, and 0% of solvent B at 0, 3, 40, 45, 50, 55, 47, and 60 min, respectively. Anthocyanins and proanthocyanins were detected at 520 and 280 nm using a diode array detector (DAD), respectively. Chemstation software (Agilent Technologies Inc.) was used for both protocol control and data processing.

The HPLC-ESI-MS analyses were made with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (m/z 150−2000), with a photodiode array (PDA) detector (200−600 nm), version 1.2, autosampler version 1.2, and Xcalibur software for data processing. The HPLC separations were carried out on a C-18 reversed-phase column (150 mm, 2.1 mm i.d., particle size = 5 μm, 90 Å) (VYDAC, Western Analytical, Murrieta, CA). The analysis was carried out using mobile phase consisting of 94.9% H_2O, 5% acetonitrile, and 0.1% formic acid (A) and of 94.9% acetonitrile, 5% H_2O, and 0.1% formic acid (B), with a step gradient of 5, 30, 60, 90, 90, 5, and 5% of solvent B at 0, 40, 45, 50, 55, and 60 min, respectively. A constant flow rate of 200 μL/min and an injection volume of 5 μL were employed. Samples were prepared by dissolving 5 mg of extract or enriched fraction in 1 mL of methanol and

filtering through 0.45 μm nylon filters before injection. Acquisition of LC-PDA-MS data was performed and processed using XCalibur Qual Browser v1.4 software (Thermo Electron Corp., Waltham, MA).

The total anthocyanin content of the two berries (*A. chilensis* and *V. floribundum*) and the commercial powder was calculated as cyanidin-3-glucoside equivalents (C3G equivalents). Three concentrations of the standard at 0.25, 0.5, and 1.0 mg/mL were used to quantify the anthocyanins using peak areas measured by HPLC at 520 nm. The identification of anthocyanins was based on comparison with published data (*7*,*8*,*13*,*24*), MS spectral data, and commercial standards. In the same way, the total proanthocyanidin content of the two berries and the commercial powder was calculated as epicatechin equivalents from the peak area measured at 280 nm with the exclusion of 520 nm wavelength peaks, which were anthocyanins.

Total Polyphenol Content (TP). Total phenolic content was measured using the Folin−Ciocalteu method, adapted to a microassay, from the method described by Chandra and de Mejia (*25*). Briefly, to a 96-well flat-bottom plate were added 50 μL of 1 N Folin−Ciocalteu phenol reagent and 50 μL of either sample, standard, or blank; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na_2CO_3. The solution was then allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multiwell plate reader (Biotek Instruments, Winooski, VT). Results were expressed as catechin milligram equivalents, using the standard curve $y = 0.011x - 0.071$, $R^2 = 1$.

Antioxidant Capacity (AC). AC was measured by the oxygen radical absorbance capacity (ORAC) assay (*26*, *27*). Briefly, aliquots of 20 μL of sample, Trolox standards dissolved in 75 mmol/L phosphate buffer, pH 7.4, or 75 mmol/L phosphate buffer, pH 7.4, blank were added to a 96-well black-walled plate. This was followed by the addition of 120 μL of 17 nmol/L fluorescein. The plate was then incubated for 15 min at 37 °C, and then 60 μL of 153 mmol/L AAPH was added. The plate was read in a Synergy 2 multiwell plate reader (Bio-Tek), at 37 °C and sensitivity 60, every 2 min for 120 min with excitation at 485 nm and emission at 582 nm. Results were expressed as millimoles of Trolox equivalents, using the standard curve $y = 0.23x + 1.11$, $R^2 = 0.99$.

Effect of Phenolic Extracts on Adipogenesis and Lipid Metabolism in 3T3-L1 Adipocytes in Vitro. *Cytotoxicity Assay*. All treatments were assayed for cytotoxicity before lipid quantification or Pref-1 assays. CellTiter 96AQueous One Solution was used to determine the number of viable cells according to the manufacturer's manual (Promega, Madison, WI). Briefly, the CellTiter 96AQueous One Solution (20 μL) was added to 100 μL of media containing wells (with cells), and then the plate was incubated in a 5% CO_2 incubator at 37 °C. After 2 h, absorbance was measured at 515 nm with a 96-well plate reader (Biotek Instruments). Cell viability was calculated using the following equation:

$$A_{\text{treatment,515nm}}/A_{\text{control,515nm}} \times 100 = \%\text{cell viability}$$

Cell Culture and Treatments. The 3T3-L1 preadipocytes were seeded at 3×10^4 cells/well in 24-well plates and cultured in DMEM containing 10 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% FBS (FBS/DMEM medium). For preadipocyte differentiation, 2 days after reaching 100% confluence, the cells were stimulated with FBS/DMEM medium containing 167 μmol/L insulin, 0.5 M IBMX, and 1 M DEX for 2 days. Cells were then maintained in FBS/DMEM medium with 167 nmol/L insulin for another 2 days, followed by culturing with FBS/DMEM medium for an additional 4 days, at which time up to 90% of cells were mature adipocytes with accumulated fat droplets. To study the effect of the phenolic extracts on lipid metabolism, mature adipocytes received a single treatment of 100 μmol/L (C3G or epicatechin equivalents) PAE, ANC, and PAC of the two berries and the commercial powder; 10 days after the initiation of differentiation for 48 h, lipid accumulation was quantified.

To monitor the effect of these extracts on adipogenesis, adipocytes were treated throughout the differentiation process at days 2, 4, 6, 8, and 10; when adipocytes matured, lipid accumulation was quantified. In addition, EGCG and C75 (100 μmol/L) were used as positive controls. All treatments were dissolved in 0.01% DMSO. The concentrations used were based on preliminary data from a cytotoxicity assay (data not shown), making sure that cell viability was at least 80%.

Lipid Quantification in 3T3-L1 Adipocytes by Oil Red O Assay. Briefly, treated mature adipocytes were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 10% formalin (in DPBS) in 24-well plates for 1 h. Then, cells were washed with 60% isopropanol and allowed to air-dry. The Oil Red O stock solution (6:4 v/v with water) was added to lipid droplets for 1 h. After Oil Red O lipid staining, cells were washed with water four times and were air-dried. Oil Red O dye was eluted by adding 100% isopropanol and then incubating at room temperature for 10 min. The absorbance of the resulting eluant was measured at 510 nm using a Synergy 2 multiwell plate reader (Bio-Tek). Inhibition of lipid accumulation in adipocytes was calculated using the following equation:

$$(A_{\text{control,510nm}} - A_{\text{treatment,510nm}})/A_{\text{control,510nm}} \times 100 = \%\text{inhibition of lipid accumulation}$$

Pref-1 Expression by Western Blotting. Preadipocytes were seeded at a concentration of 1.0×10^5 cells/well in 6-well plates and cultured with FBS/DMEM. After 24 h, the cells were treated with 100 μmol/L (C3G or epicatechin equivalent) PAE, ANC, and PAC of the two berries and the commercial powder for 24 h. Following the procedure by Kellogg et al. (*28*) treated preadipocytes were lysed in sample loading buffer (Laemmli buffer with 5% β-mercaptoethanol) and sonicated using an ultrasonic cell disruptor from Misonix Inc. (Farmingdale, NY). After lysis, the cell lysates were boiled for 5 min and separated via electrophoresis on 4−20% Tris-HCl ready gels (Bio-Rad Laboratories). Gels were run on a PowerPac 300 (Bio-Rad) at 200 V for 30 min. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 0.1% SDS) using Western sandwich assembly for 1 h at 4 °C using 110 V. The membrane was then blocked with 5% nonfat dry milk (NFDM) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at 4 °C. After blocking, the membrane was washed with TBST and incubated with 10 mL of anti-Pref-1 goat polyclonal IgG antibody (1:2000 in TBST with 1% NFDM) at 4 °C overnight. The membrane was washed again and incubated with 10 mL of ECL anti-goat IgG horseradish peroxidase conjugate (1:1000 in TBST with 1% NFDM) for 3−4 h at room temperature. The membranes were washed a final time in TBST. Pref-1 expression was visualized using chemiluminescent reagent (GE Life Sciences, Piscataway, NJ) following the manufacturer's instructions. The membrane picture was taken with a Kodak Image station 440 CF (Eastman Kodak Co., New Haven, CT). Pref-1 expression was calculated as the ratio between Pref-1 and actin band intensity.

Effect of Phenolic Extracts on Inflammatory Markers. *Cytotoxicity Assay*. All of the cell treatments were assayed for cytotoxicity before any inflammatory marker assays were performed. CellTiter 96AQueous One Solution was used (Promega) as mentioned previously.

Cell Culture and Treatments. Macrophage cell line RAW 264.7 was seeded at 2×10^5 cells/well in 6-well plates and cultured in FBS/DMEM medium at 37 °C in 5% CO_2/95% air. After 48 h of incubation, cells were treated with 100 μmol/L (C3G or epicatechin equivalent) of PAE, ANC, and PAC of the two berries and the commercial powder and 1 μg/mL of LPS for 24 h. In addition, quercetin (100 μmol/L) was used as a positive control. Treatments were dissolved in 0.01% DMSO. The concentrations used were based on preliminary data from a cytotoxicity assay (data not shown), making sure that cell viability was at least 80%. After 24 h of treatment, the spent medium was collected and analyzed for NO and PGE_2. Cell lysates were used to study the effect of the phenolic extracts on the expression of COX-2 and iNOS.

Measurement of NO and PGE_2 Production. NO production was determined by measuring the level of nitrite accumulation (the stable metabolite of NO) in the spent medium. For nitrite measurement, 100 μL of the spent medium was plated in a 96-well plate, and an equal amount of Griess reagent (1% w/v sulfanilamide and 0.1% w/v *N*-1-(naphthyl)-ethylenediamine-diHCl in 2.5% v/v H_3PO_4) was added. The plate was incubated for 5 min and the absorbance measured at 550 nm. The amount of NO was calculated using the following sodium nitrite standard curve: $y = 0.14x + 0.09$, $R^2 = 0.99$. For PGE_2 measurement, a PGE_2 ELISA kit monoclonal was used following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Table 1. Total Anthocyanins, Total Proanthocyanidins, Total Phenolics, and Antioxidant Capacity of *A. chilensis* (Maqui) Fruits and Phenolic Extracts

	anthocyanins[a] (%)	proanthocyanidins[b] (%)	total phenolics[c] (mg/g)	antioxidant capacity[d] (mmol/g)	yield[e]
freeze-dried berries	4.6 ± 0.1[f]	0.4 ± 0.3[f]	53.3 ± 5.8 d[g]	0.3 ± 0.1 d[g]	NA
post-Amberlite extract (PAE)	58.4 ± 0.7	5.1 ± 0.4	632.6 ± 5.8 b	7.5 ± 0.1 b	7.8
anthocyanin-enriched fraction (ANC)	79.8 ± 1.7	≥0.1	573.7 ± 13.0 c	9.5 ± 0.3 a	2.9
proanthocyanidin-enriched-fraction (PAC)	≥0.1	49.3 ± 3.2	973.2 ± 36.9 a	9.8 ± 0.5 a	0.6

[a] Expressed as cyanidin-3-glucoside equivalents. [b] Expressed as epicatechin equivalents. [c] Catechin equivalent estimated by Folin−Ciocalteu. [d] Trolox equivalents estimated by ORAC. [e] Grams of extract or enriched-fraction obtained per 100 g of dried berry. NA, not applicable. [f] Data from phenolic-rich extracts were converted to freeze-dried fruit weight basis. [g] The dried sample was homogenized in 80% ethanol containing 0.3% TFA, kept overnight at 4 °C, and centrifuged. The ethanolic extract was used to measure TP and AC. The data represent the mean ± SD from at least two independent studies and at least a triplicate analysis. Values within a column followed by different letters are significant at $p < 0.05$.

Table 2. Total Anthocyanins, Total Proanthocyanidins, Total Phenolics, and Antioxidant Capacity of *V. floribundum* Freeze-Dried Berries, the Commercial Powder, and Their Phenolic Extracts

	anthocyanins[a] (%)	proanthocyanidins[b] (%)	total phenolics[c] (mg/g)	antioxidant capacity[d] (mmol/g)	yield[e]
freeze-dried berries	1.1 ± 0.1[f]	0.5 ± 0.1[f]	53.0 ± 1.6 e[g]	0.2 ± 0.01 f[g]	NA
post-Amberlite extract (PAE)	11.1 ± 0.5	5.3 ± 0.5	524.4 ± 4.5 d	8.3 ± 0.4 c	9.8
anthocyanin-enriched fraction (ANC)	15.7 ± 0.2	≥0.1	711.2 ± 21.2 c	10.6 ± 0.9 a	2.1
proanthocyanidin-enriched fraction (PAC)	≥0.1	54.3 ± 2.4	869.2 ± 15.1 b	9.2 ± 0.3 b	0.4
commercial powder	0.2 ± 0.1[g]	0.5 ± 0.03[g]	18.1 ± 0.2 f	0.1 ± 0.03 g[g]	NA
post-Amberlite extract (PAE)	2.3 ± 0.6	4.6 ± 0.3	495.6 ± 9.1 d[g]	3.3 ± 0.1 d	10.2
anthocyanin-enriched fraction (ANC)	4.3 ± 0.6	≥0.1	927.2 ± 7.6 a	10.1 ± 0.1 a	0.1
proanthocyanidin-enriched fraction (PAC)	≥0.1	49.2 ± 1.1	788.1 ± 3.2 b	4.9 ± 0.4 d	0.8

[a] Expressed as cyanidin-3-glucoside equivalents. [b] Expressed as epicatechin equivalents. [c] Catechin equivalent estimated by Folin−Ciocalteu. [d] Trolox equivalents estimated by ORAC. [e] Grams of extract or enriched-fraction obtained per 100 grams of dried berry. NA, not applicable. [f] Data from phenolic-rich extracts were converted to freeze-dried fruit weight basis. All data represent the mean ± SD from at least two independent studies and at least a triplicate analysis. [g] The dried sample was homogenized in 80% ethanol containing 0.3% TFA, kept overnight at 4 °C, and centrifuged. The ethanolic extract was used to measure TP and AC. Values within a column followed by different letters are significant at $p < 0.05$.

Western Blot Analysis. COX-2 and iNOS expressions were determined in cell lysates. The primary antibodies used were COX-2 or iNOS mouse monoclonal antibodies (1:200) and anti-mouse IgG horseradish peroxidase conjugate as the secondary antibody (1:1000). All other steps were performed as described above. The expression of these enzymes was calculated as the ratio between COX-2 or iNOS and actin band intensity.

Statistical Analysis. Data were expressed as means of at least two independent replicates. Results were compared by one-way analysis of variance (ANOVA) using the proc GLM function of Statistical SAS version 9.2 (SAS Institute Inc., Cary, NC). Group means were considered to be significantly different at $p < 0.05$. Mean separation was achieved through the least significant difference (LSD) procedure in SAS.

RESULTS AND DISCUSSION

Polyphenolic Composition. Table 1 shows the total anthocyanins, total proanthocyanidins, total phenolics, and antioxidant capacity of *A. chilensis* berries and their phenolic extracts. **Table 2** shows the total anthocyanins, total proanthocyanidins, total phenolics, and antioxidant capacity of *V. floribundum* berries, the commercial powder, and their phenolic extracts.

Anthocyanins. The berry of *A. chilensis* contained seven main anthocyanin structures: delphinidin-3-sambubioside-5-glucoside (*m*/*z* 759), delphinidin-3,5-diglucoside (*m*/*z* 627), cyanidin-3-sambubioside-5-glucoside (*m*/*z* 743), delphinidin-3-sambubioside (*m*/*z* 597), delphinidin-3-glucoside (*m*/*z* 465), cyanidin-3-sambubioside (*m*/*z* 581), and cyanidin-3-glucoside (*m*/*z* 449). This was in agreement with previous reports (*7*, *8*). An HPLC chromatogram illustrating these structures in the PAE is shown in **Figure 2A**, and the peak assignment and the concentration of individual anthocyanins in the PAE and ANC for *A. chilensis* are presented in **Table 3**. Delphinidin-3-glucoside (peak 5) was the main anthocyanin component present in this berry. HPLC analysis indicated that berries from *A. chilensis* contain 45.7 mg/g of DW (C3G equivalents). This value is higher than that previously reported by Escribano et al. (*7*) and Cespedes et al. (*8*), who reported on average about 2.5 mg/g of DW (C3G equivalents). This difference in anthocyanin concentration in part may be explained by differences in the time and place of collection of *A. chilensis* berries in the previous studies. The berries analyzed for this study were collected at the most southern location in Chile. The time and place of harvest can influence growing conditions, which have been shown to affect the composition of these fruits (*29*, *30*).

Berries and commercial powder of *V. floribundum* contained the same anthocyanin profiles (**Figure 2 B**,**C**). Five anthocyanins were identified in the berries of *V. floribundum*: delphinidin-3-galactoside (*m*/*z* 465), cyanidin-3-galactoside (*m*/*z* 449), delphinidin-3-arabinose (*m*/*z* 435), cyanidin-3-glucoside (*m*/*z* 449), and cyanidin-3-arabinose (*m*/*z* 419). This was in agreement with previous studies (*13*). Delphinidin-3-arabinose (peak 2) and cyanidin-3-arabinose (peak 5) were found to be the main anthocyanins in this berry. The individual concentrations of anthocyanins present in the PAE and ANC of the berry and the commercial powder of *V. floribundum* are presented in **Table 4**. HPLC analysis indicated that the total anthocyanin content of this berry was 10.6 mg/g of DW (C3G equivalent), which is comparable to the value previously reported (*13*). The commercial powder of *V. floribundum*, although maintaining the same anthocyanin profile, showed a lower anthocyanin content (2.4 mg/g of DW C3G equivalents) as compared with the freeze-dried berry. This was consistent with several studies that indicated that processing of anthocyanin-containing foods can lead to anthocyanin degradation (*31*).

Proanthocyanidins. The berry of *A. chilensis* contained 4.0 mg/g of DW (epicatechin equivalents), whereas the freeze-dried berries and commercial powder from *V. floribundum* had slightly higher concentrations of 5.2 and 4.8 mg/g of DW (epicatechin equivalents), respectively. HPLC-MS analysis revealed a series of proanthocyanidins ranging from dimers to hexamers. The proportion of proanthocyanidin oligomers and

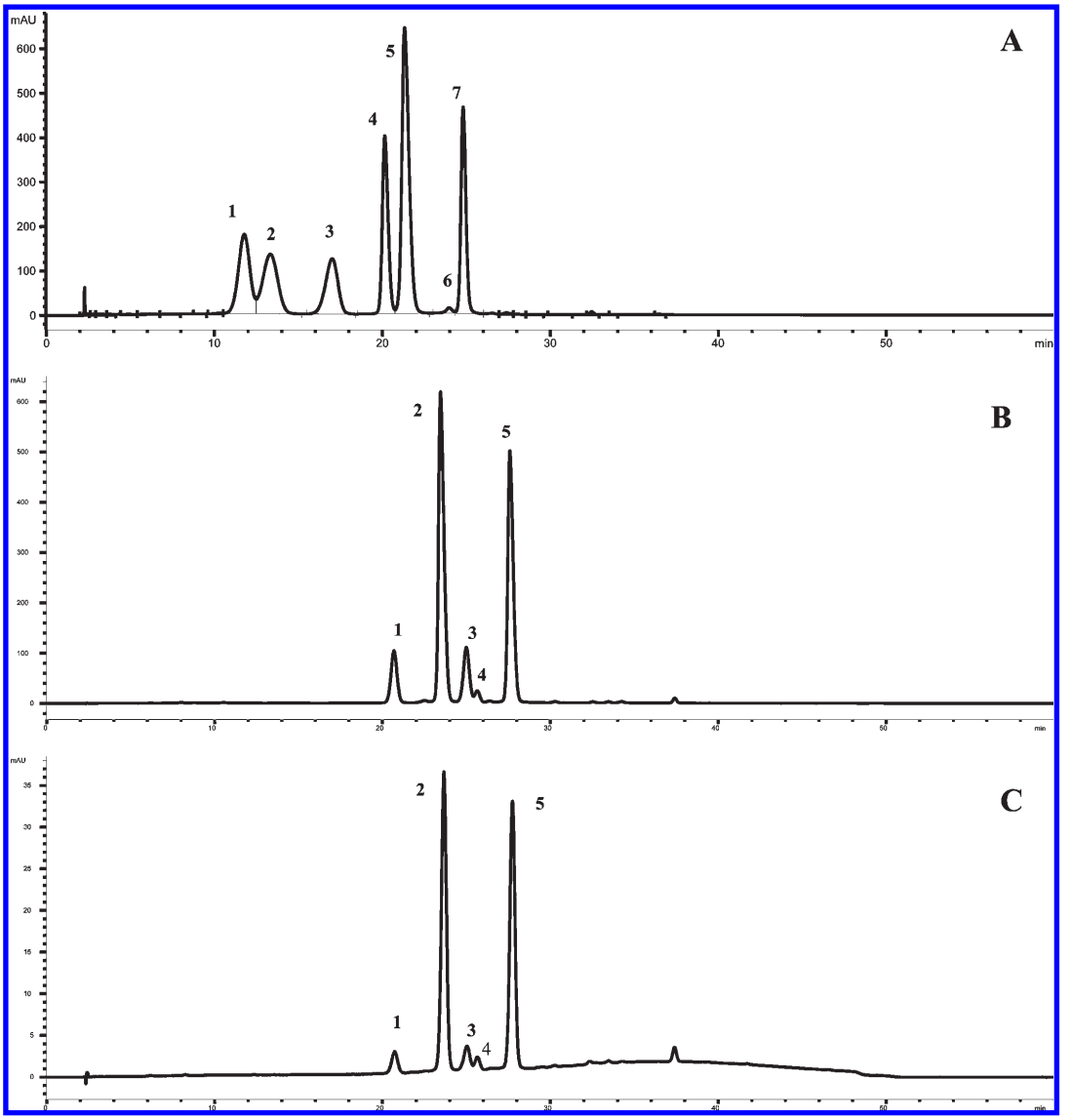

Figure 2. HPLC chromatograms of the post-Amberlite extract (PAE) of *A. chilensis* (**A**), *V. floribundum* (**B**), and the commercial powder of *V. floribundum* (**C**). The identities of the compounds associated with the anthocyanin peaks shown here are given in **Tables 3** and **4**, respectively.

Table 3. Identification and Content of Anthocyanins in the Post-Amberlite Extract (PAE) and Anthocyanin-Enriched Fraction (ANC) of *A. chilensis*

peak	anthocyanin	(*m*/*z*)	PAE[a] (%)	ANC[a] (%)
1	delphinidin-3-sambubioside-5-glucoside	759/597/465/303	8.4	11.9
2	delphinidin-3,5-diglucoside	627/465/303	7.5	11.0
3	cyanidin-3-sambubioside-5-glucoside	743/581/449/287	6.1	8.7
4	delphinidin-3-sambubioside	597/303	9.3	12.9
5	delphinidin-3-glucoside	465/303	17.5	22.3
6	cyanidin-3-sambubioside	581/287	0.3	0.4
7	cyanidin-3-glucoside	449/287	9.3	12.5
	total		58.4	79.7

[a] Percentages were calculated as cyanidin-3-glucoside equivalents.

Table 4. Identification and Content of Anthocyanins in the Post-Amberlite Extract (PAE) and in the Anthocyanin-Enriched Fraction (ANC) of the Freeze-Dried (F) and Commercial Powder (C) of *V. floribundum*

			PAE[a] (%)		ANC[a] (%)	
peak	anthocyanin	(*m*/*z*)	F	C	F	C
1	delphinidin-3-galactoside	465/303	1.0	0.1	1.4	0.3
2	cyanidin-3-galactoside	449/287	4.8	0.8	7.0	2.9
3	delphinidin-3-arabinose	435/303	1.0	0.2	1.4	0.1
4	cyanidin-3-glucoside	449/287	0.4	0.2	0.4	0.2
5	cyanidin-3-arabinose	419/287	3.9	0.9	5.5	0.8
	% total		11.1	2.2	15.7	4.3

[a] Percentages were calculated as cyanidin-3-glucoside equivalents.

polymers varied among the two berries and the commercial powder. To estimate the relative ratio of the proanthocyanidins, the HPLC-MS peaks were summed, and each peak was expressed as a percentage of the total sum (*32*). On the basis of this estimation, *A. chilensis* contained mainly dimers (56%) and trimers (14%). Small MS peaks of proanthocyanidin tetramers, pentamers, and hexamers were also detected. *V. floribundum* contained trimers (68%) and in less proportion pentamers (16%) and hexamers (8%). The commercial powder contained a larger percentage of proanthocyanidin oligomers, mainly dimers (34%) and trimers (23%). **Figure 3** illustrates the UV chromatogram, at 280 nm absorption, and the ESI/MS spectra

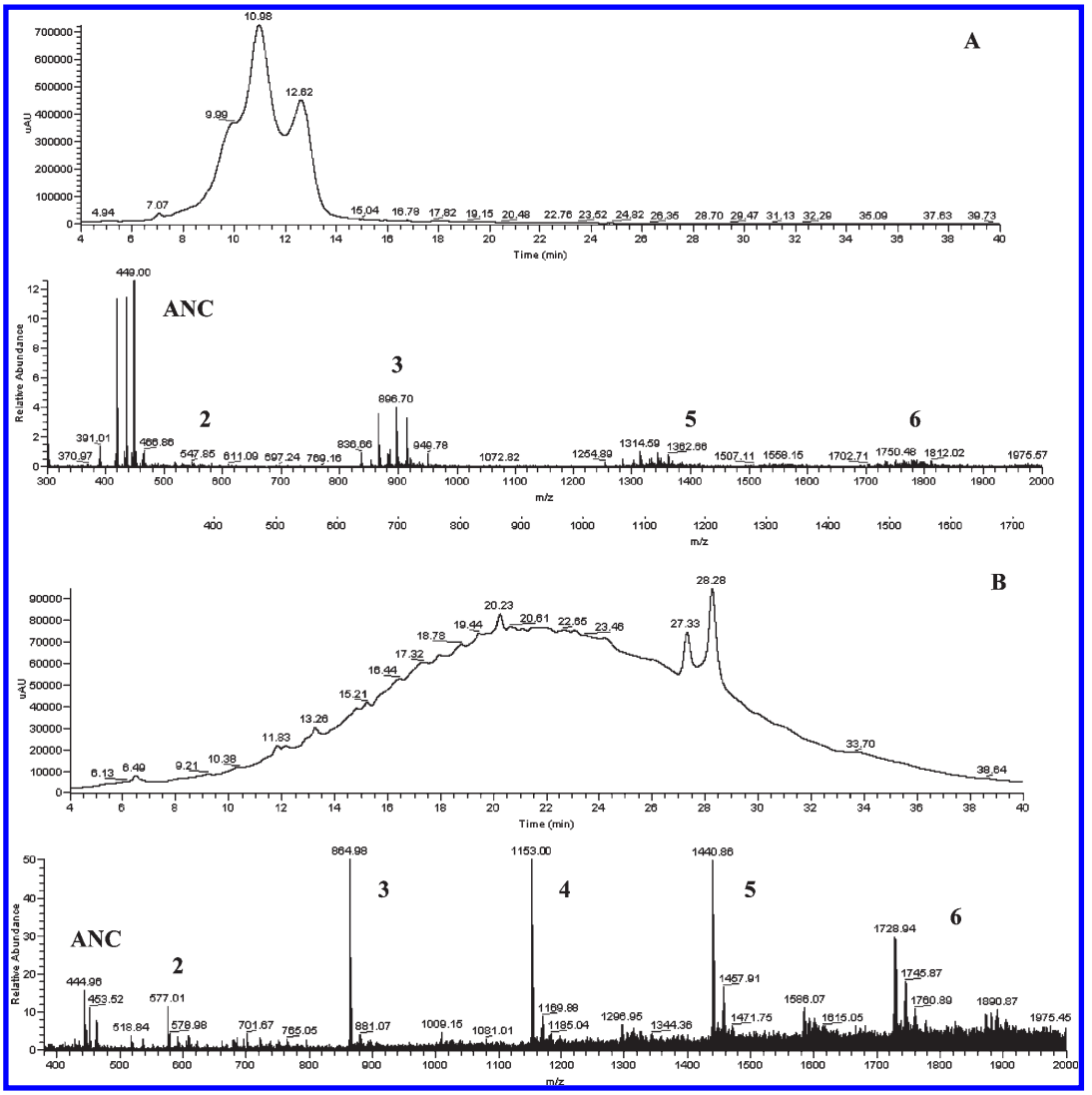

Figure 3. HPLC-ESI/MS chromatogram of *V. floribundum* (**A**) post-Amberlite extract (PAE) and (**B**) proanthocyanidin-enriched (PAC). UV chromatogram at 280 nm and ESI/MS spectra are presented. The bold numbers indicate the average degree of polymerization from dimers to hexamers, and ANC indicates the presence of anthocyanins.

of the PAE (**A**) and the PAC (**B**) of *V. floribundum*. In the PAE, anthocyanins are present at a higher proportion in comparison with proanthocyanidins oligomers and polymers (**Figure 3A**). After the enrichment process (**Figure 3B**), the proportion of proanthocyanidins increases, displaying a series of proanthocyanidins ranging from dimers to hexamers. In the UV chromatogram of the PAC (**Figure 3B**) a large hump characteristic of proanthocyanidins can be visualized.

Antioxidant Capacity. The berries of *A. chilensis* and *V. floribundum* showed similar values of total phenolics expressed as catechin equivalents (**Tables 1** and **2**). However, the AC measured as oxygen radical scavenging capacity and expressed as Trolox equivalents was higher in the berries of *A. chilensis*, indicating that the constituents of this berry have greater AC. When the berry and the commercial powder of *V. floribundum* were compared, results indicated that the phenolic content and the AC of the commercial powder decreased, and this is likely to be a consequence of processing. Total phenolics and AC correlated for *A. chilensis* and both the *V. floribundum* berries and commercial powder with R^2 values of 0.90, 0.86, and 0.78, respectively. Anthocyanins were more highly correlated with AC than proanthocyanins.

Effect of Phenolic Extracts on Lipid Metabolism and Adipogenesis in 3T3-L1 Adipocytes in Vitro. Figure 4 shows the percent inhibition of lipid accumulation for both the single treatment on mature adipocytes and treatment throughout their differentiation process with 100 μmol/L (C3G or epicatechin equivalents) of the phenolic extracts (PAE, ANC, and PAC) from the two berries and the commercial powder and at 100 μmol/L of positive controls (EGCG and C75). Lipid accumulation was inhibited from 4 to 11% when adipocytes received a single treatment and from 6 to 38% when adipocytes were treated throughout the differentiation process. The highest lipid accumulation inhibition (38%) was observed when adipocytes were treated throughout the differentiation process with PAC from *V. floribundum*. **Figure 5** shows 3T3-L1 adipocytes after treatment with PAC from *V. floribundum* in comparison to the negative control. It can be visualized that most cells remain with the shuttle shape characteristic of preadipocytes (**Figure 5A**) as compared with the oval or round adipocytes seen in the control (**Figure 5B**).

Inhibition of lipid accumulation highly correlated with AC of *A. chilensis* phenolic extracts (R^2 = 0.99) when adipocytes were treated throughout the differentiation process. On the other hand, the proanthocyanidin-enriched fraction of *V. floribundum*, the most potent to inhibit lipid accumulation, did not present high AC when compared to the other phenolic extracts. These results lead us to the conclusion that, despite AC having been associated

with a reduction in lipid accumulation (*33*), in the present study there was not sufficient evidence to state a general conclusion that AC contributes to the potential of phenolic extracts to reduce lipid accumulation; this depends on the type of phenolic compound. The potential of phenolic compounds in proving an antioxidative role in association with lipid accumulation has been previously studied (*33*). Berniakovich et al. (*33*) found that intracellular oxidative stress might accelerate aging by favoring fat deposition and fat-related disorders. They suggested that regulated generation of H_2O_2 has evolved to control energy conservation, because oxidative signals promote fat accumulation. In this regard, antioxidant capacity will reduce fat accumulation. Dietary supplementation of orange juice significantly reduced body weight gain and fat accumulation regardless of the increased energy intake because of sugar content (*34*). Only the anthocyanin extract, but not the purified C3G, slightly affected fat accumulation. They found that the antiobesity effect on fat accumulation cannot be explained only by its anthocyanin content.

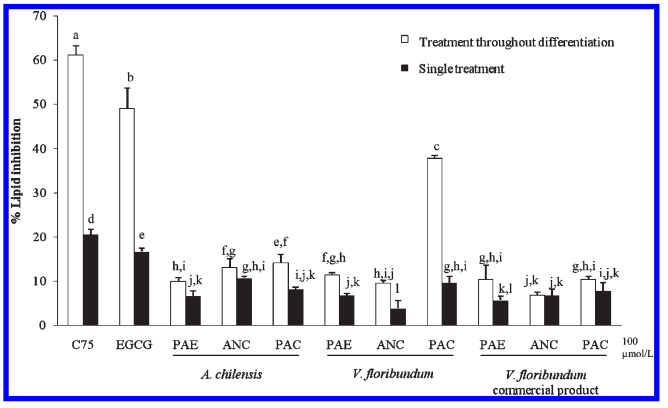

Figure 4. Effect of phenolic extracts (PAE, ANC, and PAC) of the two berries and the commercial powder and positive controls (EGCG and C75) on lipid accumulation inhibition when 3T3-L1 adipocytes were treated throughout the differentiation process and when mature adipocytes received a single treatment at mature stage. 3T3-L1 adipocytes were treated with 100 μmol/L of each phenolic extract (equivalent C3G or epicatechin) and positive controls for 48 h at 37 °C in a humified 5% CO_2 incubator. Cells were harvested 10 days after initiation of differentiation, and lipid quantification was performed by Oil Red O assay. The data represent the mean ± SD from at least two independent studies and at least a triplicate analysis. Different letters indicate significant difference, $p < 0.05$. Abbreviations: ECGC, epigallocatechin-3-gallate; PAE, post-Amberlite extract; ANC, anthocyanin-enriched; PAC, proanthocyanidin-enriched.

As shown in **Figure 4**, lipid accumulation inhibition was higher when adipocytes were treated continuously throughout the differentiation process. To further study the effect of these extracts on adipogenesis, Pref-1 expression was analyzed. Pref-1 is a transmembrane protein highly expressed in preadipocytes that inhibits the initiation of adipogenesis (*35*, *36*). The ability of a phenolic extract to increase or maintain the expression of Pref-1 can indicate its potential to inhibit adipogenesis (*37*). The effect of the phenolic extracts on Pref-1 expression in 3T3-L1 preadipocytes when treated for 24 h is shown in **Figure 6**. The PAC from *V. floribundum* showed a significant increase ($p < 0.05$) in Pref-1 expression in comparison with the control. The PAC of *A. chilensis* and of the commercial powder also increased Pref-1 expression, although these treatments were not statistically different than the control ($p > 0.05$). No correlation was found between the AC of the phenolic extracts and Pref-1. The only fraction that significantly increased the expression of Pref-1 (proanthocyanidin-enriched fraction of *V. floribundum*) did not show the highest AC; thus, it was concluded that this fraction increases Pref-1 expression by mechanisms different from AC.

The present study showed that the phenolic extracts of the two berries were more efficient in reducing lipid accumulation in vitro through the inhibition of adipogenesis than through regulation of lipid metabolism at the mature stage of adipocytes. It was also shown that PAC of *V. floribundum* is very potent in inhibiting adipogenesis, reaching inhibition levels close to that of EGCG, a known adipogenesis inhibitor (*38*). This observation is supported by previous studies that have shown that proanthocyanidin-rich extracts inhibit differentiation in vitro (*39*) and in vivo (*40*). In addition, the PAC of *V. floribundum* increased the expression of Pref-1 in preadipocytes, indicating that this is one of the potential mechanisms by which it inhibits adipogenesis. This is in accordance with other studies which show that proanthocyanidins can increase Pref-1 expression in 3T3-L1 (*39*). The PAC of *A. chilensis* and the commercial powder of *V. floribundumof* also showed a modest inhibition in adipogenesis as well as an elevation in Pref-1 expression; nevertheless, their effects were not as potent as PAC of *V. floribundum*. HPLC-MS analysis performed in this study indicated that PAC of *V. floribundum* had a higher degree of polymerization than *A. chilensis* and the commercial powder. Several studies have demonstrated that higher molecular weight proanthocyanidins have a greater biological activity than smaller weight forms (*41*). Thus, the higher degree of polymerization of proanthocyanidns found in *V. floribundum* PAC may partially explain its higher inhibitory effect on adipogenesis. Nevertheless,

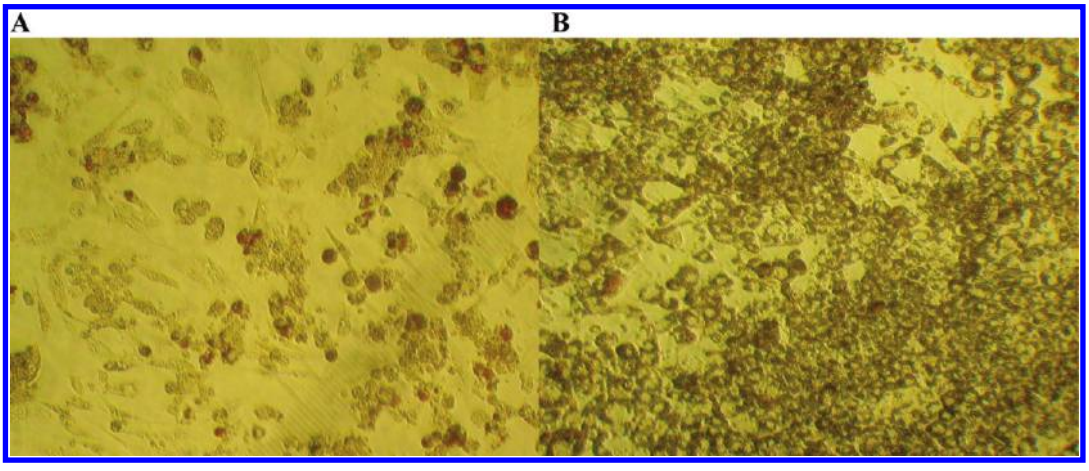

Figure 5. Visualization of 3T3-L1 mature adipocytes when treated with 100 μmol/L equivalent epicatechin of PAC from *V. floribundum* throughout the differentiation process (**A**) compared to the negative control (no PAC treatment) (**B**). Images were photographed at 20× magnification.

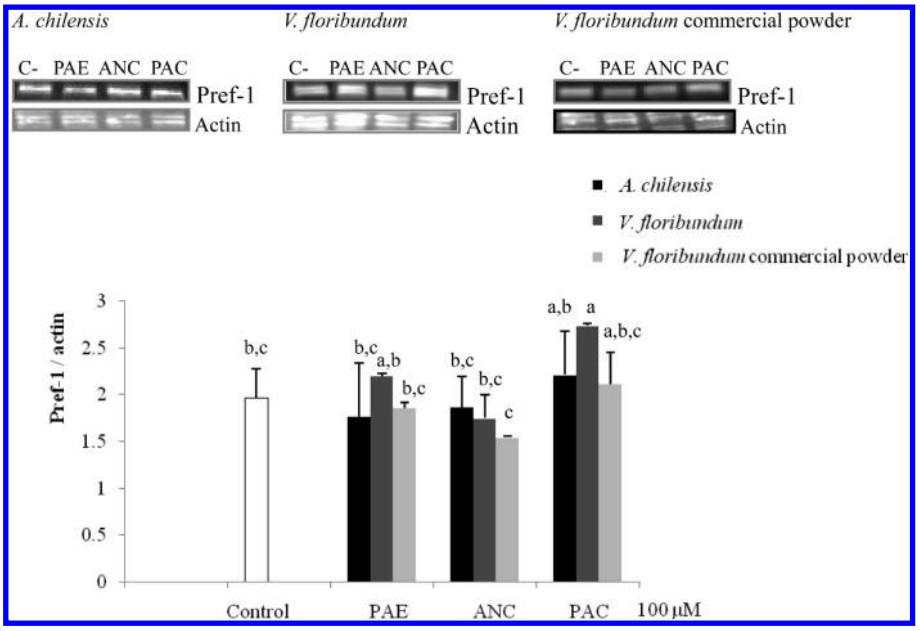

Figure 6. Effect of phenolic extracts (PAE, ANC, and PAC) of the two berries and commercial powder on Pref-1 expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with 100 μmol/L of each phenolic extract (C3G or epicatechin equivalents) for 24 h at 37 °C in a humidified 5% CO_2 incubator. Cells were harvested 24 h after treatment, and Pref-1 expression was analyzed by Western blotting. The data represent the mean ± SD from at least two independent studies and at least a triplicate analysis. Different letters indicate significant difference, $p < 0.05$. Abbreviations: PAE, post-Amberlite extract; ANC, anthocyanin-enriched; PAC, proanthocyanidin-enriched.

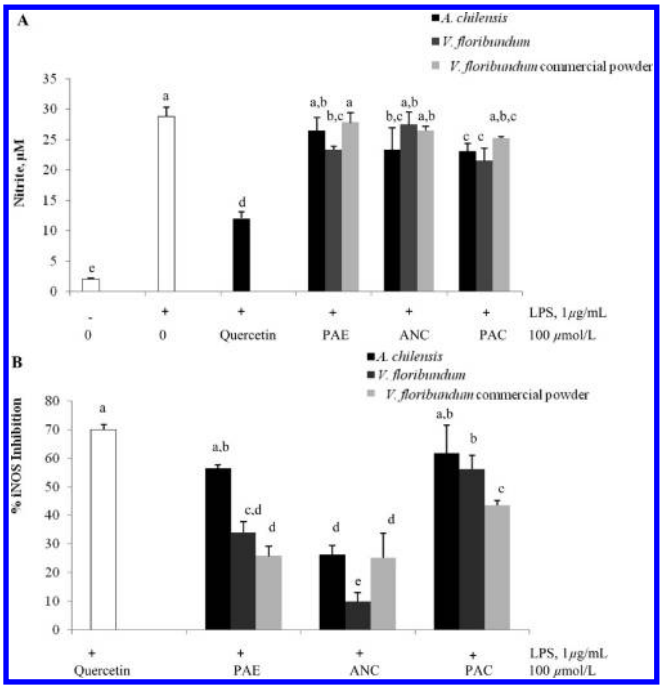

Figure 7. Effect of 100 μmol/L (C3G or epicatechin equivalents) of phenolic extracts (PAE, ANC, and PAC) of the two berries and commercial powder (quercetin was used as a positive control) on nitrite production (**A**) and iNOS protein expression by Western blot (**B**) in LPS-stimulated RAW 264.7 macrophages. The data represent the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p < 0.05$. Bars indicate SD. Abbreviations: PAE, post-Amberlite extract; ANC, anthocyanin-enriched; PAC, proanthocyanidin-enriched.

further characterization of proanthocyanidins is needed to better understand the relationship between the proanthocyanidins' structure and their role in adipogenesis (*40*, *42*).

Effect of Phenolic Extracts on Inflammatory Responses in RAW 264.7 Macrophages in Vitro. Figure 7A shows that nitrite production was significantly inhibited by four of the nine phenolic extracts as compared to positive control ($p < 0.05$). PAC fractions of both berries showed the highest inhibitory effect. In addition, all of the phenolic extracts inhibited iNOS expression from 9.8 to 61.8% (**Figure 7B**). PAC from *A. chilensis* showed the highest inhibitory effect (61.8%), which is comparable to the effect exerted by quercetin, a potent anti-inflammatory agent. To further understand the role of these phenolic extracts on inflammation, the production of PGE_2 and the expression of COX-2 were also determined on LPS-stimulated RAW 264.7 macrophages. **Figure 8** shows the effect of the phenolic extracts of the two berries and the commercial powder on the production of PGE_2 and the expression of COX-2. **Figure 8B** shows eight of the nine phenolic extracts significantly suppressed the production of PGE_2 as compared to the positive control ($p < 0.05$). The ANC fraction from *V. floribundum* showed the highest inhibition (89.1%), which was comparable to that of quercetin. In addition, all of the phenolic extracts inhibited the expression of COX-2 at levels that ranged from 16.6 to 62.0%. The PAC fraction of the commercial powder and the ANC fraction from *V. floribundum* showed the highest inhibitory effects (62 and 58%, respectively).

The results of this study showed that the phenolic extracts from the two berries and the commercial powder decreased inflammation in vitro by inhibiting LPS-induced iNOS/NO and COX-2/PGE_2 pathways in macrophages. This is in accordance with several other studies that have shown berries possess anti-inflammatory properties (*43*, *44*). Furthermore, anthocyanins have been shown to reduce the levels of inflammatory mediators in vitro (*44*−*46*) and in vivo (*47*−*50*). Proanthocyanidins have also been shown to ameliorate inflammation by modulating cytokine expression or inhibiting pro-inflammatory enzymes or through other mechanisms (*51*−*54*).

Monomeric phenols have been widely studied in vivo with regard to their absorption, bioavailability, and metabolism; however, there is not much information about bioavailability of

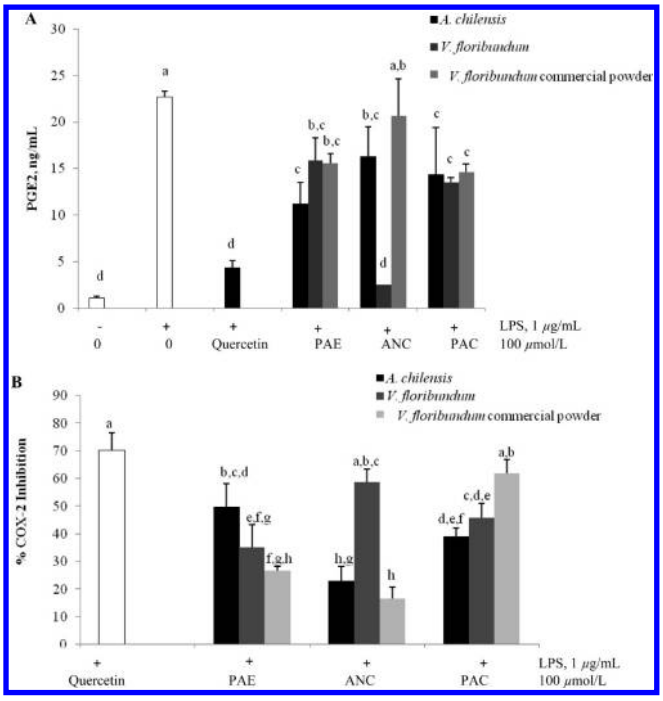

Figure 8. Effect of 100 μmol/L (C3G or epicatechin equivalents) of phenolic extracts (PAE, ANC, and PAC) of the two berries and commercial powder (quercetin was used as a positive control) on PGE_2 production (**A**) and COX-2 protein expression by Western blot (**B**) in LPS-stimulated RAW 264.7 macrophages. The data represent the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p < 0.05$. Bars indicate SD. Abbreviations: PAE, post-Amberlite extract; ANC, anthocyanin-enriched; PAC, proanthocyanidin-enriched.

polymeric proanthocyanidins, and the existing results are controversial (*55*). Previous papers have shown that supplementation of 320 mg/day/kg of C3G or 160 mg/day/kg of anthocyanins through food pellets reduced fat accumulation in mice. Tsang et al. (*56*) found catechins and proanthocyanidins up to trimers in urine of rats after oral intake of grape seed extract. Also, dimeric procyanidins were found in human plasma after the consumption of cocoa (*57*). Human volunteers consumed 80 g of chocolate, and from 2.02 mmol of total proanthocyanidin intake only 2.02 μmol was excreted in urine as microbial metabolites (*58*). A most recent study has shown that proanthocyanidins are degraded by the gut microflora before absorption in rats (*59*). However, it is possible for some types of proanthocyanidins to pass through the entire gastrointestinal tract largely intact, especially if their molecular weight is large. Also, some polyphenols may have the ability to bind strongly to various molecules in the colonocytes and dietary macromolecules in the colonic lumen, which could lead to their underestimation in human bioavailability. Clinical data on humans are still limited (*55*).

The potential role of modulation found in the present in vitro study adds interesting new data that need to be confirmed in an in vivo study.

In summary, phenolic extracts from the berries of *A. chilensis* and *V. floribundum* and the commercial powder of *V. floribundum* inhibited lipid accumulation, adipogenesis, and inflammatory mediators in vitro. PAC fraction from *V. floribundum* markedly inhibited adipogenesis in 3T3-L1 adipocytes by increasing the expression of Pref-1 in preadipocytes. Furthermore, the phenolic extracts inhibited the expression of LPS-induced iNOS/NO and COX-2/PGE_2 pathways in RAW 264.7 macrophages. The potency of these extracts to reduce inflammation in vitro depends on their phytochemical composition. The results of this study show that *A. chilensis* and *V. floribundum* contain phytochemicals that limit adipogenesis and lipid accumulation in fat cells and inflammatory pathways in macrophages in vitro, and therefore further research in vivo should be conducted on these berries.

ABBREVIATIONS USED

AAPH, 2,2-azobis(2-amidinopropane dihydrochloride); AC, antioxidant capacity; ANC, anthocyanin-enriched; C3G, cyanidin-3-glucoside; C75, trans-4-carboxy-5-octyl-3-methylene-butyrolactone; COX-2, cyclooxygenase-2; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; DW, dry weight; EGCG, epigallocatechin gallate; FBS, fetal bovine serum; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; HPLC-ESI/MS, high-performance liquid chromatography−electrospray ionization−mass spectrometry; IBMX, isobutylmethylxanthine; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; LPS, lipopolysaccharide; NO, nitric oxide; PAC, proanthocyanidin-enriched; PGE_2, prostaglandin E_2; PAE, post-Amberlite extract; PDA, photodiode array; PVDF, polyvinylidene difluoride; S, Sephadex LH-20; SDS, sodium dodecyl sulfate; SG, silica gel type G; TBST, Tris-buffered saline with Tween 20; TFA, trifluoroacetic acid; Toyopearl HW-40; TP, total polyphenol.

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