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Juice and Phenolic Fractions of the Berry *Aristotelia chilensis* Inhibit LDL Oxidation *in Vitro* and Protect Human Endothelial Cells against Oxidative Stress

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Oxidative modification of low-density lipoprotein (LDL) particles is a key event in the development of atherosclerosis. Oxidized LDL induces oxidative stress and modifies gene expression in endothelial cells. Berries constitute a rich dietary source of phenolic antioxidants. We found that the endemic Chilean berry *Aristotelia chilensis* (*ach)* has higher phenol content and scores better for total radical-trapping potential and total antioxidant reactivity in *in vitro* antioxidant capacity tests, when compared to different commercial berries. The juice of *ach* is also effective in inhibiting copper-induced LDL oxidation. In human endothelial cell cultures, the addition of *ach* juice significantly protects from hydrogen peroxide-induced intracellular oxidative stress and is dose-dependent. The aqueous, anthocyanin-rich fraction of *ach* juice accounts for most of *ach*'s antioxidant properties. These results show that *ach* is a rich source of phenolics with high antioxidant capacity and suggest that it may have antiatherogenic properties.

KEYWORDS: Oxidative stress; phenolic antioxidants; LDL oxidation; endothelial cell; *Aristotelia chilensis*

INTRODUCTION

An increasing number of studies suggest that the consumption of fruits, vegetables, and beverages rich in phenolic antioxidants protects against cardiovascular disease (*1*−*5*). The oxidation of low-density lipoproteins (LDL) is considered an early event in the development of atherosclerosis (*6*, *7*); therefore, it is tempting to relate the beneficial effects of fruit and vegetables on coronary heart decease to the capacity of their phenolics to inhibit LDL's oxidation (*8*). Oxidized LDL induces oxidative stress and modifies gene expression in endothelial cells, acting on all key events that develop the fatty streak lesion in the arterial wall (*2*, *7*, *9*). Fruit phenols also protect endothelial cells directly from oxidative stress induced by different stressors (*10*).

The numerous beneficial effects attributed to phenolics (*11*−*13*) has given rise to a new interest in finding vegetal species with high phenolic content and relevant biological activity. Berries constitute a rich dietary source of phenolic antioxidants (*14*, *15*). After a preliminary screening for antioxidant capacity, we selected the endemic Chilean berry *Aristotelia chilensis* (*ach)*, an edible black-colored fruit produced by a bush with many branches, which grows in dense thickets. Chilean traditional medicine attributes healing properties to *ach*. In addition, six indole alkaloids (*16*) were described in *ach*. Here we show that *ach* has an exceptionally high content of phenolics with high antioxidant capacity, which protect both LDL from oxidation and endothelial cells from intracellular oxidative stress.

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MATERIALS AND METHODS

Sample Preparation. Concentrated berry juices were a kind gift of Bayas del Sur S.A. (Purranque, Chile). Sugar values and varieties were as follow: *Aristotelia chilensis (ach)*, concentrate 70.0 brix, fruit 18.0 brix; blackberry (*Rubus* spp., var. Chilean wild type and Black Satin), concentrate 65.0 brix, fruit 10.3 brix; blueberry (*Vaccinium corymbosum*, var. High Bush, Elliot, Rabbit Eye), concentrate 65.0 brix, fruit 12.8 brix; cranberry (*Vaccinium macrocarpon*, var. Pilgrim, Stevens, Bergman), concentrate 50.0 brix, fruit 5.4 brix; raspberry (*Rubus idaeus*, var. Heritage, Meeker, Glen Clova), concentrate 65.0 brix, fruit 10.5 brix; strawberry (*Fragaria* spp., var. Chandler, Camarossa, Douglas, Aiko), concentrate 50.0 brix, fruit 10.4 brix. The ascorbic acid contents of the berry concentrates, given in milligrams per 100 mL, were as follow: ach, not detected; blackberry, 2.27; blueberry, 2.22; cranberry, 5.61; raspberry, 116; strawberry, 3.72. The red wine used was a Chilean Cabernet Sauvignon.

Juice fractions were prepared by a modification of the method reported by Salagoity-Auguste and Bertrand (*17*). In brief, 4 mL of juice diluted to approximately 200 mg/mL gallic acid equivalents (GAE) was adjusted to pH 7.0 with 1 N NaOH and extracted three times with 2 mL of ethyl acetate. Each extraction was performed with a 30-min agitation under darkness, collecting the organic phase. After the third extraction, the organic phase was evaporated to dryness under nitrogen and dissolved in HPLC-grade methanol. This is the neutral fraction. The remaining aqueous phase was adjusted to pH 2.0 with 1 N HCl and extracted under the same conditions as before. The organic phase obtained is the acid fraction, and the remaining aqueous phase contains the anthocyanins. Neutral and acid fractions were dissolved in methanol−0.01% HCl. Therefore, for the dichlorofluorescein (DCF) assay (see below), the solutions were prepared to expose all wells to

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equal amounts of methanol−0.01% HCl. Total phenol content in juices and fractions was measured according to a modification of the Folin−Ciocaulteau method (*18*). A 50-μL aliquot of each sample, diluted 50−300 times, was mixed with 3 mL of distilled water and 250 μL of Folin−Ciocalteau reagent (Merck, Darmstad, Germany), followed by the addition of 750 μL of 20% (w/v) Na_2CO_2 and 950 μL of distilled water. After vortex agitation, the mixture was incubated for 35 min at room temperature. Absorbance was measured at 765 nm, and concentration was calculated using gallic acid (Sigma, St. Louis, MO) as a reference standard. Values were corrected for sugar content in the diluted samples, according to standard correction factors (*19*), and were not corrected for protein content.

Cell Culture. Human umbilical vein cells (HUVECs) were isolated using a modification of the method repported by Jaffe et al. (*20*). HUVECs were cultured over a 0.2% gelatin coat in medium A: Dulbecco's Modified Eagle Medium (DMEM) containing low glucose and L-glutamine, supplemented with 100 units/mL penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL anphotericin B, 5% fetal bovine serum, 15% newborn calf serum, 50 μg/mL heparin (Sigma), and 10 ng/mL basic fibroblast growth factor (bFGF, a gift from C. J. Nascimento, Chiron Corp., Emeryville, CA). For DCF assay, medium B was used: DMEM without phenol red and without supplementation, because we have noticed that serum or additional proteins interfere with the fluorescence emission. All incubations with cells were performed at 37 °C in a humidified 5% CO_2/95% air atmosphere. Cells were used at passages 3−7.

Determination of Ascorbic Acid Content. The method of ion-pairing RP-HPLC with electrochemical detection was used for the measurement of ascorbic acid (*21*, *22*). The liquid chromatography system consisted of a Merck-Hitachi (Tokyo, Japan) pump, model L-6000, a Supelco (Bellefonte, PA) 250-mm × 4.6-mm LC-18 column, and a BAS (West Lafayette, IN) electrochemical detector, model LC-4C, equipped with a glassy carbon electrode. The detector potential was set at +0.65 V, with a sensitivity setting of 50 nA vs an Ag/AgCl reference electrode. The mobile phase was 40 mM sodium acetate, 0.54 mM Na_2EDTA, 1.5 mM dodecyltriethylammonium bromide, and 15% methanol, taken to pH 4.75 with glacial acetic acid. Elution was isocratic at a flow rate of 1 mL/min and at ambient temperature. Prior to the injection into HPLC, the diluted juice was filtered in a Sep-pack cartridge (Waters, Milford, MA) to eliminate anthocyanins and flavonoids.

Total Radical-Trapping Potential (TRAP) and Total Antioxidant Reactivity (TAR) Assays. Berry juice antioxidant capacity was evaluated from luminol-enhanced chemiluminescence measurements employing two procedures: the TRAP method developed by Wayner et al. (*23*) and the TAR method (*21*). 2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH, from Wako Chemicals, Richmond, VA) was used as the free radical source, and luminol (Sigma) chemiluminescence was used to monitor the reaction (*24*, *25*). The standard used was Trolox (Sigma), a vitamin E analogue, and the results were expressed as micromoles per liter Trolox equivalents. Buffer A (60 μM luminol, 10 mM AAPH for TRAP or 2 mM AAPH for TAR, in 50 mM glycine buffer, pH 9.40) was kept in ice under darkness before use. For each determination, 1 mL of buffer A was preincubated for 4 min at 37 °C and placed into a polystyrene cuvette (Clinicon, Stockholm, Sweden) in a luminometer (BioOrbit 1250, Turku, Finland), with the cuvette kept at 37 °C. The light emission was recorded in a BD 111 recorder (Kipp & Zonen B.V., Delft, The Netherlands). For TRAP determination, when light emission from buffer A was stabilized in the steady-state phase, 10 μL of berry juice, berry juice fractions, wine, or standard, all of them diluted in 50 mM glycine buffer, pH 9.40, was added. The cuvette contents were mixed for 10 s with the luminometer internal mixer. The induction time was taken as the time required to reach 40% of the initial luminescence. Recording was performed at 1000 mV full scale at a speed of 10 mm/min. For TAR determination, when the light emission of buffer A was stabilized in the steady-state phase, 10 μL of berry juice, berry juice fractions, wine, or standard, diluted in glycine buffer, was added. The cuvette contents were mixed for 3 s with the luminometer internal mixer. The measurement of light intensity was carried out prior to ($I°$) and after (I) sample addition. Recording was performed at 500 mV full scale at a speed of 10 mm/min. Values of TRAP and TAR were corrected for dilution and by the ratio juice brix/fruit brix and expressed as millimoles per liter Trolox equivalents.

LDL Oxidation. Human LDL (1.019−1.063 g/mL) were prepared by zonal centrifugation (*26*) from plasma obtained from normolipidemic blood donors. Prior to oxidation, EDTA in the LDL solution was removed by two passages through an Econo-Pac 10 G desalting column (Bio-Rad, Richmond, CA), hydrated and eluted with phosphate-buffered saline (PBS). Protein concentrations in LDL preparations were determined with Bradford reagent (Bio-Rad) using bovine serum albumin as a reference standard. For the oxidation assay, 50 μg/mL LDL and 5 μM $CuSO_4$ in PBS with or without berry extract were incubated in a quartz cuvette at 37 °C. Absorbance at 234 nm was recorded along time for monitoring conjugated dienes formation in a UV-1601 spectrophotometer with a CPS controller (Shimatzu, Kyoto, Japan). Lag time was calculated by drawing a tangent line to the maximum velocity region of the curve. The intersection of the tangent with the time axis was considered as the lag time.

Determination of Reactive Oxygen Species by DCF Assay. To quantify cellular oxidative stress, a modification of the method reported by Wang and Joseph was used (*27*). HUVEC were grown to confluence in 96-well plates and loaded for 45 min with 100 μL/well of medium A containing 100 μM dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR) taken from a 100 mM stock in fresh dimethyl sulfoxide (DMSO), stored under N_2 at −20 °C. Cells were washed twice with 50 μL/well of PBS and then incubated with 180 μL of medium B with or without berry extracts for 30 min. Before starting the fluorescence recording, 20 μL/well of H_2O_2 solution in PBS was added to give a final concentration of 500 μM. An LS 50B luminescence spectrometer (Perkin-Elmer) was used with excitation wavelength at 485 ± 10 nm, emission at 530 ± 12.5, and 515 nm cutoff. The fluorescence of each well was digitized and stored in Excel (Microsoft, Seattle, WA)-compatible format. The initial fluorescence of each well was subtracted from the fluorescence at the final recording time in the same well to correct for differences between wells and background fluorescence. Values for each well were then divided by the mean of the values of the control wells and multiplied by 100 to obtain the relative fluorescence increase.

Statistics. A nonpaired Student's *t*-test was used for comparison between means. The results were expressed as mean ± SD in figures and text, except where otherwise indicated in the figure legend. For the bivariate Pearson's correlation in **Figure 1**, a two-tailed test was applied to calculate the significance.

RESULTS AND DISCUSSION

Relationship between Antioxidant Potential and Phenolic Content. The content of phenols in *ach* was compared to that in different commercial varieties of berries (**Figure 1**). Red wine was also included because it is a known rich source of dietary phenols (*28*−*30*). TRAP values are a measure of the amount of free radicals that can be trapped by the sample and therefore of the total antioxidants present (*2*). When we compared these values with the total phenol content (**Figure 1A**), we found, as expected, a good correlation ($r = 0.94$, $p < 0.05$). TAR values indicate the capacity of the sample to decrease the steady-state free radical concentration and therefore are considered a better index of the antioxidant quality (*24*). We also found a good correlation between TAR and total phenol content ($r = 0.98$, $p < 0.001$) within berry species (**Figure 1B**). Total phenol content was measured by the Folin−Ciocaltau method, which is subject to interference by vitamin C (*18*) and by reducing sugars (*19*). Therefore, the data presented in **Figure 1** were corrected for sugar content. The concentration of vitamin C (ascorbic acid) in *ach* juice was undetectable (refer to Materials and Methods section), indicating that the *ach* phenol concentration is not overestimated because of vitamin C interference. In addition, the good correlation between TRAP and phenol content suggests

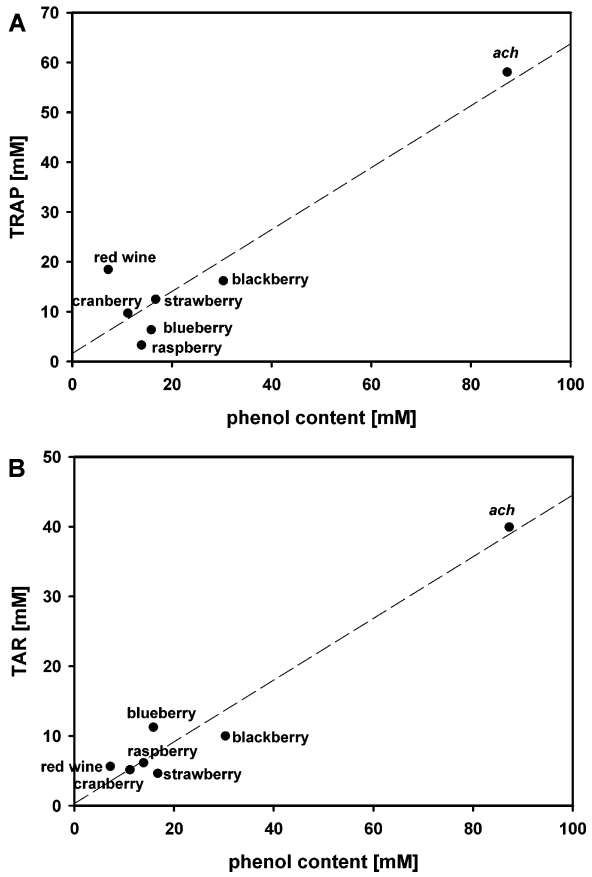

Figure 1. (A) Relationship between TRAP (millimoles per liter Trolox equivalents) or (B) TAR (millimoles per liter Trolox equivalents) and total phenol content (millimoles per liter GAE) in berries and red wine. Measurements were made using diluted berry juice or diluted wine. Values plotted were corrected for dilution and by the ratio juice brix/fruit brix. Therefore, the values represent TRAP, TAR, and phenol content in the berries or in the undiluted wine and can be compared. Total phenol content measurement was corrected for sugar interference. Values are the means of duplicate or triplicate measurements.

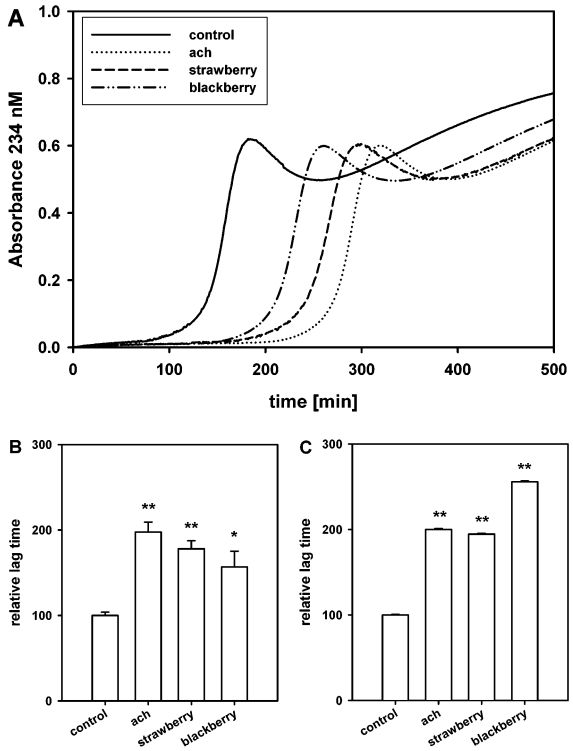

Figure 2. Protective effect of berry juice on copper-induced LDL oxidation. A 1 μg/mL amount of LDL was oxidized with 5 μM $CuSO_4$ in the absence (control) or presence of 1 μM GAE of each berry juice or 1 μM GAE aqueous phase of each berry juice. (A) Time course of the oxidation reaction in the presence of berry juice; a representative experiment is shown. (B) Lag time comparison in the presence of unfractioned berry juice or (C) aqueous fraction of berry juices. (For each bar, $n = 3$, from six different experiments). *, $p < 0.05$; **, $p < 0.001$ (the significance of the difference between control lag time and lag time in the presence of phenolic antioxidants).

that the differences found are not due to differences in vitamin C content, because TRAP is only marginally affected by vitamin C (*31*).

Berries have the highest content of phenolics among edible vegetable species (*14*, *15*). In particular, strawberry has been previously found to have the highest content of phenolics and the highest *in vitro* antioxidant activity, compared to other fruits and vegetables regularly consumed (*32*, *33*). We found that the total phenol content of strawberries was 176 mg gallic acid equivalents (GAE)/100 mg fresh weight (FW). This value is within the range reported previously (*34*) of 161−265 mg GAE/100 mg FW, depending on the method of extraction. This indicates that the strawberry juice used for our comparisons was within international standards. Therefore, the finding that *ach* has by far the highest content, with at least a comparable quality, makes *ach* an interesting source of phenolic antioxidants.

Inhibition of Human LDL Oxidation. Phenolic compounds can associate with LDL particles following incubation with plasma, exerting a protective antioxidant effect (*35*, *36*). To assess the comparative capacity of *ach* to inhibit *in vitro* copper-catalyzed LDL oxidation, we compared equal amounts (1 μM GAE) of *ach*, strawberry, and blackberry juice. The increase in extension of LDL oxidation lag time is proportional to the capacity of the antioxidant to recycle endogenous LDL antioxidants (*38*) and therefore is a measure of the antioxidant capacity of the tested compound. We found that the lag time was longer with the addition of *ach* than with strawberry (not significant) or blackberry ($p < 0.05$) juice (**Figure 2B**). It is known that some phenols have the capacity to chelate copper ions (*39*), although there is controversy regarding this point (*40*). Nevertheless, it is unlikely that the increase in lag time could account for a possible chelating capacity of *ach* phenolics, since we used five times more copper than phenolics in the assay.

Berries are anthocyanin-rich fruits (*33*); we found that 82.3 ± 0.6% of the total phenol content of *ach*, strawberry, or blackberry was in the aqueous (anthocyanin-rich) fraction of the juice (see **Figure 5** below). Therefore, we compared the antioxidant capacity of aqueous fractions in the LDL oxidation assay. **Figure 2C** shows that, when using equal amounts of phenolics (1 μM GAE), the aqueous phase of *ach* juice is more effective ($p < 0.05$) than the aqueous phase of strawberry juice and that blackberry juice aqueous phase has a higher inhibitory capacity ($p < 0.001$). When we compare unfractioned juice (**Figure 2B**) with the aqueous fraction (**Figure 2C**) of *ach*, there is no significative increase in lag time, while in the case of strawberry and blackberry we found a significant increase ($p < 0.05$ and $p < 0.001$, respectively), which reflects differences in phenolic composition between different species.

Protective Effect of *ach* Juice on HUVEC against Oxidative Stress. Although results obtained by TAR and TRAP assays and copper-catalyzed LDL oxidation give us an idea of the antioxidant potency of the juices tested, the biological relevance of this *in vitro* activity is questionable (*41*). Therefore, we used

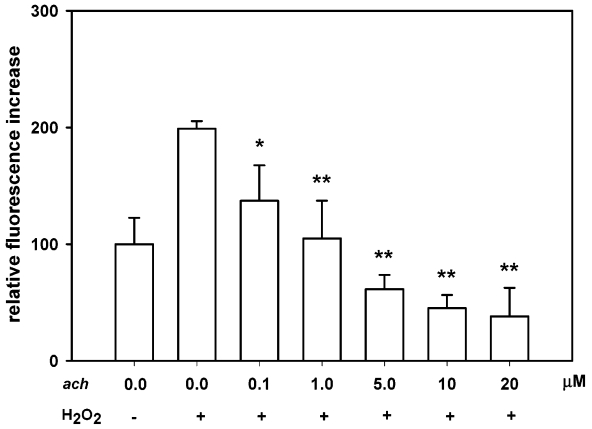

Figure 3. Protective effect of *ach* presupplementation (0.1–20 μM) of human endothelial cells for 30 min against 500 μM H_2O_2 induced oxidative stress by DCF assay. Values after 1 h exposure to H_2O_2 were normalized to the value of the control without H_2O_2; $n = 5$, except for controls without H_2O_2 and with H_2O_2 alone, $n = 10$. *, $p < 0.05$; **, $p < 0.001$ (the significance of the difference between values with *ach* juice and H_2O_2 alone). The figure shows a representative experiment that was repeated three times with equivalent results.

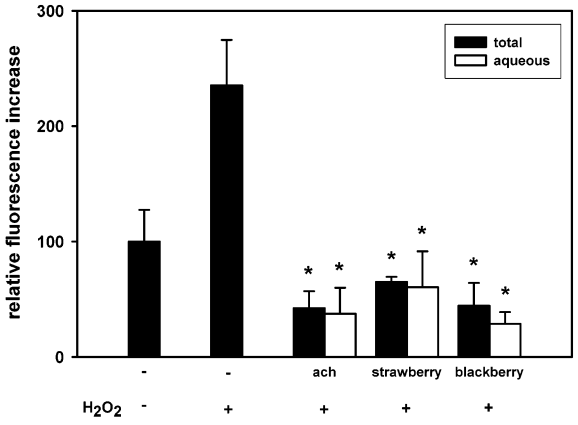

Figure 4. Comparative effect of berry juices (10 μM each) or aqueous fractions (10 μM each) presupplementation of human endothelial cells for 30 min against 500 μM H_2O_2 induced oxidative stress by DCF assay. Values after 45 min exposure to H_2O_2 were normalized to the value of the control without H_2O_2. $n = 5$, except for controls without H_2O_2 and with H_2O_2 alone, $n = 10$. *, $p < 0.001$ (the significance of the difference between values with juice and with H_2O_2 alone). The figure shows a representative experiment that was repeated three times with equivalent results.

primary human endothelial cell cultures as a physiologically relevant model. Endothelial cells form the walls of blood vessels and are in direct contact with circulating blood, thus playing a key role in normal vascular function and atherosclerosis development (*42*).

Primary cultures of human endothelial cells were exposed to H_2O_2 as a model of vascular oxidative stress. To test the protective effect of *ach*, cells were preincubated with increasing concentrations of *ach* juice for 30 min before addition of H_2O_2. The cell culture medium containing *ach* was not removed, to reassemble the presence of phenolics in plasma; therefore, these assays do not directly demonstrate incorporation of phenolics into the cells, but rather endothelial cell protection against intracellular oxidative stress. Nevertheless, incorporation probably occurs since it has been demonstrated in bovine aortic endothelial cells for elderberry anthocyanin extracts (*10*) and because anthocyanins have been detected in rat (*43*, *44*) and human (*45*–*47*) plasma and urine.

A significant increase of intracellular oxidative stress was observed with the addition of H_2O_2 ($p < 0.001$), which was inhibited by concentrations as low as 0.1 μM of *ach* phenolics (**Figure 3**). The inhibition is dose-dependent, reaching a maximum at 10 μM. Values under the control indicate that basal oxidative stress, produced by cell metabolism and probably increased during experimental handling, can also be reduced by phenolics. Of interest is the fact that the 0.1 μM concentration had an inhibition effect comparable to that of higher concentrations, such as 10 μM, until 30 min after the addition of H_2O_2. After this time, oxidative stress had a sharp increase (not shown), suggesting that the antioxidants were almost quantitatively consumed.

Comparative Protective Effect of Berry Juices on HUVEC against Oxidative Stress. To compare with the protective effect of other berry juices, a 10 μM concentration was chosen on the basis of the dose–response curve. **Figure 4** (black bars) shows a significant increase of intracellular oxidative stress with the addition of H_2O_2 ($p < 0.001$), which was inhibited to equal extents by *ach* and blackberry juice, while strawberry was a less effective inhibitor ($p < 0.05$). When the aqueous phase was tested, the difference with the total, unfractioned juice was not significative in *ach*, as well as in strawberry and blackberry

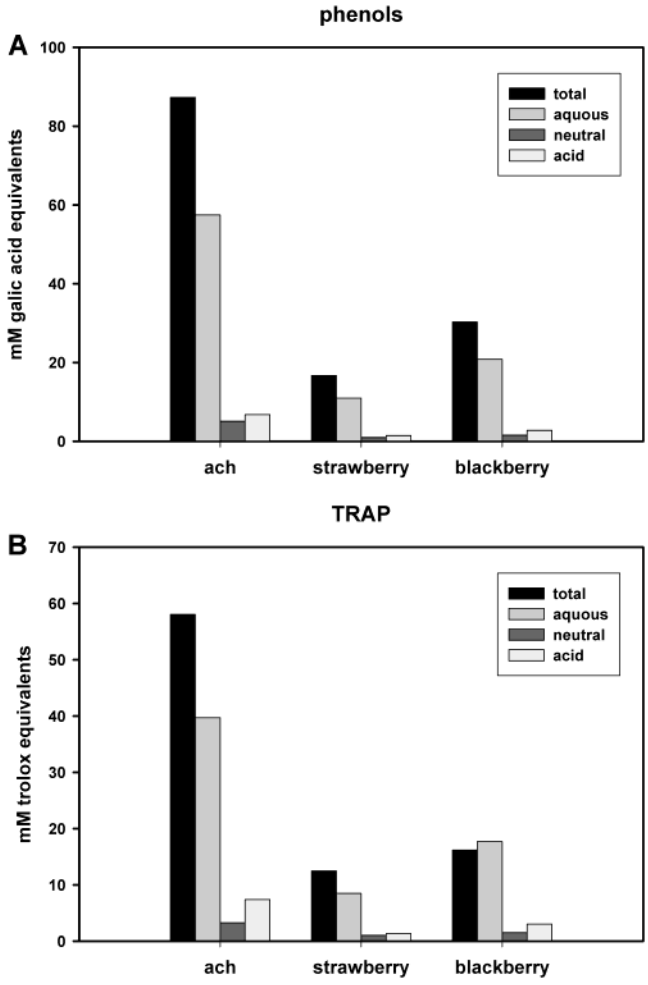

Figure 5. Comparison between phenols in total berry juice and juice fractions: (A) total phenol content; (B) TRAP. Values are the means of duplicate or triplicate measurements and were corrected for dilution and by the ratio juice brix/fruit brix.

(**Figure 4**, white bars), suggesting that the most beneficial effects are due to the 80% anthocyanins present in the sample. Although

the remaining 20% of phenolics present in the neutral and acid phase have a relatively small contribution to intracellular antioxidant protection (**Figure 5A**), we also tested the effect of *ach* fractions added at equal amounts (10 μM GAE) on endothelial cell oxidative stress protection. Similar protection was found with aqueous, neutral, and acid phases (not shown), indicating that, at equal concentrations, anthocyanins are not better at protecting against intracelular oxidative stress than other phenolic compounds that are less abundant in berries. In addition to the total phenol content (**Figure 5A**), the prevalence of the anthocyanin-rich fraction was further supported by the TRAP values in total juice and fractions (**Figure 5B**).

In conclusion, *ach* has an exceptionally high content of phenolics with high antioxidant capacity, which protect both LDL from oxidation and endothelial cells from intracellular oxidative stress, suggesting that *ach* could have antiatherogenic properties. We are currently isolating and analyzing the chemical structure of *ach* anthocyanins for further testing of their biological activity and bioavailability.

ABREVIATIONS USED

AAPH, 2,2′-Azo-bis(2-amidinopropane) dihydrochloride; *ach*, *Aristotelia chilensis*; DCF, dichlorofluorescein; LDL, low-density lipoproteins; HUVEC, human umbilical vein cells; TRAP, total radical-trapping potential; TAR, total antioxidant reactivity; var., varieties; GAE, gallic acid equivalents.

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