

Antioxidant and cardioprotective activities of phenolic extracts from fruits of Chilean blackberry *Aristotelia chilensis* (Elaeocarpaceae), Maqui

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Abstract

The methanol extract from mature fruits of *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae) showed antioxidant activities and cardioprotective effects on acute ischemia/reperfusion performed in rat heart in vivo. This extract protected animals from heart damage by the incidence of reperfusion dysrhythmias, and the no-recovery of sinus rhythm. On the other hand, the MeOH extract of the fruit was able to prevent these harmful events in the animal's heart by diminishing lipid oxidation and reducing the concentration of thiobarbituric acid reactive substances (TBARS), a lipid peroxidation index. In addition, MeOH extract of *A. chilensis* was evaluated for DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, crocin radical scavenging, oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), an estimation of lipid peroxidation in liposomes through the inhibition of formation of TBARS. MeOH extract was found to have IC₅₀ of 1.62 ppm against DPPH and 2.51 ppm against TBARS, compared with the juice, whose IC₅₀ was 12.1 ppm and 9.58 ppm against DPPH and TBARS formation, respectively. Antioxidant activities of MeOH extract were strongly correlated with total polyphenol content. Consistent with this finding, MeOH had the greatest ORAC and FRAP values as percentage of activity. These results show that these fruits could be useful as antioxidant, cardioprotective and nutraceutical sources.

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1. Introduction

Antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (Halliwell & Aruoma, 1991). This concept is fundamental to food chemistry, where synthetic antioxidants such as butylated hydroxytoluene (BHT) have long been used to preserve the good quality of food by protecting it against oxidation-related deterioration. A growing body of literature points to the importance of natural antioxidants from

many plants, which may be used to reduce cellular oxidative damage, not only in foods, but also in the human body (Prior et al., 2003). This may provide protection against chronic diseases, including cancer and neurodegenerative diseases, inflammation and cardiovascular disease (Prior & Gu, 2005). Adverse conditions within the environment, such as smog and UV radiation, in addition to diets rich in saturated fatty acids and carbohydrates, increase oxidative damage in the body. Given this constant exposure to oxidants, antioxidants may be necessary to counteract chronic oxidative effects, thereby improving the quality of life (Roberts, Gordon, & Walker, 2003).

The use of traditional medicine is widespread and plants still present a large source of novel active biological compounds with different activities, including anti-inflamma-

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tory, anti-cancer, anti-viral, anti-bacterial and cardioprotective activities. Antioxidants may play a role in these health promoting activities (Yan, Murphy, Hammond, Vinson, & Nieto, 2002). Cardiovascular disease is one of the main causes of mortality of people in the developed countries of the world. Coronary artery ischemia–reperfusion (I/R) injury which is known to occur on restoration of coronary flow after a period of myocardial ischemia and includes myocardial cell injury and necrosis (Dhalla, Elmoselhi, Hata, & Makino, 2000). Reperfusion of ischemic myocardium leads to severe damage which is indicated by release of free radicals, intracellular calcium overloading and loss of membrane phospholipids integrity (Maxwell & Lip, 1997).

The numerous beneficial effects attributed to phenolic products (Hertog et al., 1995; Stoner & Mukhtar, 1995) have given rise to a new interest in finding botanical species with high phenolic content and relevant biological activity. Berries constitute a rich dietary source of phenolic antioxidants with bioactive properties (Pool-Zobel, Bub, Schröder, & Reckemmer, 1999; Smith, Marley, Seigler, Singletary, & Meline, 2000). Chilean blackberry *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae), an edible black-coloured fruit, which ripens from December to March, is highly consumed during these months in Central and South Chile and Western Argentina. Juice and EtOH extracts of fruits of *A. chilensis* have been used in folk medicine to treat many ailments, particularly digestive and cardiac disorders, inflammation and migraine (Silva, Bittner, Céspedes, & Jakupovic, 1997). The plant grows in dense populations called “macales” and is endemic in Chile together with other two members of this family (*Crinodendron patagua* Mol and *Crinodendron hookerianum* Gay) (Vázquez & Simberloff, 2002).

We have previously reported the alkaloid composition of the leaves of *A. chilensis* (Céspedes, Jakupovic, Silva, & Tschritzis, 1993; Céspedes, Jakupovic, Silva, & Watson, 1990). Continuing with our general screening program of Chilean flora with biological activities (Céspedes et al., 2006), an examination of the MeOH extract of fruits of *A. chilensis* (Elaeocarpaceae) has been initiated. The leaves of this plant have gained popularity as an ethno-medicine for many years, used particularly as an anti-inflammatory agent, kidneys pains, stomach ulcers, diverse digestive ailments (tumors and ulcers), fever and cicatrization injuries (Bhakuni et al., 1976).

There are no previous reports relating the antioxidant properties of the plant to any bioactivity or chemical composition, such as a complete study of the phenolic content of the juice and MeOH extract of the fruits or complete phytochemical studies of the fruits of this plant.

Up-to-date some studies report that the juice (an aqueous extract) of *A. chilensis* has a good antioxidant activity against FRAP analysis but no reduction of endogenous oxidative DNA damage in human colon cells (Pool-Zobel et al., 1999). It also has an effective capacity to inhibit the copper-induced LDL oxidation in vitro and the intra-

cellular oxidative stress induced by hydrogen peroxide in human endothelial cell culture (Miranda-Rottmann et al., 2002). There is a recent study that reports the partial composition of anthocyanidin constituents of the juice (Escribano-Bailón, Alcalde-Eón, Muñoz, Rives-Gonzalo, & Santos-Buelga, 2006).

Thus, in the present investigation, we investigated the antioxidant activity of the MeOH extract from fruits of *A. chilensis*, and its relationship to the presence of phenolic compounds as well as the cardioprotective effects of 10 mg/kg body weight of MeOH extract on the I/R-induced cardiac infarct size in an in vivo rat model. Furthermore, we also determined the thiobarbituric acid reactive substances (TBARS) levels, which is widely used as a marker of oxidative stress. It is also important to mention that this fruit is profusely consumed as dried or fresh ingredient in diverse marmalades, beverages and juices in Chile and Argentina.

2. Materials and methods

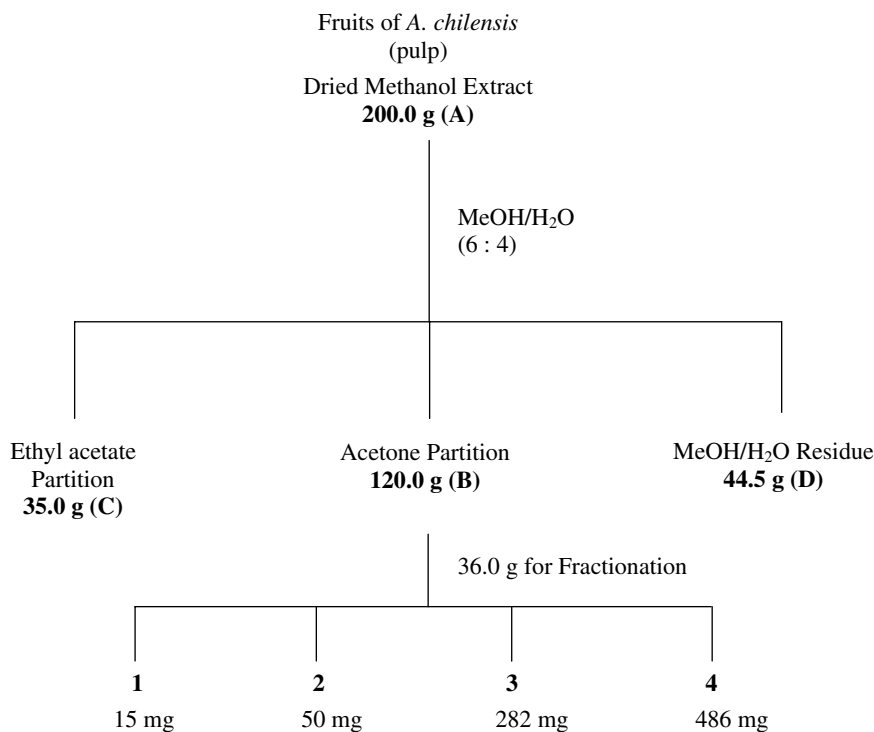
2.1. Biological material

Fruits of *A. chilensis* (Mol) Stuntz (Elaeocarpaceae) were collected from fields near the University Campus of Universidad Del Bio-Bio, Chillán City, Chile, in March, 2003. The samples of plants and fruits were botanically identified by Professor Dr. Roberto Rodríguez (Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile) and Voucher specimens were deposited at the Herbarium (CONC) of Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile. Voucher: R. Rodríguez and C. Marticorena. The collected fruits were air-dried and prepared for extraction.

2.2. Sample preparation

The fruits were divided and their main morphological parts were separated (seed and pulp), dried and then milled. The seeds were separated from the pulp, and two samples of the pulp were extracted, once with methanol containing 0.1% HCl, and a second time with pure water 100%, thus obtaining two extracts (A (MeOH) and E (water)). Then, the MeOH extract was partitioned with acetone and ethyl acetate (see Scheme 1).

Based on popular use as base for marmalade and beverages, the MeOH extract from the pulp was studied. Because most of the activity was associated with the methanol extract (data not shown), only this extract was further evaluated. The methanol extract (A) was dried and redissolved in methanol/water (6:4, v/v), then partitioned into acetone (B) and ethyl acetate (C), leaving a residue (D), as shown in Scheme 1. The acetone partition (B) showed a good antioxidant activity and was further fractionated into four sub-fractions (1–4). Elution was carried out with hexane: 100% hexane (1); hexane/ethyl acetate, (1:1, v/v)



Scheme 1. Method of obtaining extracts, partitions and fractions. Fraction 1 (hexane 100%), fraction 2 (hexane:ethyl acetate 1:1), fraction 3 (ethyl acetate:methanol 1:1), fraction 4 (methanol 100%). Extract E corresponds to 100% water.

(2); ethyl acetate/methanol, (1:1, v/v) (3); and 100% methanol (4), (see Scheme 1), by open column chromatography using silica gel (type G, 10–40 μm , Sigma-Aldrich, Toluca, State of Mexico, Mexico) as solid phase, and all fractions were analyzed by TLC as antioxidant bioautographic assay against DPPH (Dominguez et al., 2005). The MeOH extract, the acetone partition and its four sub-fractions were analyzed for oligomeric anthocyanidin contents according to Kandil et al. (2000).

The above-mentioned extracts (A–E) and partitions (1–4) were subjected to a number of analyses, including crocin, ORAC, FRAP, DPPH, and TBARS and were evaluated for total phenolic content using the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999).

2.3. Chemicals and solvents

All reagents used were either analytical grade or chromatographic grade, 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (2,2-diphenyl-1-(2,4,6-trinitrophenyl), DPPH), butylated hydroxytoluene (BHT), 2[3]-*t*-butyl-4-hydroxytoluene), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), quercetin, Folin–Ciocalteu reagent, (+)-catechin, 2-thiobarbituric acid (TBA), 2,4,6-tripyridyl-*S*-triazine (TPTZ), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, fluorescein disodium (FL) (3',6'-dihydroxy-spiro[isobenzofuran-1 [3H], 9[9H]-xanthen]-3-one), tetramethoxypropane (TMP), 1,1,3,3-tetraethoxypropane (TEP), Tris-hydrochloride buffer, phosphate buffered saline (PBS), phosphatidylcholine, FeSO_4 , trichloroacetic

acid, were purchased from Sigma-Aldrich Química, S.A. de C.V., Toluca, Mexico, or Sigma, St. Louis, MO. Methanol, CH_2Cl_2 , CHCl_3 , NaCl, KCl, KH_2PO_4 , NaHPO_4 , NaOH, KOH, HCl, sodium acetate trihydrate, glacial acetic acid, silica gel GF₂₅₄ analytical chromatoplates, silica gel grade 60, (70–230, 60 A°) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck-Mexico, S.A., Mexico D.F., Mexico.

2.4. Reduction of the 2,2-diphenyl-1-picrylhydrazyl radical

Extracts and partitions were chromatographed on TLC and examined for antioxidant effects by spraying the TLC plates with DPPH reagent. Specifically, the plates were sprayed with 0.2% DPPH in methanol (Dominguez et al., 2005). The plates were examined 30 min after spraying, and active compounds appeared as yellow spots against a purple background. In addition, TLC plates were sprayed with 0.05% β -carotene solution in chloroform, and then held under UV₂₅₄ light until the background bleached. Active components appeared as pale yellow spots against a white background (Dominguez et al., 2005). Samples that showed a strong response were selected for fractionation by open column chromatography, using solvents of increasing polarity. Furthermore each fraction was analyzed with DPPH in microplates of 96 wells as follow: extracts, partitions and fractions (50 μL) were added to 150 μL of DPPH (100 μM , final concentration) in methanol (The microtiter plate was immediately placed in a Biotek™ Model ELx808, Biotek Instruments, Inc., Winooski, VT) and their

absorbance read at 515 nm after 30 min (Domínguez et al., 2005). Catechin, quercetin and α -tocopherol were used as standards.

2.5. Oxygen radical absorbance capacity estimation

Oxygen radical absorbance capacity measures antioxidant scavenging activity of a sample or standard against peroxy radicals generated from AAPH at 37 °C using FL Trolox as standard (Domínguez et al., 2005). The assay was carried out in black-walled 96-well plates (Fischer Scientific, Hanover Park, IL), at 37 °C in 75 mM phosphate buffer (pH 7.4) (200 μ L). The following reactants were added in the order shown: Sample or Trolox (20 μ L; 7 μ M final concentration) and fluorescein (120 μ L; 70 nM final concentration). The mixture was preincubated for 15 min at 37 °C, after which AAPH (60 μ L; 12 mM final concentration) was added (final volume 200 μ L). The microtiter plate was immediately placed in a Biotek™ Model FLx800 (Biotek Instruments, Inc., Winooski, VT, USA) fluorescence plate reader set and the fluorescence recorded every minute for 120 min, using an excitation $\lambda = 485/20$ nm and emission $\lambda = 582/20$ nm, to reach a 95% loss of fluorescence. Results are expressed as μ mol Trolox equivalents (TE) per gram. All tests were conducted in triplicate (Domínguez et al., 2005).

2.6. Ferric reducing antioxidant power estimation (FRAP)

Reagents were freshly prepared and mixed in the proportion 10/1/1 (v/v/v), for A:B:C solutions, where A = 300 mM sodium acetate trihydrate/glacial acetic acid buffer pH 3.6; B = 10 mM TPTZ in 40 mM HCl and C = 20 mM FeCl₃. Catechin was used for a standard curve (5–40 μ M final concentration) with all solutions, including samples, dissolved in sodium acetate trihydrate/glacial acetic acid buffer. The assay was carried out in 96-well plates, at 37 °C, pH 3.6, using 10 μ L samples or standard plus 95 μ L of the mixture of reagents shown above. After 10 min incubation at room temperature, absorbance was read at 593 nm. Results are expressed as μ mol catechin equivalents (Cat E) per gram of sample. All tests were conducted in triplicate (Domínguez et al., 2005).

2.7. Estimation of total polyphenol content

The total phenolic content of extracts was determined using the Folin–Ciocalteu reagent: 10 μ L sample or standard (10–100 μ M catechin) plus 150 μ L diluted Folin–Ciocalteu reagent (1:4 reagent:water) was placed in each well of a 96-well plate, and incubated at RT for 3 min. Following addition of 50 μ L sodium carbonate (2:3 saturated sodium carbonate: water) and a further incubation of 2 h at RT, absorbance was read at 725 nm. Results are expressed as μ mol Cat E per gram. All tests were conducted in triplicate (Domínguez et al., 2005).

2.8. Determination of TBARS as an index of lipid peroxidation in liposomes and heart homogenate

Liposomes were prepared by sonication during 30 min of 200 mg of phosphatidylcholine from soybean (Sigma) in 2 mL phosphate buffer. The clear final solution was centrifuged at 12,000 rpm (15,000g) and filtered through a column of Sephadex G50 to eliminate all trace of metal flaking off from the tip during sonication. Lipid peroxidation of liposomes was induced by 5 μ M of CuSO₄ and 1 μ M of ascorbic acid to generate hydroxyl radicals by the Fenton reaction in the presence and absence of different plant extracts.

The thiobarbituric acid reactive substances (TBARS) assay was used to evaluate the lipid peroxidation in liposome and heart homogenate. The fluorescence method was used for TBARS quantification according to the method described by El Haffidi and Baños (1997), the procedure was carried out with 10 mg phosphatidylcholine liposomes or 10 mg protein of heart homogenate. The samples were treated with 0.05 mL methanol containing 4% butylated hydroxytoluene (BHT) in 1 mL KH₂PO₄ (0.15 M, pH 7.4), the mixture was agitated using a vortex for 5 seconds, later they were incubated for 30 min at 37 °C. At the end of incubation, 1.5 mL of 0.8% thiobarbituric acid and 1 mL 20 % acetic acid pH 3.5 were added. The mixture was heated in boiling water for one hour, immediately after the samples were placed in ice.

The TBARS were extracted adding 1 mL of 2% KCl plus 5 mL *n*-butanol. The *n*-butanol phase separated by centrifugation at 755 \times g during 2 min, they were used to measure the fluorescence in a Fluorometer (Perkin Elmer Luminescence LS-50B) at 515 nm excitation and 553 nm emission. The TBARS, reported as malondialdehyde (MDA) equivalents, were determined by means of a calibration curve, using as standard of 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, Toluca, Mexico State, Mexico).

2.9. Ischemia–reperfusion methods

Ischemia was induced in the heart of anesthetized animals, by occlusion of the left anterior descending coronary artery for 5 min, followed by reperfusion for 6 min. MeOH extract (A) (10 mg kg⁻¹) was administered 10 min before the induction of damage to the myocardium by ischemia and reperfusion to prevent lipid oxidative process during reperfusion (Carvajal, El Haffidi, & Baños, 1999).

2.9.1. Experimental groups

All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Local Committee on Animal Research. Male Wistar rats weighing 250–300 g were placed in a quiet and temperature (21 \pm 2 °C) and humidity (60 \pm 5%) controlled room in which a 12–12 h light–dark cycle was maintained. All experiments were performed between 9.00 and 17.00 h.

The rats were divided into three groups: control (SHAM), I/R + vehicle and I/R + extract. Vehicle (0.9% NaCl) or extract (100, 10 and 1 ppm/kg body weight of rat) was administered by intravenous injection 10 min before ischemia. Tissue TBARS levels were measured in eight animals for each group. MeOH extract was diluted in physiological solution (0.9% NaCl) before administering it to the rats.

2.9.2. Ischemia–reperfusion procedure

The ischemia and reperfusion damage was produced by the means of surgical occlusion of the left coronary artery as described previously (Carvajal et al., 1999).

The rats were anesthetized with sodium pentobarbital 55 mg/kg administered i.p., and artificially ventilated through a cannula inserted into the trachea. The femoral artery had a catheter inserted which was then connected to a hydrostatic pressure transducer for monitoring systemic blood pressure (BP). A surface electrocardiogram (ECG) was taken with three electrodes placed at DII standard position and a recording was taken by mean of a Grass polygraph (Model 79; Quincy, MA, USA). The chest was opened by a lateral thoracotomy, followed by sectioning the fourth and fifth ribs, about 2 mm to the left of the sternum. Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 ml/100 g body weight at a rate 60 beats/min to maintain normal pressures of CO₂, O₂ gases, and pH parameters. After the pericardium was incised, a gentle pressure on the right side of the rib cage exteriorized the heart. A 6/0 loosely silk suture thread attached to a 10 mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery and tied. The heart was then carefully replaced in the chest, and the animal was allowed to recover for 20 min. Any animal in which this procedure produced arrhythmias or a sustained decrease in mean arterial BP to less than 70 mm Hg was discarded.

A small plastic tube was gently pushed under the ligature and placed in contact with the heart. The artery could then be occluded by applying tension to the ligature, and reperfusion was achieved by removing the tube and releasing the tension.

At the end of the experiment, the heart was removed from the animals and saline solution was perfused to eliminate the blood, then it was frozen in liquid nitrogen and stored at –70 °C until required for biochemical analysis of TBARS levels.

2.10. Biochemical determination

The heart sample was homogenized in ice-cold 150 mM KCl for determination of the levels of TBARS and analyzed spectrophotometrically as described above. (Carvajal et al., 1999; El Haffidi & Baños, 1997).

2.11. Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's test for comparisons against control. Values of

$p \leq 0.05$ (*) and $p \leq 0.01$ (**) were considered statistically significant and the significant differences between means were identified by GLM Procedures. In addition, differences between treatment means were established with a Student–Newman–Keuls (SNK) test. The I_{50} values for each analysis were calculated by Probit analysis. Complete statistical analyses were performed using the MicroCal Origin 6.2 statistical and graphs PC program. *Statistics of reperfusion*: data are expressed as means \pm SEM of five different experiments; when $p < 0.05$, the difference was considered to be statistically significant. Multiple comparisons between the experimental groups were performed by one-way ANOVA with a Tukey post hoc test.

3. Results and discussion

3.1. Reducing power

Compared to the methanol extract A of pulp, the aqueous extract E was less effective at inhibiting reduction of the DPPH radical or at inhibiting TBARS formation (Table 1) within the assayed range concentrations (0.1–10.0 ppm), for this reason this extract E was not partitioned any further.

The DPPH radical scavenging assay was used first as a screen for antioxidant components within the primary extracts (Domínguez et al., 2005). As shown in Table 1, the methanol and acetone partitions (A and B, respectively) had a higher inhibitory activity against DPPH radical formation compared to the other partitions, with an IC₅₀ values of 1.62 and 2.25 ppm, respectively (Table 1). For partitions C and D, the IC₅₀ values were 20.1 and 9.43 ppm, respectively. Almost all these samples exhibited a concentration-dependence in their DPPH radical scavenging activities, particularly A, which showed the highest activity (100% inhibition) at a concentration above

Table 1
Amounts of A, B, C, D, E and fractions 1, 2, 3, 4 from acetone partition B of *Aristotelia chilensis* needed to inhibit oxidative damage by 50%^a

Sample ^b	DPPH ^c	TBARS ^d
A	1.62 \pm 0.3b	2.51 \pm 0.1b
B	2.25 \pm 0.4b	4.78 \pm 0.2b
C	20.10 \pm 3.6a	23.77 \pm 0.8
D	9.43 \pm 2.1a	10.91 \pm 3.2c
E	12.10 \pm 2.5e	9.58 \pm 0.9c
1	89.4 \pm 9.2a	> 250
2	59.3 \pm 5.3b	105.1 \pm 9.9a
3	11.2 \pm 1.8c	31.2 \pm 2.9b
4	6.1 \pm 0.9c	9.6 \pm 1.1c

^a Values expressed as $\mu\text{g/mL}$ (ppm), mean \pm SD, $n = 3$. Different letters show significant differences at ($p < 0.05$), using Duncan's multiple-range test.

^b See Scheme 1 for an explanation of extracts and partitions.

^c IC₅₀ for inhibition of DPPH radical formation.

^d IC₅₀ for inhibition of peroxidation of lipids, estimated as thiobarbituric acid reactive substances. Values are expressed as $\mu\text{g/mL}$ (ppm). See Methods for details. Mean \pm SD, $n = 3$. Different letters show significant differences at ($p < 0.05$), using Duncan's multiple-range test.

8.6 ppm (data not shown). This activity was greater than that of α -tocopherol, which at 31.6 ppm only induced 53.8% quenching and very similar to ferulic and *p*-coumaric acids with IC₅₀ values of 5.1 and 7.8 ppm, respectively (data not shown). Partition **B** was then loaded onto a silica gel open chromatography column, from which four fractions were collected (1–4). Of these, fraction **4** was the most active against DPPH reduction, with an IC₅₀ of 6.1 ppm (Table 1).

In addition to extracts **A–E**, fractions **1**, **2**, **3** and **4** of extract **B** showed considerable activity, quenching DPPH radical reduction completely (100% of inhibition, data not shown); their IC₅₀ values were 89.4, 59.3, 11.2 and 6.1 ppm, respectively (Table 1). The lowest IC₅₀ value for extract **A** (1.62 ppm), lesser than for any of the partitions from **A**, might be due to a synergistic effect of the components (mainly hydroxycinnamic acid derivatives, anthocyanidins and flavonoids) within this extract, similar to that reported for components of *Vaccinium corymbosum* and *Vaccinium angustifolium* fruits (Smith et al., 2000), where the acetone and MeOH partitions were the most active extracts.

Of the many biological macromolecules, including carbohydrates, lipids, proteins, and DNA, that can undergo oxidative damage in the presence of ROS, membrane lipids are especially sensitive to oxidation through this physiological process (Diplock et al., 1998). For this reason, liposomes were used for the investigation of lipid peroxidation as an assessment of oxidative stress. The capacity for plant extracts to prevent lipid peroxidation was assayed using TBARS (malondialdehyde equivalents) formation as an index of oxidative breakdown of membrane lipids, following incubation of liposomes with the oxidant chemical species Fe²⁺. Ferrous ion both stimulates lipid peroxidation and supports decomposition of lipid peroxides once formed, generating highly reactive intermediates such as hydroxyl radicals, perferryl and ferryl species (Ko, Cheng, Lin, & Teng, 1998). Fraction **4** was most effective, fraction **3** was least effective, but none were as effective as **A** or **B** extracts, quercetin or BHT in inhibiting lipid peroxidation. Table 1 shows the tabulated data that provide IC₅₀ values; extract **A** clearly showing the greatest activity. Thus extract **A** reduced lipid peroxidation in a dose-dependent manner, and proved to be an excellent antioxidant, reflected by its low IC₅₀ value when analyzed by both TBARS and DPPH.

When the relative contribution of each fraction to the total antioxidant activity of partition **B** was evaluated using DPPH and TBARS, all fractions except fraction **1** showed some protective effect, with IC₅₀ values between 6.1 and 59.3 ppm (Table 1). Fractions **3** and **4** were most active, with IC₅₀ values of 11.2 and 6.1, and 31.2 and 9.6 ppm, for DPPH and TBARS, respectively. Fraction **4** was substantially more active than other fractions. It is noteworthy that the IC₅₀ value for fraction **4** is very low compared with both values for flavonoids and anthocyanins in general, as well as for morin or quercetin (Diplock

et al., 1998; Makris & Rossiter, 2001). We are presently carrying out qualitative and quantitative analyses on fraction **4** and all other fractions and extracts.

It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to their phenolic content (Rice-Evans, Miller, & Paganga, 1997), suggesting a causative relationship between total phenolic content and antioxidant activity (Veglioglu, Mazza, Gao, & Oomah, 1998). Halliwell and Aruoma (1991) have defined antioxidants as substances that, when present at low concentrations compared with an oxidizable compound (e.g. DNA, protein, lipid, or carbohydrate), delay or prevent oxidative damage due to the presence of ROS. These ROS can undergo a redox reaction with phenolics, such that oxidant activity is inhibited in a concentration-dependent manner. In the presence of low concentrations of phenolics or other antioxidants, the breaking of chain reactions is considered to be the predominant mechanism (Pokorny et al., 1988), and phenolics have been suggested to be the most active substances from natural sources (Rice-Evans, 2000). Thus, we measured total phenolic content in each one of the extracts, partitions and fractions (Fig. 1). Extract **A**, which had the greatest DPPH and TBARS activities, had a significantly greater phenolic content than other extracts. The phenolic content of fractions **1–4** showed a small but significant increase in phenolic content in fraction **4** over fraction **3**, which had a similar content to that of fraction **2**; fraction **1** had significantly lower phenolic content. These findings correlate well with fraction **4** having one of the greatest activities against DPPH and TBARS, thus it could be that this fraction has also the largest content of the active components.

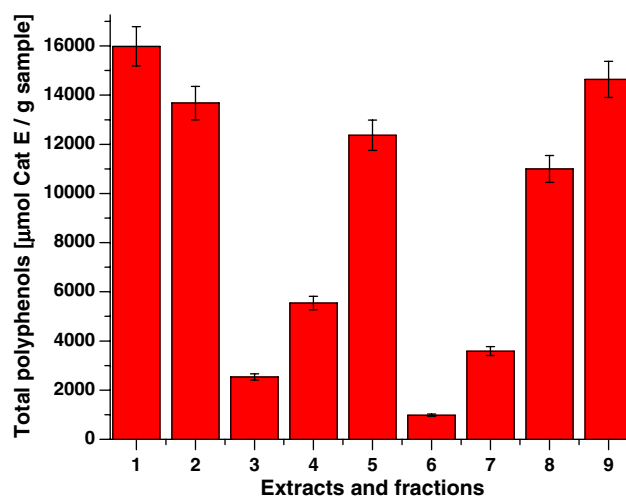


Fig. 1. Total phenolic content of extracts and fractions of *Aristotelia chilensis*. For explanation of the extracts and partitions, see Scheme 1. Values are the mean \pm SE of three replicates, ($n = 3$), different letters show significant differences at ($p < 0.05$), using Tukey test. Column: 1 = extract **A** (15,987 \pm 799.35), 2 = partition **B** (13,678 \pm 683.9), 3 = partition **C** (2534 \pm 162.7), 4 = residue **D** (5543 \pm 277.15), 5 = extract **E** (12,369 \pm 618.45), 6 = fraction **1** (980 \pm 49), 7 = fraction **2** (3589 \pm 179.45), 8 = fraction **3** (10,999 \pm 549.95), 9 = fraction **4** (14,641 \pm 732.01).

3.2. ORAC and FRAP assay

The capacity for a compound to scavenge peroxy radicals, generated by spontaneous decomposition of AAPH, was estimated in terms of Trolox equivalents, using the ORAC assay (Prior, Wu, & Schaich, 2005). A wide variety of different phytochemicals from edible plants, either purified or as an extract or fraction, has been found to be active in this assay, including alkaloids, coumarins, flavonoids, phenylpropanoids, terpenoids and phenolic acids (Aruoma, 2003; Domínguez et al., 2005). Among the extracts assayed here, the values ranged from 7200 to 29,600 $\mu\text{mol TE/g}$ extract for ORAC and from 4800 to 12,900 $\mu\text{mol catechin equivalents/g}$ extract for the FRAP assay, respectively (Table 2). The ORAC and FRAP values for *A. chilensis* extracts are given in Table 2. As with our earlier measurements, extract **A** had the highest activity in both assays, with values of 29,689.5 $\mu\text{mol TE/g}$ and 12,973.9 $\mu\text{mol catechin equivalents/g}$ for ORAC and FRAP assays, respectively. Similarly extracts **B** and **E** showed a very good potency with values of 28,489.1 $\mu\text{mol TE/g}$ and 9199.4 $\mu\text{mol catechin equivalents/g}$ for ORAC and FRAP assays for **B** and 22,560.8 $\mu\text{mol TE/g}$ and 8931.9 $\mu\text{mol catechin equivalents/g}$ for ORAC and FRAP assays for **E**, respectively. The other extracts (**C** and **D**) showed low values and intermediate potency, 7200.7 and 14,807.1 $\mu\text{mol TE/g}$ extract in the ORAC assay without significant difference ($p < 0.05$) and 6798.1 and 4810.9 $\mu\text{mol catechin equivalents/g}$ for FRAP assay without significant difference ($p < 0.05$), respectively (Table 2). Among the fractions tested, fraction **4** was more than twice as active as any other fraction (Table 2).

The FRAP assay showed greater variability (Table 2). Several extracts had very low values and only extracts **A**, partition **B** and fractions **3** and **4** showed substantial activity. Again, **A** was significantly more active than any other sample (Table 2). Fractions **3** and **4**, had higher phenolic content showing activity in the FRAP assay (Table 2).

One possible explanation for the low values obtained is that, for these samples, the reaction of the ferric–TPTZ complex was only partially completed within the 10 min reaction period. In agreement with the ORAC assay, it was extract **A**, partitions **B**, **D** and fraction **4** that showed the greatest FRAP values of 12,973.9, 9199.4, 8931.9 and 11,664.9 $\mu\text{mol catechin equivalents/g}$ extract, respectively. These data correlate well with the ORAC values, (Tables 2).

Antioxidant activities bore a direct relationship with the phenolic content of the extracts and fractions. Extract **A** and fraction **4** were the most active in ORAC and FRAP assays. These facts show a good correlation between ORAC and total polyphenolic composition of all extracts and partitions and between FRAP and total phenolic composition of fractions. The phenolic characterization suggests that the different phytochemical components of the extract and fractions, mainly anthocyanins, cinnamic derivatives and flavonoids, may be involved in the antioxidant action.

ORAC method gives us a direct measurement of the hydrophilic chain-breaking antioxidant capacity against peroxy radicals of our samples. Thus, the highest ORAC numbers of our extracts and fractions show an excellent antioxidant potential (Table 2), for instance, the extracts **A**, **B** and fraction **4**. In addition, the ORAC numbers of fractions showed a very high correlation with polyphenol content ($R > 0.95$); the same level of correlation was observed between the FRAP numbers and phenolic composition of the extracts and fractions. In the case of the extracts **A** and **B**, there is a similar level of correlation ($R > 0.98$) between FRAP numbers and their polyphenolic content (data not shown).

3.3. Ischemia and reperfusion

As the extract **A** was the most active in both the ORAC and FRAP assays, probes of scavenging capacity, it was

Table 2
Antioxidant capacity of *Aristolelia chilensis* extracts **A**, **B**, **C**, **D** and partitions **1**, **2**, **3** and **4**, measured with the ORAC assay and the FRAP assay

Sample ^a	ORAC ^b		FRAP ^c	
	[$\mu\text{g/mL}$]	$\mu\text{mol TE/g}$	[$\mu\text{g/mL}$]	$\mu\text{mol Cat E/g}$
A	10.0	29,689.5 \pm 120.2a	25.0	12,973.9 \pm 44.1b
B	10.0	28,480.1 \pm 185.8a	25.0	9199.4 \pm 11.5a
C	10.0	7200.7 \pm 29.7c	25.0	6798.1 \pm 10.9c
D	10.0	14,807.1 \pm 177.1a	25.0	4810.9 \pm 9.3b
E	10.0	22,560.8 \pm 190.1b	25.0	8931.9 \pm 12.7d
1	1.0	1639.7 \pm 135.2a	2.5	N.D.
2	1.0	8868.1 \pm 253.6b	2.5	682.4 \pm 12.9a
3	1.0	15,656.5 \pm 391.8c	2.5	998.2 \pm 19.3b
4	1.0	26,288.5 \pm 535.9d	2.5	11,664.9 \pm 123.3c

^a Extracts **A** (methanol, 100%); **B** (acetone partition); **C** (ethyl acetate partition); **D** (MeOH/H₂O residue); and **E** (water 100%) from *Aristolelia chilensis*. Fractions **1–4** from partition **B**. For detail see Methods and Scheme 1.

^b Expressed as $\mu\text{mol TE/g}$ extract, (μmol of Trolox equivalents/gram extract). Mean \pm SD, $n = 3$. Different letters show significant differences at ($p < 0.05$), using Duncan's multiple-range test. In two different experiments, one for extracts and other for fractions.

^c Expressed as $\mu\text{mol CatE/g}$ extract, (μmol of Catequin equivalents/gram extract). Mean \pm SD, $n = 3$. Values with the same letter are not significantly different ($p < 0.05$).

tested in a system of reperfusion of ischemic myocardium which leads to severe damage, indicated by release of free radicals, intracellular calcium overloading and loss of membrane phospholipid integrity. The damage can be reflected in changes observed in systolic blood pressure and in the time course of heart rate (arrhythmia). Fig. 2a shows systolic blood pressure (SBP) values which were taken from the femoral artery as basal pre-ischemic value (116 ± 8.6 mmHg). This value was approximately maintained constant in SHAM animals, suggesting that the surgery did not affect this parameter. During the first 5 min, corresponding to the ischemic period, SBP was decreased to 69 ± 13 mm Hg in control animals treated with the vehicle whereas extract A at 100 ppm, maintained the SBP high as found in SHAM animals (107 ± 21 v/s 117 ± 6 mm Hg). At the beginning of the reperfusion of the heart from animals with vehicle (min: 6), SBP decreased to 25 ± 10 mm Hg while the SBP from animals treated with 100 ppm of extract A decreased significantly at the first minute of the reperfusion but recovered the sinus rhythm and the basal arterial pressure at the seventh minute (Fig. 2a). The reperfusion did not affect the SBP at any time in the animals treated with 10 or 1 ppm of A.

Fig. 2b depicts the time course of the heart rate in the extractA-treated and control rats without any treatment during the ischemic and reperfusion period. At the occlusion time, the heart rate was normal and similar in all groups studied but as the reperfusion began, the hearts of the control group treated with vehicle developed arrhythmic beats while those of the groups treated with A did not.

3.4. Lipoperoxidation

Heart tissues from extract A-treated animals were assayed to evaluate the lipoperoxidation processes induced by ischemia and reperfusion. The extent of lipoperoxidation was measured by quantification of TBARS as MDA equivalents. It can be seen that ischemia/reperfusion increased TBARS level and that A at 100 ppm, reduced it to normal levels found in SHAM or ischemic animals without reperfusion (Fig. 3). A at 10 and 1 ppm reduced TBARS significantly but did not reach normal levels.

In the heart, the manifestations of reperfusion injury are varied and, among them, the appearance of dysrhythmias (Manning, Singal, & Hearse, 1984) and reduced contractile function (Boli et al., 1989) are more immediately identifiable. In this study, we demonstrated that the cardiac injury resulting from I/R was significantly reduced by acute administration of extract A of *A. chilensis*. I/R increased TBARS levels when compared with control and *A. chilensis* administration significantly reduced the increased TBARS values. These results suggest that *A. chilensis* is effective for the reduction of I/R-induced damage.

The reperfusion of the heart after a period of ischemia causes oxidative damage, which is indicated by generation of free radicals (Boli et al., 1989; Maxwell & Lip, 1997). It has been described that at the start of reperfusion, the

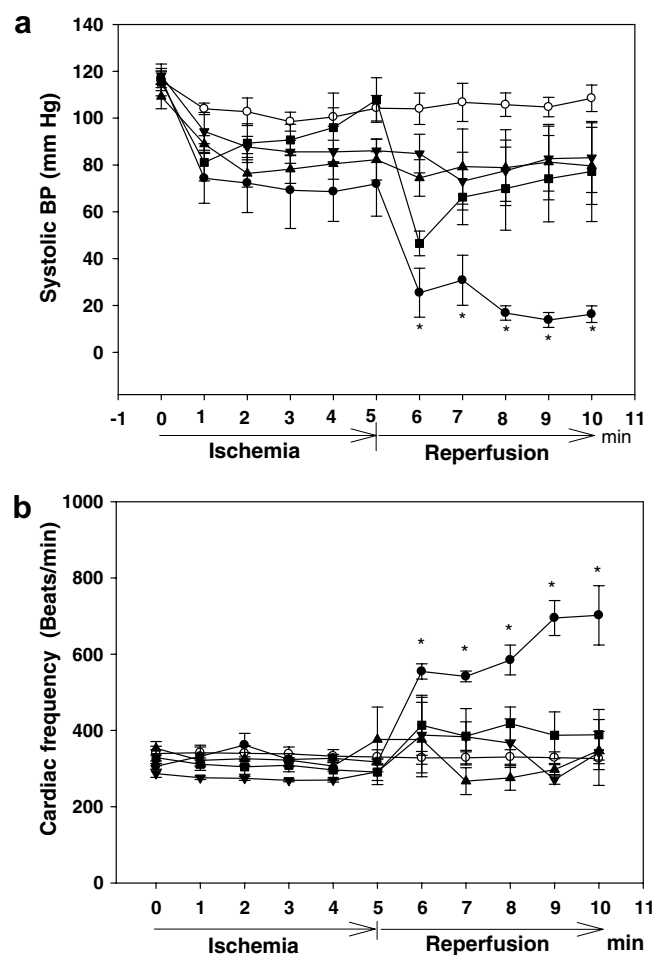


Fig. 2. Graph a: time course of SBP during ischemia–reperfusion in rats. Ischemia/reperfusion (I/R) was performed as described in Methods. At the time indicated (0 min), the left coronary artery was occluded for 5 min and then the clamp was released to allow reperfusion during five more minutes. Values are mean \pm SE from five different animals per group. ○: SHAM, ●: ischemia/reperfusion, ■: *Aristotelia chilensis* MeOH extract 100 ppm; ▲: *Aristotelia chilensis* MeOH extract 10 ppm; ▼: *Aristotelia chilensis* MeOH extract 1 ppm/kg (body weight of rat). * $p < 0.01$, Significant difference between rat with vehicle and with *Aristotelia chilensis* MeOH extract 100 ppm/kg (body weight of rat), both animal groups underwent I/R. Graph b: time course of heart rate during ischemia–reperfusion in rats. Ischemia/reperfusion was performed as described in Methods. Values are mean \pm SE from five different animals per group. ○: SHAM, ●: ischemia/reperfusion, ■: *Aristotelia chilensis* MeOH extract 100 ppm; ▲: *Aristotelia chilensis* MeOH extract 10 ppm; ▼: *Aristotelia chilensis* MeOH extract 1 ppm. * $p < 0.01$, Significant difference between rat hearts with vehicle and with *Aristotelia chilensis* MeOH extract 100 ppm/kg (body weight of rat). Both animal groups underwent I/R.

mitochondrial respiratory rate is increased markedly and more free radicals are generated (Dhalla et al., 2000).

Thus, the effect of *A. chilensis* in reducing the TBARS concentration can be attributed to its high content of polyphenols, which is efficient free radical and singlet oxygen scavengers. Phenols are well known to inhibit lipid peroxidation in in vitro systems and in experimental animals (Prior et al., 1998), as demonstrated in this paper. The mechanism by which extract A prevents heart injury is

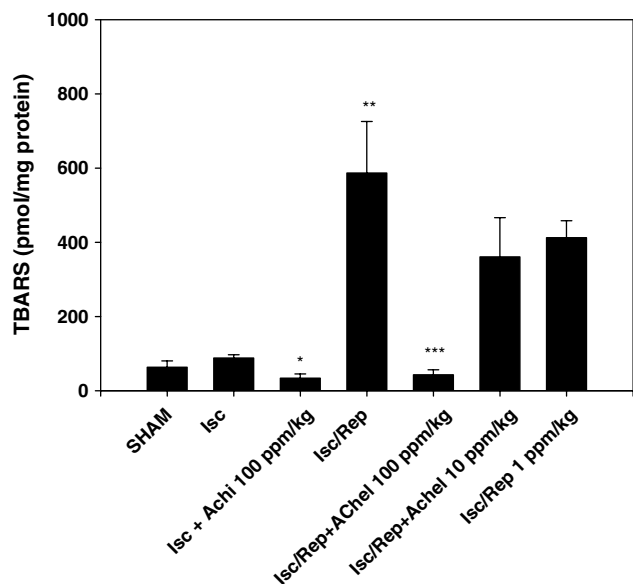


Fig. 3. Lipoperoxidation expressed as TBARS (MDA equivalent) generation in treated and untreated groups. The results are expressed as a mean \pm SE from five different tissues analyzed per group. Unit are expressed as pmol/mg of protein of heart homogenate. * $p < 0.01$ Isc vs Isc + *Aristolelia chilensis* MeOH extract; ** $p < 0.01$ Isc vs SHAM and Isc; *** $p < 0.001$ Isc/Rep vs Isc/Rep + *Aristolelia chilensis* MeOH extract.

probably by blocking the free radical formation after ischemia–reperfusion even if I/R is a complex phenomenon that involves other mechanisms such as intracellular calcium overloading and loss of membrane phospholipids (Opie, 1989) which were not assayed in this work. On the other hand, ROS induces damage on cell components by activating phospholipase A2 (Kusmic, Basta, Lazzarini, Vesentini, & Barsacchi, 2004), which induces calcium overload and increases production of lipid-derived vasoactive and chemotactic mediators, such as eicosanoids and platelet-activating factor which influence blood pressure during the period of ischemia/reperfusion. The fact that extract A at a low concentration (10 ppm and 1 ppm/kg of body weight of rats) protects heart from damage induced by I/R, makes it interesting at physiologic and pharmacologic levels to explore it in other pathological situations. In addition, until now *A. chilensis* MeOH extract has not been described to have any toxic effects.

4. Concluding remarks

The extract A of *A. chilensis* and some of its fractions exhibited substantial potency in scavenging DPPH radicals and inhibiting lipid peroxidation. Two of the four fractions isolated from B, the 3 and 4 showed efficacy as scavengers against DPPH radicals, as well as having a strong inhibitory effect against lipid peroxidation, particularly fraction 4. The antioxidant activities, total phenolic content and ORAC and FRAP assays all correlated, suggesting but not proving a causative relationship. The acetone partition B that showed this activity suggests that the phenolic com-

pounds present are probably low or medium molecular weight, with relative high polarity. Phytochemical analyses of the extract, partitions and fractions are in progress, and we expect to identify chemical structures of bioactive components that may have a role in human health maintenance.

Many cellular components are sensitive to oxidative damage, caused by the presence of nitrogen or oxygen reactive species, including a myriad of different free radicals. Rat brain homogenates rich in lipids such as polyunsaturated fatty acids can undergo peroxidation. Our findings show that extract A and acetone partition B of *A. chilensis* and several fractions of that extract, contain antioxidants that can inhibit lipid peroxidation, and that they have a high phenolic content. The relationship between total phenolics with ORAC and FRAP values in all extracts and fractions was similar to those found in other methanol and ethyl acetate plant extracts, and that values are similar to those from different known fruits and vegetables as prunes, raisins, blueberries, spinach and broccoli.

Finally, this study clearly indicates that an increase in the phenolic composition of the extract resulted in an increase in the antioxidant activities assayed.

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