Phytochemical profile and the antioxidant activity of Chilean wild black-berry fruits, Aristotelia chilensis (Mol) Stuntz (Elaeocarpaceae)

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Abstract

From ethanolic, water extracts and their fractions of mature fruits of wild black-berry Aristotelia chilensis (Mol) Stuntz (Elaeocarpaceae), different phenolic compounds were identified by chromatographic (HPLC) and unequivocal assignments by spectroscopic (UV, NMR) data analysis. Anthocyanidins, flavonoids and phenolic acids fractions were obtained using flash and open column chromatography. The main compounds gentisic acid, ferulic acid, gallic acid, p-coumaric acid, sinapic acid, 4-hydroxybenzoic acid, delphinidin, cyanidin, vanillic acid, delphinidin gallate, gallocatechin gallate, quercetin, rutin, myricetin, catechin and epicatechin as mixture 1:1, and several glycosides of anthocyanidins (delphinidin-3-sambubioside-5-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin-3-sambubioside, and cyanidin-3-glucoside), and proanthocyanidin B were detected. In addition to phytochemical analysis the antioxidant activities of extracts, partitions and fractions were strongly correlated with the highest polyphenol contents. The most active samples were the ethanolic and acetone extracts in all bioassays used and all samples were compared for activity against butylated hydroxy toluene (BHT), quercetin and tocopherol used as pattern samples. The juice (E), EtOH extract (A) and acetone partition (B) were found to have IC50 values of 4.7, 1.7 and 7.4 ppm, respectively against DPPH and 5.9, 2.1 and 3.9 ppm, respectively against TBARS formation. Additionally, the fraction F-4 showed a strong activity with IC50 of 4.9 and 6.5 ppm, against DPPH and TBARS respectively. Consistent with this finding, ETOH extract had the greatest ORAC and FRAP values as percentage of activity. On the other hand the IC50 values for the inhibitory activity against DPPH of extract B, F-3 and F-4 were 9.7, 13.2 and 10.7 ppm, respectively and against OH• were 29.1, 7.0 and 6.3 ppm, respectively. The ETOH extract protects against stress oxidative reducing the concentration of the MDA a lipid peroxidation index. These results shows that this fruit could be useful as antioxidant and nutraceutical sources.

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1. Introduction

Antioxidants are substances that delay the oxidation process, inhibiting the polymerisation chain initiated by free radicals and other subsequent oxidising reactions (Halliwell & Aruoma, 1991). This concept is fundamental to biomedical, nutraceutical, food chemistry and phytochemical sciences, where synthetic antioxidants like butylated hydroxy toluene (BHT) have long been used to preserve quality of food by protecting 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. This may provide protection against chronic diseases, including cancer and neurodegenerative diseases, inflammation and cardiovascular disease (Prior, Wu, & Schaich, 2005).

The increasing interest in the measurement of the antioxidant activity of different plant samples is derived from the overwhelming evidence of the importance of Reactive Oxygen Species (ROS), including superoxide (O2•−), peroxyl (ROO•), alkoxyl (RO•), hydroxyl (OH•−), and nitric oxide (NO•) radicals in aging and chronic disease.
Several methods have been developed to measure the antioxidant activity in biological samples, including the oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazl (DPPH) radical scavenging and inhibition of formation of thiobarbituric acid reactive species (TBARS) (Prior et al., 2005; Taruscio, Barney, & Exon, 2004).

The use of traditional medicine is widespread and plants still present a large source of novel active biological compounds with different activities, including anti-inflammatory, anti-cancer, anti-viral, anti-bacterial and cardioprotective activities. Antioxidants may play a role in all these health promoting activities (Yan, Murphy, Hammond, Vinson, & Nieto, 2002).

The numerous beneficial effects attributed to phenolic products 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 antioxidant and bioactive properties (Pool-Zobel, Bub, Schröder, & Recknemmer, 1999; Smith, Marley, Seigler, Singletary, & Meline, 2000). Chilean wild black-berry Aristotelia chilensis (Mol) Stuntz (Elaeocarpaceae), an edible black-coloured fruit, which reach its ripeness between December and March, have a popular and very high consume during these months in Central and South Chile and western of Argentina. Previously, we have reported the alkaloid composition of the leaves of Aristotelia chilensis and the botanical characteristics were reported previously (Cespedes, El-Hafidi, Pavon, & Alarcon, 2008; Silva, Bittner, Cespedes, & Jakupovic, 1997).

Up-to-date some studies reports that the juice (an aqueous extract) from fruits of A. chilensis has a good antioxidant activity against FRAP analysis but not reduce endogenous oxidative DNA damage in human colon cells (Pool-Zobel et al., 1999), an effective capacity to inhibit the cooper-induced LDL oxidation in vitro and the induction of intracellular oxidative stress induced by hydrogen peroxide in human endothelial cells culture (Miranda-Rottmann et al., 2002), other study report only the partial composition of anthocyanidins constituents of the juice (Escribano-Bailon, Alcalde-Eon, Muñoz, Rives-Gonzalo, & Santos-Buelga, 2006), and recently was reported the inhibitory activity against aldose reductase by an extract rich in anthocyanins of this fruit (Kraft et al., 2007). Subsequently, we have a recent report about the effects of MeOH extract from ripe fruits of A. chilensis on isquemic/reperfusion system, several antioxidant activities of that extract and its relationship between total phenolic levels and the cardioprotective effect. In that work, the antioxidant activities determined for MeOH extract were ORAC, FRAP, DPPH and TBARS as an index of lipid peroxidation in liposomes and heart homogenate (Cespedes et al., 2008).

In spite of those data, there are not previous ethno-botanical reports or a more complete phenolic content of the aqueous and ethanolic extracts and fractions or a more complete phytochemical study of this fruit and neither of the mechanisms involved has been evaluated until now.

In the continuation of our general screening program of Chilean flora with biological activities a re-examination of the EtOH extract of fruits of A. chilensis (Elaeocarpaceae) has been initiated. Thus, in the present investigation, we designed to re-investigate the antioxidant activities (TBARS now from brain homogenate, crocin, DPPH, superoxide dismutase through hypoxanthine–xanthine oxidase system, hydroxyl anion radical through hydrogen peroxide–peroxidase system) of the EtOH, aqueous extracts, fractions, mixtures of phenolic compounds and its relationship to the occurrence of phenolic compounds; together with a phytochemical contents (HPLC and NMR analyses) of these extracts and fractions from ripe fruits of A. chilensis. Continuously, we are working in a more complete metabolomic profile of this plant.

2. Material and methods

2.1. Biological material

In this opportunity the fruits of A. chilensis (Mol) Stuntz (Elaeocarpaceae) were collected from fields at foothills of Los Andes Mountains (900 m, 38°38′57″S, 71°52′32″W) on track at shore of “Quepe” lake very near of “Conguillio” National Park, inside of Cherqueno district, a little town near to Temuco City, Chile, January, 2006. The samples of plants and fruits were identified botanically by Professor Fernando Perich, M.Sc. (Departamento de Ciencias Químicas, U. de La Frontera, Temuco, Chile) and voucher specimens were deposited at the Herbarium (CONC) of Departamento de Botánica, Facultad de Ciencias Naturales y Oceanograficas, Universidad de Concepción, Concepción, Chile. Voucher: R. Rodriguez and C. Marticorena. The collected plants were air-dried and prepared for extraction.

2.2. Sample preparation

Fruits were separated in their main morphological parts (seed and pulp), the pulp was dried and then milled (450 g). One portion (300 g) was extracted with ethanol/water (6:4) containing 0.1% HCl and other portion (150 g) was extracted with bi-distilled water (juice). Thus was obtained two main extracts (A (EtOH/H2O) and E (water)). The EtOH/H2O extract was then partitioned with acetone (B) and ethyl acetate (C), according to Cespedes et al. (2008), see Scheme 1 (Cespedes et al., 2008).

Based on popular use as ethno-medicines and beverages, juice (water extract) and EtOH extract from the pulp were studied. Because most of the ethno-medicinal use and biological activity was associated with the ethanol extract, and that a beverage similar to wine made with EtOH is used for the treatment of different illness, this extract was used for antioxidant evaluation and phytochemical analyses. The ethanol extract (A) was concentrated and redissolved in ethanol:water (6:4), then partitioned into acetone (B) and ethyl acetate (C), leaving an EtOH-H2O residue (D), as shown in Scheme 1. The acetone partition (B) showed an excellent antioxidant activity and was further fractionated into four fractions (1–4). These extracts, partitions and all fractions were analyzed by TLC as antioxidant bioautographic assay against DPPH (Cespedes et al., 2008; Dominguez et al., 2005). The ethyl acetate, and acetone partitions and their four fractions were analyzed for phenolics, flavonoids and oligomeric anthocyanidins contents.

The above-mentioned extracts (A–E) and partitions (1–4) were submitted to a number of analyses, including crocin, radical anion superoxide (O2·−), the radical hydroxyl (OH−), 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, AAPH, DPPH, BHT, THQ, BHA, TBH, ETDIA, bovine serum albumin, Percoll, Trolox, quercetin, Folin–Ciocalteu reagent, TBA, TPTZ, ABTS, H2O2, peroxidase, FeCl3, H2O, hypoxan- thine, xanthine oxidase, dihydrouridylide (DHE), fluorescein disodium (FL), TEMP, TEP, tris-hydrochloride buffer, phosphate buffered saline (PBS), FeSO4, trichloroacetic acid, gentisic acid, gallic acid, p-coumaric acid, o-coumaric acid, propil-gallate, quercetin, myricetin, kaempferol, (+)-catechin hydrate, (−)-catechin gallate, (−)-gallocatechin, gallocatechin-gallate, α-carotene, saffron, crocin, sorbitol, tricine, and trizma-hydrochloride were...
2.4. Apparatus

60, (70–230, 60A with a HP-5MSi inert column. Method: on a GC/MS Agilent 5975-C Series instrument (70 eV) equipped with Turner filters. The electron impact mass spectrum was taken on a GC/MS Agilent 5975-C Series instrument (70 eV) equipped with Turner filters. The electron impact mass spectrum was taken with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected. Fluorimetric measurements were determined with Turner Barnstead-Thermolyne, hot-plate apparatus and remain uncorrected.

Fruits Pulp of A. chilensis Dried Ethanol Extract (A)

<table>
<thead>
<tr>
<th>Ethyl acetate Partition (C)</th>
<th>Acetone Partition (B)</th>
<th>MeOH/H2O Residue (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1,F3</td>
<td>F7</td>
<td>Vacuum chromatography on silica-gel</td>
</tr>
<tr>
<td>F4,F6</td>
<td>F9</td>
<td></td>
</tr>
<tr>
<td>Triglycerides and fatty acids</td>
<td>p-coumaric acid</td>
<td>p-hydroxybenzoic acid</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>gentisic acid</td>
<td>cyanidin-3-glucoside</td>
</tr>
<tr>
<td>rutin, catechin</td>
<td>sinapic acid</td>
<td>mixture of procyanidins</td>
</tr>
<tr>
<td>+ epicatechin</td>
<td>gallic acid</td>
<td>free</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>quercetin, myricetin</td>
<td></td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>delphinidin-3-glucoside</td>
<td></td>
</tr>
<tr>
<td>delphinidin-3,5-diglucoside</td>
<td>cyanin-3-glucoside</td>
<td></td>
</tr>
<tr>
<td>cyanin-3-sambubioside</td>
<td>delphinidin-3,5-diglucoside</td>
<td></td>
</tr>
<tr>
<td>of procyanidins</td>
<td>cyanin-3-sambubioside-5-glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>trimers and tetramers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>free</td>
<td></td>
</tr>
</tbody>
</table>

Scheme 1. Method of obtaining extracts, partitions, 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 correspond water 100%. Note A: F3–F7 together with F4–F9 were collect up and chromatographed on silica gel by vacuum chromatography, solvent system starting with n-hexane, ethyl acetate and increasing MeOH–H2O. Furthermore F8–F10 were chromatographed on Sephadex LH-20 column, solvent system starting with ETOH and going to 100% acetone.

2.5. General experimental procedures

Preliminary, high-performance liquid chromatography (HPLC) was performed on a Waters model 600E, equipped with Bondapack RP-18 column, 250 mm x 8 mm, flow rate 1.5 ml/min; UV detector (254, 235, 280, 520 nm), mobile phase MeOH/H2O 7:3 v/v. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 Merck plates, and the spots were visualised by spraying with a 10% solution of H2SO4, followed by heating at 110 °C for 3 min and with DPPH at 1% MeOH solution. Additionally, was used an HPLC Hewlett-Packard, Series 1050, with diode array detector, and UV detector at 254, 280, 365 and 520 nm, column YMC C18-Pack ODS-AM-303, AM1250S–2546 WT, 250 × 4.6 mm, ID S-Sum, 12 nm; movil phase water/methanol/acetonitrile (50:35:15), isocratic, pressure 212 bar; it was prepared 300 μl of each sample in amber vials and injected 20 μl of each sample. For comparative and separation procedures, an HPLC Hitachi Model LaChrome Elite L-2300 with a Kromasil column KR100 – 5C18 (50 × 4.6 mm), UV detector at 254, 280, 365 and 520 nm, column YMC C18-Pack ODS-AM-303, AM1250S–2546 WT, 250 × 4.6 mm, ID S-Sum, 12 nm; movil phase water/methanol/acetonitrile (50:35:15), isocratic, pressure 212 bar was used for preparative isolation of compounds.

2.6. Bioactivity-guided isolation and purification of phenolic acids, flavonoids, anthocyanins and proanthocyanins

Elution of acetone partition (B) was carried out with hexane:hexane 100% (F1); hexane:ethyl acetate, 1:1 (F2); ethyl acetate:methanol, 1:1 (F3); and methanol 100% (F4), [see Scheme 1], by open column chromatography using silica gel (type G, 10–40 μm, Sigma–Aldrich) as solid phase.

The most active fractions F3 and F4 were collected and submitted to open column chromatography using SiO2 (G 60, Merck) as solid phase and for final purification through Sephadex LH-20.
Elution of each one of these samples with n-hexane:ethyl acetate mixtures and ethyl acetate followed by methanol afforded a total of 40 subfractions (SF) [20 subfractions from F-3 (SF1–SF20) and 20 subfractions from F-4 (SF21–SF40)] that after evaporation of the solvent to dryness were analyzed by TLC, HPLC and bioautographic assay (Veglioglu, Mazza, Gao, & Oomah, 1998) using different solvent systems (n-hexane:ethyl acetate and DCM:MeOH mixtures) against pattern samples of phenolic acids, flavonoids and anthocyanins. Repeated TLC of these fractions led to the isolation of the secondary metabolites, some of which were purified by prep-TLC and prep-HPLC. All compounds were collected and identified by TLC (Rf), HPLC, GC/MS, IR, UV, 1H NMR, and 13C NMR data, compared with authentic samples (see Scheme 1).

On the other hand, an independent sample (45.0 g) of milled pulp was extracted with 0.1% HCl (v/v) in methanol for 20 h at room temperature, in darkness according to Longo and Vasapollo (2005). Briefly, the solution was filtered on a Buchner funnel, and the solid residue was washed with 0.1% HCl in methanol. The filtrate was dried using a rotatory evaporator at 30 °C. The remaining solid was redissolved on 0.01% HCl (v/v) in deionized water and successively purified through a C-18 Sep-Pack cartridge (Waters Corporation, Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (v/v). Anthocyanins and polyphenolics were adsorbed on the Sep-Pack column while sugars, acids, and other water soluble compounds were removed by washing the minicolumn with two volumes of 0.01% aqueous HCl (v/v). The anthocyanins were eluted with methanol containing 0.01% HCl (v/v), solvent was evaporated and the solid containing anthocyanins was stored at −20 °C, until used for successive analysis. Five millilitres of 2 N HCl was added to 1 mL of above solution in a screw–cap test tube, flushed with nitrogen, and capped. This solution was saponified with 5 mL of 10% KOH for 8 min in dark at room temperature, after neutralised and used for HPLC analysis and anthocyanins determination (Fig. 1).

In summary, the isolation, purification and identification of individual flavonoids, phenolic acids, anthocyanins and proanthocyanins was made by open column chromatography (silica gel G 60 (70–230, 60A¹), Merck), Sephadex LH-20 (Sigma–Aldrich), preparative TLC (silica gel GF254 analytical chromatoplates, Merck and Machery-Nagel), HPLC procedures (were carried out with three different equipments, included – HPLC/UV and diode array detection), UV, 1H NMR and 13C NMR spectra were used for chemical structure determination, for NMR were used a Varian Mercury-400, a Varian VXR-300S and VXR-500S spectrometers, and direct comparison with authentic samples.

Only for comparative effects some antioxidant bioassays were made with samples of phenolic acids, flavonoids and anthocyanins whose antioxidant potency is reported in an ample body of literature.

### 2.7. Oxygen radical absorbance capacity estimation

ORAC measures the antioxidant scavenging activity of a sample or standard against peroxyl radicals generated from AAPH at 37 °C using FL. All procedures were in according with Dominguez et al., 2005, Trolox was used as standard (Ou, Hampsch-Woodill, & Prior, 2001). Results are expressed as micromole of Trolox Equivalents (TE) per gram. All tests were conducted in triplicate.

### 2.8. Ferric reducing antioxidant power estimation

The FRAP assay was performed as previously described by Dominguez et al. (2005). Results are expressed as micromoles of catechin equivalents (Cat E) per gram of sample. All tests were conducted in triplicate.

### 2.9. Estimation of lipid peroxidation

As an index of lipid peroxidation, TBARS levels were measured using rat brain homogenates according to the method described by Ng with some modifications (Ng, Liu, & Wang, 2000), and as is described in Dominguez et al. (2005). Results are expressed as nanomoles of TBARS per milligram of protein, with percent inhibition after 30 min calculated as the inhibition ratio (IR), where (C) absorbance of the control and (E) absorbance of the test sample. These values were plotted against the log of the concentrations of individual extracts and fractions, and a decrease of 50% in peroxidation was defined as the EC50 (Dominguez et al., 2005).

### Table

<table>
<thead>
<tr>
<th>R’</th>
<th>R3</th>
<th>R5</th>
<th>Name</th>
<th>Contenta (%)</th>
<th>Peak number</th>
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</thead>
<tbody>
<tr>
<td>18</td>
<td>OH</td>
<td>Xyl-Glc</td>
<td>Delphinidin-3-sambubioside-5-glucoside</td>
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<tr>
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<td>23</td>
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<tr>
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<td>Cyanidin-3-glucoside</td>
<td>17.20</td>
<td>5.97</td>
</tr>
</tbody>
</table>

* Content in mg/100g of dried weight

Fig. 1. Anthocyanidins isolated from fruits of Aristotelia chilensis. Xyl = Xylose, Glc = glucose. Expressed in mg-equivalents of delphinidin-3-glucoside. (HPLC-DAD, and UV using λ = 254, 260, 365 and 520 nm).
2.10. Generation of the radical anion superoxide ($O_2^-$) with the system hypoxanthine–xanthine oxidase

For the specific generation of radical anion superoxide a chemical system was used that involved hypoxanthine and the specific enzyme, xanthine oxidase, whose reaction subsequently to release the radical anion superoxide (El-Hafidi & Baños, 1997).

Briefly, in 1 ml of KH$_2$PO$_4$ (10 mM, pH 7.4) containing, 5 mM of hypoxanthine, 23 mg µg of xanthine oxidase and 1 mM µM of dihydroethydium (DHE), an indicator of radical anion superoxide.

![Fig. 2. HPLC-DAD of extract B (EtOH). All peaks were identified comparing with data bases, patterns and authentic samples.](image)

![Fig. 3. H NMR (400 MHz) of EtOH (A) extract.](image)
2.11. Generation of the radical hydroxyl (OH•) by means of the system hydrogen peroxide-peroxidase

Briefly, the reaction of the \( \text{H}_2\text{O}_2 \) with the peroxidase generates radical hydroxyls (OH•) that they react with the ABTS (2,2-azino-bis(3-ethylbenzothiazolin-6-sulphonic ammonium salt)), Sigma. In the presence of radical hydroxyl ABTS forms a radical cation ABTS•+, a soluble and a green colour end product which is detected by spectrophotometry at \( \lambda = 414 \text{ nm} \). Each chemical reaction was carried out in 1 mL of sodium phosphate buffer (30 mM, pH 7.0) containing, ABTS 20 mM, \( \text{H}_2\text{O}_2 \) 200 mM and 25 mg of peroxidase. The effect of different plant extracts on the generation of radical hydroxyl was analyzed in a spectrophotometer Perkin–Elmer Lambda 18 UV–visible at \( \lambda = 414 \text{ nm} \). The results were expressed as enzymatic activity in mol/mg/min taking in account the extinction coefficient of the ABTS (36.8; Sigma–Aldrich product information; Keeseoy, 1987).

2.12. 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:water. Specifically, the plates were sprayed with 0.2% DPPH in methanol. Quercetin and \( \alpha \)–tocopherol were used as standards (Dominguez et al., 2005).

2.13. Bleaching of crocin

The solutions were placed under UV254 light. Following the decrease of absorbance, bleaching of crocin and fluorescence emission at 440 and 470 nm were monitored with time each 5 min (Cespedes et al., 2008; Dominguez et al., 2005).

2.14. Estimation of total polyphenol content

The total phenolic content of extracts was determined using the Folin–Ciocalteu reagent: 10 \( \mu \)L sample or standard (10–100 \( \mu \)M Catechin) plus 150 \( \mu \)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 \( \mu \)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 \( \mu \)mol Cat E per gram. All tests were conducted in triplicate (Singleton et al., 1999).

2.15. 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 \( \lambda_{0.05} \) values for each analysis were calculated by Probit analysis. Complete statistical analyses were performed using the MicroCal Origin 7.0 statistical and graphs PC program. Data are expressed as a mean ± 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. Phytochemical profile

Phenolic acids, some flavonoids, anthocyanins and a proanthocyanidin were identified from acetone partition (B), from which were obtained four main fractions (F-1 to F-4). F-3 and F-4 were collected obtaining 40 subfractions (SF) from which were isolated and identified the active compounds (Scheme 1). From SF-4 to SF-6 were obtained p–coumaric acid, rutin and a mixture of catechin and epicatechin; from SF-7 was isolated a mixture of p–coumaric acid and p–hydroxybenzoic acid; SF-8 to SF-10 were collected and chromatographed on Sephadex LH-20 from this column were isolated gentisic acid, sinapic acid and procyanidin B; from SF-11 to SF-15 was isolated through Sephadex LH-20 gallic acid, quercetin, myricetin, delphinidin-3-glucoside and cyanidin-3-glucoside; from SF-16 to SF-20 was isolated through Sephadex LH-20 cyanidin-3-glucoside, 4-hydroxybenzoic acid, ferulic acid, a mixture of catechin and catabase; from SF-21 to SF-25 was isolated through Sephadex LH-20 delphinidin-3-glucoside, delphinidin-3,5-diglucoside, delphinidin-3-sambubioside and cyanidin-3-sambubioside; from SF-26 to SF-30 was isolated through Sephadex LH-20 a mixture of proanthocyanidins (probably trimers and tetramers) and a dimer identified as proanthocyanidins B, together with cyanidin-3-sambubioside-5-glucoside and delphinidin-3-sambubioside-5-glucoside, and finally from SF-31 to SF-37 through Sephadex LH-20 were isolated trimers and tetramers of proanthocyanidins unidentified, from SF-38 to SF-40 was isolated a mixture of free sugars (see Scheme 1). All known structures. These compounds were identified by comparison of their retention times (Rt) in the HPLC with those from our library HPLC data (see Fig. 2) and some structures were confirmed by their spectral features (UV, IR, H NMR, and GC/MS) with our data base and with those reported in the literature and by comparison with authentic samples (see Fig. 3). (Dugo, Mondello, Morabito, & Dugo, 2003; Hillebrand, Schwarz, & Winterhalter, 2004; Lee, 2002; Mabry, 1980; Mata-Bilbao, Andres-Lacueva, Jauregui, & Lamuela-Raventos, 2004; Dugo, Mondello, Morabito, & Dugo, 2003; Hillebrand, Schwarz, & Winterhalter, 2004; Lee, 2002; Mabry, 1980; Mata-Bilbao, Andres-Lacueva, Jauregui, & Lamuela-Raventos, 2007; Merken & Beecher, 2000).

The anthocyanin composition of Maqui berries was determined by means of HPLC, UV, and GC/MS analysis. The HPLC chromatogram of the anthocyanins extract revealed that all peaks correspond to different pigments that coeluted under the conditions employed in this work, indicating the presence of eight different anthocyanins in Maqui berries. The structures the anthocyanins are shown in Fig. 1. All compounds were identified by comparison of HPLC retention times, and additionally with GC/MS, NMR and photodiode array (DAD/UV/VIS) spectroscopic data analysis.

3.2. Features of \(^1\text{H} \text{NMR} \) spectra analyses of metabolites present in \text{EtOH} extract and fractions of \text{A. chilensis}

A completely dried and lyophilised \text{EtOH} extract and their three fractions from \text{A. chilensis} were redissolved with CD\text{D}_2\text{OD}; these were analyzed by \(^{13}\text{C} \) and \(^{1}\text{H} \) NMR. The \( H \) NMR spectra of total extract \text{EtOH} (Fig. 3) show common patterns at aliphatic region, carbohydrate region and aromatic region. In the sugar region, signals from glycosidically bound glucose were found apart from ubiquitious carbohydrates such as \( \alpha \)-, \( \beta \)-glucose and sucrose. Based on its chemical shift (on the region from \( \delta = 3.1 \) to \( \delta = 5.2 \)), the type of glycosides present could be deduced (Cazor, Deborah, Moing, Rolin, & This, 2006). The chemical shift for the anomeric glucose hydrogens and it is detected by fluorescence which was carried out in the same Fluorometer described above (Turner Barnstead-Thermolyne, Quantech SS) at wavelength of 488 nm excitation and 620 nm emission. The reaction was performed at 37°C in the presence and absence of plant extracts, allowing them to incubate previously for 2 min and then the reaction began when adding the xanthine oxidase (Rahman, Ichiyanagi, Komiyama, Hatano, & Konishi, 2006; Wang & Jiao, 2000).
in aliphatic glycosides appears at δ 4.48 and 5.15 for α- and β-glucose, respectively, independently of structure of the side chain (Pereira et al., 2005). On the other hand, in the aromatic region of this 1H NMR spectrum (Fig. 3) a singlet at δ 9.53, presumably due to carboxylic proton or proton bond of flavonoid with hydroxyl group at C-5, and two doublet at δ 7.38 (J = 3.6 Hz) and δ 6.58 (J = 3.7 Hz), typical of quercetin and myricetin flavonoids. Other signal are a strong singlet at δ 3.34 assignable to methoxyl groups (ferulic acid), this signal is corroborated in everywhere fraction of the chromatograms (Fig. 2). The 13C NMR of EtOH extract correlate very well these shifts with 1H NMR, where is possible to observe aromatic, carboxylic and aliphatic carbons.

In general the NMR spectra obtained show a dominance of signals in the carbohydrate region of the spectrum. In addition to these signals, well-defined signals in both the aromatic and aliphatic regions of the spectra were present (Fig. 3). The sharp singlet at δ 3.34 was identified as ferulic acid. Similarly, other signals in relatively clear areas of the trace could be assigned to particular carbohydrates (e.g. α- and β-glucose anomeric hydrogens at δ 5.15 and 4.48), and for polyphenols region where can be observed typical signals for anthocyanins and flavonoids (δ 6.4 to 9.5).

Interestingly, in the H NMR of F-4 (spectra not show) do not appear the shifts to 7.9; 8.65 and 9.65 ppm, but is so very clear the shifts to 4.4 and 5.1 ppm for anomerics protons of β- and α-glucose, respectively, and the strong signal for O-CH3 (ferulic acid), all additional signals are very similar to H NMR of EtOH extract.

Due to methodology procedures both NMR spectra do not showed signals under 3.0 until 0.0 ppm, where is possible to observe the signals for amino acids, we are working in the elucidation of these components.

In the extracts and fractions evaluated is possible to observe the presence of 4-hydroxyflavanones (flavonols), being quercetin, myricetin, the aglicone of rutin and ferulic acid the most abundant, together with several anthocyanins. A total of eight anthocyanins were identified and quantified.

On the other hand, the GC/MS of F-4 show a peak at 24.86 min (data not shown) that cannot be compared with neither of known compounds in our data base. However, its mass spectra showed a molecular ion peak at m/z 133 and the fragmentation patterns lead us to a 3-hydroxyindole (Cespedes, Alarcon, Valdez, & Paredes-López, 2009). This compound has not been reported previously in this fruit, and the presence of this compound, although in minute amounts, show that the indole derivatives occurs also in the fruits of this plant; until now indole derivatives has been isolated only from leaves and stems (Silva et al., 1997).

3.3. Antioxidant activities

3.3.1. DPPH and TBARS evaluation

The DPPH radical scavenging assay was used first as a screen for antioxidant components within the primary extracts (Cespedes et al., 2008; Dominguez et al., 2005). As shown in Table 1, the juice, methanol and acetone partitions (E, A and B, respectively) had the highest inhibitory activity against DPPH radical formation compared to the other partitions with IC50 values of 4.7, 1.7 and 7.4 ppm, respectively. For partitions C and D the IC50 values were 30.6 and 17.9 ppm, respectively. Almost all these samples exhibited a concentration-dependence manner in their DPPH radical scavenging activities, particularly A, which showed the highest activity (100% inhibition) at a concentration of 8.6 ppm (data not shown). This action was greater than that of α-tocopherol, which at 31.6 ppm caused only 53.8% quenching and very similar to ferulic and p-cumaric acids with IC50 values of 5.1 and 7.8 ppm, respectively (data not shown). Of the four fractions only F-3 and F-4 were the most active against DPPH, with an IC50 of 7.1 and 4.9 ppm, respectively (Table 1).

In addition to samples A-D, fractions F-3 and F-4 showed considerable activity, quenching DPPH radical reduction completely (100% of inhibition, data not show). Nevertheless, F-1 and F-2 showed a moderate activity their IC50 values were 19.9 and 79.8 ppm, respectively (Table 1). The lowest IC50 value for partition A (1.7 ppm) than for any of the other partitions, might be due to a synergistic effect of the components due to extraction procedures (mainly hydroxycinnamic acid derivatives, anthocyanids and flavonoids) inside this extract, similar to that reported for components of Vaccinium corymbosum and Vaccinium angustifolium fruits (Ehlenfeldt & Prior, 2001; Lo & Cheung, 2005; 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 from this physiological process (Diplock et al., 1998). For this reason, brain homogenates 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 malondialdehyde formation as an index of oxidative breakdown of membrane lipids, following incubation of rat brain cortical and heart homogenates with the oxidant reactive substances. Values are expressed as μg/mL (ppm), mean ± SD, n = 3. Different letters show significant differences at (P < 0.05), using Duncan’s multiple-range test.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TBARS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Crocin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.70 ± 0.3b</td>
<td>2.1 ± 0.1b</td>
<td>0.97 ± 0.1a</td>
</tr>
<tr>
<td>B</td>
<td>7.40 ± 1.4b</td>
<td>3.9 ± 0.2b</td>
<td>1.1 ± 0.5a</td>
</tr>
<tr>
<td>C</td>
<td>30.6 ± 13.6a</td>
<td>20.1 ± 0.8</td>
<td>22.9 ± 2.1b</td>
</tr>
<tr>
<td>D</td>
<td>17.2 ± 9.1a</td>
<td>11.2 ± 1.2c</td>
<td>15.8 ± 2.4b</td>
</tr>
<tr>
<td>Juice</td>
<td>4.7 ± 0.8b</td>
<td>5.9 ± 0.9b</td>
<td>3.3 ± 0.5a</td>
</tr>
<tr>
<td>F-1</td>
<td>109.9 ± 9.2a</td>
<td>131.2 ± 12.5a</td>
<td>89.9 ± 10.2c</td>
</tr>
<tr>
<td>F-2</td>
<td>79.8 ± 5.9b</td>
<td>90.5 ± 9.9a</td>
<td>68.8 ± 8.8c</td>
</tr>
<tr>
<td>F-3</td>
<td>7.1 ± 1.4c</td>
<td>9.9 ± 2.7b</td>
<td>12.5 ± 2.1b</td>
</tr>
<tr>
<td>F-4</td>
<td>4.9 ± 0.5c</td>
<td>6.5 ± 1.3c</td>
<td>3.9 ± 2.2a</td>
</tr>
<tr>
<td>Quercetin</td>
<td>19.98</td>
<td>2.90</td>
<td>21.0</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>11.9</td>
<td>3.92</td>
<td>10.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values expressed as μg/mL [ppm], Mean ± SD, n = 3. Different letters show significant differences at (P < 0.05), using Duncan’s multiple-range test.

<sup>b</sup> See Scheme 1 for an explanation of extracts and partitions.

<sup>c</sup> IC50 for inhibition of DPH radical formation.

<sup>d</sup> IC50 for inhibition of peroxidation of lipids, estimated as thiobarbituric acid reactive substances. Values are expressed as μg/mL (ppm).

<sup>e</sup> IC50 for bleaching of crocin.
other fractions. It is noteworthy that the value for F-4 is very low compared with values for flavonoids and anthocyanins in general, as well as for myricetin or quercetin (data not shown) (Lo & Cheung, 2005; Makris & Rossiter, 2001).

It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to the phenolic content (Rice-Evans, Miller, & Paganga, 1997), suggesting a causative relationship between total phenolic content and antioxidant activity (Veglioglio et al., 1998). Halliwell (Halliwell & Aruoma, 1991; Halliwell & Gutteridge, 1990) has 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 the presence of phenolics has been suggested to be the most active substances from natural sources (Rice-Evans, 2000). Thus, in addition to total acidity, pH, and total sugars, we measured total phenolic content in each one of the extracts, partitions and fractions (data not shown). Extract A, which had the greatest DPPH and TBARS activities reduced MDA generation, had a significantly greater phenolic content than other extracts (17879 ± 335.7 of μmol Cat Equiv/g) and extract B showed a value of 16554 ± 501 of μmol Cat Equiv/g. The total phenolic content showed a small but significant increase for F-4 (18987 ± 755.9 of μmol Cat Equiv/g) over F-3 (13555 ± 310 of μmol Cat Equiv/g), and F-1 had significantly lower phenolic content. These findings correlate well with F-4 having one of the greatest activities against DPPH and TBARS. Because F-4 had one of the greatest activities against DPPH and TBARS formation, it could be that this fraction has the most active components.

3.3.2. ORAC and FRAP evaluations

The capacity for a compound to scavenge peroxyl radicals generated by spontaneous decomposition of AAAPH was estimated in terms of Trolox Equivalents, using the ORAC assay (Dominguez et al., 2005). A wide variety of different phytochemicals from edible plants, purified or as an extract or fraction, have been found active in this assay, including alkaloids, coumarins, flavonoids, phenylpropanoids, terpenoids and phenolic acids (Aruoma, 2003; Dominguez et al., 2005; Ng et al., 2000; Rice-Evans, Miller, Bolwelly, Bramley, & Pridham, 1995). Among the extracts assayed here, the values were found to be in the range of 1740–31926 μmol TE/g extract for ORAC and from 710 to 13937.9 μmol Cat E/g extract for the FRAP assay, respectively. 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 trials, with values of 31926.5 μmol TE/g extract and 13937.9 μmol Cat E/g extract for ORAC and FRAP assays, respectively. In similar form extract B show a very good potency with values of 29590.3 μmol TE/g and 10113.9 μmol Cat E/g for ORAC and FRAP assays, respectively. The other extracts (C, D and E) showed values from intermediate to good potency, 8941.5, 15641.1 and 22669.4 μmol TE/g extract in the ORAC assay with significantly differing (P < 0.05) and 6928.9, 5154.9 and 9930.8 μmol Cat E/g for FRAP assay with significantly differing (P < 0.05), respectively (Table 2). Among the fractions, F-4 was significantly more than twice as active as any other fraction (Table 2).

The FRAP assay showed greater variability. Several extracts had very low values and only extracts A, B, D and F-4 showed substantial activity. Again, A was significantly more active than any other samples. F-3 and F-4, those with substantial phenolic content, were the fractions showing good activity in the FRAP assay. One possible explanation for the 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, C and F-4 that showed the greatest values. Those data correlate well with the ORAC values.

Antioxidant activities bore a direct relationship with the phenolic content of the extracts and fractions. As with DPPH and TBARS activities, extract A was the most active in both the ORAC and FRAP assays. Among the fractions, F-4 was the most active in both assays. These facts can be correlated very well between ORAC and total polyphenolic composition of all extracts and partitions and between FRAP and total phenolic composition of fractions, which are shown in Table 1. The phenolic characterisation suggests that the different phytochemical antioxidants components in the active extract and fractions, mainly anthocyanins, cinnamic derivatives and flavonoids, may be involved in the antioxidant mechanism of action and the ORAC method gives us a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical of our samples. Thus, the highest ORAC numbers of our extracts and fractions showed an excellent antioxidant potential (Table 2) as in the extracts A, B and F-4, for instance. In addition, the ORAC numbers of fractions showed a very high correlation with total polyphenols content (R > 0.95) (data not shown), the same level of correlation was observed between the FRAP numbers and polyphenolic 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 its polyphenolic content (data not shown).

3.3.3. Superoxide radical (O2−) and hydroxyl radical (OH−) evaluations

In this study a hypoxanthine-XOD system was used for superoxide radical generation. Recently was confirmed that anthocyanins directly scavenged superoxide radicals even when a hypoxanthine-xod system is used (Rahman et al., 2006). Our results confirmed the findings reported by Rahman about the synergistic effects of mixtures of anthocyanins on O2−. Moreover, the extracts and fractions from A. chilensis fruits showed very good potency compared with other anthocyanins mixtures against O2− and OH− (Wang & Jiao, 2000). The IC50 values for the inhibitory activity against O2− of extract B, F-3 and F-4 were 9.7, 13.2 and 10.7 ppm, respectively and against OH− were 29.1, 7.0 and 6.3 ppm, respectively (Table 3).
Among the different extracts and fractions assayed, extract B and F-4 had the highest antioxidant capacity of inhibition of the free radical activity against \( \text{O}_2^- \) and \( \text{OH}^- \) (Table 3).

Excerpt for proanthocyanidin B and galloclatechin gallate, the antioxidant activities were also evaluated against the radical anion superoxide and the radical hydroxyl [data not shown]. In addition to F-4, the mixture of anthocyanins and the mixture of flavonoids showed the most potent antioxidant activity against \( \text{OH}^- \) with an \( EC_{50} \) of 1.29 ± 0.31 and 4.35 ± 1.1 ppm, respectively, followed by hydroxycinnamic acids with a range between 3.96 and 10.5 ppm (data not shown). The antioxidant activities of the two mixtures were comparable to the activity of quercetin and tocopherol used as standards in this study. Against \( \text{O}_2^- \) the most active was the anthocyanins mixture with an \( EC_{50} \) of 15.5 ± 2.5 ppm (data not shown). All antioxidant activities measured in this work for the isolated compounds and its mixtures are very similar to reported in an ample body of literature.

Finally, the extracts A and B of fruits of \( A. \) chilensis and some of its fractions exhibited substantial potency in scavenging DPPH-radical and inhibiting lipid peroxidation. Two of the four fractions isolated from B, F-3 and F-4 showed potency in scavenging against DPPH-radicals, as well as a strong inhibitory effect against lipid peroxidation, particularly F-4. The antioxidant activities, total phenolic content and ORAC and FRAP assays all correlated, suggesting but not proving a causative relationship. That it was the acetone partition B that showed this activity suggests that the phenolic compounds present are probably low or medium molecular weight, with relative high polarity. Additionally, the \( \text{in vitro} \) strong scavenging activity of \( \text{EtOH} \) (B) and F-4 seems to be increased probably due to the presence of a 3-hydroxylindole derivative as in other samples (Fernandes et al., 2004). The phytochemical analysis of these extract, partitions and fractions have shown been rich in phenolic composition and is the identification of its chemical structures of bioactive components that may have a future role in human health maintenance and nutrition.

4. Concluding remarks

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 and rat brain homogonates, rich in lipids such as polyunsaturated fatty acids that can undergo peroxidation. Additionally, plants which have a strong capacity to synthesise proanthocyanidins generally do not metabolize substantial quantities of esters of gallic and hexa-hydroxyphenolics acids and vice versa (Haslam, 2007). Our findings show that the extract A and acetone partition B of \( A. \) chilensis and several fractions of that extract (F-3 and F-4), contain antioxidants that can inhibit lipid peroxidation, and that they have a high phenolic content. The relationship between total phenolics with DPPH, crocin, \( \text{O}_2^- \); \text{OH}^- \), ORAC and FRAP values in all extracts and fractions were similar to those found in other methanol and ethyl acetate plants extracts, and that values are similar with those for different known fruits and vegetables as carrots, berries, prunes, raisins, blueberries, spinach and broccoli (Cespedes et al., 2008; Dominguez et al., 2005 and references there in; Jeffery & Araya, 2009; Prior et al., 1998).

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