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Original Research Article

Ramorinoa girolae Speg (Fabaceae) seeds, an Argentinean traditional indigenous food: Nutrient composition and antioxidant activity

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ABSTRACT

Ramorinoa girolae Speg. seeds were characterized in terms of phenolic and flavonoid contents, multi-element profile, fatty acid composition and antioxidant activity. The MeOH extract of seeds showed a strong scavenging capacity against the DPPH free radical, inhibition of lipid peroxidation in erythrocytes and reducing power. A significant positive correlation between the total phenolic content and the antioxidant activity of extracts suggests that such compounds could be responsible for the antioxidant capacity of the seeds. Five phenolic compounds were identified by liquid chromatography tandem electrospray ionization mass spectrometry (LC–ESI-MS/MS) and high-resolution electrospray ionization time-of-flight mass spectrometry (LC–ESI-TOF-MS): a coumaric acid derivative, epigallocatechin, catechin-*O*-glucoside, procyanidin B (catechin dimer), catechin, and two unknown compounds. The presence of catechins suggests health benefits. The seeds also contained a high concentration of monounsaturated fatty acids (≈71%), especially oleic acid, and a low percentage of polyunsaturated fatty acids (≈14%). K, Mg and Ca were major elements, potassium accounting for 59% of the element content. The main roles of these elements can be described as maintenance of pH and osmotic pressure, nerve conductance, muscle contraction, energy production and almost all other aspects of biological life. The observed phenolic content and antioxidant activity of the seeds is an indication of their ability to offer potential health benefits. This is the first report of *R. girolae* to validate its popular reputation as a nutritionally healthy food.

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1. Introduction

Native Argentinean edible fruits from trees like *Prosopis* spp., *Geoffroea decorticans*, *Ziziphus mistol* Griseb, *Ramoniroa girolae* or *Cactaceae* spp., are widely consumed in different forms in several Argentinean communities as supplementary foods and play an important role in the diet of inhabitants of rural areas.

R. girolae Speg. (Fabaceae), commonly named "chica", is an Argentinean legume plant species belonging to a monotypic genus in the Fabaceae family, whose geographical distribution includes the mountain slopes of San Juan, San Luis and La Rioja (Central Western Argentina). In the province of San Juan there are extensive forests of *R. girolae* called "Chicales", mainly located in the Caucete and Valle Fértil districts. Ethnobotanical surveys and ethnohistoric documentation (Spegazzini, 1924) indicate that the consumption of *R. girolae* seeds played an important role in the diet of Amerindians during the pre-Inca and pre-Hispanic periods. *R. girolae* seeds are still consumed by the inhabitants of small rural settlements close to the "Chicales". These populations use *R. girolae* as a food in addition to the pods of some *Prosopis* species (see Fig. 1). In these areas, human activities are based on subsistence farming, goat-herding for meat and dairy products and the marketing of other natural products obtained through a sustainable production system and believed to have medicinal or nutritional properties (Agüero et al., 2011).

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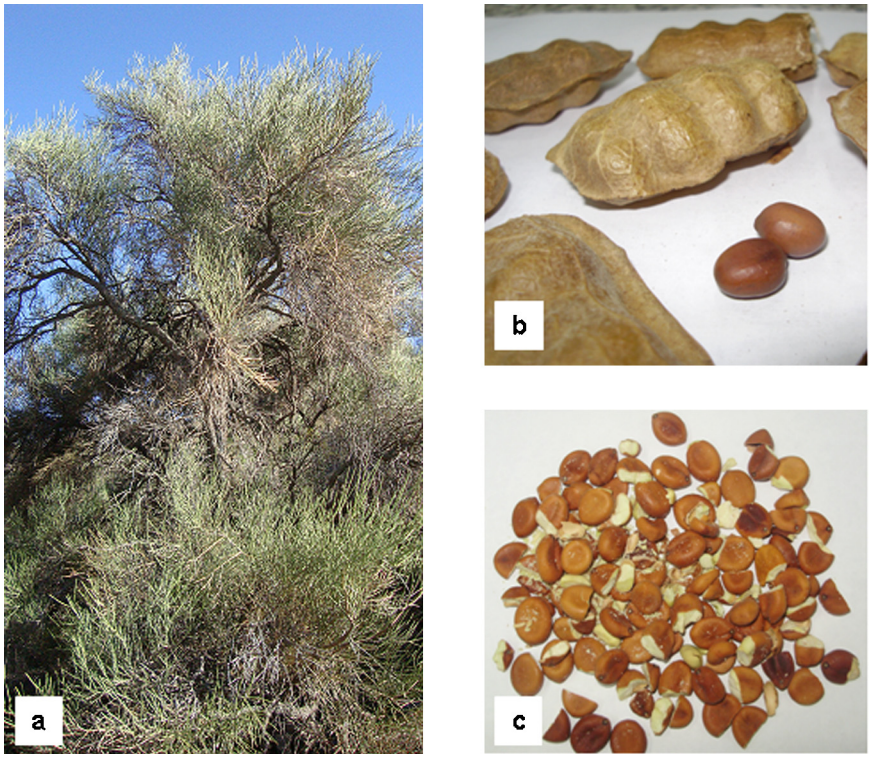

Fig. 1. Photographs of the *Ramorinoa girolae* Speg.: tree (a), fruits (b) and seeds (c). (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

Popular knowledge of the effect of plant constituents on organisms is almost always based on traditional knowledge and empirical observation from repetitive use (da Silva Nunes et al., 2011). Phenolic compounds are widely distributed in plants and they have recently gained much attention due to their antioxidant activity and free radical scavenging ability with potential beneficial implications for human health. In particular, these important food constituents may contribute to the prevention of major pathologies such as cardiovascular disease, aging and cancer. To exploit the health-promoting functionalities of locally available, culturally acceptable, and economically viable indigenous foods, it is important to focus on their bioactive compounds. Among the various bioactive substances, phenolic compounds are the most abundant antioxidants in commonly consumed foods of plant origin (Vadivel et al., 2011).

The main goal of this study was to characterize seeds of *R. girolae* to determine their phenolic profile and antioxidant activity, multi-element content and fatty acid composition.

2. Materials and methods

2.1. Chemicals

All reagents and solvents used were of analytical grade. Ultrapure water (Millipore, Milli-Q system) was used to prepare standard solutions, dilutions, and blanks. Chloroform was purchased from Fisher (Fair Lawn, NJ), and methanol was obtained from J.T. Baker (Phillipsburg NJ). Acetonitrile was from Caledon Laboratory Ltd. (Canada) and formic acid was purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu phenol reagent, aluminum chloride hexahydrate and sodium carbonate, nitric acid and sulfuric acid were also purchased from Merck (Darmstadt, Germany). The reagent 1,1-diphenyl-2-picrylhydrazyl (DPPH) was acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All glassware used was left in a sulfonitric solution overnight and then washed with ultrapure water.

2.2. Seed samples

The *R. girolae* fruit is an oval, hard and indehiscent legume containing between 3 and 7 seeds each (Fig. 1, esp. b and c). Three samples (weighing 4 kg each) of randomly chosen fruits were collected during March 2008 from three native plantations of *R. girolae*, which currently extend over approximately 2 km^2, located in the province of San Juan (Argentina). Fruits were identified by Dr. Gloria Barboza, IMBIV CONICET (Universidad Nacional de Córdoba). A voucher specimen was deposited at the Museo Botánico de Córdoba (CORD 9833), Argentina. Each sample was subjected to a standard analytical procedure of cracking to obtain three 100–250 g subsamples from different fruit pools. Thus, we prepared nine subsamples arising from three independent samples ($n = 9$). Prepared samples were subsequently stored at −40 °C until analyzed. The seeds of the fruits were then removed by breaking their cover. Samples (each containing ≈20–50 g of seeds) were homogenized by grinding with a porcelain mortar and pestle. The analyses of seed characteristics were carried out in triplicate.

2.3. Extracts

Seeds (5 g from each subsample; $n = 9$) were extracted separately and in triplicate with petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH), 250 mL in each case, using a Soxhlet apparatus. The solvents were then evaporated under vacuum to give semi-solid extracts: PEE, DCME and MeOHE, affording 34.1 ± 4.5%, 2.4 ± 2.2% and 9.5 ± 2.1%, dry weight, respectively.

2.4. LCMS analyses

Qualitative analysis by LC–ESI-MS/MS and LC–ESI-QTOF-HRMS was performed according to Torras-Claveria et al. (2007), and Vallverdú-Queralt et al. (2010). Samples of methanol extract (MeOHE) were prepared at a concentration of 1 mg mL^{-1} in acetic

acid (0.05%): MeOH (4:1) and filtered using a 0.45 μm polytetrafluoroethylene (PTFE) filter (Waters). LC analyses were carried out using a 1100 Agilent quaternary pump system (Waldbronn, Germany), equipped with an autosampler and diode array detector (DAD). A Luna C18 column (150 × 2.1 mm, 5 μm) (Phenomenex, Torrance, CA) was used for the separation of phenolic compounds. Gradient elution was performed with acetic acid (0.05%) (solvent A) and acetonitrile containing 0.05% acetic acid (solvent B) at a constant flow rate of 400 μL min^{-1}. An increasing linear gradient (v/v) of solvent B was applied: (*t* (min), %B): (0, 5), (10, 15), (30, 35), (40, 80), (45, 5). Chromatograms were recorded at 280 nm, with peak scanning between 200 and 600 nm.

For LC–ESI-MS/MS analyses, an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada) coupled online to LC was used. The mass spectrometer was equipped with a Turbo Ionspray source operating in negative mode with the following settings: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, −200 V; entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The declustering potential (DP) was – 60 and collision energy (CE) – 35, although other DP and CE potentials were also assayed. Different MS/MS experiments, such as product ion scan, precursor ion scan, and neutral loss scan, were carried out. Full scan acquisition was performed scanning from *m*/*z* 100 to 800 u in a profile mode with a cycle time of 2 s, a step size of 0.1 u, and a 2 min pause between each scan.

For LC–ESI-QTOF-HRMS analyses (HRMS = high resolution mass spectrometry), a QSTAR Elite hybrid Quadrupole-Time of Flight (QToF) mass Spectrometer (Applied Biosystems, PE Sciex, Concord, Ontario, Canada) coupled on-line to the same LC as described above was used. The instrument provided a typical accuracy ≤3 mDa. All the acquisition and data analyses were controlled by Analyst QS version 2.0 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada). QTOF-HRMS data were recorded from *m*/*z* 100 to 800 with an accumulation time of 1 s and a pause between the mass ranges of 0.55 ms, operating in the negative mode. The instrument parameter settings were the following: capillary voltage −3500 V, nebulizer gas (N_2) 50 (arbitrary units), curtain gas (N_2) 50 (arbitrary units), collision gas (N_2) 5 (arbitrary units), focusing potential −190 V, declustering potentials (DP1) −60 V and (DP2) −15 V. The drying gas (N_2) was heated to 400 °C.

2.5. Determination of total phenolic and flavonoid contents

The total phenolic (TP) content of extracts was determined using the method described by Heldrich (1990) with slight modifications. An extract dilution (1 g/L) was oxidized using Folin-Ciocalteu reagent (125 μL) and neutralized with sodium carbonate (375 μL to 20%, w/v). After 30 min, the absorbance of the resulting blue solution was measured at 765 nm using a Shimadzu UV-160A spectrophotometer. TP were determined by linear regression from a calibration plot constructed using gallic acid (0, 25, 50, 100, 150 and 250 μL/mL) and expressed as mg of gallic acid equivalents per 100 g of seeds (mg GAE/100 g seeds). All measurements were done in triplicate.

The total flavonoid content in the extracts was determined following the procedure described by Chang et al., 2002, using a colorimetric method with $AlCl_3$ hexahydrate as a complex-forming reagent, and known quercetin concentrations as a standard to construct the calibration plot. One milligram of quercetin was dissolved in 95% ethanol and then diluted to 5, 10, 25, 50 and 100 mg L^{-1}. The diluted standard solutions (250 μL) were separately mixed with 750 μL of 95% ethanol, 50 μL of 10% aluminum chloride, 50 μL of 1 M potassium acetate and 1400 μL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 250 μL of extracts were reacted with aluminum chloride to determine the flavonoid content. The absorbance of the reaction mixture was measured at 415 nm using a Shimadzu UV-160A spectrophotometer. Results are expressed as mg of quercetin equivalent per 100 g of seeds (mg QE 100 g/seeds). Data from triplicates are reported as mean ± SD.

2.6. Antioxidant activity

2.6.1. Free radical scavenger assay

Free radical scavenger activity of extracts was assessed by the fading of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical, as previously reported by Tapia et al. (2004). Extracts were assayed at concentrations of 100, 50, 10 and 5 μg/mL. Scavenging activities were evaluated spectrophotometrically at 517 nm using a Shimadzu UV-160A spectrophotometer. Quercetin was used as a reference compound. The percentage of decoloration was calculated as follows:

$$\text{percentage of decoloration} = \left[1 - \left(\frac{\text{sample absorbance} - \text{control absorbance}}{\text{DPPH absorbance}}\right)\right] \times 100$$

Values are reported as the mean ± standard deviation (SD) of three independent determinations.

2.6.2. Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay is simple, robust, speedy and inexpensive, and does not require specialized equipment (Prior et al., 2005). FRAP was determined by the direct reduction of $Fe^{3+}(CN^-)^6$ to $Fe^{2+}(CN^-)^6$ and by measuring absorbance resulting from the formation of Perl's Prussian Blue complex following the addition of excess ferric ions (Fe^{3+}) (Gülçin et al., 2010). The iron-reducing power of samples was tested according to Oyaizu, 1986. Briefly, 200 μL extract (100, 200, 500 and 1000 μg/mL) or gallic acid was added to 500 μL of phosphate buffer (0.1 M, pH 6.6) and 500 μL of potassium ferricyanide (1%, w/v). Afterwards, the mixture was incubated at 50 °C for 20 min, 500 μL of 10% (w/v) trichloroacetic acid (TCA) was added and it was vortex shaken for 20 s. One millilitre of this solution was mixed with 1 mL of distilled water and 200 μL $FeCl_3$ (0.1%, w/v). After 30 min incubation, the absorbance was read at 700 nm using a Shimadzu UV-160A spectrophotometer. Analyses were performed in triplicate. Increased absorbance of the reaction means an increased reducing power.

2.6.3. Lipid peroxidation in human erythrocytes

The evaluation of lipid peroxidation in human erythrocytes was carried out as described by Tapia et al. (2004) with minor modifications. Human red blood cells, obtained from healthy non-smoking adult individuals after informed consent, were washed three times in cold phosphate buffered saline (PBS) by centrifugation at 3500 rpm. After washing, cells were suspended in PBS, regulating the density to 1 mM hemoglobin in each reaction tube. The final cell suspension was incubated with different concentrations of the test compounds and dissolved in DMSO and PBS for 10 min at 37 °C. The final concentration of samples and controls in DMSO was 1%. After incubation, cells were exposed to tert-butylhydroperoxide (1 mM) for 15 min at 37 °C under vigorous shaking. Then, lipid peroxidation was determined indirectly by the TBARs formation. Results are expressed as percentage of inhibition compared to controls. Each determination was performed in quadruplicate.

2.7. Fatty acid analysis

For fatty acid composition, PEE (0.5 g) was subjected to alkaline saponification by reflux (45 min) using 30 mL 1 N KOH in methanol. Unsaponifiable matter was extracted with n-hexane (3 × 30 mL). The fatty acids were converted to methyl esters (FAMEs) by reflux (45 min) using 50 mL 1 N H_2SO_4 in methanol and analyzed by GC (Perkin-Elmer Clarus 500, Shelton, CT, USA) using a fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) CP Wax 52 CB (Varian, Walnut Creek, CA, USA); carrier gas N_2 at 1 mL min^{-1}; column temperature programmed from 180 °C (5 min) to 240 °C at 4 °C/min; injector and detector temperatures at 250 °C, FID. FAME identification was carried out by comparison of their retention times with those of reference compounds (Sigma-Aldrich, St. Louis, MO, USA).

Iodine values (IV) were calculated from fatty acid percentages by using the formula:

$$IV = (\%\,\text{oleic acid} \times 0.899) + (\%\,\text{linoleic acid} \times 1.814) + (\%\,\text{eicosenoic acid} \times 0.818)$$

2.8. Multi-element analysis

Multi-element content was analyzed according to Di Paola-Naranjo et al. (2011). Twenty nine elements were quantified in the seeds of *R. girolae*: Li, Be, B, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Ag, Cd, Te, Ba, Tl, Pb and Bi. Multi-element analyses were carried out on a Quadrupole Inductively Coupled Plasma Mass Spectrometer (Q-ICPMS) (Agilent Technology 7500 cx Series), equipped with an ASX-500 series autosampler model (Agilent Technology). The sample introduction system consisted of a microflow concentric nebulizer, Peltier cooled spray chamber and 2.5 mm ID fixed injector torch. The RF power was set to 1500 W for all the experiments and the interface was fitted with Ni sampling and skimmer cones, designed for low polyatomic formation. Two operation modes were used: with and without collision cell technology (CCT). CCT mode measurements were performed for Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr and Mo. For the CCT mode, the collision cell was flushed with the collision gas (He, 5.0 grade). Li, Be, B, Ag, Cd, Te, Ba, Tl, Pb and Bi were measured without operating the collision cell.

The oxide ratio and double charged species were maintained below 1% for both operation modes. All the measurements were performed using Sc, In, and Re as internal standards. Instrumental and procedural blanks were determined, together with samples. Three replicates were obtained for each sample. Full quantitative analysis was performed against calibration standards for each element. All samples were analyzed in duplicate (two independent fractions of the same sample, each measured in triplicate; $n = 2$).

Sodium measurements were carried out by Flame Atomic Absorption Spectrometry (FAAS) using a Perkin Elmer 3030 in an air-acetylene flame. All samples were diluted tenfold using HNO_3 (2% in ultrapure water) before Q-ICPMS measurements. Standards and blanks were prepared using the same mixture (HNO_3 2%).

Seeds were prepared for multi-elemental analysis as follows: seeds (≈0.5 g) were powdered using a ceramic mortar and pestle. Fine, powdered samples (0.2 g) were then introduced into quartz vessels, followed by the addition of 8 mL concentrated nitric acid (sub boiling grade). After fumes were released (2–3 h), vessels were closed with PTFE caps, mineralized in a microwave oven (Anton Paar 3000) and heated using the following power sequence: starting a 15 min ramp until reaching 600 W, holding for 45 min (maximal $T = 169$ °C; max pressure = 75 bar) and a final 15 min step disabling power to reach pressure equilibration. Mineralized samples were quantitatively transferred to 25 mL volumetric flasks, completing the volume with ultrapure water, followed by filtration using 0.45 μm filters. Spiked samples were also prepared by adding variable amounts of individual standard solutions (1000 mg L^{-1} in 1% nitric acid), doubling the starting concentration for each element. The rest of the procedure was the same used for nonspiked samples. All recoveries were between 84 and 116%. A certified reference material (CRM: NIST 2548 a – typical diet) was analyzed for quality control using the same procedure. Recovery of elements measured in this work from CRM was between 80 and 110% of certified values.

2.9. Statistical analysis

Values are expressed as means ± standard deviations of replicate determinations. Statistical analysis was done with one-way variance analysis. The Pearson correlation coefficient (R) and P-value were used to show correlations and their significance (SPSS 16.0 for Windows, SPSS Inc., IL, USA). Probability values of $P < 0.05$ and $P < 0.01$ were considered as statistically and extremely significant, respectively.

3. Results and discussion

Pictures of *R. girolae* are shown in Fig. 1. The tree can measure up to 10 m (Fig. 1a) and the fruits (Fig. 1b) have 3–7 brown-red seeds (Fig. 1c).

3.1. Total phenolic and flavonoid contents

In the DCM and MeOH extracts of *R. girolae* seeds, total phenol contents were 55 and 1548 mg GAE/100 g seeds, respectively, and flavonoid contents were 31 and 1048 mg QE/100 g seeds, respectively (Table 1).

The two major classes of phenolic compounds known for their antioxidant activity are phenolic acids and flavonoids, which occur widely in plants, and especially in fruits and vegetables (Wojdylo et al., 2007).

3.2. Antioxidant activity

The assays evaluating antioxidant activity can be roughly classified into two types: a) those based on hydrogen atom transfer (HAT) reactions (DPPH assay) and b) those based on electron transfer (ET) (FRAP and Total Phenolic assay by Folin–Ciocalteu reagent) (Huang et al., 2005), which involve the following transfer reaction:

$$\text{probe (oxidant)} + \text{e (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant}$$

DPPH free radical scavenger activity and Ferric-reducing antioxidant power (FRAP) assay

DPPH is widely used for quickly assessing the ability of polyphenols to transfer labile H atoms to radicals, a likely mechanism of antioxidant protection (Prior et al., 2005). The

Table 1
Total phenolic and flavonoid contents of extracts from *R. girolae* seeds.

Extract	Total phenolics (mg GAE/100 g seeds)	Flavonoids (mg QE/100 g seeds)
PE	n.d.	n.d.
DCM	55 ± 23	31 ± 19
MeOH	1548 ± 711	1048 ± 184

PE: petroleum ether, DCM: dichloromethane, MeOH: methanol. GAE: gallic acid equivalent; QE: quercetin equivalent. Each value is expressed as mean ± SD (standard deviation) ($n = 3$). n.d.: not determined.

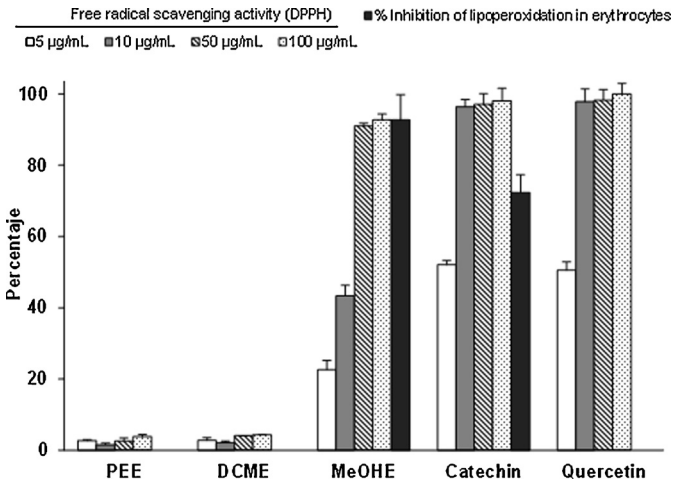

Fig. 2. Free radical scavenging activity (DPPH) at 5, 10, 50 and 100 µg/mL and lipid peroxidation in erythrocytes (at 500 µg/mL and 100 µg/mL of extracts and compounds, respectively) of extracts of *R. girolae* Seeds.

results of the DPPH scavenging assay are presented in Fig. 2. The MeOHE of *R. girolae* seeds showed high scavenging activity (>90% at 50 µg/mL), equivalent to that of the standard quercetin (>90% at 50 µg/mL). This could be due to the presence of hydrogen-donating compounds, which are more likely to be present in polar extracts (Middleton et al., 2005).

Regarding the FRAP assay, the reducing power of DCME and MeOHE of *R. girolae* seeds at various concentrations (100, 200, 1000 and 2000 µg/mL) are presented in Fig. 3. The best reducing power was obtained for MeOHE at 500 and 1000 µg/mL, with values that were higher than and equivalent to gallic acid at 10 and 25 µg/mL, respectively, while the DCME at 500 µg/mL was equivalent to 10 µg/mL gallic acid.

3.2.1. Lipid peroxidation in human erythrocytes

Human erythrocytes were used as a cell-based model to elucidate the biological relevance of the antioxidant activities of *R. girolae* seed extracts. As shown in Fig. 2, the highest activity (>90%) was found in the MeOHE at 500 µg/mL. The MeOHE prevented the hemolysis caused by the rupture of cell membranes induced by lipid peroxidation. Since lipid peroxidation inhibition by antioxidant compounds is crucial to mitigate the induction and/or propagation of oxidative stress related diseases (Mendes et al., 2011), the strong lipid peroxidation inhibition by *R. girolae* extracts may be an indicator of their high therapeutic potential.

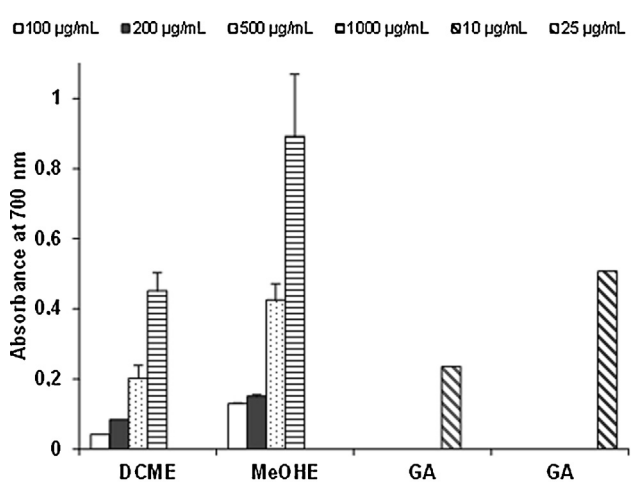

Fig. 3. Reducing power of extracts (FRAP) from *R. girolae* seeds at various concentrations and compared with standard gallic acid at 10 and 25 µg/mL.

Our results show that the level of TP significantly correlated with values from the FRAP assay at 500 µg/mL ($r = 0.959$, $P = 0.01$; $n = 9$). The excellent linear correlations usually found between TP and FRAP may be due to the similarity of chemistry between the two assays (Huang et al., 2005). Moreover, TP content also correlated with values obtained from the DPPH assay at 50 µg/mL ($r = 0.890$, $P = 0.01$; $n = 9$). An extremely significant positive correlation by means of Pearson's coefficient between the TP content and antioxidant activity suggests that phenolic compounds could be responsible for the antioxidant capacity of *R. girolae* seeds.

3.3. LC–ESI-MS/MS and LC–ESI-TOF-MS characterization of the main compounds in MeOHE

LC–ESI-MS/MS analysis showed the presence of catechin derivatives, along with other minor phenolic compounds. The MeOHE chromatogram showed the seven main signals corresponding to compounds **1**–**7** (Fig. 4). A second analysis by LC–ESI-TOF-MS allowed the tentative identification of five compounds: coumaric acid derivative **1**, epigallocatechin **4**, catechin-*O*-glucoside **5**, procyanidin B (catechin dimer) **6** and catechin **7** (Table 2). The four catechin derivatives **4**, **5**, **6** and **7** (Table 2) showed fragmentation patterns similar to those reported in the literature (Del Rio et al., 2004; Touriño et al., 2008; Zywicki et al., 2007). The profiles observed for catechin and procyanidin B are in accordance with those from the MassBank database (www.massbank.jp). Table 2 shows the tentative molecular formula that matches the exact mass of each ion. Compound **1** was tentatively identified as a coumaric acid derivative due to the presence of characteristic ions at m/z 163 and 119 (Regos et al., 2009). In addition, other minor compounds were detected whose fragmentation indicated they could be derived from unidentified catechins and coumaric acid. The phenolic compounds identified in MeOHE, epigallocatechin **4**, catechin-*O*-glucoside **5**, and catechin **7** (see Fig. 5), have shown antioxidant activity in different trials (Schmeda-Hirschmann et al., 2003; Naghma and Hasan, 2007). In previous work, we reported an IC_{50} of 75.6 µg/mL for catechin isolated from *Peumus boldus* Mol. in the lipid peroxidation assay (Schmeda-Hirschmann et al., 2003). The antioxidant effect of catechin on lipid peroxidation has been reported by Gorelik and Kanner (2001), who found that catechin at 500 mM decreased membrane lipid peroxidation by 90% and oxymyoglobin oxidation by 50%. Using erythrocyte membranes, Liao and Yin (2000) compared the antioxidant effect of several flavonoids, including catechin. The antioxidant activity followed the order of catechin > epicatechin > rutin > quercetin > myricetin. Catechins and quercetin are peroxyl and hydroxyl free-radical scavengers and show protective effects against lipid peroxidation (Zhang et al., 1997). It has also been reported that catechin is an inhibitor of COX-1 and COX-2 enzymes, which are involved in prostaglandin biosynthesis (Noreen et al., 1997).

On the other hand, procyanidins are reported to be potent antioxidants. Some studies in animal models show that procyanidins from grape seeds inhibit chemically induced lipid peroxidation, DNA fragmentation, and subsequent apoptosis in a dose-dependent manner (Bagchi et al., 1998). Rats fed with a procyanidin-enhanced diet had a higher plasma antioxidant activity and their hearts were less susceptible to ischemia/perfusion damage induced by iron and copper ions (Maffei Facino et al., 1999). Human studies show that a diet rich in procyanidins decreases/inhibits lipid peroxidation of LDL cholesterol and increases free radical scavenging capacity (Fuhrman et al., 1995; Natella et al., 2002). Procyanidins appear to have an affinity for vascular tissue and they play a role in the protection of critical proteins in connective tissue (collagen and elastin) by strongly inhibiting several enzymes involved in the degradation of both

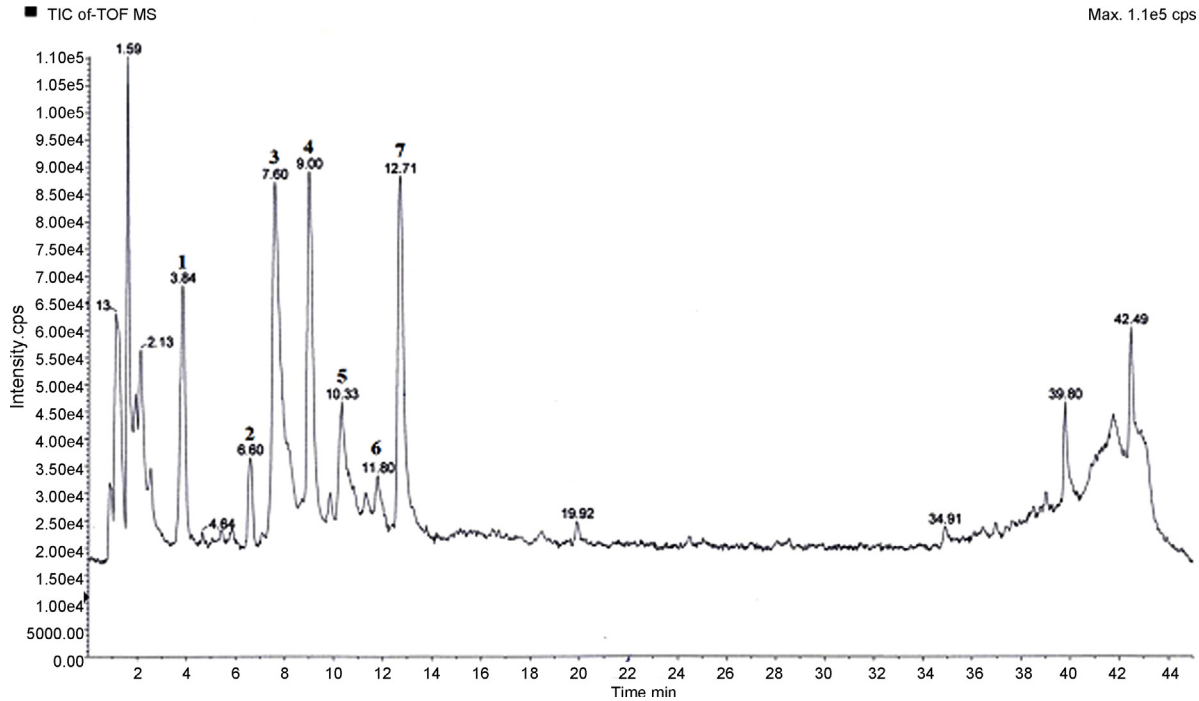

Fig. 4. LC–ESI-QTOF-MS Chromatogram of MeOHE *Ramorinoa girolae* seeds full scan experiment showing the seven main compounds identified.

protein and hyaluronic acid (Maffei Facino et al., 1994)). The identification of several polyphenols in the seed extracts supports our hypothesis that the potent antioxidant activity exhibited by the MeOHE of *R. girolae* seeds could be partially due to the presence of these compounds. Furthermore, to our knowledge, this is the first report of the polyphenol profile of methanolic extracts from *R. girolae* seeds, presenting conclusive phenol characterization by LC–ESI-MS/MS and LC–ESI-TOF-MS, which provide a powerful tool for identifying and quantifying phenolic compounds in plant extracts by means of soft ionization, using electrospray ionization (ESI) and MS/MS techniques (Torras-Claveria et al., 2007).

Thus, considering the presence of recognized antioxidant compounds in *R. girolae* seeds, in addition to their high FRAP and DPPH values, it is possible to think their regular consumption can have a beneficial effect on human health, which validates the use of the plant in popular medicine.

3.4. Fatty acid analysis

The seeds from *R. girolae* fruits contained a high concentration (≈71%) of monounsaturated fatty acids, especially oleic acid (OA), and a low percentage of polyunsaturated fatty acids (≈14%). Seven fatty acids were identified in PEE from *R. girolae* seeds (Table 3). The main feature of their fatty acid profile is the high content (about 71%) of OA, which is one of the highest amounts found in vegetable oils, being comparable to OA levels in some varietal olive oils (Torres and Maestri, 2006). Polyunsaturated fatty acids were only represented by linoleic acid (9,12-octadecadienoic) (14.47%). Saturated acids were found to be hexadecanoic (9.75%, palmitic, 16:0) and octadecanoic (3.13%, stearic, 18:0) acids. Eicosanoic (arachidic, 20:0), eicosenoic (gadoleic, 20:1) and docosanoic (behenic, 22:0) acids (0.58, 0.50 and 0.60%, respectively) were detected in small quantities. Additionally, some parameters used as a means of predicting shelf life and oil stability, such as the Oleic/Linoleic (O/L) ratio, saturated and unsaturated fatty acid ratio (S/U) and iodine index, were calculated. The degree of unsaturation is inversely proportional to the quality of the fat fraction, including the increase of oxidative rancidity, associated with high levels of polyunsaturated acids. The values obtained for *R. girolae* seed oil were O/L, 4.93; iodine index, 86.49 and S/U, 0.164, indicating that it could have higher oxidative stability than peanut oil (Nepote et al., 2009).

OA has been reported to reduce coronary heart disease risk by 20–40%, mainly *via* LDL-cholesterol reduction. Other beneficial effects on risk factors for cardiovascular disease, such as those related to thrombogenesis, *in vitro* LDL oxidative susceptibility and

Table 2
Tentative identities of main compounds detected in the antioxidant methanol extract.

	Compounds	Rt (min)	Detected mass m/z $[M-H]^-$	Theoretical mass m/z $[M-H]^-$	MS/MS fragments	$[M-H]^-$ MF	Error (mDa)
1	Coumaric acid derivative	3.84	309.1121	309.1132	180.072/163.047/128.044/119.055	$C_{19}H_{17}O_4$	−1.13
2	unknown	6.60	296.1530		222.124/195.074/177.065/149.074/133.072/118.054	$C_{23}H_{20}$	0.15
3	unknown	7.60	255.0524		219.869/201.855/195.037/165.066/149. 071/123.052	$C_{11}H_{11}O_7$	1.37
4	(Epi) Gallocatechin	9.00	305.0682	305.0666	219.068/179.037/139.041/125.024	$C_{15}H_{13}O_7$	1.52
5	Catechin-*O*-Glucoside	10.33	451.1261	451.1245	289.076/245.0898	$C_{21}H_{23}O_{11}$	1.51
6	Procyanidin B (catechin dimer)	11.80	577.1356	577.1351	451.122/425.099/407.089/289.084/245.081	$C_{30}H_{25}O_{12}$	0.45
7	Catechin	12.71	289.0730	289.0717	245.084/205.057/123.054	$C_{15}H_{13}O_6$	1.24

MF: Molecular formula.

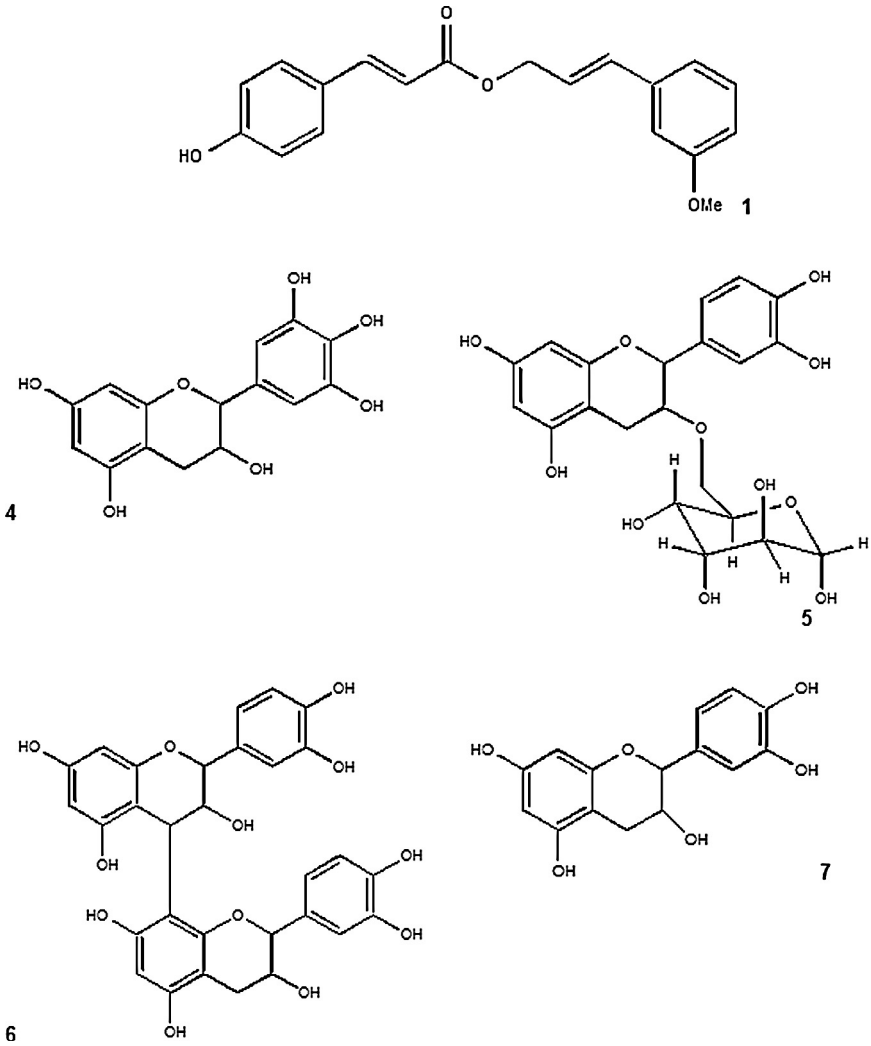

Fig. 5. Main phenolics identified in MeOHE *Ramorinoa girolae* Seeds.
1: Coumaric acid derivative (tentative estructure), **4**: epigallocatechin, **5**: catechin-*O*-glucoside, **6**: procyanidin B (catechin dimer), **7**: catechin.

insulin sensitivity, have also been reported (Wahrburg, 2004; Vessby et al., 2001). An increase in OA intake may also be beneficial as it limits the intake of saturated fat, which in many countries is higher than the recommended levels (Hulshof et al., 1999). Several lines of evidence support the protective effects of OA against Alzheimer's disease and other neurological disorders. Recently, Cardoso et al. (2011) proposed that OA can modulate inflammatory and immune responses in skin lesions. This fact results in differential wound repair and suggests that monounsaturated fatty acid n-9 may be a useful tool in the treatment of cutaneous wounds, especially in cases of skin burns and diabetic or pressure ulcers.

Table 3
Fatty acid composition (% of total fatty acids) from PEE[a] from *R. girolae seeds* (mean value ± SD, $n = 3$).

Fatty acid	Composition (g/100 g fatty acid)
Saturated	
Palmitic acid (C16:0)	9.8 ± 0.2
Stearic acid (C18:0)	3.1 ± 0.4
Arachidic acid (C20:0)	0.58 ± 0.01
Behenic acid (C22:0)	0.60 ± 0.03
Monounsatured	
Oleic acid (C18:1)	71.0 ± 1.0
Eicosenoic acid (C20:1)	0.50 ± 0.02
Polyunsaturated	
Linoleic acid (C18:2)	14.5 ± 1.0
Oleic/Linoleic ratio	4.9 ± 0.4
IV (iodine value)	86.5 ± 1.0

[a] PEE: petroleum ether extract.

Thus, the fatty acid profile of *R. girolae* seed oil also has good nutritional properties, which, together with the previously discussed antioxidant activity, reinforce the beneficial health effects of the seeds extensively used by the population living close to "chicales".

3.5. Multi-element analysis

The multi-element content of *R. girolae* seeds is presented in Table 4. Results show that K, Mg, and Ca are major elements in the seeds, while Na, Fe, Zn, B, Cu, Mn, Al, Sr, Rb, Mo and Be are present in a low proportion. Quantitatively, potassium was the most important, accounting for 59% of the total element content, with a concentration of 8174.76 μg/g. Magnesium and calcium were present in moderate amounts, accounting for 22% and 17% of the total elements, respectively. Fifteen out of twenty nine elements evaluated were above the LOD, but Li, V, Cr, Co, Ni, Ga, As, Se, Cd, Ag, Te, Ba, Tl, Pb and Bi were below (Table 4).

Magnesium (Mg) is the fourth most abundant cation in the body and the second most prevalent intracellular cation. In terms of its

Table 4
Mineral content of *Ramorinoa girolae* seeds (μg/g).

Mineral	Concentration	LOD[a]	LOQ[b]
Li	<LOD	0.010	0.309
Be	0.38 ± 0.02	0.010	0.031
B	25.86 ± 3.18	0.823	1.871
Na	48.25 ± 12.20	1.195	2.183
Mg	3085.00 ± 249.55	1.720	3.732
Al	8.82 ± 2.90	0.281	0.720
K	8174.76 ± 767.91	4.913	9.801
Ca	2320.16 ± 136.21	41.385	119.082
V	<LOD	0.005	0.008
Cr	<LOD	0.559	0.619
Mn	14.98 ± 1.64	0.019	0.045
Fe	35.15 ± 2.97	0.588	3.292
Co	<LOD	0.010	0.031
Ni	<LOD	0.397	1.091
Cu	18.86 ± 1.28	0.010	0.031
Zn	31.47 ± 2.64	1.126	2.626
Ga	<LOQ	0.006	0.021
As	<LOD	0.031	0.051
Se	<LOD	0.033	0.079
Rb	2.65 ± 0.84	0.003	0.008
Sr	5.57 ± 0.30	0.037	0.094
Mo	2.06 ± 0.17	0.065	0.068
Ag	<LOQ	0.002	0.017
Cd	<LOQ	0.010	0.031
Te	<LOD	0.074	0.327
Ba	<LOQ	0.042	0.126
Tl	<LOD	0.441	1.373
Pb	<LOQ	0.010	0.031
Bi	<LOD	0.010	0.031

Values are expressed as mean ± standard deviation (from three replicates).
[a] LOD: Limit of detection (μg/g)
[b] LOQ: Limit of quantification (μg/g)

physiological role, Mg is essential for many enzymatic reactions (Rude and Gruber, 2004). The amount of Mg present would provide 308.5 mg per 100 g of seed consumed, exceeding the amount required by the Dietary References Required (DIRs), which is 250 mg (WHO, 2002).

Copper (Cu) can be found in many enzymes, some of which are essential for Fe metabolism. Cu deficiencies are infrequent, but various studies have reported a direct correlation between the dietary Zn/Cu ratio and the incidence of cardiovascular disease (Cabrera et al., 2003). The recommended daily intake of Cu for adults is 900 μg (WHO, 2002). Cu contribution from the seeds of *R. girolae* is 1900 μg per 100 g of seeds. On the other hand, zinc (Zn) enzymes participate in a wide variety of metabolic processes like the synthesis or degradation of carbohydrates, lipids and proteins. This element is required for deoxyribonucleic and ribonucleic acid synthesis; it may also play a role in stabilizing plasma membranes. Zn has been recognized as a co-factor of the superoxide dismutase enzyme, which is involved in protection against oxidative processes. The net delivery of Zn to an organism is a function of the total amount of this element in foods and of its bioavailability. The recommended daily consumption of Zn for adults is 7.0 mg (WHO, 2002) and 100 g of *R. girolae* seeds produce a Zn intake of 3.15 mg, representing 45% of the required daily dose.

4. Conclusion

In conclusion, this work has shown that *R. girolae* seeds have a significant content of total phenols (including characterized catechins) and flavonoids, which act as sources of dietary antioxidants. Other potential health benefits are provided by the identified content of fatty acids and multi-elements. We can therefore conclude that *R. girolae* seeds represent a useful food in the human diet, which supports their traditional consumption by native populations of the provinces of San Juan, San Luis and La Rioja (Central Western Argentine). Finally, the present study looks to enhance the use of *R. girolae* as a food for human nutrition, mainly by considering its polyphenolic profile as a source of antioxidant compounds, its high oleic oil content, and a multi-element profile rich in potassium.

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