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PII: S0308-8146(16)30498-8
DOI: http://dx.doi.org/10.1016/j.foodchem.2016.03.115
Reference: FOCH 18999

To appear in: *Food Chemistry*

Received Date: 11 November 2015
Revised Date: 16 February 2016
Accepted Date: 29 March 2016

Please cite this article as: Cattaneo, F., Costamagna, M.S., Zampini, I.C., Sayago, J., Alberto, M.R., Chamorro, V., Pazos, A., Thomas-Valdés, S., Schmeda-Hirschmann, G., Isla, M.I., Flour from *Prosopis alba* cotyledons: a natural source of nutrient and bioactive phytochemicals, *Food Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.foodchem.2016.03.115

Flour from *Prosopis alba* cotyledons: a natural source of nutrient and bioactive phytochemicals

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Running title: Characterization of cotyledons flour of *Prosopis alba*

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Abstract

The *Prosopis alba* seed is a waste material in the process to produce pod flour. To suggest a potential use of these seeds it is necessary to determine the nutritional, phytochemical and funtional quality of cotyledon flour from *Prosopis alba*. This flour showed high level of proteins (62 %), low content of total carbohydrate and fat. Free polyphenol (1150 ± 20 mg GAE/100 g flour) and carotenoids (10.55±0.05 mg β-CE/100 g flour) compounds were the dominant compounds. The main identified constituents in the polyphenolic extracts were C- glycosyl flavones, including schaftoside, isoschaftoside, vicenin II, vitexin and isovitexin. The extract enriched in polyphenolic compounds exhibited $ABTS^{\bullet+}$ reducing capacity and scavenging activity of H_2O_2, and was able to inhibit phospholipase, lipoxygenase and ciclooxygenase, three pro-inflammatory enzymes. According to our results, the *P. alba* cotyledon flour could be considered as a new alternative in the formulation of functional foods or food supplements.

Keywords: *Prosopis alba,* cotyledon flour, nutritional properties, antioxidant activity, anti-inflammatory capacity, genotoxicity.

Introduction

In Argentina, the *Prosopis* species are characteristic of the Monte phytogeographical region from the province of Salta to the province of Chubut. The pods of "algarrobo" (*Prosopis alba* Griseb.) are used to prepare fermented and non-fermented beverages (aloja and añapa) and food products such as syrup, flour and sweets (arrope, patay, jam) (Cardozo, Ordóñez, Zampini, Cuello, Dibenedetto & Isla, 2010; Pérez et al., 2014; Cattaneo et al., 2014). The main fraction from the *Prosopis* pods used to prepare flour is the mesocarp. In previous papers the nutritional and biological properties of mesocarp flour (Cardozo et al., 2010; Pérez, et al., 2014) were reported. The seeds plus endocarp of *Prosopis* pods are discarded and constitute food waste. Nowadays, food wastes are substrates for the recapture of functional compounds and the development of new products with a market value (Galanakis, 2013; Galanakis, 2012). On the other hand, some phytochemical (phenolics compounds and carotenoids) obtained from food waste could be used as natural food or beverage preservatives (Galanakis, 2012). However, the main phytochemicals, the nutritional quality, and the biological activity of *P. alba* cotyledon flour remain unknown. To suggest a potential use of these material is needed to determine the nutritional, phytochemical and funtional quality of cotyledon flour from *P. alba*. At present, only a report indicated that the isolated proteins of cotyledons showed, all essential amino acids, biological activities (antioxidant and anti-inflammatory activities), and functional properties such as solubility at different pH, capacity to produce emulsions, oil binding capacity and water adsorption capacity (Cattaneo et al., 2014). For this reason, cotyledon proteins from *P. alba* could be used as functional food or food additive.

The goal of this study was to assess the phytochemical composition, proximate composition, phenolic profile and determine some biological activities of the *P. alba* cotyledon flour.

2. Materials and Methods

2.1. Phytochemical extraction from *Prosopis alba* cotyledon flour

Ripe *Prosopis alba* (Griseb.) pods were collected in Amaicha del Valle (Tucumán, Argentina). The cotyledon flour analysis was carried out according to Cattaneo et al., (2014).

Differential extractions from cotyledons flour was realized to obtain sugar (Costamagna, Ordóñez, Zampini, Sayago & Isla, 2013) and storage proteins (albumin, globulin, prolamin and glutelin). The proteins were obtained from cotyledon flour based on their solubility (Ferreira, Franco & Teixeira, 2000).

Free phenolic compounds were obtained from cotyledons flour according to Pérez et al., (2014). The methanolic extract enriched in free phenolic compounds (ME-enriched in FPC) was concentrated under reduced pressure using a rotary evaporator (Buchii, China) to dryness at 40 °C and then filled up with methanol to a final volume of 10 mL. After extraction of free phenolics, the bound-phenolics were extracted from residual material according to Pérez et al., (2014) by alkaline and acid hydrolysis. The released phenolic acids were extracted with ethyl acetate and then, were evaporated to dryness. Then, the bound phenolic compounds were reconstituted in 10 mL of methanol and stored at -20 °C until use.

Phenolic compounds such as tannins and anthocyanins were extracted according to Cardozo, Ordóñez, Alberto, Zampini & Isla (2011). Tannins were extracted with acetone: water (70:30, v/v) to obtain acetone-water extract enriched with tannins (AWE-enriched in tannins). Anthocyanins were extracted with 1% HCl in methanol to obtain the anthocyanins extract (AE).

The carotenoid and vitamin C extractions were carried out according to Costamagna et al., (2013).

2.2. Chemical composition

2.2.1. Macronutrient determination

The proximate composition of the cotyledon flour was assessed according to the Association of Official Analytical Chemists (AOAC, 2000) methods, as follows: moisture, ashes, total protein, total carbohydrate, fat and dietary fiber. The results were expressed in g/100g of flour.

Total neutral soluble sugars and reducing soluble sugars were measured according to Costamagna et al., (2013).

Storage proteins (albumin, globulin, prolamin and glutelin) were obtained from flour based on their solubility (Ferreira et al., 2000). The process started with the removal of albumin with 10 mM $CaCl_2$ and 10 mM $MgCl_2$ with agitation during 2 h at 4 °C. Then, the suspension was centrifuged at 10,000 *xg* during 1 h. The pellet was resuspended in a solution of 100 mM Tris-HCl, 10% NaOH, 10 mM EDTA and 10 mM EGTA, pH 7.5 during 4 h at 4 °C and centrifuged at 10,000 *x*g for 1 h and globulins were thus obtained. The pellet was resuspended in 75% ethanol for extracting prolamines and maintained during 4 h. Then, it was centrifuged at 9,000 x*g* for 1 h. Finally the precipitate was subjected to a final extraction for the glutelins with H_3BO_3 solution containing 50 mM β-mercaptoethanol, 1% (v/v), sodium dodecyl sulfate (SDS) and then was centrifuged at 9,000 *xg* for 1 h. Protein fractions were stored at -20 °C for further characterization.

2.2.2. Amino acid analyses

Flour samples of cotyledon were subjected to acid hydrolysis according to Creamer & Matheson, (1976) and the amino acid concentrations were determined using a Biochrom 30 series amino acid analyzer (http://www.biochrom.co.uk) in cation-exchange resin column. L-Norleucin was used as internal standard.

2.2.3. Mineral analyses

The mineral analysis of cotyledon was carried out by quadrupole inductively coupled plasma mass spectrometry (Q-ICPMS) (ISIDSA, Córdoba, Argentina). The mineral content and composition of ashes was determined by atomic absorption spectroscopy according with the AOAC (2000) recommendations. The following ions were analyzed: sodium, magnesium, potassium, calcium, and iron. The phosphorus analysis was performed by AOAC Spectrophotometric method (AOAC, 2000). The results were expressed in mg/g of flour. All analyses were performed in triplicate.

2.2.4. Fat acid analysis

Fatty acid methyl esters were quantified using an Agilent Technologies (Model 6890N) GC with flame ionization detector.

The fatty acid composition of the cotyledon oil was investigated by gas chromatography of the corresponding methyl esters (FAME). A sample of the oil (100 mg) was saponified with 15 mL of alcoholic KOH for 20 min. After addition of 15 mL of 20% BF_3 in MeOH, the reaction mixture was refluxed for 2 h. After cooling, water and 5 mL of petroleum ether (PE) were added. The methyl esters dissolved in the PE fraction. The mixture was analyzed by gas chromatography and the FAME were identified by comparison of the retention times with known FAME mixtures. GC analysis was carried out using a Shimadzu GC-9A instrument with a capillary Supelco SP 2330 column (30 m x 0.25 mm i.d.). To reconfirm the identity of the FAME, GC-MS analysis was undertaken using a Perkin Elmer Turbo Mass equipment (USA) with a capillary Elite 5MS column (column length 30 m, internal diameter 0.25 mm). The chromatographic conditions were as follows. Time: 45 min. Delay time: 0.0 min; initial temperature: 80°C for 2 min. Ramp 1: 4.0 °C/min to 200 °C hold for 0.0 min. Ramp 2: 6.0 °C/min to 250°C hold for 6.676 min.

The standard used (Supelco) contained the following fatty acids (as methyl esters): caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0); tridecanoic acid (C13:0), myristic acid (C14:0), myristoleic acid (C14:1n9c); pentadecanoic acid (C15:0); palmitic acid (C16:0); palmitoleic acid (C16:1n9c); heptadecanoic acid (C17:0); stearic acid (C18:0), elaidic acid (C18:1n9t); oleic acid (C18:1n9c); linoleic acid (C18:2n6c); arachidic acid (C20:0); cis-11-eicosenoic acid (C20:1); linolenic acid (C18:3n3); behenic acid (C22:0) and erucic acid (C22:1n9).

2.2.5. Secondary metabolites analyses and fiber

Free and bound polyphenol were determined by Folin–Ciocalteau's reagent (Pérez et al., 2014).

Flavonoid content in ME-enriched in FPC was determined according to Pérez et al.. (2014).

Total proanthocyanidin in AWE-enriched in tannins was determined with 4-dimethylaminocinnamaldehyde according to Cardozo et al., (2011).

Gallotannin content was determined in AWE-enriched in tannins after acid hydrolysis with 4 mL of 2N H_2SO_4 at 100 °C for 26 h. The gallic acid released was determined according to Cardozo et al., (2011) and the gallotannin was calculated as:

Gallotannins (mg GAE) = [GH(AWE)]- [G(AWE)]

where [GH(AWE)] is the amount of gallic acid present in the hydrolyzed extract [H(AWE)] and [G(AWE)] is the amount of gallic acid present in the non-hydrolyzed extract.

Total anthocyanins and carotenoids were determined according to Cardozo et al., (2011); ascorbic acid and fiber according to Costamagna et al., (2013).

2.2.6. Profile of phenolic compounds by HPLC-DAD

The polyphenolic extract from *P. alba* cotyledons was analyzed by HPLC coupled to a diode array detector (HPLC-DAD) to set the conditions for HPLC-MS analysis. The HPLC system used for DAD analysis was a Shimadzu equipment (Shimadzu Corporation, Kyoto, Japan) consisting of a

LC-20AT pump, a SPD-M20A UV diode array detector, CTO-20AC column oven and a LabSolution software. A MultoHigh 100 RP 18–5µm (250 × 4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany) maintained at 25 °C was used. Approximately 5 mg/mL of the polyphenolic extract was filtered through a 0.45 µm filter (Waters, Milford, MA, USA) and injected into HPLC-DAD and HPLC-ESI-MS/MS. The compounds were monitored at 254 and 330 nm, and UV spectra from 200 to 600 nm were recorded for peak characterization. The HPLC analyses were performed using a linear gradient solvent system consisting of water:acetic acid 99.9:0.1 v/v (A) and methanol:acetic acid 99.9:0.1 v/v (B) as follows: 90% A to 43% A over 45 min, followed by 43 to 0% A from 45.0 to 60 min and 100% B from 60 to 65 min. The flow rate was 0.5 mL/min and the volume of injected sample was 20 µL. The mass spectrometer consisted of a HPLC HP1100 (Agilent Technologies Inc., Santa Clara, CA, USA) connected through a split to the mass spectrometer Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltonik GmbH, Bremen, Germany). Ionization was performed at 3000 V assisted by nitrogen as nebulizing gas at 50 psi and as drying gas at 365 °C and a flow rate of 10 L/min. Negative ions were detected using full scan (*m/z* 20–2200) and normal resolution (scan speed 10300 *m*/*z*/*s*; peak with 0.6 FWHM/*m*/*z*). The trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms. Collision induced dissociation (CID) was performed by collisions with helium background gas present in the trap and automatically controlled through Smart Frag option.

The compounds were identified by interpretation of the mass spectra and UV data as well as by comparison with reference compounds. For the quantification of the main flavonoids, standards of schaftoside (code 83325) and vitexin (code 89290) (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) were used. Plots were build relating area and concentration in the

range of 10-750 ppm. The coefficients r^2 were 0.999 for schaftoside and 0.993 for vitexin, respectively. The results are expressed as mg equivalents of the standard compounds employed per gram of extract

2.2.7. Identification of phenolics by HPLC-ESI-MS/MS

The identification of *Prosopis* cotyledon flour phenolics was carried out by interpretation of the spectral properties (UV and ESI-MS and MS/MS) of the compounds and comparison with those of reference samples and literature data. The main constituents in *Prosopis* mesocarp flour were flavonoid C-glycosides as described by Pérez et al., (2014) and Schmeda-Hirschmann et al., (2015).

2.3. Measurement of antioxidant capacity

The ABTS cation radical scavenging capacity of ME-enriched in PC (1 to 50 µg/mL) was determined as described Cardozo et al., (2011) with an end-points at 6 min. Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and ME- enriched in PC (2.9 to 60 µg GAE/mL) to produce hydroxyl radicals from the Fe^{+3}/ascorbate/ EDTA/H_2O_2 system (Cardozo et al., 2011). The hydrogen peroxide scavenging activity was determined according Aruoma, Grootveld, & Halliwell, (1987). The commercial synthetic antioxidant (BHT) and the natural antioxidant quercetin were used as positive control.

2.4. Anti-inflammatory activity

The inhibitory effect of ME-enriched in PC (until 30 µg GAE/mL) on lipoxygenase (LOX) activity and on secretory phospholipase A_2 ($sPLA_2$) activity was determined according to D'Almeida, Isla, Vildoza, Quispe, Schmeda-Hirschmann & Alberto (2013).

The inhibitory activity of ME-enriched in PC on cyclooxygenase 2 (COX-2) was measured using a COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) according of manufacturer's instructions.

2.5. Mutagenicity and antimutagenicity assays

The mutagenic and antimutagenic effects of ME-enriched in PC (25-100 µg GAE/plate) were evaluated on two *Salmonella typhimurium* strains (TA98 and TA100) according to Maron & Ames (1983) with and without an exogenous metabolic system, the S9 fraction (Moltox – Molecular Toxicology Inc). The positive controls employed were 4-nitro-o-phenylenediamine (4-NPD, 10 µg/plate) and 2-aminofluorene (2-AF, 10 µg/plate).

2.6. Statistical analysis

Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation (SD). The correlation between two variants by Pearson test was analyzed using Infostat software package with the level of significance set at $p < 0.05$ and of variance (ANOVA) with Tukey post-test at a confidence level of 95%.

3. Results and discussion

In Argentina, *P. alba* flour is produced from whole dry pods. The hardness of the endocarp prevents seed grinding with the consequent yield loss. An efficient method to separate cotyledons from endosperm and episperm of *P. alba* seeds was recently reported by Cattaneo et al., (2014) and used in these research. The endocarp fraction represents 47.7 ± 4.8% of pod dry weight, seeds constitute 12.8 ± 1.3% and cotyledon fractions make up 6.1 ± 0.6%. Cotyledon flour was obtained by grinding (Figure 1) and was then characterized.

3.1. Macronutrients, amino acid composition, mineral and secondary metabolites in Prosopis cotyledon flour

Macronutrients

By comparing the cotyledon flour with mesocarp flour of *P. alba* pods it can be observed that the mesocarp flour exhibit higher carbohydrate content (52.08 ± 0.09 g/100 pod flour, Cardozo et al.,

2010) than cotyledon flour (8.97±0.05 g/100 g cotyledon flour, Table 1). The content of soluble reducing sugar (0.21±0.07 g/100g cotyledon flour) was lower than mesocarp flour (3.73 g/100 g pod flour). On the other hand, the fat content was higher (12.20 ± 0.05 g/100 g flour) in cotyledon flour (Table 1).

The crude protein was the major component of cotyledon flour (62.09%) while, the content of proteins of mesocarp flour was only 4.2% (Cardozo et al., 2010) (Table 1). Protein values from *P. alba* cotyledon flour were higher than those of soybeans (34.6%), lentils (25.4%), peas (22.9%), chickpeas (18.5%) (Cosiansi, Milanesi, Da Riva, & Hayipanteli, 2002). Albumin (44.59±1.70%) and globulin (30.82±1.64%) were the major proteins. Recently, the biological activity of isolate protein and protein hydrolysate obtained from *Prosopis alba* cotyledon were described (Cattaneo et al., 2014).

The results suggested that due to its high protein content with biological activity and the low content of fat and carbohydrate, the cotyledon flour could be considered a new alternative in the formulation of foods or food supplements with low calories, alone or combined with cereal proteins (cereal bars, cookies, coffee substitutes).

Amino acids

Table 2 shows the essential amino acid pattern of *P. alba* cotyledons flour compared to wheat, soy, skim milk and the reference values of FAO/WHO (1990) for evaluating proteins. In general, animal proteins are considered good sources of complete proteins. On the other hand, plant proteins (including those of cereals) are often called incomplete proteins because they generally lack of one or more essential amino acids. Despite this, *P. alba* cotyledon proteins contained all essential amino acids such as soy and wheat (Table 2). The amino acid profile of cotyledon flour showed that the concentration of essential amino acids, such as isoleucine and valine, normally deficient in other

grains, were higher than the reference pattern (FAO/WHO, 1990), while the level of other essential amino acids was adequate. *P. alba* cotyledon proteins contain amino acids with sulphur (cys 1.47%), which are the limiting amino acids in other grains (Johnson & Aguilera, 1980) (Table 2).

Fatty acids

Nine fatty acids, including saturated fatty acids (SFAs), mono-unsaturated (MUFAs) and poly-unsaturated fatty acids (PUFAs), were determined in cotyledon flour. The main total fatty acids (TFA) components were PUFA (linoleic acid, 60.62%), followed by MUFA (oleic acid, 18.08%), and SFA (palmitic acid, 15.87%). Linoleic acid (omega-6 fatty acids) belongs to the essential fatty acids that humans and other animals are unable to synthesize and must be acquired from food sources. They act as structural components of membranes and as precursors of eicosanoids, which modulate renal and pulmonary function, vascular tone, and inflammatory responses. Linoleic acid, significantly benefits human health, induces the expression of uncoupling proteins and coordinately up regulates several dozen genes related to oxidative energy metabolism. Subsequently, PUFAs stimulate mitochondrial biogenesis and enhance energy reserves, leading to stabilized synaptic function (Mori & Hodgson, 2013). Consequently, the cotyledon flour could also be used as a potential supplement of essential fatty acids.

Minerals

As regards minerals, the cotyledon flour was high in K (7.5 mg/g flour) but low in Na (0.09 mg/g flour). A high K/Na ratio makes *Prosopis* cotyledon flour interesting for diets with a defined electrolytic balance. The high content of K can be utilized beneficially in the diets of people who take diuretics to control hypertension. Minerals are also important as constituents of bones, teeth, soft tissues, haemoglobin, muscle, blood and nerve cells and are vital to mental and physical welfare. One hundred grams of cotyledon flour provide the 91% and 71% of the daily requirements

of Mg (2.9 mg/g flour) and P (9.1 mg/g flour). The content of Ca and Fe in cotyledon flour was 1.07 and 0.10 mg/g flour, respectively.

Phytochemicals

Phenolic compounds

The free polyphenol content of cotyledon flour extracted with 70% aqueous/methanol (1150 ± 20 mg GAE/100 g flour) was higher than those from mesocarp flour (180 to 410 mg GAE/100 g flour, Cardozo et al., 2010; Pérez et al., 2014) and white wheat flour (4.4–14 mg GAE/100 g flour, Hung, Maeda, Miyatake, & Morita, 2009). The free phenolic content was similar to fruits flour from the Argentine native plants *Ziziphus mistol* (1190 ± 68 g GAE/100 mg DW, Cardozo et al., 2011) and *Geoffroea decorticans* (1240 ± 30 mg GAE/100 g DW, Costamagna et al., 2013). Vasco, Ruales & Kamal-Eldin, (2008) classified the fruits according to their polyphenolic content in low (<100 mg GAE/100 g), medium (100–500 mg GAE/100 g) or high (>500 mg GAE/100 g) categories. *Prosopis* cotyledon flour would be considered to be in the high level of free phenolic compound. C-glycosyl flavonoids were the main free phenolics in the cotyledon flour (396 mg QE/100 g flour) followed by tannins (Table 1). Pérez et al., (2014) reported high flavonoid content in *Prosopis* mesocarp flour (3-6 %). In our working conditions, hydrolizable tannins and anthocyanins were not detected in cotyledon flour. Phenolic compounds play an important role controlling oxidative stress in the human body by maintaining a balance between oxidants and antioxidants and for this reason it has been associated with possible prevention of chronic illnesses such as cancer, atherosclerosis or cardiovascular disease (Joven et al., 2013). The bound phenolic content in cotyledon flour was 230 mg GAE/100 g cotyledon flour. The content of bound phenolic was similar to that found in wheat flour grade 1 (Hung, Maeda, Miyatake, & Morita, 2009). The bound phenolics were considered to have more health benefits because they may escape from upper gastrointestinal

digestion conditions along with cell wall materials and are absorbed into blood plasma during digestion of intestinal microflora (Andreasen, Kroon, Williamson & Garcia-Conesa, 2001).

HPLC-ESI-MS/MS analysis

The UV spectra of the main compounds detected in the phenolic-enriched extract of *P. alba* cotyledon flour showed maxima in the range of 324-338 nm, suggesting the occurrence of flavones or *C*-glycosyl flavones. The characteristic UV maxima for flavone *C*-glycosides is around 330 nm for the flavonoid Band 1 while in the MS/MS spectra, the flavone *C*-glycosides present losses of 120, 90 and 30 atomic mass units, according to literature (Pérez et al., 2014; Quispe, Petroll, Theoduloz, & Schmeda-Hirschmann, 2014; Schmeda-Hirschmann et al., 2015). Four sets of isomers with $[M-H]^-$ of 725, 593, 563 and 431 atomic mass units (amu) were found in the sample, with the compounds **5** and **6** as main constituents. The compounds **1**-**8** showed the characteristic loss of 120 amu and differ in the relative proportion of the daughter ions in support of *C*-glycosyl flavones. The isomers, differing in the placement of the sugars either at C-6 or C-8 can be differentiated by the relative proportion of the MS/MS ions.

The HPLC chromatogram is show in Figure 2 and extracted ion MS^2 spectra of compounds **1**-**6** and **8** are shown in Figure 3. The compounds 1 and 2, eluting at Rt 34.9 and 35.8 min showed a *m/z* ion at 725 amu and the neutral loss of 162 amu (hexose) leading to a flavone C-glycoside, in agreement with schaftoside/isoschaftoside. The compounds **1** and **2**, differing in the Rt are isomers and were identified as schaftoside/isoschaftoside hexosides. Both flavonoids are related to the 4'-O-β-D-glucopyranoside of 6´-O-β-glucopyranoside of schaftoside/isoschaftoside, reported from *Ceratonia siliqua* or *Stellaria holostea*, respectively (Dictionary of Natural Products in DVD, 2015). However, the exact placement of the O-glycoside cannot be given. The mass spectra of compounds **3** and **4** show a characteristic fragmentation pattern of flavonoids C-glycosides and were identified as

Vicenin II/Isomer (Schmeda-Hirschmann, Quispe, Soriano, Theoduloz, Jiménez-Aspee, Pérez, Cuello, & Isla, 2015). The main phenolics in the cotyledon flour were the flavonoids **5** and **6**, identified as isoschaftoside and schaftoside, respectively (Pérez et al., 2014; Schmeda-Hirschmann et al., 2015). The compounds **7** and **8** were the C-glycosyl flavones vitexin and isovitexin, identified by the UV spectra, MS data and co-injection with standards. The compounds **3**-**8** were previously reported from the pod mesocarp flour from *Prosopis nigra* and *P. alba* (Pérez et al., 2014), and Chilean *Prosopis* species (Schmeda-Hirschmann et al., 2015). The main constituents identified in this extracts were C- glycosyl flavones, including schaftoside, isoschaftoside, vicenin II, vitexin and isovitexin. The compounds are based on the aglycone apigenin and were previously described from *P. alba* mesocarp and *Prosopis* pod syrups (Pérez et al., 2014; Quispe et al., 2014). HPLC-DAD-MS data of the phenolic identified in methanol extracts of *P. alba* cotyledons are presented in Table 3. The apigenin di-C-glycosides (ACGs) have been reported to contribute to the yellow colour of Asian alkaline noodles made from bread wheat (*Triticum aestivum* L) flour (Asenstorfer, Wang, & Mares, 2006). As a consequence, they are a potential target for wheat breeders attempting to develop new cultivars with improved noodle colour. An increase in the levels of these endogenous compounds would enable manufacturers to reduce the use of colour additives that are currently in common usage. Flavonoid *C*-glycosides have shown to present antioxidant, anti-inflammatory, antiplatelet activity, anti-cancer, enzyme converting angiotensin (ECA) inhibitors, among others (Zhang, Luo, Han, Xu, & Kong, 2015; Barreca, Belloco, Leuzzi & Gattuso, 2014; Carvalho, Silva, Silva, Valent, Andrade, & Bastos, 2010; Zucolotto, Goulart, Montanher, Reginatto, Schenkel & Frode, 2009; Velozo, Ferreira, Santos, Moreira, Guimaraes, Emerenciano et al., 2009; Piccinelli, Garcia Mesa, Armenteros, Alfonso, Arevalo, Campone et al., 2008). They are rapidly absorbed after oral administration and distributed by plasma in different tissues. Furthermore, its distribution

indicates that long-lasting therapeutic effect on liver disease may be expected (Wang, Jiang, Dua, Liang, Wang, Zhang, Zhang, Yeb, & Li, 2012).

Carotenoids and ascorbic acid

Carotenoids were the dominant compounds in cotyledon flour (10.55±0.05 mg β-CE /100 g flour) while ascorbic acid (0.33 mg L-AA/100 g flour) was detected in very low levels (Table 1). Carotenoids and ascorbic acid are good antioxidants and carotenoids also can be precursors of vitamin A. In addition, both phytochemicals could be applied as natural food or beverage preservatives since they extend the shelf-life of the product by delaying the formation of off-flavors and rancidity compounds (Galanakis, 2012).

Fiber

The insoluble fiber content of *Prosopis* cotyledon flour was 9.06% . Thus, it could be considered a "source of fiber", since its contents surpass 3 g/100 g (Table 1). Further research would be desirable to assess the physiological effects of this fiber in the human body.

3.2. ***Antioxidant activity of extracts enriched with free phenolic compounds***

The antioxidant activity of organic extracts enriched with polyphenolic compounds (derivates C-glycosil apigenin) obtained from cotyledons flour was analyzed in the present study.

The preparations exhibited $ABTS^{\bullet+}$ reducing capacity with SC_{50} values of 7.1 µg GAE/mL. A dose-response relationship ($r^2>0.90$, $p<0.05$) between the scavenging activity percentage and phenolic compound content was observed. The $ABTS^{\bullet+}$ scavenging activity of phenolic extract was similar than commercial natural and synthetic antioxidants used in food industry such as quercetin (SC_{50}= 1.4 µg/mL) and BHT (SC_{50} = 3.52 µg/mL).

During aerobic metabolism, reactive oxygen species (ROS), such as radicals and non-radicals are produced as by-products. Between them, hydroxyl radicals ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2).

Although hydrogen peroxide itself is not very reactive, can diffuse across biological membranes and, it can generate the highly reactive $HO^{\bullet}$ through the Fenton reaction (Halliwell, 1991). Hydrogen peroxide can deactivate enzymes involved in cellular energy production such as glyceraldehyde-3-phosphate dehydrogenase found in glycolytic pathway (Hyslop et al., 1988) as well as α-ketoglutarate dehydrogenase found in Krebs cycle (Tretter & Vizi, 2000) by oxidation of essential thiol (-SH) groups. Therefore, scavenging of hydrogen peroxide could be considered as an important feature of antioxidants. The ME-enriched in FPC showed scavenging activity of H_2O_2 with SC_{50} values of 25 µg GAE/mL. The potency was similar than a natural antioxidant such as quercetin (50 µg/mL). The polyphenolic extract does not scavenge or promote the formation of $HO^{\bullet}$ radicals (data not shown).

The antioxidant activity of the phenolic extract could be attributed to C-glycosil flavonoids, compounds with demonstrated antioxidant capacity (Picerno, Mencherini, Lauro, Barbato, & Aquino, 2003).

3.3 Anti-inflammatory activity

The inhibition of the LOXs, COX-2 and sPLA2 activities would be an important treatment to many inflammatory diseases (D'Almeida et al., 2013).

The ME- enriched in PC was able to inhibit the LOX activity in a concentration dependent manner. The inhibition was 12, 43, 63 % for 10, 20 and 30 µg GAE/mL of ME-enriched in PC, respectively. The IC_{50} value was 24 µg GAE/mL. A phenolic acid used as control, caffeic acid at 30 µg/mL showed lower inhibition (31%) than the extract.

The $sPLA_2$ activity was also affected (39% with 30 µg GAE/mL). A similar effect (40.2% of inhibition) was reached with 50 µg/mL of the anti-inflammatory drug, acetylsalicylic acid. On the other hand, 20 µg GAE/mL of ME- enriched in PC was able to inhibit 98.5% the COX-2 activity.

Plant extracts with a percentage inhibition above 70%, from 40% to 70%, and below 40% were considered as showing high, moderate or low inhibitory activity, respectively (Tunón, Olavsdotter & Bohlin, 1995). At the concentration of 20 µg/mL, the ME- enriched in PC exhibited high COX-2 inhibition.

The reference drug nimesulide showed an IC_{50} value of 0.39 µg/mL. The extract enriched with polyphenols was more effective to inhibit COX-2 and LOX enzymes than $sPLA_2$ enzyme. In the search for new anti-inflammatory drugs, the simultaneous blockage of both the COX and LOX pathways by dual inhibitors has been suggested as a possible alternative approach to selective COX-2 inhibition (Pferschy-Wenzig, Kunert, Presser & Bauer, 2008). Compounds or extracts possessing these characteristics are believed to be more effective, with lower gastric toxicity due to a stronger anti-inflammatory effect.

3.4. Mutagenic and antimutagenic activities

The extract enriched with phenolic compounds (C-glycosyl apigenin derivatives), was not mutagenic nor antimutagenic on TA98 nor TA100 strains.

4. Conclusions.

The flour of cotyledons from *P. alba* pod seeds could be used as functional food or food additive by its high content of functional phytochemicals (proteins, carotenoids, phenolic compounds and linoleic acid) and fiber. Therefore, our studies add value to this species and could promote their propagation, conservation and sustainable management in arid areas.

Acknowledgements

We thank FONDECYT Project 1120096, PCCI12067 and MINCYT (CH/11/13) "Valorización de frutos nativos sudamericanos", ANPCyT (PICT 1959) "Evaluación de las propiedades nutricionales y funcionales de frutos nativos del noroeste argentino para su revalorización y aprovechamiento en el diseño de alimentos funcionales", CONICET (PIP) and SCAyT-UNT (PIUNT G-533) for financial support.

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Figure Legends

Figure 1. *Prosopis alba* pods (A); seeds and cotyledons (B); cotyledon flour (C).

Figure 2. HPLC chromatogram of polyphenols from *P. alba* seed flour. Detection: $UV_{254\ nm}$. Compounds: **1**: isoschaftoside hexoside; **2**: schaftoside hexoside; **3**: Vicenin II (Apigenin-di-C-hexoside) /Isomer; **4:** Vicenin II/Isomer; **5**: Isoschaftoside (Apigenin-C-hexoside-C-pentoside); **6**: Schaftoside; **7**: Vitexin; **8**: Isovitexin.

Figure 3. Extracted ion MS^2 spectra of compounds **1**-**6** and **8** identified in the phenolic-enriched cotyledon flour extract.

Figure 1

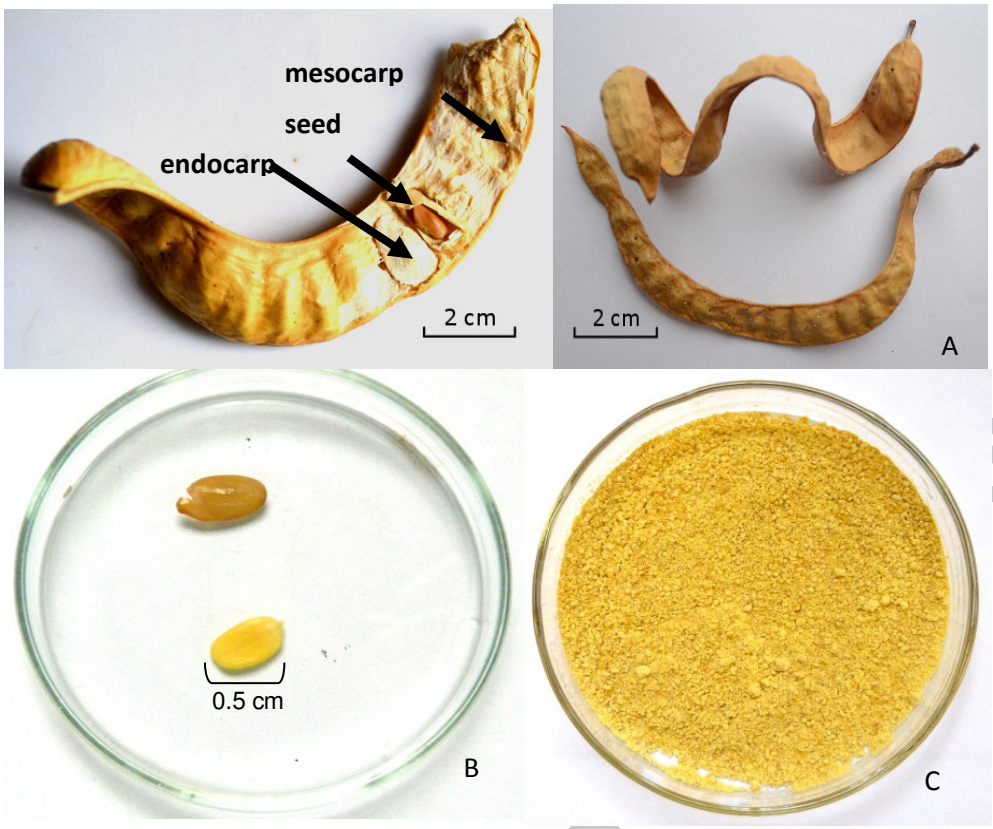

Figure 2

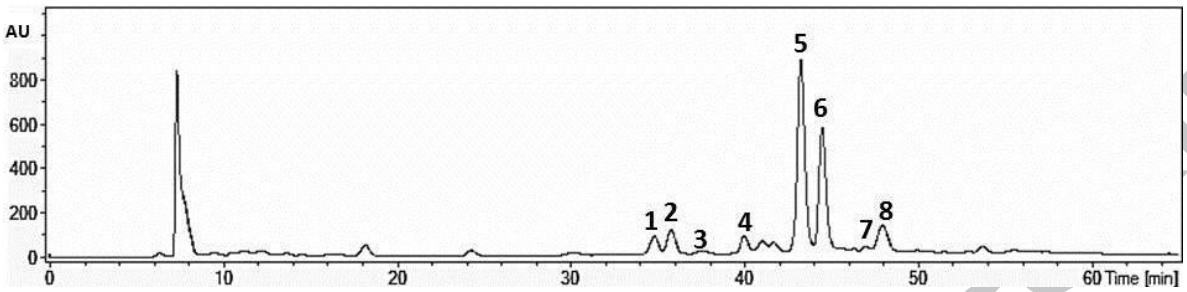

Figure 3

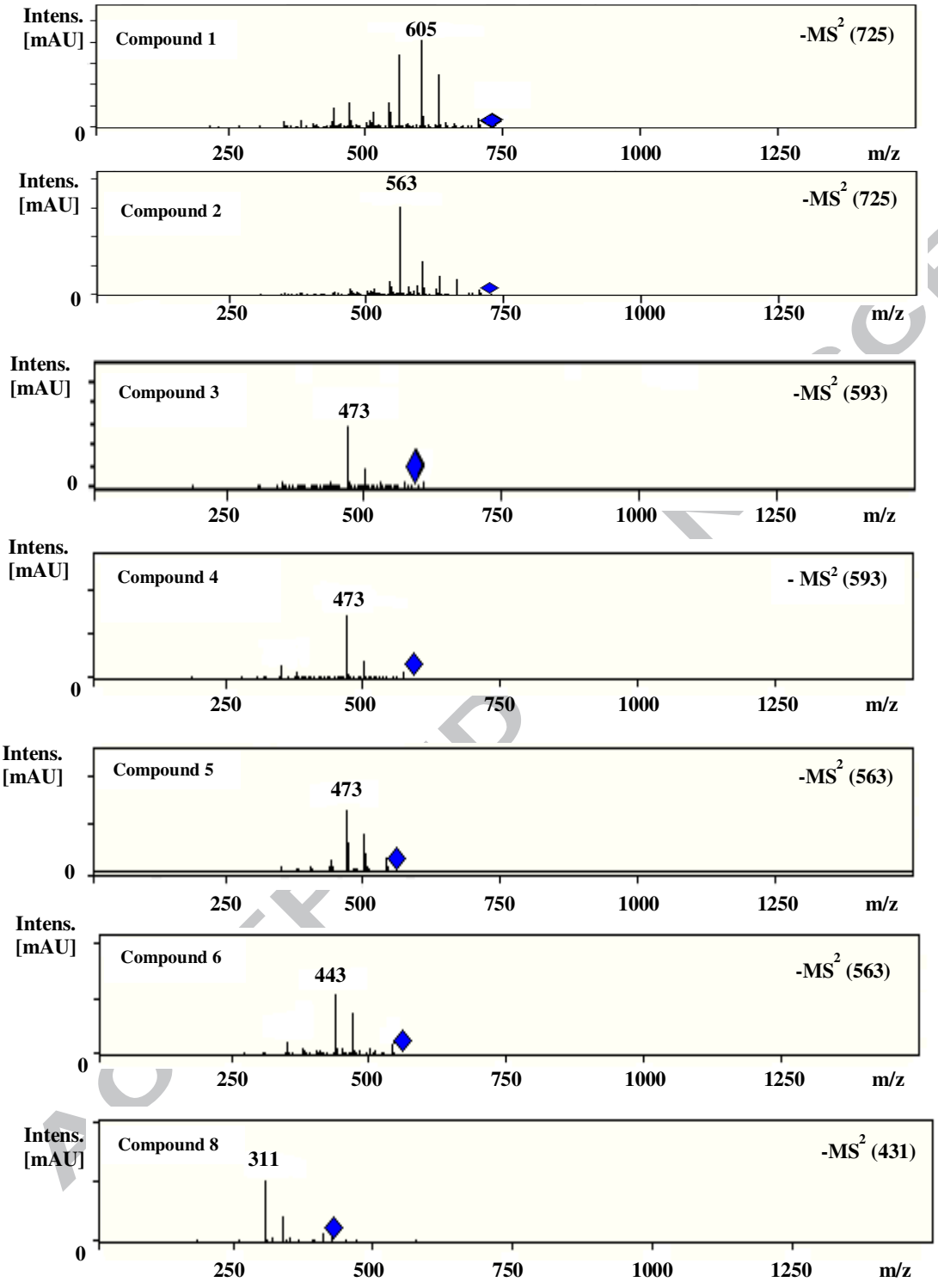

Table 1. Chemical characterization of *Prosopis alba* cotyledons flour

Macronutrient Content of flour		Phytochemical Content of flour	
Total carbohydrate (g/100 g sample)	8.97±0.05	Free phenolics (mg GAE/100 g DW)	1150±20
Total soluble sugars (g GE/100 g DW)	2.57±0.35	Bound phenolics (mg GAE/100 g DW)	230±2
Reducing sugars (g GE/100 g DW)	0.21±0.07	Flavonoids (mg QE/100 g DW)	396±10
Total proteins (g/100 g DW)	62.09±6.21	Condensed tannins (mg PB2E/ 100g DW)	175±15
Fat (g/100 g DW)	12.20±0.05	Hydrolyzable tannins (mg GAE/100 g DW)	BDL
Fiber (g/100 g DW)	9.06±1.00	Anthocyanins (mg C3GE/100 g DW)	BDL
Ash	4.22 ±0.01	Ascorbic acid (mg L-AA/100 g DW)	0.33±0.01
Moisture (g/100 g sample)	3.46±0.10	Carotenoids (mg β-CE /100 g DW)	10.55 ±0.05

BDL: below detection limit. DW: dry weigh. GE: glucose equivalents; GAE: gallic acid equivalents; QE: quercetin equivalents. C3GE: cyanidin-3-glucoside equivalents; L-AA: L-ascorbic acid; β-CE: β-carotene equivalents; PB2E: procyanidin B2 equivalents

Total carbohydrate=100-weight in grams [protein+fat+ash+moisture+fiber]

Table 2. Amino acid composition of *Prosopis alba* cotyledons compared to soy, wheat, skim milk and FAO/WHO reference values

Amino acid	FAO[a]	Amino acid content (g/100 g protein)			
		Cotyledon flour	Soy[b]	Wheat[b]	Skim milk[b]
Ile	2.8	2.55±0.09	4.7	3.8	5.6
Leu	6.6	7.40±0.22	7.0	6.6	9.8
Lys	5.8	4.41±0.12	6.3	2.5	8,2
Phe	-	4.11±0.19	4.6	4.5	4.8
Tyr	6.3	3.32±0.13	3.6	3.0	5.0
Cys	2.5	1.47±0.07	1.4	2.2	0.9
Met	-	0.81±0.03	1.4	1.7	2.6
Thr	3.4	1.77±0.09	3.9	2.9	4.6
Val	3.5	7.07±0.16	4.9	4.7	6.9
Asp	-	9.44±0.35	-	-	-
Ser	-	5.80±0.11	-	-	-
Glu	-	18.49±0.76	-	-	-
Gly	-	4.44±0.12	-	-	-
Ala	-	4.48±0.13	-	-	-
His	-	3.47±0.17	-	-	-
Trp	-	0.66±0.03	-	-	-
Orn	-	0.35±0.01	-	-	-
Arg	-	14.52±0.03	-	-	-
Pro	-	5.40±0.21	-	-	-

Reference values of FAO/WHO (1990)[a] and Johnson & Aguilera, (1980)[b] .

Table 3: Identification and content of the main flavonoids in the phenolic-enriched extract of *Prosopis alba* cotyledons from the Argentinian Chaco. Compounds **1**, **2**, **5** and **6** were quantified with a reference curve of schaftoside. Compounds **7** and **8** were quantified with a reference curve of vitexin. The results are expressed as mg equivalents of the standard compounds employed.

Compound	Rt (min)	UV (nm)	MW	$[M-H]^-$ and fragment ions (MS/MS, *m/z*, percent relative intensity)	Tentative identification/ Identification*	mg/g extract
1	34.9	324, 273	726	725; 635 (61), 605 (100), 563 (83), 473 (28), 353 (6)	Isoschaftoside hexoside	2.43
2	35.8	324, 273	726	725; 665 (16), 635 (22), 605 (38), 563 (100)	Schaftoside hexoside	3.33
3	37.1	-	594	593; 503 (28), 473 (100)	Vicenin II/Isomer	0.67
4	40.0	335, 271	594	593; 575 (6), 503 (27), 473 (100), 353 (13)	Vicenin II/Isomer	2.34
5	43.3	338, 271	564	563; 545 (22), 503 (57), 473 (100), 443 (18)	Isoschaftoside	23.67
6	44.5	337, 272	564	563; 545 (11), 473 (68), 443 (100), 353 (18)	Schaftoside*	14.86
7	47.0	-	432	431; 311 (100)	Vitexin*	0.46
8	47.9	334, 272	432	431; 341 (38), 311 (100)	Isovitexin	2.09

- The *Prosopis alba* cotyledons flour showed nutritional and functional properties
- The *P. alba* cotyledons flour was source of fiber, carotenoid and phenolic compounds
- Five bioactive C-glycosyl flavones were identified,
- The *P. alba* cotyledons flour showed antioxidant and antiinflammatory activity
- The cotyledon flour of *P. alba* could be used in the formulation of functional foods