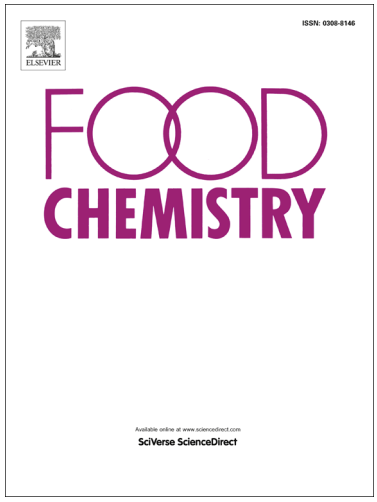

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Morphological, histological, chemical and functional characterization of *Prosopis alba* flours of different particle sizes

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Running title: Characterization of *Prosopis alba* pericarp flours of different particle sizes

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Abstract

Prosopis alba (algarrobo) flours are traditional food resources from Argentina. The aim of this work was to determine the effect of particle sizes of *Prosopis* flour on its chemical composition and functional properties. Flours were obtained by mechanical methods (F1 to F4, > 840, 840 to 500, 500 to 149 and <149 µm, respectively). F1 and F2 contain 40% mesocarp while F3 and F4 show 80 and 90%, respectively. Mesocarp reserve parenchyma is rich in free and bound phenolic compounds, carbohydrates and fibers of the vascular system, $CaCO_3$ crystals, and minerals (Fe, Ca, Mg, K). Apigenin C-glycosides and phenylpropanoids acids were identified in all fractions whereas F4 showed the highest level. All fractions showed functional properties. F3 and F4 showed the highest activity as H_2O_2 and $HO^{\bullet}$ scavenger. F1 and F2 showed the highest activity as reductor agents and lipoperoxidation inhibitors. Flours with different granulometry may be suitable as functional ingredient or nutraceutical whereas flours with a smaller particle size (F3 and F4) are more interesting as functional ingredients because of their high content of bound phenolic compounds and particle homogeneity and could be used either alone or together.

Key words *Prosopis alba*; pericarp flour; free phenolics; bound phenolics ; granulometries

1. Introduction

Prosopis is a genus with almost 45 species distributed in America, Africa and Asia. Argentina is the country with the greatest diversity (nearly 27 species) all over the world (Burkat, 1976), and there, *Prosopis* is distributed in approximately 23,000,000 hectares of native forests of the Chaco and Monte Regions. *Prosopis* tree presented environmental benefits, it grows in arid and semi-arid zones where other crops hardly prosper, tolerates extreme temperatures, drought, salinity, and brings nitrogen to the soil, thus allowing its regeneration (Velarde, Felker & Gardiner, 2005). *Prosopis* tree has an economic potential since it is a promising crop. *Prosopis* does not require annual plantings and can accompany species which, in general, are used as monocultures and decreases the quality of the soils. The approximate cost of afforestation per hectare, with a density of 450 trees installed per year is approximately US$ 7,000. It would be amortized with the production of fruits (pod being composed by the pericarp and seeds) without added value (flours, beverages) between 7 and 12 years of age, since the estimated yield is about 10,000 kg pod per hectare (Verga, 2005). When the tree productivity decreases, its high quality wood can be marketed.

In Argentina, the National Program of Algarrobo (NPA) promotes the cultivation of this species in order to decrease the pressure on the native forests, thus contributing to regional development under a social, environmental and economic sustainability approach. Our work aims to revalue our forest resources, promote the sustainable cultivation of *Prosopis* species to avoid the migration of native population to the urbanized centers with the consequent loss of its cultural identity.

Previous studies have been carried out on the nutritional composition and bioactivity of flours obtained from *Prosopis alba* and *Prosopis nigra* pods (Cardozo, Ordóñez, Zampini, Cuello, Di Benedetto & Isla, 2010; Pérez , Cuello, Zampini, Ordóñez, Alberto,

Quispe, Schmeda-Hirschmann & Isla, 2014; Cattaneo, Sayago, Alberto, Zampini, Ordoñez, Chamorro, Pazos & Isla, 2014; Cattaneo, Costamagna, Zampini, Sayago, Alberto, Chamorro, Pazos, Thomas-Valdés, Schmeda-Hirschmann & Isla, 2016). Flours obtained from *Prosopis* pericarp (exocarp and mesocarp without endocarp) could be used, due to its high sugar content and flavor (taste and aroma), as ingredients in bakery while seed flours could be used as food, because of their high protein content and essential aminoacids (Cattaneo et al., 2014). C-glycosyl flavonoids and O-flavonol glycosides were found to be the main constituents identified in phenolic-enriched extracts of *Prosopis* seed and pod flours (Pérez et al., 2014; Cattaneo et al., 2016). Eight and fourteen flavonoid glycosides were identified in *P. alba* and *P. nigra* pod extracts, respectively. These molecules exhibited antioxidant and anti-inflammatory activities *in vitro* (Cardozo et al., 2010; Pérez et al., 2014; Cattaneo et al., 2014; 2016). So far, there are not studies focused on the effect of particle size distribution on the chemical composition, morphological characteristics and functional parameters of *Prosopis* pericarp flour.

Particle size significantly affects physicochemical properties of flours by increasing the surface area per volume unit and it could be expected different distribution of macronutrients and functional components; an increase being observed in bioavailability by raising digestion rate which would readily affect human nutrition and health. Such a fact has been confirmed in barley and sorghum flour in which the starch digestion kinetics by alfa amylase was dependent on the particle size of flours (Al-Rabadi, Gilbert & Gidley, 2009). De la Hera, Talegon, Caballero & Gomez (2013a) reported the influence of particle size of corn flour on gluten-free bread performance concluding that coarser-grained flours (>180 μm) provide bread with higher volume and softer crumbs due to their ability to retain carbon dioxide during proofing, whereas

finer-grained flours (<106 μm) are more suitable for cakes (De la Hera, Martínez & Gomez, 2013b).

The aim of the present work was to determine botanical constituents and chemical composition as well as functional properties of *P. alba* pericarp flours of different particle sizes.

2. Materials and Methods

2.1. Sample preparations and processing

Prosopis alba ripe pods were collected from Amaicha del Valle, Tucumán, Argentina. Plant material was identified by PhD Cuello and incorporated to the herbarium LIL at the Miguel Lillo Foundation. Chromatic parameters were measured with a Chroma meter CR-400 (Konica Minolta, Tokio, Japan) colorimeter by using the CIELab system, results being expressed as chromaticity coordinates L*, a* and b* (objective parameter). The coordinated L* represents lightness (contribution of black or white varying between 0 and 100); a* represents the contribution of green or red (negative or positive), and b* represents the contribution of blue or yellow (negative or positive). Samples were dried at 50 °C, ground in a Helix mill (Metvisa®,Mod MP-200-Power ½ HP-0.75 Kw-Brasil) and sieved. Two fractions were obtained, one was constituted by epicarp and mesocarp, and the other by the endocarp with the seeds. The first fraction was passed through three sieves 840, 500 and 149 μm. First recovered fraction was named F1 (flour with particle size >840 μm), second fraction F2 (flour with particle size between 840 and 500 μm), third fraction, F3 (flour with particle size between 500 and 149 μm) and four fraction F4 flour with particle size <149 μm. Granulometric degree was selected taking into account the provisions of the Argentine Food Code (Código

Alimentario Argentino, CAA) for soybean proteinic semolin (CAA, 2010) and the plant proteinic flours (CAA, 2010).

2.2 Morphological and histological analysis

In order to analize morphologically and histologically the different flours, pods and fractions F1, F2, F3 and F4 were studied under stereoscopic and optical microscopy.

To understand *P.alba* pod general anatomy, middle segment (comprising one seed) of three fruits obtained from different specimens of *Prosopis* were studied.

For anatomical characterization, each pod segment was mounted on dental wax supports and sectioned with a rotary microtome (MicromHM315). The cuts (thickness range 25–30 μm) were cleared with 50% NaClO solution, washed with distilled water, colored in two successive steps with astra blue-safranin dye and mounted on a 50% glycerol solution (Zarlavsky, 2014).

Epicarp, mesocarp and endocarp segments and seeds were mechanically separated under a stereoscopic magnifying glass and subjected to maceration for 2 to 48 hours, according to the nature of the material, in 10% KOH (Dizeo de Strittmatter, 1973), washed with distilled water, mounted on glycerinated water (1: 1) and observed under optical microscope. For comparative purposes 1 g of each flour sample was subjected to the same treatment.

Pericarp sections and flours samples were subjected to histochemical tests with Sudan IV for lipids, 10% $FeCl_3$ for phenolic compounds, KI and polarized light for starch (Johansen, 1940) and 5% Toluidine Blue O (TBO) for other carbohydrates and polysaccharides (Atia, Debez, Barhoumi, Abdelly & Smaoui, 2009).

All tissues and flour obtained from different sieves were seen with a Zeiss Stemi 2000-C stereoscopic microscope, and a Zeiss Axiolab optic microscope both equipped with a

Zeiss Axiocam ERc 5s digital camera. Measurements were made by using the AxioVision software version 4.8.2 (Carl Zeiss Ltd, Herts, UK).

For scanning electron microscopy (SEM), dry pod samples and fractions F1, F2, F3 and F4 obtained from flour were coated with gold (Fine Coat Ion Sputter JEOL JFC-1100). Scanning electron microscopy (SEM) of gold coated samples was performed by using a ZEISS SUPRA-55 VP field emission scanning electron microscope at Centro Integral de Microscopía Electrónica (CIME), CONICET-UNT.

2.3. Proximal composition

2.3.1. Sugar analysis

Four flour samples were extracted with 80% ethanol at 75 ºC for 10 min and ultrasound assisted. Then, extracts were centrifuged at 9000 x g for 5 min. Pellets were extracted several times with the same solvent. All organic extracts were combined and then evaporated. Sucrose, glucose and fructose were determined according to Orqueda, Rivas, Zampini, Alberto, Torres, Cuello, Sayago, Thomas-Valdes, Jimenez-Aspee, Schmeda-Hirschman and Isla (2017). Results were expressed as glucose, fructose or sucrose g/100 g flour. Sweetness index (Obando-Ulloa, Eduardo, Monforte & Fernández-Trujillo, 2009) was used to estimate total sweetness perception, expressed as sucrose equivalents:

$$\text{Sweetness index} = 1 \times [\text{Sucrose}] + 0.74 \times [\text{Glucose}] + 1.73 \times [\text{Fructose}]$$

2.3.2. Resistant and soluble starch analysis

The method used was proposed by Goñi, García-Díaz, Mañas and Saura-Calixto (1996). Triplicate portions (100 mg) of flour samples were preincubated with a pepsin solution containing 1 g of pepsin (No 7000, Sigma-Aldrich, St Louis, MO, USA) at pH 2, for 60

min at 40 °C under stirring. Then, samples were taken out of water bath and cooled to room temperature and added with KCl–HCl buffer (pH 7, 0.1 M) and 0.10 g/100 g solution of sodium azide. Furthermore, starch was hydrolysed at 37 °C for 16 h by adding 1 mL of α-amylase solution (40 mg de α-amylase -No 3176, Sigma-Aldrich, St Louis, MO, USA- per mL of tri-maleate buffer) pH 7. After α-amylase hydrolysis, samples were centrifuged at 5000 rpm for 5 min and two fractions were obtained (supernatant and precipitate). Supernatants (Digestible starch, DS) were collected and stored in a volumetric flask. Precipitate (isolated resistant starch, RS) was dispersed in water before adding KOH (2 M) at room temperature with constant shaking for 30 min. DS and RS were incubated at pH 4.7 and 60 °C for 30 min with 80 µL of amyloglucosidase from *Aspergillus niger* (N° 7420, Sigma-Aldrich, St Louis, MO, USA) with constant shaking. Then, both samples (DS and RS) were centrifuged at 5000 rpm for 5 min, supernatants were collected and residue was discarded. Glucose concentration in both supernatants was determined by using a kit (GAGO20, Sigma-Aldrich, St Louis, MO, USA). After being mixed well and left for 30 min in a water bath at 37 °C, colour absorption was measured at 500 nm by using a spectrophotometer (Spectronic Instruments, Spectronic® 20 Genesys™, Chicago, IL, USA), and digestible starch concentration of sample was calculated as mg of glucose.

2.3.3 Fiber analysis

Fiber content was determined according to Jaafar, Rahman, Mahmod and Vasudevan (2009) by acid and alkaline digestion. Digested material was placed in a crucible and dried for 12 h at 120 °C. The crucible was heated in a muffle oven at 550 °C for 12 h and crucible weight was recorded.

2.3.4 Fat analysis

Fat content determination in *P.alba* flour samples was carried out following the methodology proposed by the AOAC (1998) .

2.3.5. Total protein analysis

Total proteins (N x 6.25) were determined by the Kjeldahl method (AACC 46-10, 2000) by using a Kjeldahl distillation unit.

2.3.6 Ash analysis

Ash content was determined by incineration method (AACC 08-01, 2000).

2.3.7. Minerals analysis

Flours mineral analysis was carried out by quadrupole inductively coupled plasma mass spectrometry (Q-ICPMS) (ISIDSA, Córdoba, Argentina). Mineral content and ash composition were determined by atomic absorption spectroscopy in accordance with AOAC (2000) recommendations. Sodium, magnesium, potassium, calcium, and iron ions were analyzed. Results were expressed in μg/g of flour. All analyses were performed in triplicate.

2.3.8. Moisture analysis

Moisture content was determined by drying for 12 h at 120 °C (AOAC, 1998 Official Method 925.09).

2.4. Extraction and determination of free and bound polyphenolic compounds content

Prosopis flour (10 g) was extracted six times with methanol in an ultrasonic bath for 90 minutes at 25 °C. Extracts were filtered, two fractions being obtained (soluble fraction and insoluble residue). Soluble fractions were combined, and the MeOH evaporated under vacuum (40 °C). The resulting dried extract was suspended by using an ultrasound bath in 1 mL methanol and called soluble (free) phenolics. The insoluble residue (cell-wall fraction containing bound phenolic) obtained after free phenolic

extractions was mixed with 20 mL of 2 M NaOH, ascorbic acid 1% and EDTA 10 mM with shaking at 30 °C for 30 min. Then, pH was adjusted to 3 with 4 M HCl, and 300 mg of NaCl was added. Mixture was filtered and extracted six times with ethyl acetate. Pooled ethyl acetate fraction was dried under reduced pressure by using a cold trap (Nardini & Ghiselli, 2004). Dried extract was suspended in 1 mL MeOH and called bound phenolic extract. All extracts were stored at −20 °C until analysis. Free and bound phenolic contents were determined in each extract by Folin-Ciocalteu reagent, according to Singleton, Orthofer, & Lamuela-Raventos (1999). The non-flavonoid phenols (NFP) were measured by determining total phenol content remaining after precipitation of flavonoids with acid formaldehyde (Isla, Salas, Danert, Zampini & Ordoñez, 2014) in each extract. The flavonoid phenols (FP) were determined by difference between total free phenols and non-flavonoid phenols (FP=TP-NFP). Results are expressed in mg equivalents of gallic acid (mg GAE) per 100 g flour weight.

2.5. Profile of free and bound phenolic compounds

Free and bound polyphenolic extracts of *Prosopis* flours were analyzed by HPLC coupled to a diode array detector (HPLC-DAD) (Waters Corpo-ration, Milford, MA, USA) in an analytical C18 column (XBridge) by using a linear gradient solvent system consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol as follows: 35% A to 20% A over 10 min, 20% A to 0% A from 10 to 20 min, 0% A for 10 min. Compounds were monitored at 254 nm, and UV spectra of 220 to 540 nm were recorded for peak characterization. Polyphenol quantification was based on external calibration curves from available phenolic standards (HPLC grade - Sigma-Aldrich (MO, USA), Fluka Chemical Corp. (USA) and Indofine Chemical Company, Inc.). Results were expressed as μg equivalents of standard compounds employed per mg of phenolic dry weight.

Phenolic compound identification was carried out by HPLC-ESI-MS/MS. The HPLC system was connected to a QTOF mass spectrometer (rmicroTOF-QII Series, Bruker), with an electro spray ionization (ESI) source. Mass spectra were recorded in negative ion mode between *m/z* 50 and 1000. Ionization was performed at 4500 V assisted by nitrogen as a nebulizer gas at 4.0 bar, and as a drying gas at 200°C and a flow rate of 8.0 $L.min^{-1}$. Argon was used as a collision gas. MS detector was programmed to perform MS and alternative MS/MS from the three most abundant ions obtained with a collision energy of 12 eV. Data acquisition and processing were performed by using a Compass Version 3.1 software and a Data Analysis Version 4.0 software, respectively (Bruker Daltonics, MA-USA).

2.6. Biological activities of bound phenolic compounds

2.6.1. Cation-radical ABTS scavenging activity

Antioxidant capacity assay was carried out according to an improved $ABTS^{\bullet+}$ method described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999). One hundred microliters of ABTS•+ solution was added to different concentrations of free and bound polyphenolic extracts (0.75–15 µg GAE/mL). Inhibition percentage was measured after 1 min reaction. The SC_{50} was defined as the concentration of extracts, in micrograms gallic acid equivalents per milliliter (µgGAE/mL) necessary to scavenge 50% of ABTS•+. Quercetin was used as a positive control (7–25 µg/mL).

2.6.2. β-Carotene-linoleic acid assay

Antioxidant activity of *P. alba* free and bound polyphenolic extracts was determined by Prieto, Vázquez and Murado (2012) method. Different concentrations of phenolic extracts (2-20 µg/mL) were mixed with a β-carotene emulsion and incubated at 50 °C. The oxidation was monitored spectrophotometrically at 470 nm for 120 min. Quercetin (1.18–27.3 µg GAE/mL) and BHT (2.3–9.1 µg/mL) were used as a positive control. The

concentration necessary to inhibit 50% of β-carotene bleaching (IC_{50}), expressed in micrograms gallic acid equivalents per milliliter (μg GAE/mL), was determined.

2.6.3. Hydroxyl radical scavenging

The hydroxyl radical scavenging capacity was carried out according to Chobot (2010) methodology by using different concentrations of polyphenolic extract (0.5–10 μg GAE/mL). Results are shown as SC_{50} values in μg GAE/mL required to inhibit by 50% of 2-deoxy-D-ribose degradation.

2.6.4. Hydrogen peroxide scavenging

For H_2O_2 scavenging activity determination, the methodology described by Chamira and Preethi (2015) was followed. Different concentrations of free and bound polyphenolic extracts from *P. alba* flour (1-10 μg GAE/mL) were contacted with a mixture containing phenol (12 mM), 4-aminoantipyrene (0.5 mM), H_2O_2 (0.7 mM) and phosphate buffer at pH 7 (84 mM), horseradish peroxidase (0.1 U / mL, 40 mL) and incubated at 37 °C for 30 minutes. Then, absorbance was registered at 504 nm. Results are shown as SC_{50} values in μg GAE/mL required to scavenging by 50% of hydrogen peroxide.

2.7. Statistical analysis

Sampling and analyses were performed in triplicate, and 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 analysis of variance (ANOVA) with Tukey post-test at a confidence level of 95%.

3. Results and discussion

Fruit chromatic characteristics were determined to select those in the same ripening grade (L*= 58.41±10; a*= 3.42±1 y b*= 33.43±2). *P.alba* pericarp flours with different

particle sizes (<149 until > 840 µm) present different yield percentage, 4%, 24%, 45% and 27% to F1, F2, F3 and F4, respectively. Thus, selected milling process was quite simple, and this method allowed to obtain flours from pericarp pods with almost 100% of recovery.

3.1. Macroscopic and microscopic characters of pods and flours

P.alba pod is an indehiscent drupaceous legume classified as lomentum drupaceum, formed by a pericarp or fruit itself and seeds (Fig.1A and 1B). Pericarp is composed of three layers, epicarp, mesocarp and endocarp (Fig.1A-F).

Epicarp presented a papiraceous yellowish appearance shiny on the upper side to opaque on the underside (Fig.1D), polyhedral epidermal cells with straight to slightly curved anticlinal walls and anomocytic stomata (36.5 ± 7.3 µm long., 23.6 ± 2.6 µm lat.) (Fig. 1G). In a cross section it is formed by a thick cuticle, one layer of epidermal cells (Fig. 1H) is formed with sunken stomata with respect to epidermal surface; occlusive cells showed a cuticular ridge (Fig. 1I).

Mesocarp is a pulpy and edible brown mass with a sweet characteristic aroma accompanied by fibers belonging to vascular system (Fig. 1E). In a cross section it is formed by 2-3 layers of peripheral sub-epidermal tangential collenchyma that can be lignified at the marginal end of the pod (Fig.1C and 1H), collateral vascular bundles with strong fibers, sclereids reinforcements towards phloem (Fig.1C and 1O-P), and an abundant reserve parenchyma (Fig. 1C and 1J-L). Near vascular bundles, abundant calcium oxalate prismatic crystals were observed (Fig. 1O). It was difficult to distinguish native starch grains in this tissue due to its opacity, whereas free starch granules were rarely observed; these grains correspond to regular grains with faceted surfaces with a filamentous centric hilum and a maltese cross with linear arms (Fig.

1M). Similar grains were previously described for *P. flexuosa* and *P. chilensis* (Giovannetti, Lema, Bartoli & Capparelli, 2008). Carbohydrate and sugar composition of *Prosopis* pods have been subject to a lot of debate. Giovannetti et al. (2008) have previously reported that the main constituents of *Prosopis* are sugars and that starch is present but in lower concentrations. The presence of other sugars was detected with TBO in parenchymatic storage cells from mesocarp (Fig. 1L). Also, $FeCl_3$ revealed phenolic compounds presence in these cells (Fig. 1K). No lipids were detected.

Endocarp is hard and segmented into one-seeded joints (Fig. 1F), formed exclusively by lignified fibers arranged perpendicularly to the fruit longitudinal axis (Fig. 1C and 1P-R).

Prosopis alba flours, fractions F1, F2, F3 and F4, were formed exclusively by fragments of epicarp and mesocarp in different proportions (Fig. 2A-L). Fractions F1 and F2 showed approximately 60% of epicarp and 40% of mesocarp (Fig.2A-B, 2E-F and 2I-J), whereas fractions F3 and F4 showed greater proportions of mesocarp, representing up to 80% and 90% of the sample (Figure 2C-D, 2G-H and 2K-L). Particularly in F4, remains of reserve parenchyma cells and fragments of vascular bundles were the main constituents. In a micrograph (Figure 2K) homogeneity of flour particles of F4 is noticed. According to Hoseney (2010) flour particle homogeneity is very important in baking because it favors a better distribution of water by the dough, reduces mixing time and improves some sensory characteristics (appearance, taste and texture).

3.2. Chemical characterization of P. alba flours with different particle sizes

Moisture, protein, fat, ash, sugar and starch content for *Prosopis* flours were determined as shown in Table 1. Moisture content was similar regardless of flours granulometry. These values were higher than those of wheat flour (9-13%) (Nasir et al, 2003) but were

similar to the ones reported for *P. alba* and *P. pallida* flour (25.7 and 33.9%, respectively) (Felker, Grados, Cruz & Prokopiuk,2003). Ash content was higher in flour with the smallest particle size (F4) (Table 1). These values are higher than those reported by Pereira de Gusmao, Cavalcanti-Mata, Moreira, Martins Duarte & Souza Gusmao (2016) for mesquite flour.

Total carbohydrate content was similar in all flours (around 70%). Sucrose was the major sugar present in *P. alba* flours of different particle sizes. Sucrose content showed the same behavior as total sugars, the highest sucrose content were observed in flours with the lowest particle size (26.09 to 35.31%). Sweeteness index (Table 1) would indicate that flour of the lowest granulometry has a sweeter taste. Starch percentage in *Prosopis* flour (2.00-6.92 %) was much lower than sorghum, millet and corn (72.2–73.8%), quinoa, amaranth and wheat (66.3 – 68.1%), (Srichuwong, Curti, Austin, King, Lamothe & Gloria-Hernandez, 2017). Soluble digestible starch was higher in flour with the largest particle size. Resistant starch content was below 2% in every flour fractions (Table 1). These results suggested that starch is not the main energy source of flour and are in agreement with those found by micrographic analyses.

The highest fiber content was found in F3 and F4. These results are coincident with the micrographic analysis that showed a highest content of fibrous mesocarp in F3 and F4 samples. Total fiber values found in this study were 7.05 to 10.75%. According to European Parliament Regulation (EC) Number 1924/2006, these flours could be considered as a food with high fiber content. *Prosopis* flours could be extremely favorable to be used for enriching foods with fibers, mainly the finest-grained flour. Moreover, these flours were associated with a higher content of fiber, which is related to a higher concentration of bioactive compounds such as bound polyphenols. *Prosopis* flour showed higher fiber content than wheat flour (Durazzo, Turfani, Narducci, Azzini,

Maiani & Carcea, 2014). These results would suggest that they can be healthier than wheat flour. F4 fraction also showed the lowest protein level.

Regarding minerals, potassium was the most abundant element in *Prosopis* flour, followed by Ca, Mg, and Fe. Fraction F4 showed the highest K content. F3 showed the highest Fe content. Both fractions are rich in mesocarp, which would indicate that the mesocarp is probably enriched by these minerals. The content of Zn and Cu was not influenced by particle size and were the minority minerals. These results would indicate a distribution in all tissues of *Prosopis* pods. The consumption of 100 g of flour would cover approximately 20-35% of Mg, Ca, and Fe daily requirements. The Mg and Ca content in carob tree flour was higher than *Prosopis juliflora* flour (Pereira de Gusmao et al., 2016). *Prosopis* flour can be regarded as a food without sodium since it has values lower than 5 mg / 100g flour (Table 2).

3.3. Phenolic compounds in P. alba flour with different particle sizes

Bound phenolic compounds were more abundant than free phenolics in *Prosopis alba* flours as well as in wheat flour (Hung, Maeda, Miyatake & Morita, 2009). Bound phenolic compound content was dependent on particle size. Fraction F4 exhibited a higher content of bound phenolic compounds than F1, F2 and F3. Variations in bound phenolic compounds content could probably be related to the proportion of fiber in the different fractions (Acosta-Estrada, Gutiérrez Uribe & Serna Saldívar, 2014; Segundo, Román, Gómez & Martínez, 2017). F4 and F3 showed a higher fiber content than other fractions. In addition, extraction efficiency could increase with the decrease in particle size due to the larger contact surface that allows a greater interaction with the solvent. Taken together, these results indicate an unequal distribution of the individual phenolic compounds in the *Prosopis* flour, and provide an explanation for the increase of bound phenolic compounds in F4.

Following the same behavior as bound phenolic compounds, F4 was richer in free phenolic compounds. Bound phenolics were considered to have more health benefits since they may escape from upper gastrointestinal digestion conditions along with cell wall materials and absorbed into blood plasma during digestion by intestinal microflora (Andreasen, Kroon, Williamson & Garcia-Conesa, 2001). Therefore, consumption of F4 fraction might increase absorbed free and bound phenolics. Free phenolics content of samples (0.20-0.35 g GAE/100 g flour) was higher than that of other flours such as black rice and quinoa flours (0.15 and 0.09 g GAE/ 100 g, respectively, (Rocchetti, Chiodelli, Giuberti, Masoero, Trevisan & Lucini 2017) and similar to that of wheat flours (0.17-0.20 g GAE/100 g flour, (Lv, Yu, Lu, Niu, Liu, Costa & Yu, 2012). Bound phenolic/free phenolic ratio was between 5.7 (F4) and 1.0 (F1) (Table 3). Flavonoid and non-flavonoid phenolic compound proportion was also analyzed. In F1, F2, y F3 fractions bound flavonoid phenolic compounds were more abundant than bound non-flavonoid phenolics, whereas F4 showed more bound non-flavonoid phenolics. Free non-flavonoid phenolic compounds were more abundant than free flavonoids phenolics. In both, the FP / NF-P ratio was higher in flours with lower particle size (F3 and F4). Flours with different granulometry may be suitable as a functional ingredient but flours with less particle size (F3 and F4) could be healthier because of their high content of bound phenolic compounds and could be used either alone or together.

3.4. Identification of polyphenolic compounds

Apigenin-based C-glycosides were identified and quantified in free phenolic-enriched extracts obtained from *P. alba* flours. C-glycosides content was lower in flour with larger granulometries, even disappearing completely Schaftoside in F1 and F2. Isovitexin (1.12±0.04 µg/mg dry extract), Vicenin II (1.07±0.03 µg/mg dry extract),

Vitexin (0.91±0.08 µg/mg dry extract) and Schaftoside (0.42±0.03 µg/mg dry extract) were the major components in flours with lower granulometry (Table 3). These compounds exhibit antioxidant, anti-inflammatory, antiplatelet activity, anti-cancer, enzyme converting angiotensin (ECA) inhibitors, hypoglycemic activity, among others (Cattaneo et al., 2014; Xiao, Capanoglu, Jassbi & Miron, 2016). Besides, they are rapidly absorbed after oral administration and distributed by plasma in various tissues (Xiao et al., 2016).

Ferulic acid (4.01±0.36 µg/mg dry extract) and Coumaric acid (3.94±0.35 µg/mg dry extract), two phenylpropanoids, were identified and quantified in bound phenolic-enriched extracts obtained from all fractions of *P. alba* flour, but the highest content was obtained from F3 and F4 fractions mainly in F4 (Table 3). Antioxidant activity of ferulic acid has been associated with several positive health effects, mainly anti-carcinogenic, neuro-protective, cardiovascular effects (Bento-Silva, Vaz Patto & Bronze, 2018), protection of hepatocyte and myocardial injury induced by diabetes, oxidative stress neutralization in cells with high-glucose content, lipoperoxidation reduction, increase glutathione levels and antioxidant enzyme activity in pancreas, inhibition of NFkB activation and modulation of redox immunity, inflammatory, and balance response in cells (Seo et al., 2015). Apigenin derivatives were not detected in any bound phenolic extracts obtained from different fractions.

3.5. Antioxidant capacity

Among reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) is a relatively stable, non-radical oxidant, which can diffuse across biological membranes and generate $HO^{\bullet}$, the most potent ROS that produces damage tissue and exacerbates oxidative process (Prior, Wu & Schaich, 2005). Antioxidant molecules capable of scavenging ROS could

help to attenuate redox unbalance and reduce damage. Table 4 shows antioxidant activity of free and bound phenolic compounds obtained from *Prosopis* flours.

It was found that with increasing concentrations of phenolics, H_2O_2 linearly decreased, indicating that these compounds could effectively scavenge H_2O_2. SC_{50} values of bound phenolics were between 2.94 to 3.90 µg/mL (Table 4). The H_2O_2 scavenging activity of bound phenolic compounds was higher than that of free phenolic compounds. Free phenolic compounds obtained from F4 showed greater effectiveness (SC_{50} =10 µg/mL) than the other fractions. This effect could be probably due to the highest content of schaftoside in F4.

Moreover, hydroxyl radical scavenging activity of bound phenolics was higher than that of free phenolics (Table 4). Only free phenolic compounds from F4 fraction showed antioxidant activity (SC_{50} values of 6 µg/mL).

Data showed a positive correlation between bound phenolic compound content and $HO^{\bullet}$ scavenging activities, but no correlation was observed among SC_{50} values and particle size.

All tested flour samples showed $ABTS^{\bullet+}$ scavenging capacity (Table 4) with SC_{50} values between 1.3-13 for free phenolics and 1.16 to 3.50 µg GAE/mL for bound phenolics. Free phenolics obtained from flour with smaller particle size was less active against ABTS cation radical. This may be related to the flavonoid ratio of each fraction (vicenin II: vitexin: isovitexin: schaftoside). In addition, Pearson's correlation test showed that $ABTS^{\bullet+}$ scavenging capacity was correlated with a total phenolic content.

Flours with different particle size showed inhibitory activity of peroxidation on lipids. IC_{50} values demonstrated that flour with higher particle size were more active than those of F4 (Table 4).

Conclusion

The present study allowed to go deep into the botanical and chemical characteristics of different fractions of *Prosopis alba* flour. Thus, F4 and F3 fractions have an homogeneous aspect with a high mesocarp proportion, rich in phenolic compounds mainly bound phenolic compounds (coumaric and ferulic acid), derivates C-glycosides from apigenin as well as fibers and K content. Also, these fractions showed high ROS scavenging activity. F2 and F1 fractions were less homogeneous and were enriched in proteins and starch. F1 showed the highest content of soluble starch, reducing capacity and lipid peroxidation inhibitory capacity. Therefore, *P. alba* flour with different particle sizes has a great potential to be used as a functional ingredient in pro-health food production. This study would contribute to promote the sustainable use and conservation of this species.

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Figures

Figure 1. *Prosopis alba* fruit, morphological and anatomical aspects. **A.** SEM of pericarp and seed. **B.** Pericarp and seed under stereoscopic microscopy. **C.** Fruit anatomy, cross section under light microscopy. **D-F.** Isolated parts of the fruit under stereoscopic microscopy. **D.** Isolated epicarp aspect. **E.** Isolated mesocarp aspect. **F.** Isolated endocarp aspect. **G-R.** Fruit anatomy under light microscopy. **G.** Epicarp epidermal cells with anomocytic stomata. **H.** Epidermis cross section details. **I.** Sunken stomata. **J.** Mesocarop reserve parenchyma. **K.** $FeCl_3$ revealed the presence of phenolic compounds in reserve parenchyma. **L.** Polycasacharides stained with TBO in mesocarp parenchymatic storage cells. **M.** Starch grain under bright and polarized light. **N-O.** Fibers, sclereids and calcium oxalate prismatic crystals (arrow) observed in the mesocarp under bright and polarized light, respectively. **P.** Endocarp cross section details. **Q-R.** Endocarp fibers in macerated tissues.

Abbreviations: c, cuticle; emb, embryo; en, endocarp; ep, epicarp; epi, epidermis; lco, lignified collenchyma; me, mesocarp; nlfi, non lignified fibers; oc, occlusive cell with cuticular ridge; rp, reserve parenchyma; s, stomata; sc, sclereid; se, seed; vb, vascular bundle; vb-fi, vascular bundle fibers.

Figure 2. *Prosopis alba* fruit flours under stereoscopic microscopy, SEM and optical microscope. **A-E-I.** F1, flour with particle size >840 µm. **B-F-J.** F2, flour with particle size between 840 and 500 µm. **C-G-K.** F3, flour with particle size between 500 and 149 µm, **D-H-L.** F4: flour with particle size <149 µm.

Abbreviations: ep, epicarp; me, mesocarp; fi, fibers; brp, broken reserve parenchyma.

Figure 1:

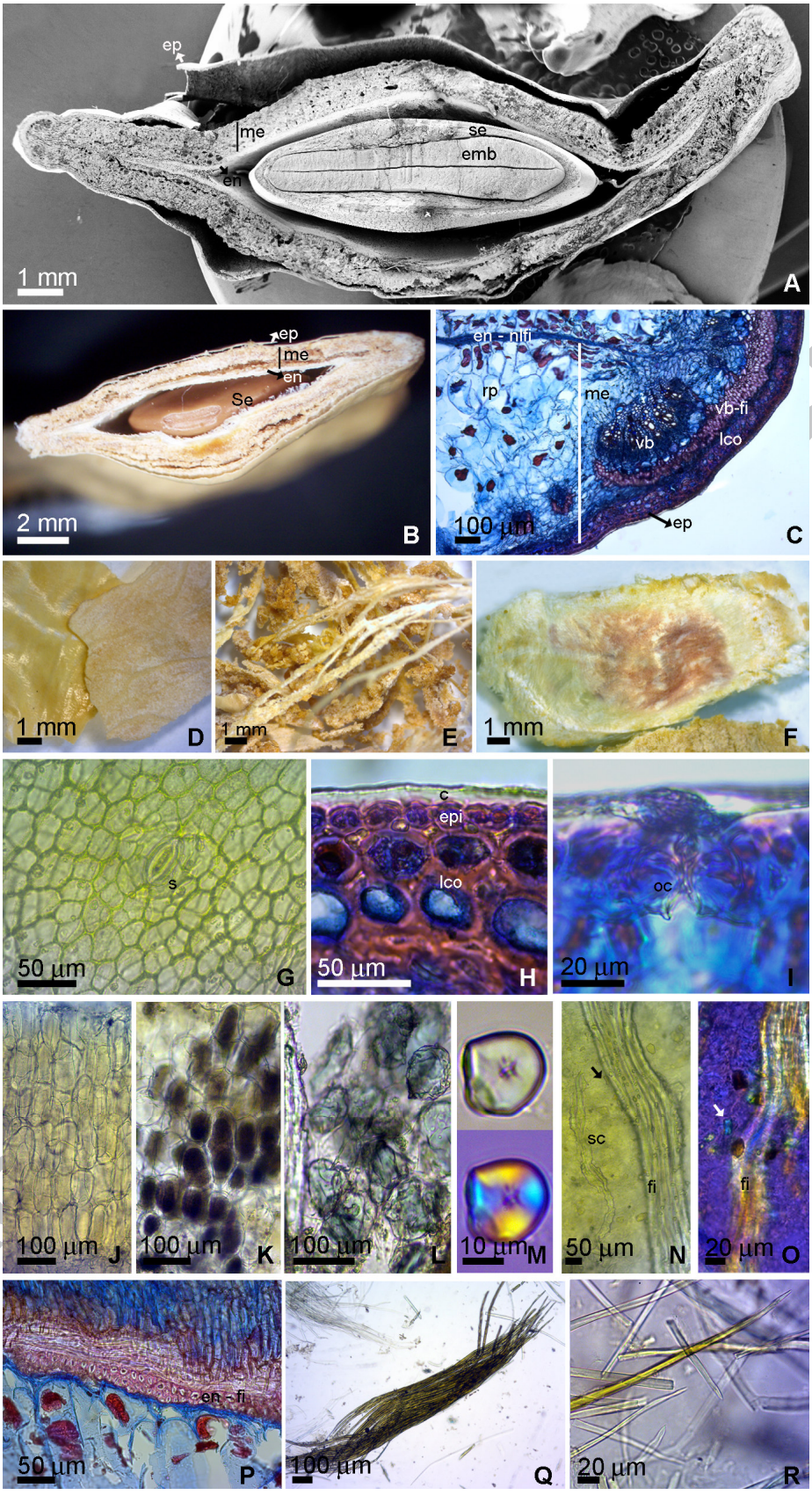

Figure 2:

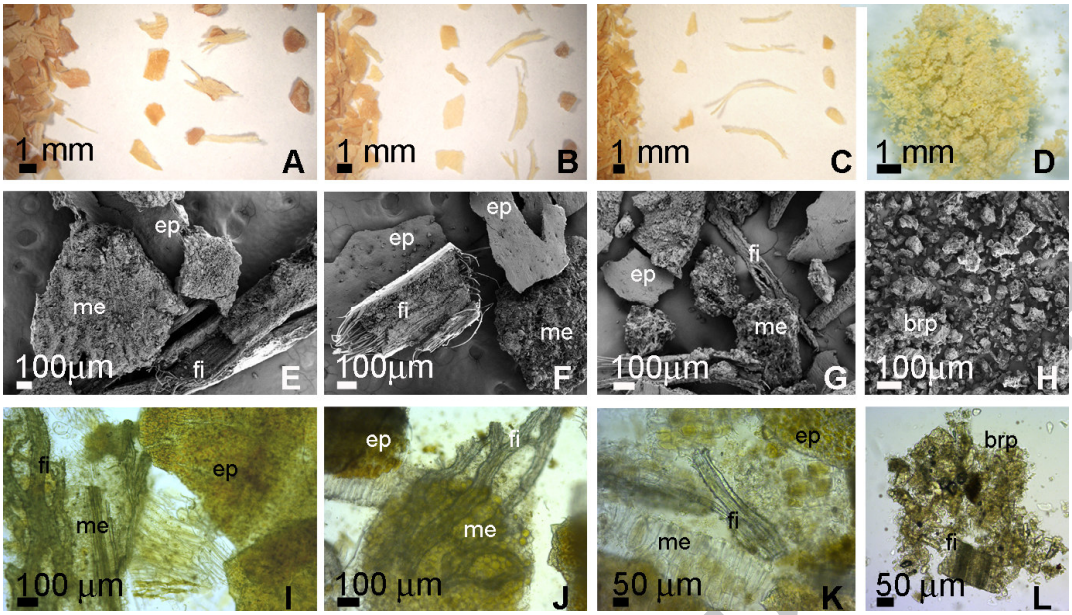

Table 1: Macronutrient content of *Prosopis alba* pericarp flour with different particle sizes.

Samples	F1-PA	F2-PA	F3-PA	F4-PA
	(g /100 g flour)			
Total carbohydrates	75.56±1.00^{B}	74.29±1.00^{B}	70.72±1.50^{A}	76.71±0.24^{B}
Glucose	0.46±0.01^{B}	0.48±0.02^{B}	0.55±0.01^{B}	0.12±0.01^{A}
Fructose	10.92±0.15^{A}	9.81±0.06^{A}	7.59±0.54^{A}	10.31±0.66^{B}
Sucrose	31.21±0.33^{A}	26.09±0.33^{B}	29.01±0.15^{C}	35.31±0.98^{D}
Sweetness index	50.44±1.00^{B}	43.41±1.20^{A}	42.55±0.50^{B}	53.24±1.00^{C}
Resistant Starch	1.24±0.12^{B}	0.93±0.10^{AB}	0.76±0.20^{A}	1.22±0.10^{B}
Digestible Starch	5.68±0.31^{C}	2.30±0.23^{B}	1.24±0.03^{A}	1.24±0.10^{A}
Total starch	6.92	3.23	2.00	2.46
Ash	5.59±0.03^{B}	5.38±0.03^{A}	5.75±0.03^{B}	8.10±0.12^{C}
Moisture (%)	34.91±2.38^{AB}	31.96±1.39^{A}	31.28±0.55^{A}	38.57±1.02^{B}
Proteins	10.67±0.42^{B}	11.39±1.11^{B}	11.20±0.37^{B}	3.39±0.20^{A}
Fats	1.13±0.11^{A}	1.14±0.11^{A}	1.58±0.14^{B}	1.69±0.14^{B}
Fibers	7.05±0.62^{A}	7.80±0.71^{A}	10.75±0.54^{B}	10.05±0.81^{B}
Minerals	(µg/g flour)			
Mg	574.00±10.00^{B}	507.00±8.00^{A}	573.00±4.00^{AB}	534.00±2.00^{AB}
K	14566.00±271.00^{C}	12613.00±218.00^{A}	13299.00±205.00^{B}	15400.00±100.00^{D}
Na	16.00±1.00^{B}	10.40±0.20^{A}	14.30±0.40^{B}	11.00±2.00^{A}
Ca	3616.00±106.00^{C}	3003.00±39.00^{B}	3410.00±44.00^{C}	1100.00±100.00^{A}
Cu	3.10±0.20^{C}	2.34±0.08^{B}	2.65±0.01^{B}	2.43±0.07^{A}
Fe	46.00±2.00^{A}	46.00±2.00^{A}	59.40±0.70^{B}	41.00±4.00^{A}
Zn	4.30±0.10^{C}	3.90±0.20^{B}	4.24±0.09^{BC}	4.42±0.08^{A}

F1: flour with particle size >840 µm, F2: flour with particle size between 840 and 500 µm, F3: flour with particle size between 500 and 149 µm, F4: flour with particle size <149 µm "semolina". PA: *Prosopis alba*. Different letters (A, B, C) in the same line for each assay show significant differences according to Tukey's test ($p \leq 0.05$).

Table 2: Phenolics components from *P. alba* fruit flour with different granulometry

Samples	Free phenolic compounds (mgGAE/100 g flour)	Non-flavonoid phenolics (mgGAE/100 g flour)	Flavonoid phenolics (mgGAE/100 g flour)	FP/NF-P	Bound phenolic compounds mgGAE/100 g flour)	Bound Non-flavonoid phenolics (mgGAE/100 g flour)	Bound Flavonoid phenolics (mgGAE/100 g flour)	FP/NF-P	Bound/free phenolics
F1-PA	210.00±1.00^{B}	0.19±0.00^{B}	0.02±0.01^{A}	**0.11**	690.00±1.00^{B}	0.21±0.01^{A}	0.48±0.05^{A}	**2.29**	**1.00**
F2-PA	198.00±4.00^{A}	0.19±0.00^{B}	0.01±0.02^{A}	**0.05**	320.00±2.00^{A}	0.12±0.00^{A}	0.20±0.01^{B}	**1.67**	**1.60**
F3-PA	228.00±1.00^{C}	0.15±0.01^{A}	0.08±0.01^{B}	**0.53**	1330.00±4.00^{C}	0.51±0.05^{B}	0.82±0.10^{C}	**1.61**	**5.70**
F4-PA	350.00±3.00^{D}	0.23±0.01^{C}	0.12±0.03^{C}	**0.52**	1700.00 ±10.00^{D}	1.04±0.06^{C}	0.66±0.07^{C}	**0.63**	**4.80**

F1: flour with particle size >840 µm, F2: flour with particle size between 840 and 500 µm, F3: flour with particle size between 500 and 149 µm, F4: flour with particle size <149 µm "semolina". PA: *Prosopis alba.* Different letters (A, B, C) in the same column for each assay show significant differences among effect of polyphenols on enzyme activity according to Tukey's test ($p \leq 0.05$).

Table 3: Phenolics content of *P. alba* metanolic extracts enriched in free and bound phenolic compounds fruit flour with different granulometry.

	Free phenolic compounds				Bound phenolic compounds	
Samples	**Vicenin II**	**Vitexin**	**Isovitexin**	**Schaftoside**	**Coumaric acid**	**Ferulic acid**
	µg/mg DW					
F1-PA	0.19±0.01^{A}	0.64±0.02^{B}	0.63±0.02^{B}	0.00±0.00	0.47±0.02^{B}	0.28±0.02^{A}
F2-PA	0.34±0.02^{B}	0.78±0.02^{B}	0.81±0.02^{B}	0.00±0.00	0.44±0.02^{B}	0.30±0.02^{A}

F3-PA	0.42±0.01^{B}	0.47±0.01^{A}	0.48±0.01^{A}	0.13±0.00^{A}	0.33±0.02^{A}	0.30±0.02^{A}
F4-PA	1.07±0.03^{C}	0.91±0.08^{C}	1.12±0.04^{C}	0.42±0.03^{B}	3.94±0.35^{C}	4.01±0.36^{B}

F1: flour with particle size >840 μm, F2: flour with particle size between 840 and 500 μm, F3: flour with particle size between 500 and 149 μm, F4: flour with particle size <149 μm "semolina". PA: *Prosopis alba.*

Table 4: Antioxidant activity of free and bound phenolic enriched extracts obtained from *P. alba* fruit flour with different granulometry

	Samples	HO˙ scavenging assay	H_2O_2 scavenging activity	$ABTS^{\bullet+}$ radical scavenging	β-carotene/linoleic acid assay
		SC_{50} (µg GAE/mL)			IC_{50} (µg GAE/mL)
Free phenolics	**F1-PA**	ND	51.00±5.66 ^{C}	1.80±0.00 ^{B}	4.60±0.50 ^{AB}
	F2-PA	ND	47.00±6.97 ^{C}	1.80±0.10 ^{B}	3.55±0.30 ^{A}
	F3-PA	ND	29.50±3.54 ^{B}	1.30±0.10 ^{A}	2.15±0.20 ^{B}
	F4-PA	6.00±0.00	10.00±0.00 ^{A}	13.00±0.0 ^{C}	11.00±0.80 ^{C}
Bound phenolics	**F1-PA**	1.26±0.01 ^{A}	3.63±0.24 ^{B}	1.65±0.06 ^{A}	5.17±0.02 ^{A}
	F2-PA	1.36±0.07 ^{A}	3.18±0.05 ^{A}	2.65±0.15 ^{A}	6.37±0.13 ^{B}
	F3-PA	1.24±0.01 ^{A}	2.94±0.11 ^{A}	1.16±0.02 ^{A}	6.40±0.24 ^{B}
	F4-PA	1.00±0.00^{A}	3.90±0.28^{B}	3.50±0.10^{B}	13.00±0.50^{C}
Reference compounds	**Quercetin**	30.00±2.81	13.90±0.00	3.60 ± 0.28	7.30 ± 0.21
	BHT				3.50 ± 0.21

F1: flour with particle size >840 µm, F2: flour with particle size between 840 and 500 µm, F3: flour with particle size between 500 and 149 µm, F4: flour with particle size <149 µm "semolina". PA: *Prosopis alba*. ND: not detected until 200 **µg GAE/mL**. Different letters (capital letters to bound phenolic; lowercase letter to free phenolic) in the same column for each antioxidant assay show significant differences according to Tukey's test ($p \leq 0.05$).

Highlights

- *Prosopis alba* flours are traditional food resources of Argentina.
- Flour particle size affects botanical, chemical and functional properties.
- All flours showed antioxidant activity.
- *Prosopis* flours may be suitable as functional ingredient or nutraceutical.