Prosopis nigra Mesocarp Fine Flour, A Source of Phytochemicals with Potential Effect on Enzymes Linked to Metabolic Syndrome, Oxidative Stress, and Inflammatory Process

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Abstract: This work is part of the search in native food matrices from arid regions of Argentina of interest to improve human health. *Prosopis* species are ethnic food resources in South America capable of growing in arid and semi-arid environments. This work was focused to determine the nutritional and phytochemical composition of *Prosopis nigra* fine flour and to evaluate its biological properties. Flour showed a high level of sucrose (30.35 g/100 g flour), fiber (6.34 g/100 g flour), polyphenols (0.45 g GAE/100 g flour), and minerals (potassium, calcium, and magnesium). Apigenin C glycosides and phenylpropanoid acids were identified in free and bound phenolic enriched extracts, respectively. Polyphenols (especially free polyphenols) were able to inhibit enzymes associated with the metabolic syndrome, including α-amylase (IC_{50} 30.1 μg GAE/mL), α-glucosidase (IC_{50} 22.5 μg GAE/mL), while bound phenolics may control lipase activity (IC_{50} 33.5 μg GAE/mL) and exhibit antioxidant activity by different action mechanisms (SC_{50} between 16 and 93 μg GAE/mL). Both extracts were more effective to inhibit cyclooxygenase-2 than phospholipase A_2 and lipoxygenase, proinflammatory enzymes. Polyphenolic extracts did not show any mutagenic effect. Our studies add value to this non-conventional flour as a promising food resource that could be used as a functional food or functional ingredient in formulations to reduce the risk of the development of obesity. These studies revalue our native resources by promoting their conservation, their use and their propagation.

Keywords: bound phenolics, free phenolics, mesocarp flour, metabolic syndrome, *Prosopis nigra*

Practical Application: Pods of *P. nigra* are traditional food resources in South America. The non-conventional flour obtained from them is a food that inhibits enzymes linked to carbohydrates metabolism and lipids metabolism, show antioxidant activity and anti-inflamatory activity, principally on COX-2. This natural product is a promising resource that could be used as a functional food or as functional ingredient in food formulations for reduce the risk of the development of obesity. Our studies are relevant to stimulate a sustainable management of this specie and for its development as potential new crops.

Introduction

Argentina presents an extraordinary biodiversity with various food plant species. Two food species, *Prosopis alba* (Griseb.) and *Prosopis nigra* (Griseb.) Hieronymus are extensively distributed in Northwestern Argentina and grow in arid and semiarid regions (Burkart, 1976). Their wood is highly used for furniture manufacture, for this reason their forests are being devastated at present. The pods of both species show different shapes and colours, *P. alba* being brown while *P. nigra* purple (Figure 1). Different traditional food products are made from pods without seeds of both *Prosopis* species: drinks (añapa, aloja, and chicha), syrup, flour, and sweets (arrope, patay, jam) (Pérez et al., 2014). Although, the fruit of *P. nigra*, the seed flour, patay and añapa are included in the Argentine Food Code (Código Alimentario Argentino), there are so far, few studies on them. The preliminary nutritional characterization and total phenolic compound content of alcoholic and aqueous extracts obtained from *P. nigra* pod flour was reported (Cardozo et al., 2010). Pérez et al. (2014), carried out the preliminary characterization of phenolic compounds from *P. nigra* mesocarp flour and reported the presence of hydrosoluble pigments such as anthocyanins, responsible of its colour. A better characterization of the nutritional composition and functional properties of Argentine phytogenetic resources is relevant for a sustainable management and for the development of local species as potential new crops.

Overweight and obesity are death risk factors in the world. The most vulnerable population to the effects of obesity in Latin America and the Caribbean is mainly the poorest people, especially women and children under five years old (FAO, 2013). Indeed, obesity is associated with the development of metabolic disorders, cardiovascular diseases and chronic diseases such as some cancers, and inflammation-based pathologies. Global strategies to slow obesity development are focused on dietary and lifestyle modifications. Natural products could reduce the risk of the development of obesity and type-2 diabetes by the regulation of various pathways, including fat absorption, intestinal catabolism of complex carbohydrates, decrease of associated inflammatory processes.

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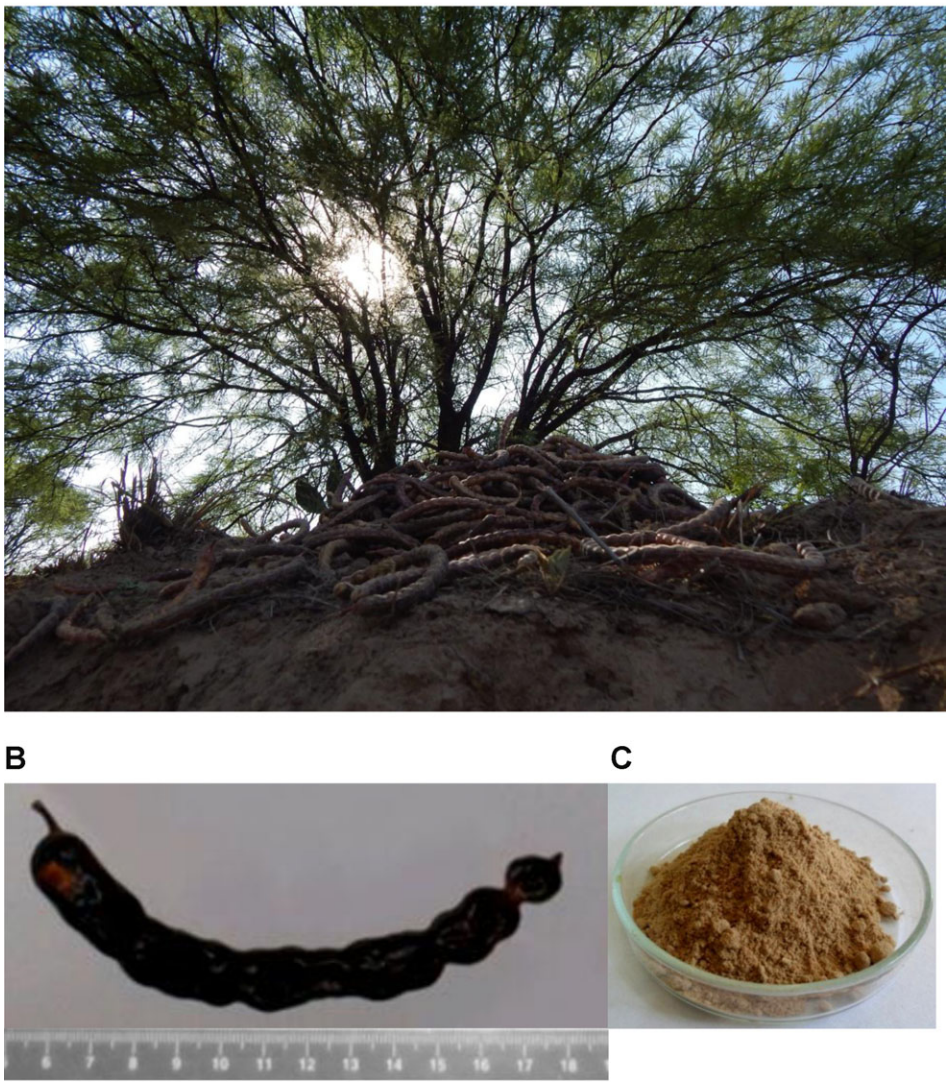

Figure 1–(A) *P. nigra* tree with pods; (B) *P. nigra* pod; (C) *P. nigra* mesocarp fine flour (granulometry <149 μm).

The aim of the present study was to determine the nutritional composition and phytochemical characterization of *P. nigra* mesocarp fine flour and to evaluate its anti-obesity properties associated with antioxidant activity, anti-inflamatory activity and inhibitory capacity of metabolic syndrome enzymes.

Materials and Methods

Chemical substances

All reagents were of analytical grade and were provided from Sigma Aldrich Chemical Co® (St Louis, MS, U.S.A.), Merck® (Darmstadt, Germany), Cicarelli® (Santa Fe, Argentina), and Anedra® (Buenos Aires, Argentina). The phenolic compounds used as standards were provided from Indofine Chemical Company® (NJ, U.S.A.) and Sigma Aldrich®.

Sample preparations and processing

P. nigra (Grisebach) Hieronymus ripe pods were collected in Amaicha del Valle, Tucumán, Argentina in March 2015. The plant material was identified by Dra. Soledad Cuello. The voucher specimens were deposited in the INBIOFIV herbarium. The pods were harvested manually from different plants in the same area. In each case were collected around 500 g per plant of 20 plants (Figure 1). The ripening stages for all samples were selected in agreement with those at which the fruit are usually consumed (subjected parameters). After collecting, the fruits were transported to the laboratory at room temperature according with the traditional storage. Chromatic parameters were measured with a colorimeter Chroma meter CR-400 (Konica Minolta, Tokio, Japan) using the CIELab system and the results were expressed as chromaticity coordinates L*, a*, and b* (objective parameter). The coordinated L* represents lightness (contribution of black or white varyng 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).

The pods were brushed and dried at 50 °C until reaching constant weight. Then, they were ground to fine flour (Helix mill, Metvisa) and sifted. Two fractions were obtained: pericarp flour (epicarp + mesocarp) and seeds with endocarp. The pericarp flour was passed through three sieves: 840, 500, and 149 μm to obtain *P. nigra* fine flour (granulometry <149 μm).

Nutritional composition

The proximate composition of the mesocarp fine flour was assessed according to the Association of Official Analytical Chemists methods using differential extractions in each case. All analyses were performed in triplicate.

Sugar determination. Flour was extracted exhaustively with 80% aqueous ethanol (1 g: 4 mL) at 75 °C. Then, the extracts were combined and then evaporated. Total sugars, reducing sugars, glucose, fructose and sucrose were determined according Costamagna, Ordoñez, Zampini, Sayago, and Isla (2013). Results were expressed as g of glucose/100 g flour.

Determination of starch was carried out according to Holm, Björck, Drews, and Asp (1986) with some modifications. Flour (0.1 g) was mixed with 10 mL chlorhidric acid (0.1 M) and 0.1 g pepsin to removal of protein (pH 1.5, 40 °C during 1 hr) and was added sodium azide (0.1 g). Then, a hydrolysis using 0.5 g of α-amylase (pH 6.9, 37 °C, during 16 hr) was carried out. Following the digestion step, samples were centrifuged. The supernatants contained digestible starch and the residues contained resistant starch. The last one was dispersed in KOH (2 M) about 30 min at room temperature. Then, digestible and resistant starch, were digested using amyloglucosidase in a 60 °C water bath for 30 min (pH 4.75). The glucose content was determided by enzymatic glycemia assay.

Total protein and amino acid composition. Crude protein content was calculated from the total nitrogen (N) content determined in the flour by Kjeldahl method using a conversion factor of 6.25 (AOAC 920.87).

Flour samples 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 in cation-exchange resin column (https://www.biochrom.co.uk).

Fat measurement. Crude fat from an aliquot of 1 g of flour was determined by extracting with petroleum ether (40 to 60 °C) in a Soxhlet apparatus during 4 hr (AOAC 920.85).

Determination of fiber. Fiber content was determined according to Jaafar, Rahman, Mahmod, and Vasudevan (2009). The algarrobo flour was put into a conical flask and 1.25% sulphuric acid solution was added. The sample was heated for about 30 min, filtered and washed. After acid digestion, the sample was treated with 3.52% NaOH solution. The sample was heated again for 30 min. It was filtered using a vacuum filter and washed with water. The whole material was placed in a crucible and dried for 12 hr at 120 °C. The crucible was heated in a muffle oven at 550 °C for 12 hr and weight of the crucible was recorded.

Determination of moisture, ash and minerals composition. The moisture content was determined by evaluating the difference in weight between the fresh sample and the sample dried at 40 °C until constant weight. The dried sample was placed in a muffle (500 °C) until the ashes were obtained. The mineral analysis was carried out by quadrupole inductively plasma mass spectrometry at the Instituto Superior de Investigación Desarrollo y Servicios en Alimentos, Córdoba, Argentina.

Determination of phytochemicals

Extraction and determination of free and bound polyphenolic compounds content. *Prosopis* flour was extracted with methanol: water (MeOH:H_2O), 70:30 (1:10 w/v) ultrasound assisted for 1 hr at 25 °C. The extracts were filtered and the MeOH evaporated "in vacuo" (40 °C). The residual water was freeze dried. The dry extract was named as total dry extract (TDE). Then, TDE was suspended using an ultrasound bath in 50 mL distilled water and were extracted with ethyl acetate to obtain an extract enriched in free phenolic compounds and free of sugar (free-phenolic extract [Free-PE]).

The residual unsoluble material was treated with 2N NaOH and then, with 6N HCl, filtered and extracted several times with ethyl acetate. The ethyl acetate fraction was dried under reduced pressure and then reconstituted with 1 mL of MeOH to obtain bound-phenolic enriched extract (Bound-PE).

The phenolic content was determined by Folin-Ciocalteu reagent, according to Costamagna et al. (2013). The total flavonoid content in all preparations was determined according to Min, Gu, McClung, Bergman, and Chen (2012).

Extraction and determination of condensed and hydrolizable tannins content. *Prosopis* flour was extracted with acetone: H_2O (70:30, 1:10 w/v) and the total condensed tannins content in each sample was determined according to Costamagna et al. (2013). Results were expressed as mg procyanidin B2 equivalent (PB2E)/100 g flour.

Acetone was evapored and the aqueous fraction was separated in two fractions. One of them was submitted to acid hydrolysis. The total gallic acid content was measured with the rhodanine method. In the other fraction, free gallic acid was measured. The content of hydrolyzed tannins was determinated by difference between them. Results were expresed as g of gallic acid equivalents (GAE)/100 g of flour.

Extraction and determination of anthocyanins content. The flour was extracted with acidified methanol (1%) ultrasound assisted during 10 min every time, several times until the food matrix was exhausted. The anthocyanin content was measured using pH differential method (Abdel-Aal, Young, & Rabalsky, 2006). Data were expressed as g of cyanidin-3-glucoside equivalents (g C3G-E)/100 g flour.

Extraction and determination of ascorbic acid content. Flour (0.2 g) was extracted with 0.5 mL of H_3PO_4 2% ultrasound assisted during 10 min and ascorbic acid content determined according to Costamagna et al. (2013). Vitamin C was expressed as g L-ascorbic acid (g L-AA)/100 g flour.

Extraction and determination of carotenoid content. Flour (1 g) was extracted with 10 mL of petroleum ether:acetone (1:1, v/v) and the total carotenoid content was calculated according to Costamagna et al. (2013). The results were expressed as g of β-carotene equivalents (g β-CE)/100 g of flour.

Extraction and determination of alkaloid content. Total alkaloids from flour were measured using bromothymol blue (BTB) as the colouring agent according Önal, Kepekçi, and Öztunç (2005). Total alkaloids were calculated as g of apomorfine chlorhydrate equivalents (g AC-E)/100 g of flour.

Free and bound phenolics profile. Polyphenols were analyzed by HPLC-DAD and HPLC-MS/MS method using an Agilent Technologies (Inc., Calif., U.S.A.) 1200 Series UPLC equipped with a gradient pump (Agilent G1312B SL Binary), solvent degasser (Agilent G1379 B) and autosampler (Agilent G1367 D SL+WP). The chromatographic separation was achieved on a AXBridgeTM C18 column (4.6 × 150 mm, 5 μm; Waters corporation, Milford, Mass., U.S.A.) by using a linear gradient solvent system consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in MeOH (B) as follows: 90% A to 43% A over 45 min, followed by 43% A to 0% A from 45 to 60 min, and remaining in 0% A for 5 min at 35 °C. The flow rate was set at 0.4 mL/min and the injection volume was 40 μL. The HPLC system was connected to a photodiode array detector (Agilent G1315 C Starligth). Compounds were monitored at 254 and 330 nm,

and UV spectra from 200 to 600 nm were recorded for peak characterization. The quantification of polyphenols was based on external calibration curves from available phenolic standards. The standards HPLC grade were from Sigma-Aldrich (MO, U.S.A.), Fluka Chemical Corp. (U.S.A.), and Indofine Chemical Company, Inc. Plots were built relating area and concentration in the range of 1 to 500 ppm. Results were expressed as mg equivalents of the standard compounds employed per gram of extract.

The HPLC system was connected subsequently to a QTOF mass spectrometer (rmicroTOF-QII Series, Bruker), equipped with 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 nebulizer gas at 4.0 bar, and as drying gas at 200 °C and a flow rate of 8.0 L/min. Argon was used as collision gas. The MS detector was programmed to perform MS and alternative MS/MS from the three most abundant ion obtained with a collision energy of 12 eV. Data acquisition and processing were perfomed using Compass Version 3.1 software and Data Analysis Version 4.0 software, respectively (Bruker Daltonics, MA, U.S.A.).

Measurement of antioxidant capacity

The antioxidant assays were carried out in the total dry extract and in the phenolic compounds-enriched extracts (Free-PE and Bound-PE).

ABTS free radical scavenging activity. Assay was carried out by the improved $ABTS^{\bullet+}$ method as described by Re et al. (1999). The end-points of the bleaching assays after the addition of different *Prosopis* extracts (phenolic compounds concentrations up to 50 μg GAE/mL) were at 6 min. Results were expressed as the concentration of polyphenols necessary to scavenge 50% of ABTS cation radical (SC_{50}). Quercetin (0.1 to 2 μg/mL) was used as reference compound.

Hydrogen peroxide scavenging activity. 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) was prepared in separate tubes for each incubation period (5 to 60 min) and for each polyphenol extract concentration (up to 50 μg GAE/mL). Then, horseradish peroxidase (0.1 U/mL) was added to each tube and incubated at 37 °C. The absorbance was measured at 504 nm (Chamira Dilanka & Preethi, 2015). Results are presented as SC_{50} values in μg GAE/mL required to inhibit 50% of H_2O_2 degradation. Quercetin (1 to 30 μg/mL) was used as reference compound.

Hydroxyl radical scavenging activity. The HO radical scavenging activity was determined according to Cattaneo et al. (2016). The reaction mixture contained 2-deoxy-D-ribose/ $FeCl_3$ and different extract concentrations (up to 50 μg GAE/mL) with and without EDTA. The reaction was started with H_2O_2 and ascorbic acid. The mixture was incubated at 37 °C for 60 min. Then, 2-thiobarbituric acid was added and then, was incubated at 100 °C for 20 min. The absorbance was measured at 532 nm. Results are presented as SC_{50} values in μg GAE/mL required to inhibit 50% of 2-deoxy- D-ribose degradation. Quercetin (5 to 50 μg/mL) was used as reference compound.

Nitric oxide scavenging activity. The capacity of nitric oxide scavenger was determined according to Kumaran and Karunakaran (2006) with some modifications. Briefly, different concentrations of the extracts (up to 400 μg/mL) were mixed with 30 μL of sodium nitroprusside 10 mM, and sodium phosphate buffer (0.1 M, pH 7.5) to a final volume of 150 μL. The reaction mixture was incubated for 1 hr at room temperature. Finally, 150 μL of Griess reagent was added to each well. The absorbance was measured at 550 nm to determine the amount of produced nitrite. Ascorbic acid (10 to 100 μg/mL) was used as positive control. Results are presented as SC_{50} values in μg GAE/mL required to scavenge 50% of nitric oxide.

Protection of lipid against oxidative damage: β-carotene bleaching assay. Antioxidant activity was determined according to Ordoñez, Gomez, Vattuone, and Isla (2006) using β-carotene-linoleic acid system. The initial absorbance at 470 nm was registered at time zero and during 120 min each 10 min. Antioxidant activity (AA%) of extracts (up to 200 μg GAE/mL) and reference compound (quercetin, 1 to 50 μg/mL) was calculated as the percent inhibition relative to control of β-carotene-linoleic acid without polyhenols extract. SC_{50} values denote the μg GAE/mL required to inhibit β-carotene bleaching by 50%.

Superoxide radical scavenging activity. *Non-enzymatic assay*. Superoxide radicals were generated in a phenazin methosulfate (PMS)-β-nicotinamide adenine dinucleotide (reduced form, NADH) system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 300 μL of sodium phosphate buffer (19 mM, pH 7.4) which contained 30 μL of NBT (500 μM), 40 μL of PMS (60 μM), 40 μL NADH (2 mM), and sample at different concentrations. The color reaction was detected at 550 nm using a Microplate reader (Valentão et al., 2002). The scavenging ratio (%) was calculated using the following formula: Scavenging ratio % = (C−S)/C × 100 where C is the absorbance of the control, and S the absorbance of the test sample. SC_{50} values denote the μg GAE/mL required to scavenge 50% of superoxide free radicals.

Enzymatic assay. Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following Cos et al. (1998) with some modifications. The reaction consisted in adding until 90 μL of sample at different concentrations, 30 μL of xanthine oxidase (0.1 U/mL) in a phosphate buffer solution (pH 7.50, 0.2 M) and 60 μL of xanthine (1 mM). The mixture (180 μL) was incubated for 30 min at 37 °C. Then we measured the UV absorbance at 290 nm.

Measurement of anti-inflammatory capacity

The results of the anti-inflammatory effect of total dry extract (TDE) and phenolic compounds-enriched extracts were expressed as IC_{50} values, defined as the test extract concentration (μg GAE/mL) able to produce 50% inhibition of enzymatic activity.

Cyclooxygenase inhibition studies. The ability of the extracts to inhibit human recombinant cyclooxygenase 2 (COX-2) was determined using a COX inhibitor screening assay kit (No. 560131; Cayman Chemical®, Ann Arbor, MI, U.S.A.). PGF2α produced from PGH2 by reduction with stannous chloride was measured by enzyme immunoassay (EIA). The commercial anti-inflammatory drug nimesulide (0.10-2 μg/mL) was used as reference.

Lipoxygenase inhibition studies. Lipoxygenase (LOX) activity was determined using a continuous spectrophotometric method, based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide (D'Almeida et al., 2013). The assay mixture containing soybean lipoxygenase (948 U/mL), linoleic acid (50 μM), sodium borate buffer (200 mM, pH 9.0) and phenolic compounds enriched-extracts (up to 400 μg GAE/mL) was incubated at 25 °C during 5 min. The absorbance at 234 nm was measured during 5 min using a spectrophotometer. Naproxen (up to 25 μg/mL) was used as reference compounds.

Phospholipase A_2 inhibition studies. Secretory phospholipase A_2 activity was determined using 1,2-diheptanoylthioglycerophosphocholine (1,2 dHGPC) and Triton X-100 as substrates (D'Almeida et al., 2013). The reaction mixture contained 50 μL of buffer Tris–HCl (10 mM, pH 8), 10 μL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (10 mM), 10 μL of $sPLA_2$ enzyme (1 μg/mL) and 25 μg GAE/mL of the assayed samples dissolved in DMSO. The reaction was initiated by the addition of 150 μL of 1,2 dHGPC (1.66 mM) and maintained for 20 min at 25 °C. The absorbance was read at 414 nm during 20 min every 2 min in a microplate reader. Commercial anti-inflammatory drug (acetylsalicylic acid, 20 to 80 μg/mL) was used as reference compound.

Inhibitory activity of enzymes related to metabolic syndrome

The effect on metabolic syndrome enzymes was evaluated for TDE, Free-PE, and Bound-PE. In all cases, the inhibition percentage of enzyme activity (α-amylase, α-glucosidase and lipase) and IC_{50} values were calculated. IC_{50} values denote the μg GAE/mL required to inhibit the enzymatic activity by 50%.

α-Glucosidase inhibition. The inhibition of α-glucosidase activity was determined using p-nitrophenyl-α-D-glucopyranoside as substrate according to Costamagna et al. (2016). The reaction mixture contained 160 μL of 0.1 M sodium phosphate buffer (pH 6.9), 5 μL of enzyme (5.46 U/mL), polyphenolic extract (4 to 30 μg/mL) and 5 μL of 25 mM p-nitrophenyl-α-D-glucopyranoside. The reaction was incubated 15 min at 37 °C. Then, 80 μL of 0.2 M sodium carbonate was added. The absorbance was measured at 405 nm in a microplate reader. Acarbose (1 to 50 μg/mL) was used as standard.

α-Amylase inhibition. The α-amylase inhibitory activity using starch as substrate was assayed using Amilokit® (Wiener Lab Group, Rosario, Argentina). The reaction mixture contained 800 μL of 0.01 M sodium phosphate buffer (pH 7.4), 5 μL of α-amylase and polyphenolic extracts (up to 100 μg GAE/mL). After pre-incubation of the reaction mixture on ice for 5 min, the enzyme reaction was started by adding 500 μL of reagent A (substrate) into the reaction mixture. Then, the reaction was incubated at 37 °C for 7 min. After that, 500 μL of reagent B (iodine solution) was added for color development and the reaction mixture was taken to a final volume of 5.3 mL with water. The absorbance was measured at 640 nm in a spectrophotometer (UV2400 PC). Acarbose (0.1 to 5 μg/mL) was used as standard.

Lipase inhibition. Lipase activity was assayed by measuring the enzymatic hydrolysis of p-nitrophenyl palmitate to p-nitrophenol according to Costamagna et al. (2016). Lipase solution (1.0 mg/mL) was mixed with the polyphenolic extracts (up to 100 μg GAE/mL) and pre-incubated on ice for 5 min. The reaction mixture for standard assay contained 330 μL of sodium phosphate buffer 0.1 M (pH 7) supplemented with 0.6% (w/v) Triton X-100 and 0.15% (w/v) arabic gum, and 20 μL of 10 mM substrate (p-nitrophenylpalmitate). The reaction was started by adding 50 μL of the lipase/polyphenolic extract solution and incubated at 37 °C for 20 min. Orlistat (0.1 to 20 μg/mL) was used as reference compound.

Mutagenicity

***Salmonella* mutagenicity assay.** The mutagenic effect of polyphenolic extracts (TDE, Free-PE, and Bound-PE) was evaluated on two *S. typhimurium* strains (TA98 and TA100). According to Maron and Ames (1983), the plate incorporation assay was performed by adding 0.1 mL of the overnight bacterial culture, 0.1 mL of polyphenolic extracts at different concentrations (up to 500 μg GAE/plate) and 2 mL of top agar on minimal agar. Then plates were incubated at 37 °C for 48 hr. After incubation, His^+ revertant colonies were counted and compared to the number of revertant colonies in the controls. The positive controls employed were 4-nitro-O-phenylenediamine (4-NPD; Aldrich Chemical Co.) (10 μg/plate). Solvent control was carried out adding 100 μL DMSO/plate. An extract would be considered mutagenic when the revertants average was the double or higher than two-fold the negative controls. Three plates per experiment and two separate experiments were used in all cases.

Statistical analyses

Analyses were conducted at least three times with three different samples. Each experimental value is expressed as the mean ± standard deviation (SD). The statistic software InfoStat (Student Version, 2011) was used to evaluate the significance of differences between groups. The one-way ANOVA with Tukey posttest at a confidence level of 95% was used for the comparisons between groups. The criterion of statistical significance was taken as $p \leq 0.05$.

Results and Discussion

Pods were collected from different trees in Amaicha del Valle, Tucuman, Argentina and were classified on the basis of their colour as follows: (1) brown with purple spots, and (2) purple. The fruit chromatic characteristics were determined to select to those in the similar ripening grade ($L^* = 46.41 \pm 10$; $a^* = 9.71 \pm 1$: $b^* = 4.23 \pm 1$). *P. nigra* mesocarp fine flour (Figure 1) was produced from dry pods without seeds by grinding and sifting in filters of different granulometry. *P. nigra* mesocarp fine flour was characterized.

Phytochemicals in *Prosopis nigra* mesocarp fine flour

Macronutrients, aminoacid composition and mineral composition. Total carbohydrates (77.12%) were the major component of *P. nigra* mesocarp fine flour. Flour exhibits higher carbohydrate content than aqueous and ethanolic extracts obtained from *P. nigra* pericarp flour (46.37 ± 0.08 and 7.51 ± 0.09 g/100 g pod flour, respectively (Cardozo et al., 2010). The major soluble sugar was sucrose, 30.35 ± 0.02%, unlike other native fruit flours such as mistol, in which fructose turned out to be the most abundant (Orqueda et al., 2017). Its digestible starch (DS = 0.25%) and resistant starch (RS = 0.09%) content was lower than wheat flour (60% to 80% of total starch and 30% to 40% of resistant starch) (Świeca, Dziki, & Gawlik-Dziki, 2017). On the other hand, *P. nigra* fine flour could be considered a fiber-rich food (6.34 ± 0.19%). The high fiber content of *P. nigra* flour can interfere with intestinal fat absorption and convert these flours in potential functional ingredients to formulations of anti-obesity products.

P. nigra mesocarp fine flour showed a low-fat content (Table 1), similar to *P. alba* pericarp flour (Sciammaro, Ferrero, & Puppo, 2016) and lower than the content reported for mistol pulp (12.3 g/100g fine flour) (Orqueda et al., 2017). The crude protein content (7.5%) was higher than *P. alba* pericarp flour (Cardozo et al., 2010) (Table 1) and chañar flour (5.06%) (Costamagna et al., 2016). Its amino acid pattern (Table 2) showed mainly aspartic acid (30.3% of total protein), proline (17.1% of total protein), and glutamic acis (7.47%), following by Ser (5.57%), Ala, Val, Tyr, Lys, and Leu (4.05 to 4.80%), Arg and Gly (around 3%), Hist, Ile, and Phe (around 2.5%), Cys, Met, and Thr (around 1%) while amino acids, tryptophan, and ornitin were limiting amino acids. Among

Table 1–Chemical characterization of *Prosopis nigra* mesocarp fine flour.

Macronutrient content (g/100 g flour)		Phytochemical content	
Total carbohydrates	77.12 ± 0.80	Free phenolics (g GAE/100 g flour)	0.45 ± 0.004
Reducing sugars	2.37 ± 0.08	Bound phenolics (g GAE/100 g flour)	0.17 ± 0.00
Glucose	0.12 ± 0.01	Flavonoids (g QE/100 g flour)	0.68 ± 0.07
Fructose	5.28 ± 0.35	Condensed tannins (g PB2E/100 g flour)	0.03 ± 0.00
Sucrose	30.35 ± 2.79	Hydrolyzable tannins (g GAE/100 g flour)	BDL
Digestible starch	0.25 ± 0.03	Anthocyanins (g C3GE/100 g flour)	47.47 ± 0.53
Resistant starch	0.09 ± 0.02	Ascorbic acid (g L-AA/100 g flour)	0.08 ± 0.00
Total proteins	7.50 ± 0.20	Carotenoids (g β-CE/100 g flour)	BDL
Fat	3.44 ± 0.15	Alkaloids (g ACE/100 g flour)	0.02 ± 0.00
Fiber	6.34 ± 0.19		
Ash	5.61 ± 0.05		
Moisture	39.70 ± 0.27		

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

Table 2–Amino acid composition of *Prosopis nigra* mesocarp flour.

Amino acid	gram/100 g of protein
Ile	2.14 ± 0.02
Leu	4.76 ± 0.03
Lys	4.05 ± 0.02
Phe	2.35 ± 0.01
Tyr	4.48 ± 0.02
Cys	1.33 ± 0.01
Met	2.24 ± 0.03
Thr	1.02 ± 0.03
Val	4.25 ± 0.02
Asp	30.27 ± 0.01
Ser	5.57 ± 0.02
Glu	7.50 ± 0.03
Gly	3.32 ± 0.02
Ala	4.60 ± 0.04
His	2.45 ± 0.03
Trp	0.00
Orn	0.00
Arg	3.17 ± 0.02
Pro	16.50 ± 0.04

the main determined minerals, potassium (1830 ± 20 mg/100 g flour) was the one present in great proportion, followed by magnesium (117 ± 1 mg/100 g flour) and calcium (88 ± 3 mg/100 g flour). Flour was high in K but low in Na. A high K/Na ratio (7.8 ± 0.8 mg/100 g flour) makes *P. nigra* flour interesting for diets with a defined electrolytic balance. The high content of K could be utilized beneficially in the diets of people who take diuretics to control hypertension. The Fe content was around 3.8 ± 0.2 mg/100 g flour. Intake of *P. nigra* mesocarp flour would contribute significantly to the supply of important minerals for human health.

Secondary metabolites. The free polyphenol content of *P. nigra* fine flour (450 ± 4 mg GAE/100 g flour) was higher than the values obtained in ethanolic extracts of *P. nigra* and *P. alba* pods (Cardozo et al., 2010) and white wheat flour (4.4 to 14 mg GAE/100 g flour) (Hung, Maeda, Miyatake, & Morita, 2009) and it was lower than the flour of other Argentine native fruits such as *Ziziphus mistol* (792.20 ± 28.02 mg GAE/100 g DW) (Cardozo et al., 2010) and *Geoffroea decorticans* (1240 ± 30 mg GAE/100 g DW) (Costamagna et al., 2013). Flavonoids were the main free phenolics in flour (Table 1). Four apigenin-based C-glycosides were identified (Table 3) and quantified in the free phenolic-enriched extracts obtained from *P. nigra* fine flour: Vicenin II (0.34 ± 0.01 μg/mg dry extract); isoschaftoside (0.27 ± 0.01 μg/mg dry extract); schaftoside (0.24 ± 0.01 μg/mg dry extract) and isovitexin (0.81 ± 0.10 μg/mg dry extract). Flavonoid *C*-glycosides have shown to present antioxidant, anti-inflammatory, antiplatelet activity, anti-cancer, enzyme converting angiotensin (ECA) inhibitors, hypoglycemic activity, among others. In addition, they are rapidly absorbed after oral administration and distributed by plasma in different tissues (Xiao, Capanoglu, Jassbi, & Miron, 2016). In previous papers, apigenin based C-glycosides were also described in methanolic extracts from pericarp and seed of *P. alba* and pod syrups (Cattaneo et al., 2016; Pérez et al., 2014; Quispe, Petroll, Theoduloz, & Schmeda-Hirschmann, 2014).

Other major phenolic compounds detected in *P. nigra* mesocarp fine flour were anthocyanins (Table 1). Hydrosoluble pigment levels were higher than other anthocyanin-rich foods such as blueberries (1.3 to 3.8 mg/g FW) and purple corn (6.8 to 82.3 mg/g FW) (He et al., 2016; Lao & Giusti, 2017). Pérez et al. (2014), reported in *P. nigra* pericarp flour the presence of cyanidin-3-hexoside and other anthocyanins.

Table 3–Identification of free and bound phenolic compounds in *P. nigra* flour by HPLC-DAD-ESI-MS data.

Rt (min)	UV λmax (nm)	[M-H]	Fragmentation pattern	Identification
Free phenolic compounds				
21.4	332, 271	593	575, 503, 473, 383, 353	Vicenin II
24.9	336, 271	563	545, 503, 473, 443, 383, 353	Schaftoside
27.2	336, 271	563	503, 473, 443, 383, 353	Isoschaftoside
29.0	338, 296 sh, 270	431	311, 431	Isovitexin
Bound phenolic compounds				
9.1	259, 293	153	109	Protocatechuic acid
23.4	227, 310	163	119	Coumaric acid
25.1	235, 322	193	134	Ferulic acid

Recently, it was demonstrated that purple corn, enriched in cyanidin-3-glucosides reduced visceral adiposity, body fat mass, and blood pressure, improved glucose tolerance, and cardiovascular structure and function, decreased plasma triglycerides and total cholesterol compared to control rats (Bhaswant, Shafie, Mathai, Mouatt, & Brown, 2016).

Phenolics bound to cell wall components are considered healthy because they may escape from upper gastrointestinal digestion and may have effect against colon cancer and other chronic diseases (Min et al., 2012; Ti et al., 2014). The bound phenolic content in mesocarp flour (0.2 g GAE/100 g flour) was lower than free phenolic content. The three major components of bound phenolics were identified as protocatechuic acid (0.33 ± 0.00 μg/mg dry extract), ferulic acid (4.47 ± 0.33 μg/mg dry extract) and coumaric acid (8.16 ± 0.63 μg/mg dry extract), Table 3. In wheat flour the major bound phenolic compound was identified as ferulic acid (33.7 to 625 μg/g of flour) (Lv et al., 2012; Moore et al., 2005). With respect to ascorbic acid, 100 g of fine flour are sufficient to cover the recommended daily allowance (Levine, Wang, Padayatty, & Morrow, 2001).

Antioxidant activity

During aerobic metabolism, reactive oxygen species (ROS) are produced as by-products. The hydrogen peroxide itself is not very reactive, but it can diffuse across biological membranes and generate the highly reactive $HO^{\bullet}$. The $HO^{\bullet}$ could act on lipid, proteins, nucleic acid and sugar and produce oxidative stress. Both polyphenols enriched preparations were active as scavenger $ABTS^{\bullet+}$, H_2O_2, $HO^{\bullet}$ with SC_{50} values between 8.25 and 23.5 μg GAE/mL (Table 4). The superoxide anion scavenging capacity of all extracts was lower than other oxygen reactive species. l *Z. mistol* radical scavenging capacity reported by Orqueda et al. (2017) (75 μg GAE/mL) was similar to activity showed by free phenolic enriched extract. Nevertheless, all extracts inhibited the enzyme xanthine oxidase and consequently, they efficiently inhibited the generation of superoxide anion (IC_{50} values to TDE, free and bound phenolics were 4.25 ± 0.06; 1.4 ± 0.10 and 4.8 ± 0.07, respectively). Bound phenolics enriched extracts were more active on $HO^{\bullet}$, H_2O_2, $O_2^{\bullet-}$, NO scavenging than free phenolics enriched extracts. In addition, bound phenolic enriched extract was almost four-fold more active as inhibitor of lipoperoxidation (β-carotene assay) than free phenol enriched extract (Table 4) with values similar to mistol (Costamagna et al., 2016). Therefore, polyphenolic compounds principally the bound-phenolic compounds obtained from fine flour of *P. nigra* could efficiently avoid oxidative pathologies associated with obesity.

Anti-inflammatory activity

Inhibition of LOX, COX-2, and sPLA2 activities would be an important treatment to many inflammatory diseases (D'Almeida et al., 2013). The extract enriched in free and bound phenolic compounds was able to inhibit the LOX activity in a concentration dependent manner with IC_{50} values without significant difference, 309.00 ± 4.24 and 267.50 ± 7.78 μg GAE/mL, respectively, while the inhibition of LOX by *G. decorticans* flour was higher (IC_{50} values of 124 μg GAE/mL,Costamagna et al., 2016) than *P. alba* flour. The $sPLA_2$ activity was also inhibited (55% with 25 μg GAE/mL) by free polyphenolic extract. The effect was higher than chañar flour (225 μg GAE/mL, Costamagna et al., 2016) and similar to anti-inflammatory drug, acetylsalicylic acid (65 μg/mL). Bound phenolic enriched extract was less active than free phenolic enriched extract. On the other hand, both

Table 4–Effect of polyphenols enriched extract of *Prosopis nigra* fine flour and reference compounds on enzymes related to carbohydrate metabolism, fat metabolism and oxidative processes.

	Enzymes of metabolic syndrome			Antioxidant capacity				
	α-Amylase	α- Glucosidase	Lipase	$ABTS^{\bullet+}$	H_2O_2	$HO^{\bullet}$	$O_2^{\bullet-}$	NO	β-carotene
Sample	IC_{50}					SC_{50}			IC_{50}
TDE	97.0 ± 0.7*	4.5 ± 1.0[a]	37.0 ± 1.4[a]	17.00 ± 1.42[a]	12.80 ± 2.27[b]	6.0 ± 0.0[a]	140.00 ± 2.01[c]	7.0 ± 0.6[a]	14.25 ± 2.05[a]
Free-PE	30.0 ± 1.1[a]	22.5 ± 2.5[b]	82.5 ± 8.6[b]	16.00 ± 1.41[a]	17.15 ± 0.35[c]	16.0 ± 0.4[c]	93.00 ± 6.00[b]	74.0 ± 4.1[c]	86.80 ± 8.67[c]
Bound-PE	70.0 ± 1.3[b]	29.0 ± 5.6[b]	33.5 ± 4.9[a]	23.50 ± 3.54[b]	8.25 ± 0.35[a]	9.0 ± 0.4[b]	57.00 ± 3.74[a]	27.0 ± 0.3[b]	24.05 ± 0.21[b]
Reference compound	Acarbose	Acarbose	Orlistat	Quercetin	Quercetin	Quercetin	Quercetin	Ascorbic acid	Quercetin
IC_{50} (μg/mL)	1.25 ± 0.11	25.0 ± 0.80	6.00 ± 0.03	1.30 ± 0.18	13.90 ± 0.00	30.00 ± 2.81	60.50 ± 4.70	37.19 ± 0.33	18.00 ± 1.40

SC_{50} (μgGAE/mL): Concentration of polyphenolic extract necessary to scavenge 50% of ABTS; H_2O_2; $HO^{\bullet}$; $O_2^{\bullet-}$; NO.
IC_{50} (μgGAE/mL): Concentration of polyphenolic extract necessary to inhibit 50% of β-carotene bleaching, α-amilase, α-glucosidase, lipase activities.
*IC_{35} (μgGAE/mL): Concentration of polyphenolic extract necessary to inhibit 35% of α-amilase activity.
TDE: total dry extract with sugar from *P. nigra*; Free-PE = free polyphenols enriched extracts from *P. nigra* mesocarp; Bound-PE = bound polyphenols enriched extracts from *P. nigra* mesocarp. 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 ($p \leq 0.05$).

extracts were able to inhibit 50% the COX-2 activity with low concentration of phenolic compounds (1 μg GAE/mL) showing their great inhibitory potency similar to nimesulide, a commercial antiinflammatory (IC_{50} values of 0.39 μg/mL). The consumption of foods with strong inhibitory properties of COX-2 could have a positive effect on obesity-associated inflammation.

Effect on key enzyme involved in the development of metabolic syndrome

α-Glucosidase and α-amylase inhibition. The increase of blood post-prandial glucose can be decreased through inhibition of enzymes involved in glucose release from food such as α-glucosidase, an enzyme located in the intestinal epithelium. The continuous administration of oral α-glucosidase commercial inhibitors (acarbose, voglibose, and miglitol) may cause abdominal discomfort, diarrhea, flatulence, and hepatotoxicity (Abdul-Ghani, Tripathy, & DeFronzo, 2006). Therefore, novel inhibitors of α-glucosidase are necessarily given as a therapeutic treatment of type II diabetes mellitus. Free and bound polyphenolic enriched extracts obtained from *P. nigra* flour were active toward α-amylase and α-glucosidase with IC_{50} values of 30 and 70 μg GAE/mL for amylase and 22.5 and 29 μg GAE/mL for glucosidase, respectively. In other native fruit flours such as chañar, similar inhibitory effect for α-amylase was observed with 25 μg GAE/mL (Costamagna et al., 2016). Free phenolic compounds were more active than bound phenolics for α-glucosidase (Table 4). Results suggest that *P. nigra* extracts might be able to reduce glucose uptake/absorption. In this sense, flour may be a dietary complement to control hyperglycemia in diabetic patients.

Pancreatic lipase. Pancreatic lipase is an enzyme that plays an important role in metabolic syndrome, lipid hydrolysis to form fatty acids in such a way that they can be absorbed in human digestive tract. The inhibition of pancreatic lipase with oral inhibitors (orlistat) is the main prescribed treatment for weight management and obesity (Birari & Bhutani, 2007). With the final purpose to find alternative natural sources for reduce the risk of the development of obesity, we evaluated the free and bound phenolic enriched extract effect on lipase activity. Results are shown in Table 4. The inhibitory activity of bound phenolic extract on lipase (IC_{50}: 33.5 μg GAE/mL) was higher than the free phenolic extract (IC_{50}: 82.5 μg GAE/mL) and higher than the inhibition reported for white and green tea polyphenols, natural products with recognized antiobesity activity (Gondoin, Grussu, Stewart, & McDougall, 2010).

Mutagenic activities

The extracts enriched with phenolic compound, were not mutagenic on TA98 or TA100 strains.

Conclusions

Fine flour of mesocarp from *P. nigra* could be used as a functional ingredient due to its high fiber and mineral content. Furthermore, polyphenols (especially free phenolic compounds) from *P. nigra* are glycosidase and amylase inhibitors and consequently may control hyperglycemia. On the other hand, bound phenolic compounds may control lipase activity. Free and bound phenolic compounds are antioxidants and inhibitors of pro-inflamatory enzymes. Thereby, our studies add value to *P. nigra* fine flour as a functional ingredient or functional food. The demonstration of the nutritional and functional quality could promote the cultivation and commercialization of the specie.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Conceived and designed the experiments: Isla, Zampini; Performed the experiments: Pérez, Zampini, Alberto; Analyzed the data: Isla, Alberto, Pérez, Zampini; Wrote the paper: Isla; and Conceived and initiated the project: Isla.

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