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PII: S2212-4292(24)00208-6

DOI: https://doi.org/10.1016/j.fbio.2024.103778

Reference: FBIO 103778

To appear in: *Food Bioscience*

Received Date: 5 September 2023

Revised Date: 9 February 2024

Accepted Date: 19 February 2024

Please cite this article as: Hoffmann E., Boeri P., Monasterio R., Fontana A., Puppo M.C., Barrio D.A. & Piñuel L., Bioactivity of *Prosopis alpataco* and *Prosopis flexuosa* flours: Healthy alternatives as ingredients for nutritional foods, *Food Bioscience* (2024), doi: https://doi.org/10.1016/j.fbio.2024.103778.

Bioactivity of *Prosopis alpataco* and *Prosopis flexuosa* flours: healthy alternatives as ingredients for nutritional foods

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Abstract: Pods of some *Prosopis* species are a staple food among rural communities. The nutritional composition, antioxidant activity and *in vivo* biological properties of *P. alpataco* Phil. and *P. flexuosa* DC., underutilized *Prosopis* species, were studied. High amounts of dietary fiber (>50%) and polyphenols (0.45-0.50 g of gallic acid equivalents/100 g flour) were detected. The amino acid content, mineral composition, and phenolic compound profile were analyzed. Among the most abundant phenolic compounds, (-)-epigallocatechin gallate, trans-picein and ε-viniferin were identified for the first time in *Prosopis* species. *In vivo* study with zebrafish model revealed that pod extracts resulted in lower reactive oxygen species generation (up to 30%) compared to the control group. Also, *P. alpataco* extract was able to inhibit enzymes associated with metabolic syndrome: α-amylase, α-glucosidase, and lipase (half maximal inhibitory concentration: 1.93, 2.94 and 22 μg of gallic acid equivalents/ml, respectively). The results evidence the potential value of this non-conventional flour as a promising ingredient for functional foods.

Keywords: *Prosopis* spp., Phenolic compounds, (-)-epigallocatechin gallate, trans-picein, ε-viniferin, Zebrafish, Metabolic Syndrome, Proximate composition

List of abbreviations: GAE, Gallic acid equivalents; PC, Phenolic compounds; ROS, Reactive oxygen equivalents; IC50, Half maximal inhibitory concentration; FAO, Food and Agriculture Organization; DCFH-DA, 2‘,7’-Dichlorodihydrofluorescein diacetate; AA, Amino acids; DPPH•, 2,2-Diphenyl-1-picrylhydrazyl; $ABTS^{\bullet+}$, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; AAPH, 2,2′-Azobis(2-amidinopropane) dihydrochloride; DMSO, Dimethylsulfoxide ; AEA, Acetone extract of *P. alpataco*; AEF, Acetone extract of *P. flexuosa*; MEA, Methanol extract of *P. alpataco*; MEF, Methanol extract of *P. flexuosa*; TPC, Total polyphenol content; dw, Dry weight; TAA, Total antioxidant activity; RDI, Recommended daily intake.

1. Introduction

The consumption patterns that sustain today's society require new food sources and improvements in the production of high-quality foods. The Food and Agriculture Organization (FAO) states that biodiversity is essential for ensuring food security, sustainable development, and the provision of ecosystem services (United Nations, 2019). Plant genetic resources offer a wide range of natural ingredients that are beneficial to human health. Historically, they have been used as a source of subsistence, especially in communities that inhabit areas of high vulnerability, such as arid and semi-arid regions of the planet. The exploration of new food sources of plant origin and the sustainable development of innovative food products have allowed the utilization and valorization of underutilized resources, thus expanding the possibility of bringing development to different territories. The food implications of *Prosopis spp*. (recently renamed *Neltuma*, Hughes et al., 2022) have been recognized on several occasions around the world. Thus, the pods of these legumes have been proved to be alternative ingredients to improve fiber and mineral content of food products (Bigne et al., 2018). In addition, the flours of the pods of different *Prosopis* species present health benefits, and their biological properties are related to the presence of secondary metabolites, such as phenolic compounds (PC) (Rodríguez et al., 2019). Moreover, in situations of environmental stress, such as in arid and semi-arid regions of the world, plants restrict their photosynthetic activity so that non-structural carbohydrates tend to accumulate. Thus, the synthesis of carbon-based secondary defense metabolites is increased, which has been confirmed in several species subjected to low nutrient or water availability (Sharma et al., 2022). Furthermore, an increase in these metabolites also influences the human health benefits associated with the presence of these bioactive compounds in plant-based foods. These effects include antioxidant, anti-inflammatory, antiplatelet aggregation, anticancer, angiotensin-converting enzyme inhibition, hypoglycemic activity, and effects on enzymes

associated with metabolic syndrome (Isla et al., 2022). Due to the role of reactive oxygen species (ROS) in aging and various pathologies, there has been an increasing interest in assessing the antioxidant potential linked with PC found in food sources. (Liu et al., 2018). A method for investigating the impact of PC on ROS is the 2‘,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay. Using this method allows for the evaluation and quantification of ROS production at the cellular level, facilitating the assessment of PC efficacy in mitigating oxidative stress. This methodology can be applied under *in vivo* conditions using the zebrafish (*Danio rerio*) model, which allows the validation of biological activities previously evaluated under *in vitro* conditions. Given the importance of the *Prosopis* species in the world and the potential use of its flour as a functional ingredient, the objective of this study was to determine the nutritional and biological properties of two dryland *Prosopis* species from Argentinean Patagonia, that are underutilized and undervalued: *P. alpataco* and *P. flexuosa.* For that purpose, the nutritional composition of flours of both species, together with the PC, amino acids (AA) and mineral profiles were analyzed. The bioactivity of obtained extracts was evaluated *in vitro* (antioxidant activity, α-amylase, α-glucosidase and lipase activity inhibition) and *in vivo* (ROS generation at the cellular level using the zebrafish model).

2. Materials and Methods

2.1.Materials

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), gallic acid standard, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox reactive), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), dichloro-dihydro-fluorescein diacetate (DCFH-DA), ProtaSea Fucoidan, and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St Louis, MO, USA). For mineral analysis, the following standards were used: PlasmaCAL-

SPC-15-AES for Varian Vista Tuning solution, nº 140-130-355; Iron Standard Solution CertiPUR, nº 1.19781.0100; Calcium Standard Solution CertPUR, nº 1.19778.0100; Magnesium Standard Solution CertiPUR, nº 1.19788.0100. For AA analysis, the standard was L-2-Aminobutyric acid ≥99% from Sigma-Aldrich (SKU: A1879). The following standards were used to identify and quantify the phenolic compounds: procyanidin B1; (+)-catechin, trans-piceid, ε-viniferin, quercetin-3-galactoside, (-)-epigallocatechin, (-)-epigallocatechin gallate, naringin, myricetin, quercetin, cyanidin 3-O-p-coumaroylglucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, peonidin 3-O-p coumaroyl glucoside, malvidin 3-O-p coumaroyl glucoside with values of purity between 90% and 99.5%, all compounds were obtained of Sigma-Aldrich.

2.2. Sample preparation

Ripe pods of *P. alpataco* and *P. flexuosa* were collected in the arid regions from Rio Negro, Argentina (40° 39' S 64° 2' W). Samples were taken from at least 10 bushes of each species and stored at room temperature (10-20°C) until use. After collection, they were washed with distilled water and dried for 5 days at 60°C in a natural convection oven (SL60CDB model, San Jor, Buenos Aires, Argentina). The dried samples were milled using a grinder (FW100, Faithful Instrument Co, Zhejiang, China) until a homogeneous flour was obtained, which was stored in air-tight plastic bags at -20°C.

2.3. Nutritional composition of wholemeal flours

The nutrients for proximate composition determination were analyzed according to standardized specifications (AOAC 2016). Moisture, crude protein, lipid, ashes, and total dietary fiber were measured according to AOAC 934.06, 976.05, 963.15, 900.02 and 985.29, respectively.

Mineral content was measured as described by Cindrić et al. (2012). The flour was broken down by microwave-assisted digestion and then analyzed in an inductively coupled plasma by

atomic emission spectrometry (Agilent 720, Agilent technologies, USA), and compared against standards of minerals.

Free sugars of 1 g of defatted samples were determined according to Sciammaro et al. (2015). One gram of defatted flour sample was added to 13 ml of milliq water, 1 ml of potassium hexacyanoferrate (II) trihydrate 3.6% w/v, and 1 ml of 7.2% w/v zinc sulfate heptahydrate. The mixture was left shaking for 30 min at 70°C. It was left to cool down at room temperature, then 10 ml of acetonitrile was added, and the solution was thoroughly mixed and then centrifuged (countertop centrifuge, CMH-28, Presvac, Buenos Aires, Argentina) for 10 min at 2655 g at room temperature. The supernatant was filtered with a 0.44 µm pore membrane. The extract was analyzed by HPLC with a refractive index detector. The column used was a Hypersil Gold Amino 250 x 4.6 with a 5µm particle size. The mobile phase was acetonitrile:water 80:20. Standards of glucose, fructose and sucrose were used to make a calibration curve.

The AA profile was measured according to Cian et al. (2020). Flour samples were hydrolyzed with 6 M hydrochloric acid maintained at 110°C for 24 hours in nitrogen-purged tubes. The resulting amino acids were derivatized at 50°C for 50 min with an excess of diethyl ethoxymethylenemalonate, and subsequently analyzed by HPLC. The analysis was performed using an LC-20AT Prominence Liquid Chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a reversed-phase column (Novapack C18, 300 mm × 3.9 mm i.d., 4 µm particle size, Waters®, Milford, Massachusetts, USA) at 18°C. For elution, the flow rate was 0.9 ml/min. The eluted amino acids were detected at 280 nm, and α-aminobutyric acids served as internal standards for quantification. Data processing was carried out using Shimadzu LC Solution software.

2.4. Antioxidant extracts

2.4.1 Preparation of antioxidant extracts

Two separate extraction experiments were conducted using 1 g of wholemeal flour with 7 ml of solvent mixture, which were either acetone:water 70:30 v/v or methanol:water 50:50 v/v. The mixture was shaken for 45 min at room temperature and then separated by centrifugation at 6000 g for 5 min at room temperature on a countertop Presvac centrifuge. The pellet was extracted twice with the same solvent mixture and the supernatants were pooled and evaporated using a rotary evaporator at 50°C. The concentrated extracts were freeze-dried and stored at -20°C until analysis. The extracts were coded as acetone extract of *P. alpataco* (AEA), acetone extract of *P. flexuosa* (AEF), methanol extract of *P. alpataco* (MEA) and methanol extract of *P. flexuosa* (MEF).

2.4.2. Total phenolic content

The total polyphenol content (TPC) was measured according to Kim et al. (2003) with modifications. Briefly, the sample was dissolved in water, and then 50 µl were mixed with 2.3 ml of water and 50 µl of Folin-Ciocalteu reagent. After 5 min, 100 µl of basic solution was added (20% w/v sodium carbonate in 0.1 N sodium hydroxide) and the mixture was kept in darkness at room temperature for 90 min. Then, absorbance was measured at 765 nm in a spectrophotometer (SP-2100UV, Shanghai Spectrum, Shanghai, China). The results were compared with a gallic acid standard solution and expressed as g of gallic acid equivalents (GAE)/100 g of flour in dw.

2.4.3. $ABTS^{\bullet+}$ radical scavenging capacity

The radical scavenging activity of $ABTS^{\bullet+}$ was measured according to the method described by Re et al. (1998), with modifications. Briefly, 1 ml of 0.28 mM $ABTS^{\bullet+}$ and 0.2 mM ammonium persulfate in ethanol 80% with an absorbance of 0.700 ± 0.005 at 734 nm was mixed with 25 µl of the sample dissolved in methanol. The mixture was kept in darkness for 6 min, and its absorbance was measured at 734 nm. The results were compared with a Trolox standard solution prepared in methanol and expressed as mmol Trolox equivalents/100 g flour

dw. The scavenger concentration 50% (SC50) of $ABTS^{\bullet+}$ radicals was estimated and expressed as mg of dry extract/ml.

2.4.4. DPPH• radical scavenging capacity

The radical scavenging activity of DPPH• was measured according to Zhang et al. (2015) with modifications. Briefly, 775 µl of 0.1 M DPPH• in methanol were mixed with 25 µl of sample dissolved in methanol and kept in darkness for 30 min at room temperature. Then, its absorbance was measured at 517 nm and compared with a Trolox standard solution dissolved in methanol and expressed as mmol of Trolox equivalents/100 g flour dw. SC50 of DPPH• radicals was estimated and expressed as mg dry extract/ml.

2.4.5. Total antioxidant activity

Total antioxidant activity (TAA) was measured according to Prieto et al. (1999). A volume of 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with 100 µl of sample dissolved in water, and kept in darkness for 90 min at 95°C. Then, it was left to cool down at room temperature, and its absorption was measured at 765 nm. Ascorbic acid was used as standard, and the results were expressed as mmoles of ascorbic acid equivalents/100 g of flour dw.

2.4.6. Polyphenol content - PC determination

The identification and quantification of individual PC in the extracts was performed by high-performance liquid chromatography coupled with diode array and fluorescence detectors (HPLC-DAD-FLD). The equipment used consisted of a Dionex Ultimate 3000 (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) equipped with a vacuum degasser unit, an autosampler, a quaternary pump, a chromatographic oven, a diode-array (Dionex DAD-3000 (RS)) and a dual-channel fluorescence detector (FLD-3400RS Dual-PMT) connected in series. The software Chromeleon 7.1 was used for controlling the system and processing the data. The conditions used for the analysis were those previously reported

193 by Ferreyra et al. (2021). Briefly, the separation of PC was performed with a reversed-phase
194 Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) using as
195 mobile phase a 0.1% formic acid aqueous solution (A) and acetonitrile (B). The gradient was
196 the following: 0 - 1.7 min, 5% B; 1.7 - 10 min, 30% B; 10 - 13.5 min, 95% B; 13.5 - 15 min,
197 95% B; 15 - 16 min, 5% B; 16–19.5% B. The total flow rate was set at 0.8 ml/min and the
198 column temperature at 35°C. The injection volume was 5 μl. The conditions of detectors,
199 identification, and quantification of PC in samples were similar to those reported previously.
200 *2.5. In vivo antioxidant activity*
201 The animal study was reviewed and approved by the Institutional Commission for the Care
202 and Use of Laboratory Animals (CICUAL) of National University of Río Negro. The
203 zebrafish maintenance and embryo collection followed the description in the OECD 236
204 guidelines (OECD, 2013). Zebrafish husbandry, the drug and food treatment protocols were
205 followed as per previous studies (Boeri et al., 2020). *In vivo* antioxidant activity was
206 performed according to Dai et al. (2020). Briefly, zebrafish embryos at 4–6-hr post-
207 fecundation (hpf) (n = 8) were placed in 24-well sterile plates in 500 μl of media containing
208 0.1% DMSO at 28°C. The AEA and AEF samples were added to the wells at concentrations
209 of both 5.7 μg GAE/ml of embryo medium with 0.1% v/v DMSO. After 4 h, the eggs were
210 rinsed and left in embryo media with 25 mM AAPH and 0.1%v/v DMSO overnight. The
211 following day, the eggs were rinsed and left in embryo media with a solution of the oxidation-
212 sensitive fluorescent probe dye DCFH-DA for 2 h. After rinsing, the sections were
213 dechorionated, observed, and photographed under a microscope. The fluorescence intensity of
214 individual larvae was quantified using the ImageJ software. The results were compared with
215 those of a control without AAPH and a blank without the sample.
216 *2.6. Inhibition of enzymes related to metabolic syndrome*
217 *2.6.1. Amylase inhibition*

Amylase inhibition was measured by a commercial kit (Amilokit, Wiener lab, Rosario, Santa Fe, Argentina) which contained reagent A (500 mg/l of starch solution, buffered to pH 7 with 0.1 M phosphate buffer in 0.15 M sodium chloride) and reagent B (0.01 M of iodine in 0.02 M hydrochloric acid). Briefly, 10 µl of extract (concentration between 20 and 100 µg GAE/ml) were added to 150 µl of reagent A solution, mixed, and left at 37°C for 5 min. A volume of 10 µl of amylase solution (concentration 2 U/ml, with 1 amylase unit defined as the quantity of amylase that will hydrolyze soluble starch at the rate of 10 mg in 30 min at 37°C) were added, mixed, and left in a 37°C water bath. A volume of 150 µl of reagent B solution and 1.5 ml of water were added to stop the reaction. The absorbance of the mixture was measured at 640 nm. The negative control corresponded to the reaction mixture without the addition of the extract and was used to calculate the reaction speed, whereas the positive control corresponded to the reaction mixture without the addition of the extract and with fucoidan at 0.5 mg/ml as inhibitor (Kim et al., 2014). Half maximal inhibitory concentration (IC50) was calculated using Probit. The reaction rate was calculated according to Equation 1 by following the absorbance change through time.

% Inhibition = Reaction rate of the sample * 100 / Reaction rate of the control (1)

2.6.2. α-glucosidase inhibition

The inhibition of α-glucosidase was measured according to Pérez et al. (2018). Briefly, 20 µl of α-glucosidase 5 U/ml (one glucosidase unit liberates 1.0 µmol of D-glucose from p-nitrophenyl α-D-glucoside/min at working conditions) were left in an ice bath with 200 µl of sample (2 to 20 µg GAE/ml) and 460 µl of pH 6.9 0.1 M sodium phosphate buffer. Then the reaction was initiated adding 20 µl of 25 mM *p*-nitrophenyl-α-d-glucopyranoside as substrate. It was left to react for 15 min in a water bath at 37°C. The reaction was stopped with an ice bath and 320 µl of 0.2 M sodium carbonate were added for the development of color. Finally, the absorbance was measured at 405 nm. The negative control corresponded to the reaction

mixture without the addition of the extract, whereas the positive control corresponded to the reaction mixture without the addition of the extract and with fucoidan at 1 µg/ml as inhibitor (Kim et al., 2014). Inhibition was expressed as a percentage and calculated as Equation 2 based on Pérez et al. (2018).

% Inhibition = (Absorbance of the control - Absorbance of the sample) * 100 / Absorbance of the control (2)

2.6.3. Lipase inhibition

Lipase inhibition was measured according to Pérez et al. (2018). Lipase solution (1.0 mg/ml) was mixed with polyphenolic extracts (10-200 µg GAE/ml) and pre-incubated on ice for 5 min. The reaction mixture for standard assay contained 330 µl of pH 7 0.1 M sodium phosphate buffer with 0.6% w/v Triton X-100 and 0.15% w/v arabic gum, and 20 µl of 10 mM substrate (p-nitrophenyl palmitate). The reaction was started by adding 50 µl of the lipase/polyphenolic extract solution and incubated at 37°C for 20 min. The reaction was stopped in an ice bath and the color was developed with the addition of 50 µl of a basic solution (20% w/v Na_2CO_3 in 0.1 N NaOH). The absorbance was read at 400 nm. The positive control corresponded to the reaction mixture without the addition of the extract. Inhibition was expressed as a percentage, and calculated according to Equation 3, as Pérez et al. (2018).

% Inhibition = (Absorbance of the control - Absorbance of the sample) * 100 / Absorbance of the control (3)

2.7. Statistical analysis

All assays were performed in triplicate. Data generated from the experiments were analyzed using OriginPro 8. The one-way ANOVA with Fisher LSD post hoc test at a significance level of $p \leq 0.05$ was employed for intergroup comparisons. The SC50 and IC50 were calculated using Probit.

3. Results and discussion

3.1. Composition analysis

3.1.1. Proximate composition, sugar, and mineral content

The nutritional content of *P. alpataco* and *P. flexuosa* wholemeal flours are presented in Table I. The main component was total dietary fiber (55.2-57.4%), followed by crude protein (10.6-11.8%), ash (~3.3%), lipid (2.8-3.8%), and moisture (0.5-0.7%). Thus, the energy value was calculated using the Atwater system, it was approximately 180 kCal/100 g flour, and the nutritional contents were of the same order as those previously reported for *P. alpataco* pods (Boeri et al., 2017). These crude protein values were calculated using the Kjeldahl factor of 6.25 and are similar to those of other *Prosopis* species and almost as high as those of wheat flour. Moisture correlates with both microbiological and chemical degradation processes, and these moisture values are sufficiently low that *Prosopis* flour can be used in baked goods (< 15%). The total dietary fiber content represents 70 and 66% of the total carbohydrates of *P. alpataco* and *P. flexuosa*, respectively, so these flours could be added to low-fiber foods to improve their fiber profile. Sucrose is the main available carbohydrate (10.3-12.5%); however, both flours showed a relatively high content of mono and oligosaccharides compared to other legumes, with values approximately between 4-11%, but lower than that reported for other *Prosopis* species (Gonzales-Barron et al., 2020; Sciammaro et al., 2015). In contrast, the glucose and fructose levels found in the studied species were higher than those found in other species of the genus. The high content of free sugars (> 15%) contributes to the sweet taste and palatability of wholemeal flour, and their presence is of particular importance for baking, as they contribute to Maillard reactions and fermentation (Sciammaro et al., 2015). Given these characteristics, pod flour could be an interesting clean label sweetener and a fiber-enriching ingredient.

The most abundant macrominerals in the wholemeal flours of these species were Ca and Mg. The Ca content was of the same order as that of almonds (220 mg/100 g) and soybeans (270 mg/100 g), foods considered to be important sources of this essential nutrient, applied to reduce the risk of osteoporosis. The recommended daily intake (RDI) of Ca is 1000 mg for women between 30 and 50 years of age (USDA, 2021); thus, the consumption of 100 g of wholemeal flour from *Prosopis* pods represents 24% of the RDI. The Mg content of these flours was three times higher than the values reported for wheat flour (25 mg/100 g flour) and 4-6 times (0.9 mg/100 g flour) for Fe. In addition, the Zn content of both species was about 1 mg/100 g, lower than the value for whole wheat flour (2.96 mg/100 g) but higher than the value for refined wheat flour (0.85 mg/100 g) (USDA, 2021). These are essential trace minerals for human nutrition, as their deficiency in children can affect neurotransmitter systems, with a consequent risk of developing mental illness. Mn and Cu are minerals involved in blood and bone health; they were abundant, and the values in 100 g of flour represent approximately 40% of the RDI of those minerals. As, Cd, Cr and Pb, known as metal contaminants, were detected in quantities < 50 µg/100 g; however, other studies found that *Prosopis* species have a tendency to bioaccumulate these metals (Muro-González et al., 2020). Detecting them in less than threatening concentrations is a positive aspect of their cultivation for human consumption. Adequate mineral intake is necessary for all the essential biological functions, from cellular oxygenation to bone maintenance. Alternative flours have proven to be valid substitutes for improving the mineral content of different bakery products (Sciammaro et al., 2015). Therefore, they could be potential ingredients for dietary mineral supplementation.

3.1.2. Amino acids and protein nutritional quality

Proteins are an important component of food from a nutritional point of view; in this sense, the search for new protein sources, especially those of vegetable origin, has increased. As

shown in Table I, the crude protein content of both species was of the same order as that of other *Prosopis* flours (González-Barron et al., 2020; Rodriguez et al., 2019). Additionally, the crude protein contents of *P. alpataco* and *P. flexuosa* wholemeal flours were comparable to those reported for cereals (9-12%), but lower than those found in other seed legumes, such as pea and soybean (22%-40%). Table II displays the full aminoacids-AA profiles of the wholemeal flours, revealing the differences between the two species. In particular, the proline content of *P. alpataco* flour was 20 times higher than that of *P. flexuosa* flour. Proline is an imino acid produced in plants as an adaptive mechanism in response to water stress (Bhaskara et al., 2015). Therefore, the higher accumulation of proline in *P. alpataco* flour may be related to the drought tolerance of this species. Moreover, *P. flexuosa* presented higher values for all essential AA than *P. alpataco*, except for phenylalanine, which was 1.8 times lower (50.7 and 88.4 mg/g protein, respectively). In addition, the lysine content was relatively high, mainly in *P. flexuosa* (142 mg/g), almost twice as high as in *P. alpataco* (82.4 mg/g). In this sense, it could be said that these proteins have a complete AA profile, unlike cereals, which are particularly deficient in lysine (FAO, 2013). Additionally, in both wholemeal flours, the content of total sulfur AA (methionine and cysteine) was significantly higher than the value recommended by the FAO (23 mg/g), and even higher than that reported in other species of the same genus (Astudillo et al., 2000). Similarly, the tryptophan content of both wholemeal flours was significantly higher than the minimum requirement. Usually, plant proteins are deficient in this essential AA, which is a precursor of metabolites such as serotonin and nicotinamide. In this regard, *Prosopis* wholemeal flours represent a valuable nutritive ingredient because they have a complete AA profile, including those that are essential for human nutrition.

3.2. Phytochemical analysis

3.2.1. Total polyphenols and antioxidant activity

As shown in Table III, the TPC obtained from the wholemeal flour extracts was different according to the extraction solvent used; thus, methanol extracts had a statistically lower TPC ($P<0.05$) than the acetone extracts. The TPC values observed in these species were similar to those reported for methanol extracts of *P. nigra* (Pérez et al., 2014; 2018) and higher than the values obtained from the ethanolic extracts of *P. nigra* and *P. alba* pods (Cardozo et al., 2010). Furthermore, the antioxidant activity of the extracts was evaluated using $ABTS^{\bullet+}$, DPPH•, and total antioxidant activity assays, and it was found that the acetone extracts (AEA and AEF) exhibited significantly higher antioxidant activity than the methanol extracts (MEA and MEF). The SC50 by extracts derived from wholemeal flours was also assessed (Table III). Acetone extracts demonstrated the highest scavenging activity for both methods ($ABTS^{\bullet+}$ and DPPH•), with SC50 values ranging from 1.68 to 4.9 mg dw/ml. The antioxidant activity of the acetone extracts was lower than that of the aqueous extracts obtained from *P. alba* and *P. nigra* but higher than that of the ethanol extracts of these species (Cardozo et al., 2010). As such, these flours can be considered potential functional ingredients, as both the total phenolic content and antioxidant activity were significantly higher than those of whole wheat flour (2-fold and 10-fold, respectively) (Yu et al., 2013). Furthermore, when comparing the extraction solvents, the results indicated that the AEA and AEF exhibited significantly higher TPC and antioxidant activities than methanol extracts. Therefore, the acetone extracts were used for PC analysis.

3.2.2 Polyphenol Components-PC profile

A total of 10 non-anthocyanin PC were identified and quantified in the flours (Table IV, Supplementary data). Figure 1S shows the chromatograms of the samples with the identification of PC found in *Proposis* samples. To our knowledge, 3 compounds are reported herein for the first time from *Prosopis* genus, trans-piceid, ε-viniferin, (-)-epigallocatechin gallate. Additionally, all the non-anthocyanin compounds identified in *Prosopis* pod have

never been reported before, with the exception of quercetin-3-galactoside (Harzallah-Skhiri & Ben Jannet, 2005).

For both species, (-)-epigallocatechin and (-)-epigallocatechin gallate were the most abundant flavonoids. The combined percentage of these two compounds accounted for 59% and 38.5% of the non-anthocyanin PC present in AEA and AEF, respectively. The concentration of both compounds was approximately twice higher in AEA than in AEF. Catechins possess oxidative potential primarily related to the hydroxyl groups they contain. Additionally, the delocalization of electrons between the carbon rings is prevented by saturation of the heterocyclic ring (Legeay et al., 2015). Numerous studies have demonstrated that galloylated catechins exhibit superior scavenging effects than non-galloylated catechins. Among these, only (-)-epigallocatechin gallate has garnered significant attention in the field of medicinal chemistry because of its exceptional antioxidant properties (Higdon & Frei, 2003). However, these compounds have also been shown to exhibit a pro-oxidant effect under typical physiological conditions (pH 7.4, 37°C) owing to the auto-oxidation of (-)-epigallocatechin gallate, which generates substantial levels of ROS. The pro-oxidant effects of (-)-epigallocatechin gallate have been suggested as a potential mechanism underlying its anticancer properties. However, the dual nature of (-)-epigallocatechin gallate, as an antioxidant and pro-oxidant, is heavily influenced by dosage and biological environment.

Another important compound in terms of concentration identified in *Prosopis* flour was the stilbene *trans*-piceid, which accounts for 24% of the non-anthocyanin PC in AEF (Table IV). *Trans*-piceid is a trans-resveratrol glycoside, being its main form found in nature, which is noteworthy for its glycosylation at the least reactive positions, retaining the trans-resveratrol biological activity (Stojanoviç & Brede, 2002). It is also important to highlight the presence of another stilbene, ε-viniferin. This compound is a trans-resveratrol dimer which is also associated with antioxidant properties of different natural extracts such as grape pruning

residues (Ferreyra et al., 2022). The combination of resveratrol and ε-viniferin had hepatoprotective effect in rats with severe acute liver failure. This mixture exhibited a protective role in the antioxidant pathway, thus opening a new perspective on the utilization of this stilbene combination in functional diets (Fernandes et al., 2021).

Additionally, the PC profile of *Prosopis* in this study also presented some similarities to that of coffee, which also contains (-)-epigallocatechin gallate, quercetin, and quercetin glycosides (Król et al., 2020). However, *Prosopis* pods do not contain caffeine, making them a possible substitute for decaffeinated coffee. Other minor compounds were detected, such as procyanidin B1 and (+)-catechin. Procyanidin B1 has the most potent antioxidant and anti-inflammatory effects among the dimeric procyanidins (Chen et al., 2022) and can be commonly found in cinnamon, peach, and *Vitis vinifera* tissues. Catechins can regulate gene and protein expression in neurons and can serve as therapeutic drugs for aging and related diseases (Sharifi-Rad et al., 2021). Additionally, four anthocyanins were identified in AEF, but AEA exhibited a total anthocyanin content that was 13 times greater than that of AEF, with only cyanidin 3-*O*-*p*-coumaroyl glucoside being detected. Previous studies have suggested a correlation between anthocyanin content and darker pod color (Pérez et al., 2014). However, in this study, the lighter-colored pod (*P. alpataco*) contained the highest anthocyanin concentration, possibly because of the presence of only one type of anthocyanin, whereas the identified anthocyanins in *P. flexuosa* contributed differently to the final pod color. These anthocyanins have been described in other *Prosopis* species (Pérez et al., 2014; Schmeda-Hirschmann et al., 2015).

3.2.3. Inhibition of enzymes related to metabolic syndrome

Natural foods enriched with PC could help to treat metabolic syndrome and improve the health of the human population, as they can inhibit digestive enzymes, such as α-amylase, α-glucosidase, and pancreatic lipase (Isla et al., 2022). AEA was active against α-amylase and

α-glucosidase with IC50 values of 1.93 μg GAE/ml and 2.94 μg GAE/ml, respectively. AEF was only active against α-glucosidase, with an IC50 value of 3.26 μg GAE/ml. The IC50 values obtained were ten times lower than those observed in extracts of *P. nigra* (Pérez et al., 2018), suggesting that wholemeal flour, in particular from *P. alpataco*, could be a dietary supplement to control hyperglycemia in patients with diabetes. *Prosopis* wholemeal flour could be an alternative natural resource to reduce the risk of obesity. Inhibition of pancreatic lipase activity was evaluated, and the inhibitory capacity of AEA was superior to that of AEF, with IC50 values of 26 and 55 μg GAE/ml, respectively. The IC50 value of the reference inhibitor (orlistat) for the enzyme pancreatic lipase was 12.5 μg/ml. As can be observed, the results agreed with the higher content of PC found in the *P. alpataco* sample. In particular, several in *vivo* and *in vitro* studies have shown that the epigallocatechin-3-gallate present in green tea may have several health benefits. Although regular intake showed no effect on insulin resistance, there was a reduction in diastolic blood pressure (Brown et al., 2008). Besides individual effects, the potential synergic effect of PC should also be taken into account. Therefore, regular intake of *Prosopis* wholemeal may have different health-promoting effects in addition to a potential reduction in body weight and a decrease in metabolic syndrome risk factors such as inflammation, oxidative stress, and diabetes prevention, as was evidenced in this work by different *in vitro* studies.

3.2.4. ***Protective effect of PC against AAPH-induced oxidative stress in in vivo zebrafish embryo model***

The zebrafish model has several advantages for modern biotechnological research and is used as an *in vivo* model for bioactivity evaluation. In this study, the zebrafish model was used to measure the antioxidant activity of AEA and AEF against AAPH-induced oxidative stress. The presence and reduction of intracellular ROS were quantified by measuring green fluorescence. Figure 1(A) (a-d) shows the zebrafish embryos after the DFCH-DA assay. The

fluorescence intensity of the embryos treated with the extracts (AEA and AEF, 5.7 µg GAE/ml) showed significantly reduced levels of ROS generation (150 and 153% respectively) when compared to the intensity of the AAPH-treated embryo group (181 ± 14%) (Figure 1 (B)). Previous studies have also tested the ability of PC extracts to reduce ROS production in zebrafish embryos. Barberry (*Berberis microphylla*) and red algae (*Pyropia yezoensis*) extracts at concentrations of 5 to 100 µg/ml showed similar protective effects (Boeri et al., 2020, Dai et al., 2020). Therefore, the zebrafish model has enabled the evaluation of the antioxidant properties of wholemeal flour extracts derived from both *Prosopis* species.

4. Conclusion

The wholemeal flours of *P. alpataco* and *P. flexuosa* species have shown to be rich in proteins and have an adequate balance of essential AA. They have low lipid content and contain pleasant, sweet sugars, abundant fiber, and significant quantities of nutritious minerals. These attributes make it a possible choice as an ingredient for more health-conscious diets, demanded by society. The nutritional profile of the flours did not exhibit significant differences from that of other flours of the same genus. Furthermore, the qualitative phytochemical profiles of these species were similar to each other and distinct from those of *Prosopis* from other regions of the world. Several PC were reported herein for the first time from *Prosopis* genus (trans-piceid, ε-viniferin, (-)-epigallocatechin gallate) and the content of PC was associated with different *in vitro* and *in vivo* effects. The evaluated bioactive properties demonstrated that they could help to regulate hyperglycemia and oxidative stress, which are important factors in metabolic syndrome progression. In conclusion, wholemeal flours of *P. alpataco* and *P. flexuosa* could be used as functional ingredients to develop alternative for healthier foods, especially in underprivileged regions of the world that have these plants as a natural resource.

5. Supplementary data:

Chromatogram obtained for (a) Non-anthocyanin phenolic compounds and (b) Anthocyanin phenolic compounds quantified in this work.

6. Declaration of Competing Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

7. Acknowledgement

This work was supported by ANPCyT (Grant number: BID PICT 2020- 3243) and PI-UNRN 40-C-877, PIP-CONICET 2020–Nº 1252, to E.H. have a CONICET scholarship. A.F., R.M., C.P. D.B. and L.P. are fellows of CONICET. The authors would like to acknowledge Leal Claudia Adriana from the CCT CONICET CENPAT for conducting the mineral analysis, and Drago Silvina Rosa from the University National of Litoral for doing the AA analysis.

8. CRediT

Elizabeth Hoffmann: Investigation, Data Curation, Writing - original draft, Writing - review & editing. Patricia Boeri: Conceptualization, Resources, Writing - review & editing, Funding acquisition. Romina Monasterio: Investigation, Writing - review & editing. Ariel Fontana: Investigation, Writing - review & editing. Maria Cecilia Puppo: Conceptualization, Writing - review & editing, Resources, Funding acquisition. Daniel Alejandro Barrio: Conceptualization, Resources, Funding acquisition. Lucrecia Piñuel: Conceptualization, Resources, Writing - original draft, Writing - review & editing, Funding acquisition.

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Tables

Table I: Nutritional content of *Prosopis* flours derived from mature pods expressed in dw basis (mean ± SD). Results are expressed per 100 g of flour dw.

Nutritional content (g)			Minerals (mg)		
	P. alpataco	*P. flexuosa*		*P. alpataco*	*P. flexuosa*
Moisture	0.56 ± 0.19a	0.77 ± 0.00a	Ca	231 ± 14	248 ± 15
Crude protein	11.8 ± 0.7a	10.6 ± 0.2a	Mg	71.0 ± 3.6	84.5 ± 4.4
Lipid	3.84 ±0.08a	2.85 ± 0.09b	Zn	1.39 ± 0.00	1.42 ± 0.02
Ash	3.34 ± 0.00a	3.32 ± 0.04a	Fe	3.73 ± 0.18	5.90 ± 0.30
Fructose	3.99 ± 0.14b	2.87 ± 0.05a	As	<0.01	<0.01
Glucose	4.37 ± 0.33a	3.24 ± 0.15a	Cd	<0.01	<0.01
Sucrose	10.3 ± 0.6a	12.5 ± 0.4a	Cr	<0.01	<0.01
Trisaccharides	0.82 ± 0.06a	0.60 ± 0.06a	Pb	<0.01	<0.01
Total dietary Fiber	57.4 ± 1.9a	55.2 ± 0.8a	Cu	0.35 ± 0.09	0.32 ± 0.07
Energy (kJ)	752 ± 42	758 ± 23	Mn	0.70 ± 0.08	0.73 ± 0.08
Energy (kCal)	180 ± 10	181 ± 6			

Data expressed as average ± standard deviation (n = 3 analytical replicates); Different letters for each district in a row indicate statistically significant differences ($p<0.05$) between means.

653 Table II. AA profile of *P. alpataco* and *P. flexuosa* wholemeal flours (mg/g protein).

AA	*P. alpataco*	IA[a]	*P. flexuosa*	IA[a]	[b]FAO, 2013
Serine	59.5 ± 2.9		85.9 ± 1.4		
Histidine	20.0 ± 1.0	1.51	24.0 ±3.7	1.25	16
Glycine	51.0 ± 2.6		65.8 ± 2.5		
Threonine	46.3 ± 2.6	2.54	63.6 ± 1.9	1.85	25
Arginine	59.4 ± 3.0		67.3 ± 3.5		
Alanine	55.4 ± 0.5		64.3 ± 0.6		
Proline	246 ± 1		11.8 ± 0.6		
Tyrosine	43.9 ± 0.1		77.4 ± 2.9		
Valine	42.3 ± 0.5	1.40	55.8 ± 1.5	1.06	40
Methionine	36.9 ± 1.1		41.9 ± 1.0		
Cysteine	35.9 ± 0.6		54.6 ± 1.1		
Isoleucine	41.0 ± 0.8	1.68	50.6 ± 1.3	1.37	30
Tryptophan	46.2 ± 2.1	11.97	79.0 ± 0.9	7.00	6.6
Leucine	91.4 ± 0.8	2.22	136 ± 1	1.50	61
Phenylalanine	88.4 ± 0.8		50.7 ± 0.7		
Lysine	82.4 ± 2.5	2.96	142 ± 1	1.72	48
Phe + Tyr	132.3	3.12	128.1	3.23	41
Met + Cys	72.9	3.17	96.5	4.20	23
Asp + Glu	206 ± 11		169 ± 13		
Amino acid score:		100		100	

654 Data expressed as average ± standard deviation. [a] IA: AA index of essential AA. [b] FAO-
655 recommended AA scoring patterns for humans aged older than 3 years. The amino acid score
656 was calculated using the ratio of a gram of the limiting amino acid in the food to the same
657 amount of the corresponding amino acid in the reference diet multiplied by 100.

658 Table III: Total Polyphenol Compounds-TPC and antioxidant activity of extracts from *P.*

659 *alpataco* and *P. flexuosa* pod flours.

Measure	Method	MEA	MEF	AEA	AEF
Antioxidant activity‡	TPC†	0.32 ± 0.02c	0.42 ± 0.01a	0.45 ± 0.04ab	0.50 ± 0.05b
	ABTS•+	3.1 ± 0.3b	2.4 ± 0.4b	4.8 ± 0.5a	5.1 ± 0.8a
	DPPH•	1.1 ± 0.0b	1.5 ± 0.0b	3.8 ± 0.2a	4.0 ± 0.5a
	TAA	40 ± 0.2c	52 ± 1b	59 ± 7b	79 ± 5a
SC50 (mg extract dw/ml)	ABTS•+	4.66 ± 1.16ab	2.91± 0.7b	1.68 ± 0.25a	2.38 ± 0.32a
	DPPH•	11.07± 0.57	6.21± 0.47c	3.32 ± 0.35a	4.90 ± 0.25a

660 †TPC are expressed in g GAE/100 g flour. ‡ABTS•+ and DPPH• are expressed as mmoles of

661 Trolox equivalents/100 g flour. TAA are expressed as mmoles ascorbic acid equivalents/100 g

662 flour. SC50: Scavenger concentration 50%. Different letters in each row indicate statistically

663 significant differences ($p<0.05$) between means.

664 Table IV: Polyphenol compounds-PCs quantitative composition of AEA and AEF.

Compounds	AEA	AEF
Non-anthocyanin phenolic compounds (µg/100 g flour dw)		
Procyanidin B1	749 ± 17	1280 ± 4
(+)-catechin	509 ± 10	1350 ± 40
trans-piceid	3150 ± 40	8360 ± 179
ε-viniferin	1920 ± 50	2190 ± 10
Quercetin-3-galactoside	1860 ± 40	1160 ± 40
(-)-epigallocatechin	10900 ± 300	7720 ± 40
(-)-epigallocatechin gallate	6390 ± 10	5790 ± 70
Naringin	2320 ± 40	4580 ± 80
Myricetin	n.d.	860 ± 10
Quercetin	1520 ± 30	1680 ± 30
Total	29300	34970
Anthocyanin phenolic compounds (µg/100 g flour dw)		
Cyanidin 3-*O*-*p*-coumaroylglucoside	546 ± 3	7.3 ± 0.2
Petunidin 3-*O*-glucoside	n.d.	7.7 ± 0.4
Peonidin 3-*O*-glucoside	n.d.	7.5 ± 0.2
Malvidin 3-*O*-glucoside	n.d.	17.9 ± 0.8
Peonidin 3-*O*-*p* coumaroylglucoside	n.d.	7.3 ± 0.6
Malvidin 3-*O*-*p* coumaroylglucoside	n.d.	11.5 ± 0.8
Total	546	59.2

665 Data expressed as average ± standard deviation. n.d. Not detectable

666 **Figure Legend**

667

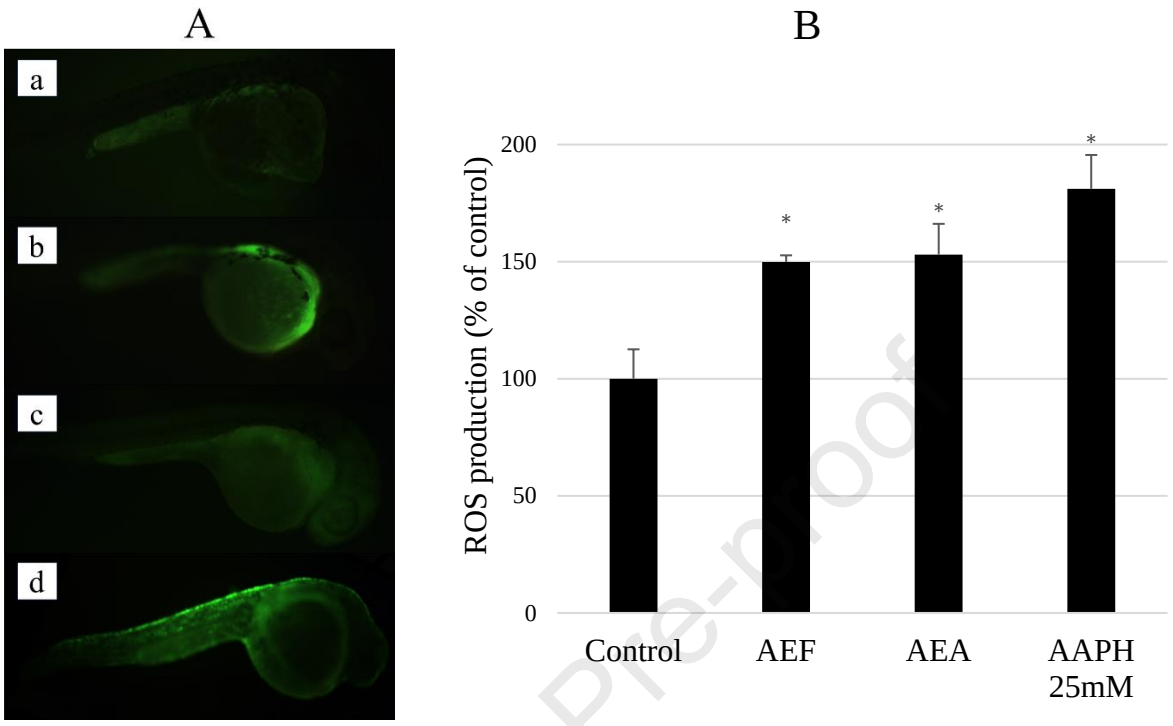
668 Figure 1: (A) Micrographs of reduction of ROS in zebrafish embryos. a) water (control without
669 AAPH). b) AEF 5.7 µg GAE/ml + 25 mM AAPH, c) AEA 5.7 µg GAE/ml + 25 mM AAPH,
670 d) AAPH 25 mM (positive control). (B) Protective effect of AEA and AEF on AAPH-treated
671 reactive oxygen species (ROS) production in zebrafish. ROS levels were measured by ImageJ.
672 Experiments were performed with 4 replicates. * Significantly different from the non-treated
673 control group ($P<0.01$).

674

675 Figure 1S: Chromatograms corresponding to *P. alpataco* and *P. flexuosa* pods extracts
676 obtained for (a) Non-anthocyanin PCs and (b) Anthocyanin PCs identified and quantified in
677 this work (Table IV). Pet 3-G: Petunidin 3-O-glucoside, Peo 3-G: Peonidin 3-O-glucoside,
678 Mal 3-G: Malvidin 3-O-glucoside, Cya 3-p cou: Cyanidin 3-O-p-coumaroylglucoside, Peo 3-
679 p cou: Peonidin 3-O-p-coumaroylglucoside and Mal 3-p cou: Malvidin 3-O-p-
680 coumaroylglucoside.

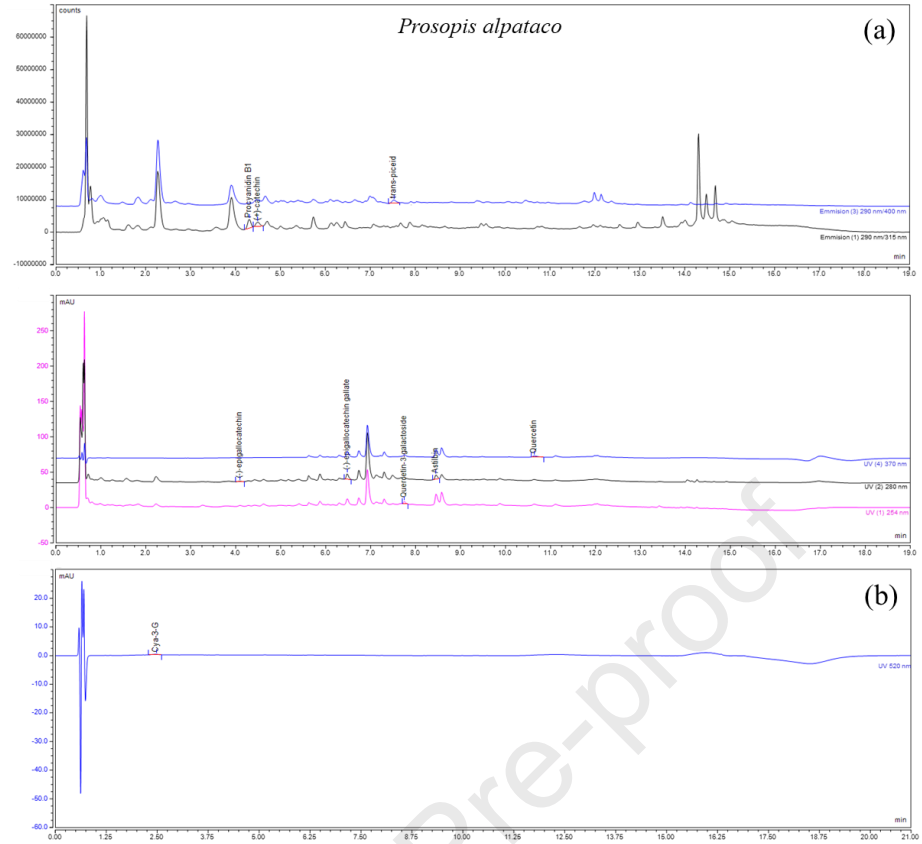
681 **Figures**

682



683

684 Figure 1



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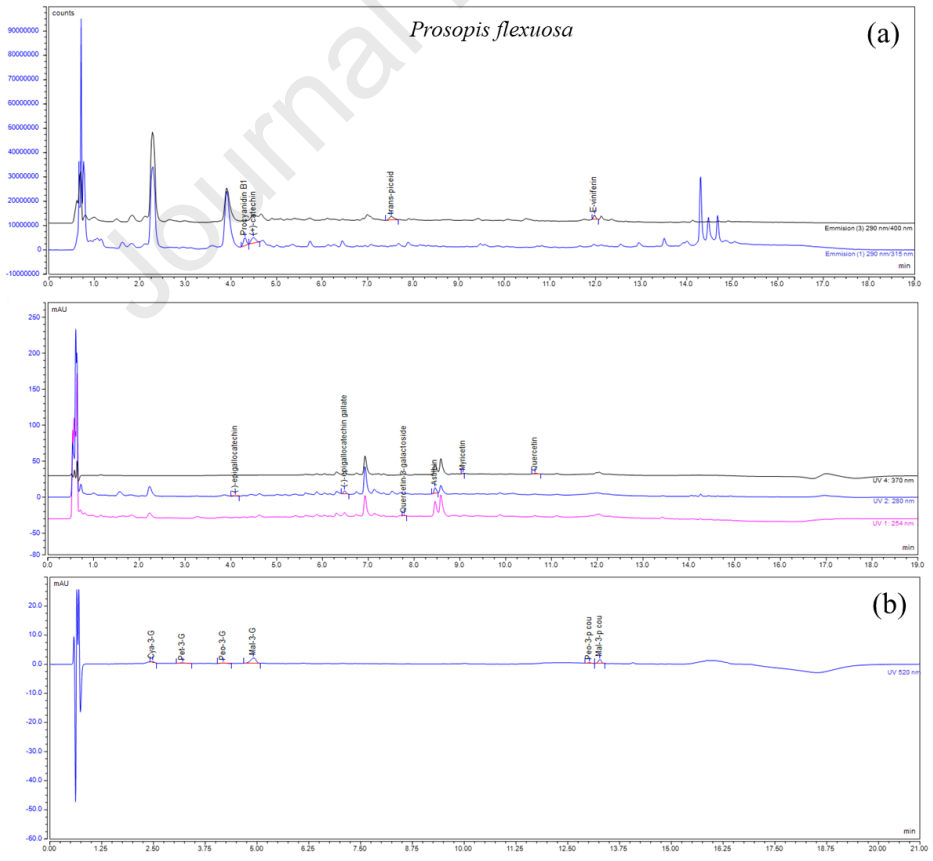

686

687 Figure 1S

1. Wholemeals from Patagonian Prosopis are potential ingredients for functional foods.

2. The pods of the species studied are rich in fiber and polyphenols.

3. (-)-epigallocatechin gallate, trans-picein and ε-viniferin - were first described in Prosopis.

4. Aminoacidic score 100, high mineral content, heavy metals undetected.

5. Extracts inhibit enzymes associated with metabolic syndrome.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: