Microencapsulated Chañar phenolics: a potential ingredient for functional foods development

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Abstract

In this work, a *Geoffroea decorticans* (chañar) extract very rich in polyphenols and with antioxidant and inhibitory activity against pro-inflammatory enzymes, was microencapsulated in a zein matrix using electrospraying to improve its stability and facilitate its handling as food ingredient. The electrosprayed microcapsules were characterized in terms of morphology, encapsulation efficiency, water sorption ability and release properties at different pH and during an *in-vitro* digestion process. The bioactivity of the encapsulated extract in terms of inhibitory activity on enzymes related to the metabolic syndrome were also evaluated and compared to that of the non-encapsulated ingredient. Results showed that microencapsulation did not affect biological activity and effectively protected the bioactive polyphenols during the simulated digestion process. Therefore, this encapsulation structures could be considered a potential ingredient for functional food development.

Keywords: *Geoffroea decorticans*; chañar; polyphenolic compounds; zein; microcapsulates; metabolic syndrome

1. Introduction

Geoffroea decorticans (Gill.ex Hook. et Arn.) Burk (Fabaceae), known as chañar, grows in the native forests of the Gran Chaco region in Argentina as well as in the Paraguayan and Bolivian Chaco and northern Chile (Scarpa, 2009). The ripe fruits (drupes) are still part of the diets of animals and aboriginal communities (Arena & Scarpa, 2007). The fruits, seeds and flour are used raw, roasted, boiled and/or fermented (beverage), in soups and in sweet jelly-like syrup called arrope. Chañar fruits flour and arrope can be used as functional foods, since apart from having high levels of sugar and fiber, they contain polyphenolic compounds with biological activities (Costamagna, et al., 2013; Reynoso, et al., 2013; Costmagna et al., 2016). The compounds indentified in phenolic extracts from chañar were caffeic acid glycosides, simple phenolics (protocatechuic acid and vanillic acid), a glycoside of vanillic acid, *p*-coumaric acid and its phenethyl ester as well as free and glycosylated flavonoids (Costamagna et al., 2016).

The metabolic syndrome (MetS) is an emergent pathology that affects around 25% of the world population. The reduction of postprandial hyperglycemia by inhibition of enzymes involved in carbohydrate or lipid metabolism (α -glucosidase and α -amylase or lipase, respectively), inhibition of oxidative stress and delay of inflammatory processes are the most common therapeutic approaches (Prasad, et al., 2012). The chañar polyphenols enriched extract with and without gastroduodenal (GD) digestion inhibited enzymes associated with metabolic syndrome, exhibited antioxidant activity and inhibited the pro-inflammatory enzymes (Costamagna et al., 2016). These findings add evidence that the extract may be considered as a multipurpose bioactive ingredient. However, phenolic compounds are unstable in solution and, thus, it is necessary to eliminate the solvents present in the extracts in order to stabilize them prior to their

incorporation in food products and facilitate their handling as food ingredients. Furthermore, polyphenols are highly susceptible to epimerization at high temperature and alkaline pH which also impact on the polyphenols activity (Kim et al., 2007). Additionally, polyphenols are very astringent and bitter in taste, making difficult their direct incorporation in food products or their use as nutraceuticals. Microencapsulation of polyphenols may help to overcome these problems. This process also facilitates the production of bioactives in powder form, allowing controlled and targeted release of the core material (Ezhilarasi et al., 2013) and it has demonstrated to prevent polyphenol degradation and epimerization under different pH conditions and even during simulated *in-vitro* digestion (Gomez-Mascaraque et al., 2015, 2016).

Microencapsulation through electrospraying has been explored in the last years as an alternative to well-established industrial encapsulation methods such as spray drying due to the ability of this technique to process the samples at room temperature and the possibility, after proper solution adjustment, of using food-grade solvents (Aceituno-Medina et al., 2013; Pérez-Masiá et al., 2014). An important aspect for the encapsulation matrix selection is the compatibility of the substance to be incorporated, not only with the matrix itself, but also with the solvent used in the process. Among the wall materials, proteins are ideal because they are generally regarded as safe (GRAS) and contain a high nutritional value (Chen et al., 2006). Most proteins can be easily digested by the human gastrointestinal tract (Joye & McClements, 2014). Both animal and plant proteins have been used as encapsulating matrices (Jia, Dumont, & Orsat, (2016). Plant-derived proteins offer some advantages over animal proteins for forming biopolymer particles since the risk of contamination and infection is reduced, they are generally cheaper, and they can be used in vegetarian or vegan products (Joye & McClements, 2014). Zein, a major corn protein, unlike most natural materials, has an

amphiphilic molecular structure where over 50% of the amino acids are hydrophobic. For this reason, it is insoluble in water but readily disperses in ethanol–water mixtures (Dong et al., 2016). Zein is well applied in food and pharmaceutical industries because of its good biocompatibility and bioavailability (Dong et al., 2016). This protein can be processed into capsules through electrospraying from ethanolic aqueous solutions, having the materials obtained by this means good thermal and stability properties (Torres-Giner et al., 2008). Therefore, this food-grade renewable material constitutes a good option for the microencapsulation of ethanol-soluble polyphenolic.

The main objective of this study was to evaluate whether microencapsulation of a chañar polyphenolic extract within a zein matrix affected its functional properties, not only as a food ingredient, but also after an *in-vitro* digestion process. Therefore, chañar extract-containing electrosprayed zein capsules were obtained and characterized in terms of morphology, encapsulation efficiency, water sorption ability and release properties, in aqueous and non-aqueous environment and during an *in-vitro* digestion process. The bioactivity of the encapsulated extract in terms of inhibitory activity on enzymes related to the metabolic syndrome and pro-inflammatory enzymes were also evaluated and compared to that of the non-encapsulated ingredient.

2. Material and Methods

2.1. Plant material

Fruits of *Geoffroea decorticans* were collected in January of each year from 2011 to 2014 in Departamento Fernández, Provincia de Santiago del Estero, Argentina. The fruits were air-dried and ground to powder (Helix mill, Metvisa). Then, the *Chañar* flour was passed through 4 mm mesh sieves.

2.2. Materials

Zein from corn (grade Z3625, 22-24 kDa), buffer solutions of pH 7.4 (phosphate buffered saline system, PBS) and pH 6.1 (2-(N-morpholino) ethanesulfonic acid hemisodium salt, MES), and potassium bromide FTIR grade (KBr) were obtained from Sigma-Aldrich (Madrid, Spain). Ethanol of 96% v/v purity was purchased from Panreac (Barcelona, Spain). Pepsin from porcine gastric mucosa, bile extract porcine and pancreatin from porcine pancreas were all obtained from Sigma-Aldrich (Madrid, Spain). Pefabloc® was purchased from Fluka-Sigma-Aldrich. All products were used as received without further purification.

2.3- Polyphenols enriched extract preparation

The flour sample (630 g) was extracted four times with ethanol:water 70:30 (1:2, w/v) in an ultrasonic bath for 30 min at 25°C. Then, all extractions were joined and concentrated under reduced pressure and then, lyophilized. The lyophilized extract was suspended in distilled water (acidified with HCl to pH 2) and mixed with Amberlite-XAD 7 resin (500 g) for 1 hour under stirring. The resin was filtered through a Büchner funnel, washed three times with the acidified water and the polyphenols bound to the resin were desorbed with ethanol. The phenolic-enriched ethanol solution was concentrated under vacuum to afford a dry crude ethanol extract that was stored at -20°C until use.

2.4- Development of electrosprayed zein microcapsules containing polyphenolic compounds from chañar flour

Zein solutions with a concentration of 12% (w/v) were prepared by dissolving the protein in ethanol 85% (v/v) at room temperature under magnetic agitation. The chañar extract was added to the previous solutions in a proportion of 10% w/w of the total solids content and the mixtures were stirred until complete solution.

The final solutions were electrosprayed using a homemade electrospraying apparatus at a steady flow-rate of 0.15 mL/h, an applied voltage of 13 kV and a syringe tip-collector distance of 10 cm. Processed samples were collected on a stainless-steel plate.

2.5. Analysis of microcapsules

2.5.1 Morphological analysis of the microcapsules

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 7-10 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination.

2.5.2 Fourier transform infrared (FT-IR) analysis

Samples (ca. 1-2 mg) of free and encapsulated polyphenolic extract were grounded and dispersed in about 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the samples at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm⁻¹ resolution.

2.5.3 Water uptake capacity of the microcapsules

The water-uptake capacity of the capsules at high relative humidity (RH) was assessed. For this purpose, the samples (ca. 25 mg) were previously dried by storing them in a desiccator at 0% RH for 48 h and the mass of dried capsules (m_0) was determined. The samples were then stored in another desiccator at 75% RH and weighted after different time intervals. The water uptake at 75% RH (WU) was calculated using Eq. (1), where m_t is the mass of the capsules at time t after being stored at 75% RH. Experiments were performed in independent triplicates.

$$WU_t (\%) = \frac{m_t - m_0}{m_0} \times 100 \quad \text{Eq. (1)}$$

2.5.4 *In-vitro* release of the chañar polyphenolic compounds

The *in-vitro* release of the chañar polyphenolic compounds from the zein microparticles was assessed following a method adapted from (Gómez-Mascaraque et al., 2015). Briefly, the capsules (5 mg/mL) were suspended in two different release media and stored at 4°C. Two different release media were used: MES aqueous buffer (pH=6.1) and PBS aqueous buffer adjusted to pH=4.1 with phosphoric acid, simulating fruit juices. At different time intervals the dispersions were centrifuged at 4200 rpm and 4 °C during 5 min using a centrifuge 5804 R from (Hamburg, Germany), and 0.15 mL aliquot of the supernatant was removed for sample analysis. The aliquot volume was then replaced with fresh release buffer. The extracted aliquots were analyzed by HPLC–DAD and the major compounds were identified and quantified.

Experiments were performed in independent triplicates.

2.6. Microencapsulation efficiency

Three different solvents were used for the extraction of phenolic compounds from microcapsules. On one hand, 1 g of the microcapsules was weighed into a screw-caped vial and extracted with distilled water for 5 min by ultrasonic treatment. Subsequently, it was centrifuged at 14,000 rpm and the supernatant was freeze-dried to obtain the aqueous dry extract. On the other hand, the same amount of sample was extracted with ethanol or ethyl acetate by ultrasonic treatment during 5 min at room temperature. Subsequently, it was centrifuged at 14,000 rpm and the supernatant was evaporated to obtain a dry extract. The phenolic content was determined as described by Singleton et al., (1999) using Folin-Ciocalteau reagent. The results were expressed as mg gallic acid equivalents (GAE)/ g microcapsules.

The microencapsulation efficiency, E, was then calculated using Eq. (2) based on dry matter content:

$$E = [\text{Phenolics eluted from microcapsule} / \text{Theoretical phenolics content of microcapsule}] \times 100$$

2.7. Release study of the encapsulates during simulated *in-vitro* digestion

In-vitro GD digestion and release of phenolic compounds from the microcapsules was determined using the method reported by Tenore et al. (2015) with minor modifications. Briefly: for the salivary digestion, the microcapsules (50 mg) or the non-encapsulated extract (5 mg GAE) were mixed with 6 mL of saliva at pH 6.8 (KCl [89.6 g/L], KSCN [20 g/L], NaH₂PO₄ [88.8 g/L], Na₂SO₄ [57.0 g/L], NaCl [175.3 g/L], NaHCO₃ [84.7 g/L], urea [25.0 g/L] and α -amylase [48.3 mg/mL]). The mixture was incubated for 3 min at 37 °C. Then, for the gastric digestion, pepsin (14,800 U) dissolved in HCl 0.1 M was added, the pH was previously adjusted to 2 and the mixture was incubated at 37 °C during 2 h. Finally, for the pancreatic (duodenal) digestion, the pH was adjusted to 6.5 with NaHCO₃ (0.5 M). Then, pancreatin (8 mg/mL) and bile salts (50 mg/mL) (1:1, v/v) dissolved in water (20 mL) were added and the mixture was incubated at 37 °C for another 2 h.

At the end of the *in-vitro* digestion processes, polyphenols were extracted with ethyl acetate and the organic phase was dried and re-suspended in dimethyl sulfoxide (DMSO).

2.8. Bioactivity assays

2.8.1 Inhibitory activity of enzymes related to metabolic syndrome

The inhibition of α -glucosidase, α -amylase and lipase was determined according to Costamagna et al., (2016). The reaction mixture contained chañar polyphenolic extract or polyphenols extracted from microcapsules, before or after the *in vitro* GD digestion (0.5- 60 μ g GAE/mL released from 0.08-9.6 mg microcapsules). In all cases, the IC₅₀ values denote the μ g GAE/mL required to inhibit the enzyme by 50%.

2.8.2. Inhibition of pro-inflammatory enzymes

The polyphenolic concentration causing 50% inhibition of pro-inflammatory enzymes (IC_{50}) was calculated from the concentration–inhibition response curve by regression analysis.

Cyclooxygenase

The ability of the chañar polyphenolic extracts and polyphenols released from microcapsules to inhibit the conversion of arachidonic acid to prostaglandin (PG) by human recombinant cyclooxygenase-2 (COX-2) was determined by enzyme immune assay, EIA (Kit no.560131; Cayman Chemical, MI, USA) (D’Almeida, et al., 2013). The assays were performed in the presence of chañar polyphenolic extract or polyphenols extracted from microcapsules, before or after *in vitro* GD digestion (25 μ g GAE/mL released from 4 mg microcapsules) or a commercial anti-inflammatory drug selective for COX-2 (nimesulide).

Lipoxygenase

Lipoxygenase (LOX) activity was determined using a spectrophotometric method based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide (Costamagna et al., 2016). Different concentrations of chañar polyphenolic extract or polyphenols extracted from microcapsules, before or after *in vitro* GD digestion (25 μ g GAE/mL released from 4 mg microcapsules) were used. Naproxen was used as a reference anti-inflammatory compound.

Phospholipase A₂

Secretory phospholipase A₂ (sPLA₂) activity was determined using 1,2-diheptanoylthio-glycerophosphocholine (1,2 dHGPC) and Triton X-100 as substrates (D’Almeida, et al., 2013). Polyphenols (25 μ g GAE/mL) released from 4 mg microcapsules were used. Acetylsalicylic acid was used as positive control.

2.8.3 Antioxidant activity

ABTS radical scavenging

The assay was carried out by the improved ABTS^{•+} method as described by Re et al., (1999). ABTS^{•+} solution (1mL; absorbance of 0.7 ± 0.02 at 734 nm) was added to the chañar phenolic extract or microcapsules, before or after GD digestion (0.17 to 11.36 µg GAE/mL released from 0.02-1.82 mg microcapsules) and mixed thoroughly. Absorbance was recorded at 734 nm after 6 min. The percentage of inhibition was calculated. Results are presented as SC₅₀ values in µg GAE/mL required to scavenge 50% ABTS free radicals.

Hydroxyl radical scavenging

The reaction mixture contained 50 µL of 10.4 mM 2-deoxy-D-ribose, 100 µL of 50 µM FeCl₃ and chañar polyphenolic extract or microcapsules before and after *in vitro* GD digestion (0.30-17 µg GAE/mL released from 0.048-2.73 mg microcapsules). The reaction was carried out with and without 100 µL of 52 µM EDTA. To start the Fenton reaction, 50 µL of 10 mM H₂O₂ and 50 µL of 1.0 mM ascorbic acid were added. The mixture was incubated at 37°C for 60 min. Then, 500 µL of 2-thiobarbituric acid (1%, w/v) dissolved in trichloroacetic acid (3%, w/v) was added. The tubes were heated at 100°C for 20 min. The absorbance was measured at 532 nm. The positive control was the H₂O₂/Fe³⁺/ascorbic acid system mixture lacking the chañar polyphenolic extract or polyphenols extracted from microcapsules (Zampini et al., 2008). The negative control contained the full reaction mixture without 2-deoxy-D-ribose. A modification was carried out without the addition of ascorbic acid. Results are presented as SC₅₀ values in µg GAE/mL required to inhibit by 50% the degradation of 2-deoxy-D-ribose.

Hydrogen peroxide scavenging

A solution of hydrogen peroxide (4 mM) was prepared in PBS, pH 7.4. Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230

nm using the molar absorptivity $81 \text{ M}^{-1}.\text{cm}^{-1}$. Samples (0.73-25 μg GAE/mL of polyphenols released from 0.097-4 mg microcapsules) were added to the hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing polyphenolic extracts without hydrogen peroxide (Zampini et al., 2008). Results are presented as SC_{50} values in μg GAE/mL required to inhibit by 50% the degradation of H_2O_2 .

2.9. Identification of phenolic compounds

The phenolic compounds extracted with ethyl acetate from microcapsules before and after *in-vitro* GD digestion and the compounds released in aqueous systems at two pHs during different times were analyzed by HPLC coupled to a diode array detector and compared with the ethanolic extract obtained from non-encapsulate chañar flour. The HPLC system used for DAD analysis was a Waters equipment (Water Corporation, Milford, Massachusetts) consisting of a binary pump 1525, a UV diode array detector 2998, Water X-bridge C18 column ($150 \times 4.6 \text{ mm i.d.}; 4.6 \mu\text{m}$). The HPLC-DAD analyses were performed using a linear gradient solvent system consisting of 9% acetic acid in water (A) and methanol (B) as follow: 90% A to 43% A over 45 min, followed by 43% A to 0% A from 45 to 60 min, and finally 0% A from 60 to 65 min. The flow rate was 0.5 mL/min and the volume injected was 20 μL . The detection was in UV at 254, 280 and 330 nm. The Empower 2TM software was used. The compounds were identified by retention time, UV spectra (from 200 to 600 nm), and by co-injection with standards as determined in Costamagna et al. (2016). The recovery efficiency was estimated as the ratio of the mean peak area to the peak area of standard. A calibration curve was prepared using commercial standards of caffeic acid, protocatechuic acid, vanillic acid, *p*-coumaric acid, and isorhamnetin to determine the relationship between

the peak area and concentration. The concentration was expressed as μg phenolic acid/g phenolic compounds. Three replicates were performed for all samples.

2.10. Statistical analysis

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

3. Result and discussion

Currently, polyphenols are of great significance not only for functional food development, but also in the nutraceutical and pharmaceutical industries. Previous studies have shown that microencapsulation technologies can lead to improve stability and bioavailability of polyphenols both *in-vivo* and *in-vitro*, (Jia, Dumont, & Orsat, 2016). However, it is unclear whether the bioactivity of these microencapsulated compounds, especially when used as extracts containing a combination of molecules which can interact with the encapsulation matrices, is compromised. Initially, the chañar polyphenolic extract was microencapsulated in zein and the microparticles developed were characterized. Later on, the bioactivity of the microencapsulated polyphenol-enriched chañar extract (M-PEE) was analyzed and compared to that of the non-encapsulated polyphenol-enriched chañar extract (NE-PEE).

3.1 Morphological characterization of the microcapsules

Figure 1 shows the morphology of zein-encapsulated chañar extract obtained using electrospraying. The electrospraying technique has previously shown to yield smaller particles than other common encapsulation techniques used in the food industry such as

spray-drying despite requiring higher polymer concentrations for processing. Zein microcapsules exhibited shrunken-like structures, attributed to the high volatility of ethanol, which causes a rapid evaporation of the solvent in the zein solutions, giving rise to more collapsed structures (Torres-Giner et al., 2008).

INSERT FIGURE 1 ABOUT HERE

3.2 Fourier transform infrared (FT-IR) analysis

FT-IR spectroscopy was used to compare the molecular organization of the chañar extract-loaded microcapsules with the unloaded microencapsulation structures, produced using the same protocol in absence of chañar extract. The obtained spectra, together with that of the free extract, are shown in Figure 2.

INSERT FIGURE 2 ABOUT HERE

The spectra of unloaded encapsulation structures showed the characteristic bands of proteins, centred at 3311 cm^{-1} (Amide A), 3065 cm^{-1} (Amide B), 1657 cm^{-1} (Amide I), 1541 cm^{-1} (Amide II) and 1241 cm^{-1} (Amide III). The spectrum of the extract-loaded microcapsules was very similar to that of its unloaded counterpart, given that the proteins account for the 90% of the total solids content in the microcapsules. However, small spectral variations were observed upon addition of the extract. For instance, a higher relative absorbance was observed in the bands between $3015\text{--}2850\text{ cm}^{-1}$ and $1185\text{--}1020\text{ cm}^{-1}$ when zein was loaded with the extract (insets in Figure 2). Moreover, the Amide A band of the proteins narrowed when the extract was incorporated within the structures (arrow in Figure 2), which is usually related to changes in the hydrogen

bonding structure of the proteins (Doyle et al., 1975). These changes might be attributed to potential intermolecular interactions taking place between the proteins and the extract, via the hydroxyl groups of the polyphenolic compounds.

3.3 Water uptake capacity of the microcapsules

A low water uptake capacity (WUC) of microencapsulates is desired to ensure storage stability. Hence, the WUC of the samples at 75% RH was calculated according to Eq. (1). The equilibrium was reached after only 3 hours of storage under the selected conditions, both for the loaded and unloaded microcapsules, and the WUC after that period was $7\% \pm 1\%$ in both cases. Moisture contents in the range of 3-10% are considered to be adequate for dry foods to have a good stability during shelf life (Klaypradit & Huang 2008). As no significant differences were observed between the water sorption capacities of the loaded and unloaded microparticles, the low values obtained were attributed mainly to the water uptake capacity of the protein. Similar moisture contents had been previously obtained for spray-dried zein microcapsules (Xue, Li et al. 2013). The limited water sorption observed for the zein microparticles suggested that this protein provided a good moisture barrier for the chañar polyphenolic extract. In fact, alcohol-soluble proteins such as zein have been described to exhibit lower water vapor permeability values than other proteins (Wang & Padua, 2004) and, thus, it constitutes an attractive food-grade matrix for protection in high moisture applications.

3.4 Microencapsulation efficiency and stability of phenolic compounds from chañar flour

An important application of encapsulation is related to controlling the release of compounds along the gastrointestinal tract. As foods are mostly absorbed in the intestines, it is desirable that the encapsulation system prevents an early release of encapsulated compounds (Chen et al., 2006). The behaviour of the capsules in a simulated GD medium depends on the microcapsule matrix composition, its resistance or susceptibility to digestive enzymes as well as on the GD conditions like pH (Saura-Calixto, Serrano, & Goñi, 2007; Siracusa et al., 2011). Hydrophobic proteins, such as zein, have been suggested to be good candidates as encapsulation matrices for polyphenols (Jia, Dumont, & Orsat, 2016). In order to determine the encapsulation efficiency, the polyphenols from chañar were released from zein microcapsules by different extraction techniques, i.e. by ultrasonic-assisted ethanolic and aqueous extraction and with ethyl-acetate before and after simulated *in-vitro* GD digestion of microcapsules. The release of polyphenols from zein particles was between 39.18 and 98.45% depending on the solvent used (Table 1). The release of polyphenols can occur by a diffusion process through the polymeric materials (using water as solvent) or by solubilization of the wall material (using ethanol as solvent). No significant differences were observed between the phenolic content released from microcapsules before and after simulated GD digestion (cf. Table 1), suggesting that the microcapsules effectively protected the polyphenolic compounds during the *in-vitro* digestion process.

INSERT TABLE 1 ABOUT HERE

HPLC-DAD was used to analyze the individual phenolic compounds from the polyphenols-enriched extract and polyphenols released from the microcapsules, to ascertain whether encapsulation affected their stability. The chromatography profile

showed five main phenolic compounds (caffeic acid hexoside; protocatechuic acid, vanillic acid, *p*-coumaric acid, Isorhamnetin/Rh hexoside) both in the digested and non-digested M-PEE and in the NE-PEE, but the recovered phenolic acid percentage was between 16 to 40% and 28 to 94 % in non-digested and digested microcapsules , respectively (Table 2, Figure 3). The greater recovery after *in vitro* GD digestion may be explained by the disruption of non covalent interactions between aminoacids from zein and the phenolic compounds of the encapsulated extract during the digestion process.

INSERT TABLE 2 ABOUT HERE

INSERT FIGURE 3 ABOUT HERE

The *in-vitro* release profiles of total phenolic compounds from loaded zein microcapsules in buffer system, was evaluated at two different pH values (Figure 4) and then determined by Folin Ciocalteu method. After an initial burst release in the solution with pH 4.1 (80%), the remaining polyphenols diffused to the solvent solution in a more sustained way. In contrast, a more sustained polyphenol release at pH 6.1 was observed during the first hours. Probably, this behavior may be ascribed to different non- covalent interactions between phenolic acids and the zein matrix affected by the pH of the release solution (electrostatic, hydrogen bond and hydrophobic interactions). With the increase in pH values of the buffer solution, the enhanced electronegativity of carboxyl group of phenolic acid would increase the electrostatic and hydrogen bond interactions (Zheng et al., 2014) and more energy will be needed to break the hydration shells when each pair of (phenolic acids) anions approaches each other.

INSERT FIGURE 4 ABOUT HERE

Interestingly, the quantities of each released phenolic acid were different at both pH conditions (Table 3). Vanillic acid, *p*-coumaric acids and isorhamnetin/rhamnetin hexoside were released in the same relation at pH 4.1 and 6.1, while protocatechuic acid was only released at pH 4.1 and caffeic acid hexoside was not released at pH 4.1 nor at 6.1. Thus, the release of phenolic compounds seems to be influenced by pH.

INSERT TABLE 3 ABOUT HERE

3.5 Antioxidant activities of microencapsulated phenolic compounds of chañar extract before and after simulated *in-vitro* digestion

Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide. Superoxide can be produced from molecular oxygen and rapidly dismutates into H_2O_2 , either spontaneously or enzymatically and the, H_2O_2 can be converted into extremely strong hydroxyl radicals that attack sensitive cellular targets, like lipids, proteins, and nucleic acids, causing their degradation. Oxidation of phospholipids and fatty acids produces reactive lipid peroxides, which in turn initiate chain reactions of lipid peroxidation in cellular membranes. Growing evidence suggests that the ROS, including free radicals generated during cellular metabolism or oxidation of lipids and proteins, play a causative role in the pathogenesis of cancer and coronary heart disease. In a previous report it was demonstrated that, the chañar polyphenolic extract was active on ABTS, H_2O_2 and $HO^\bullet$ (Costamagna et al., 2016). In this study, the antioxidant capacity of the phenolic compounds recovered from microcapsules (M-PEE) before and after simulated *in-vitro* GD digestion in comparison with NE-PEE, before and after simulated *in-vitro* GD

digestion was evaluated. The polyphenols released from microcapsules (before and after digestion) showed electron or hydrogen donor capacity on ABTS with SC₅₀ values similar to chañar polyphenolic extract non-encapsulated (Costamagna et al., 2016). These results indicate that the microcapsules were able to maintain its antioxidant activity (Table 4). However, the activity against ABTS decreased almost three times after digestion probably because some metabolites responsible for this activity could undergo transformations during digestion. The same behavior was demonstrated in microcapsules for H₂O₂ scavenger while hydroxyl radical scavenging activity was higher after digestion.

INSERT TABLE 4 ABOUT HERE

3.6. Effect of microencapsulated phenolic compounds after simulated *in-vitro* digestion on pro-inflammatory enzymes and metabolic syndrome-associated enzymes

Costamagna et al. (2016) previously reported, the activity of chañar flour polyphenolic extract before and after simulated *in-vitro* digestion towards enzymes associated with metabolic syndrome (α -glucosidase and α -amylase, pancreatic lipase and hydroxyl methyl glutaryl CoA reductase) and on pro-inflammatory enzymes (COX-2, LOX and sPLA₂). The aim of this work was to evaluate whether the microencapsulation process affect the extract bioactivity. From Table 5, it can be observed that the microcapsules containing polyphenolic compounds from chañar flour (M-PEE) were not active on the enzymes associated with the metabolic syndrome, however after simulated *in-vitro* digestion, the polyphenolic compounds released were active on the three enzymes assayed (α -glucosidase, α -amylase and lipase). However, the encapsulated dry extract

maintained the activity on lipase and amylase and decreased its activity on α -glucosidase.

The microcapsules were more effective to inhibit COX-2 than LOX and sPLA₂ enzymes. The microencapsulation process did not affect the extract inhibitory activity on COX-2 enzyme. A light decrease in the inhibitory effect was observed for LOX enzyme in the microcapsules, however the M-PEE after *in vitro* GD digestion showed almost a 50% inhibition at the polyphenolic concentration of 25 μ g GAE/mL. LOX and COX-2 inhibitors are the main drugs of current therapy aimed to modulate inflammation, pain, and to control fever and have a therapeutic potential in a variety of inflammatory and allergic diseases. These findings, together with the antioxidant activity observed in the polyphenols released from microcapsules suggest that its consumption may contribute to the reduction of inflammatory mediators that lead to many diseases, such as rheumatoid arthritis, inflammatory bowel disease, asthma, cancer and atherosclerosis and also could prevent or ameliorate oxidative stress related diseases.

Conclusions

The zein particles were able to protect entrapped chañar bioactive polyphenols and could be added to different food matrixes.

Results showed that microencapsulation did not affect biological activity and effectively protected the bioactive polyphenols during the simulated digestion process. Therefore, this encapsulation structures could be considered a potential ingredient for functional food development. The importance of this work is that microcapsules even after simulated GD digestion retain their beneficial properties for human health like antioxidant, anti-inflammatory activities and also present inhibitory activity on metabolic syndrome enzymes.

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Figure 1. Scanning electron microscopy images of chañar extract-loaded zein microparticles at two different magnifications. Scale bars: A) 2 μm and B) 5 μm .

Figure 2. FT-IR spectra of the chañar extract, together with unloaded and extract-loaded zein microencapsulation structures.

Figure 3. Chromatographic profile at 300 nm of phenolic compounds enriched extract (NE-PEE) obtained from chañar flour (A), phenolic compounds released from zein microcapsules loaded with the chañar extract (M-PEE) after (B) and before *in vitro* GD digestion (C). Peak (1) Caffeic acid hexoside; (2) protocatechuic acid, (3) vanillic acid and (4) p-coumaric acid.

Figure 4. Polyphenolic compounds released from zein microcapsules containing chañar extract at different times at (■) pH 4.1 and (♦) pH 6.1

Table 1. Phenolic compounds released from zein microcapsules containing 10% chañar extract (0.1 g PEE/g microcapsule; 7.12 mg GAE/g microcapsules) using different solvents (aqueous and organic) before or after GD digestion

Extract	Total polyphenols released	Polyphenolic recovery
	from microcapsules	%
	(mg GAE/g microcapsule)	
Ethanol extract	6.18±0.21 ^a	86.79±2.70 ^b
Aqueous extract	2.79±0.11 ^b	39.18±3.00 ^a
Ethyl acetate extract before GD digestion	6.22±0.2 ^a	99±1.20 ^b
Ethyl acetate extract after GD digestion	7.01 ±0.31 ^a	98.45±0.20 ^b

PEE: polyphenol-enriched extract; GD: gastroduodenal. Values are mean ± SD of three determinations. Different letters within each line represent significance difference ($p < 0.05$). 0.1 g of DE (dry extract) contain 7.12 mg GAE (gallic acid equivalents)

Table 2. Quantification of phenolic acids extracted from microcapsules submitted or no to GD digestion.

Peak	Compounds	*Phenolic acid extracted from microcapsules after GD digestion	Recovery (%)	*Phenolic acid extracted from microcapsules before GD digestion	Recovery (%)	*Phenolic acid content in the PEE
1	Cafeic acid hexoside	14.08±1.00	93.9±1.00	5.81±0.20	38.76±100	14.99±1.00
2	Protocatechuic acid	14.82±1.40	85.96±8.20	6.92±0.62	40.00±2.00	17.24±1.74
3	Vanillic acid	8.06±0.45	59.90±2.70	4.33±1.15	32.20±2.22	13.44±9.40
4	Coumaric acid	37.45±2.47	52.47±5.58	20.44±1.98	28.64±2.66	71.33±5.50
5	Isorhamnetin/rhamnetin hexoside	9.34±1	28.44±1	5.34±1	16.26±1	32.84±2

PEE: polyphenol-enriched extract

*Results expressed as µg phenolic acid/mg of total phenolic compounds.

The polyphenol extraction from microcapsules was carried out with ethyl acetate and the quantification was done by HPLC-DAD

Table 3. Quantification of phenolic acids released from microcapsules at two different pH values

Compounds	pH 4.1			pH 6.1		
	Release time (h)					
	1	4	8	1	4	8
Caffeic acid hexoside	0	0	0	0	0	0
Protocatechuic acid	0	1.84±0.15	1.38±0.04	0	0	0
Vanillic acid	3.47±0.16	5.48±0.18	5.40±0.17	2.54±0.12	4.70±0.44	5.00±0.88
<i>p</i> - coumaric acid	6.31±0.98	10.45±5.87	17.27±5.47	18.47±8.54	23.76±9.99	28.74±9.57
Isorhamnetin/rhamnetin hexoside	0.62±0.10	1.08±0.10	1.00±0.2	1.24±0.20	1.19±0.20	1.20±0.20

*The extraction was realized with buffers at two pHs during different times. Results are expressed in µg phenolic acid/mg of total phenolic compounds

Table 4. Antioxidant activity of phenolic compounds from the extract and released from microcapsules measured by different methods

Sample	Extraction	ABTS	H ₂ O ₂	OH•
		SC ₅₀ (μg GAE/mL)		
Microcapsules with 10% chañar PEE (M-PEE)	before GD digestion	3.5±0.2 ^a	3.7±0.3 ^a	3±0.2 ^b
	after GD digestion	9±3 ^b	6±1 ^a	1±0.1 ^a
Non-encapsulated PEE (NE-PEE)	before GD digestion	2.8±0.1 ^a	23±3 ^b	0.3±0.01 ^a
	after GD digestion	3±0.3 ^a	20±1 ^b	2.1±0.1 ^b

GD: gastroduodenal; PEE: polyphenol-enriched extract

Values are mean ± SD of three determinations. Different letters within each column represent significant difference ($p < 0.05$).

Table 5. Effect of phenolic compounds released from microcapsules before and after GD digestion on pro-inflammatory and metabolic syndrome enzymes.

Sample	Extraction	α -amylase	α -glucosidase	lipase	COX-2	LOX	sPLA2
		IC ₅₀ (μg GAE/mL)			Inhibition* (%)		
Microcapsules with 10% chañar	before GD digestion	--	--	--	99.42±5.4 ^a	33.7±3.9 ^a	--
PEE (M-PEE)	after GD digestion	28±3 ^a	5.5±0.5 ^b	5.5±0.5 ^a	97.75±5.2 ^a	47±4.6 ^b	--
Dry extract from chañar	before GD digestion	25±1 ^a	0.68±0.05 ^a	4.0±0.2 ^a	93.71±4.5 ^a	53±5.8 ^b	3.2±1.9 ^a
(NE-PEE)	after GD digestion	58±5 ^b	0.8±0.3 ^a	4.5±0.7 ^a	99.9±6.3 ^a	82±4.2 ^c	17.2±4.3 ^b
Controls							
Orlistat				0.08±0.01			
Acarbose		1.25±0.10	25.0±1.0				
Nimesulide					0.39±0.10**		
Naproxen						14.0±1.0**	
Acetylsalicylic acid							65.0±1.0**

GD: gastroduodenal. PEE: polyphenol-enriched extract. * Percentage inhibition with 25 μg GAE/mL of sample. ** IC₅₀ values (μg/mL)

Values are mean ± SD of three determinations. Different letters within each column represent significance difference ($p < 0.05$).