Food Chemistry 289 (2019) 204–214

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Research Article

Digested total protein and protein fractions from chia seed (*Salvia hispanica* L.) had high scavenging capacity and inhibited 5-LOX, COX-1-2, and iNOS enzymes

Mariana Grancieri[a,b], Hercia Stampini Duarte Martino[a], Elvira Gonzalez de Mejia[b,*]

[a] Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, MG, Brazil
[b] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, IL, USA

ARTICLE INFO

Keywords:
Chia seeds
Peptides
Simulated gastrointestinal digestion
Inflammation
Antioxidant
Atherosclerosis

ABSTRACT

The objective was to identify and characterize peptides from digested total protein (DTP) and isolated protein fractions (DPF), and their potential antioxidant, anti-inflammatory and anti-atherosclerotic effects, from chia seed (*Salvia hispanica* L.). Total protein and protein fractions from chia seed underwent simulated gastrointestinal digestion. The sequence, physicochemical properties, and biological potential of peptides were determined using chemical, *in silico*, and biochemical assays. Peptides from DTP (n = 9) and DPF albumin (n = 12), globulin (n = 11), prolamin (n = 5) and glutelin (n = 17) had interaction with cyclooxygenase-2 (COX-2), p65- nuclear factor kappa B, lipoxygenase-1 (LOX-1) and toll-like receptor 4 ($p < 0.05$). DTP, and digested albumin, globulin, and glutelin showed scavenging capacity for superoxide, hydrogen peroxide, nitric oxide and DPPH (1,1-diphenyl-2-picrylhydrazyl), and inhibition of 5-LOX, COX-1-2, and inducible nitric oxide synthase (iNOS) enzymes ($p < 0.05$). Chia seed proteins has peptides with potential beneficial health effects highlighting the importance of chia consumption.

1. Introduction

Cardiovascular disease (CVD) is the main cause of morbidity and mortality worldwide, representing around 17.3 million deaths per year (Sacks et al., 2017). Inflammation and oxidative stress are directly related to the etiology of these diseases (Chakrabarti, Jahandideh, & Wu, 2014). In the inflammatory process, the receptors present in immune cells, as toll-like receptor (TLR) and lipoxygenase (LOX) can be activated, sending signals for activation of cellular molecules, such as nuclear factor kappa B (NF-κB) (Morettini, Storm, Sacchetti, Cappozzo, & Mazzà, 2015). This promotes the activation of the inducible nitric oxide syntase enzyme (iNOS) and cyclooxygenase (COX-2), which will produce nitric oxide (NO) and prostaglandins (PGE_2), respectively, which will propagate the inflammatory process (Khodabandehloo, Gorgani-Firuzjaee, Panahi, & Meshkani, 2016). This process can be aggravated by reactive oxygen species (ROS), as superoxide and hydrogen peroxide. The LOX receptor may also induce platelet and macrophages aggregation, which together with the excess of ROS, leads to the formation of atheroma plaques, which are directly associated with the complications of CVD (Khodabandehloo et al., 2016).

Chia (*Salvia hispanica* L.) is an herbaceous plant native to northern Guatemala and southern Mexico, which supplies small seeds that stand out due to their high nutritional and functional value (Ixtaina, Nolasco, & Tomas, 2008). This seed contains protein (about 19%) greater than other traditional grains (da Silva et al., 2017), and is therefore a promising source of bioactive peptides (Coelho, Soares-Freitas, Areas, Gandra, & Salas-Mellado, 2018).

The peptides are formed naturally by gastrointestinal digestion on the human organism due to the action of specific enzymes, as pepsin, pancreatin and quimiotripsin. Each enzyme cut the bonds between specific types of amino acids, breaking the proteins in peptides and releasing them, as well as free amino acids. These new compounds can be absorbed by the intestinal lining and passed into the circulatory system (Fruton, 2002). Based on their composition and amino acid sequences, bioactive peptides can exert anti-hypertensive, hypocholesterolemic, anti-thrombotic, immunomodulatory and antioxidants effects, among others (Cicero, Fogacci, & Colletti, 2017).

The main storage protein fractions present in chia are prolamin, glutelin, albumin and globulin, being the last two found in higher concentrations (Kačmárová, Lavová, Socha, & Urminská, 2016; Orona-Tamayo, Valverde, Nieto-Rendón, & Paredes-López, 2015). Research has shown that digested protein of chia inhibited the angiotensin-

* Corresponding author at: 1201 W Gregory #228, Urbana, IL 61801, USA.
E-mail addresses: hercia@ufv.br (H.S.D. Martino), edemejia@illinois.edu (E. Gonzalez de Mejia).

https://doi.org/10.1016/j.foodchem.2019.03.036
Received 17 November 2018; Received in revised form 7 March 2019; Accepted 9 March 2019
Available online 12 March 2019
0308-8146/

converting enzyme (ACE-enzyme) (Orona-Tamayo et al., 2015; Segura-Campos, Salazar-Vega, Chel-Guerrero, & Betancur-Ancona, 2013), had high antioxidant, and antibacterial effects (Coelho et al., 2018; Segura-Campos et al., 2013) and showed inhibition of cholesterol synthesis (Coelho et al., 2018).

There is still a need to identify the peptide sequences resulting from the simulated gastrointestinal digestion, of both, total proteins and each one of the constituent proteins. Our objectives in this study were to identify and characterize peptides from digested total protein (DTP) and digested protein fractions (DPF) from chia seeds (*Salvia hispanica* L.) and determine their potential antioxidant, anti-inflammatory and anti-atherosclerotic effect.

2. Materials and methods

2.1. Chemical composition of chia seeds

The chia seeds used in this study were traditional seeds harvested in farms from Rio Grande do Sul, Brazil, crop of 2017. The seeds showed brown pericarp and approximate diameter of 2.5 mm. Previous work with chia showed that the state of Rio Grande do Sul, Brazil, has temperate climate, with average temperature of 26 °C and relative humidity ranging from 60 to 80%, regular rainfall, and clay soil. The chia plantation was on January and harvested on June (da Silva et al., 2017). Moisture was determined using an oven (Nova Ética®, model 400/6ND, São Paulo, Brazil) at 105 °C. Ash was quantified using a muffle furnace (Quimis, Q320 M model, Brazil) at 550 °C. Protein content was determined through micro-Kjeldhal. Protein was determined as percent nitrogen × 6.25 (AOAC, 2012; Capitani, Spotorno, Nolasco, & Tomás, 2012). Total dietary fiber (soluble and insoluble fiber) of dry and fat-free samples was determined by the gravimetric non-enzymatic method (AOAC, 2012). α-Amylase, proteases, and thermoresistant amyloglucosidase enzymes (total dietary fiber assay kit, Sigma®) were used to perform enzymatic hydrolysis. The total dietary fiber content was obtained through the sum between the soluble and insoluble fractions. Lipid content was determined by Soxhlet method (AOAC, 2012). Carbohydrates were calculated by difference, using the following equation: [100 − (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. All chemical analyses were performed in triplicate.

2.2. Sample preparation

Chia seeds were processed according to Orona-Tamayo et al. (2015), with modifications (Fig. 1A). Briefly, the seeds were immersed in distilled water in a proportion 1:10 (g: ml) for 1 h until mucilage formation, frozen overnight (− 80 °C) and freeze-dried (LabCoco Freeze Dryer 4.5; Kansas, MO, USA). The mucilage was manually removed from the seeds with the aid of a sieve (500 μm/35 mesh).

The mucilage-free seeds were ground (Mr. Coffee®) and sieved (500 μm/35 mesh) to obtain a uniform flour. This was then degreased using hexane (1:10 g: ml) at 60 °C for 2 h under constant stirring. The mixture was centrifuged (6000 g, 15 min, 4 °C), the supernatant discarded and the flour was left overnight under a flow rate hood and stored at 4 °C until use.

2.3. Extraction of proteins

The mucilage and fat-free flour were mixed with deionized water (1:20 w/v), the pH adjusted to 8 with 0.1 M sodium hydroxide and placed under constant stirring at 35 °C for 1 h. The mixture was centrifuged (5000 g; 15 min; 25 °C), the precipitate was discarded and the supernatant (concentrated protein) was freeze-dried and stored at − 20 °C (Alves, Vasconcelos, Bassinello, de Mejia, & Martino, 2016; Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014). This extract will be called total protein to contrast with the different protein fractions also obtained; the term total protein does not refer to protein yield, but inclusion of different protein fractions.

2.4. Extraction of protein fractions

Proteins from chia flour were fractioned using slight modifications of the method reported by Orana-Tamayo et al. (2015) and Sandoval-Oliveros and Paredes-López (2013). Briefly, the mucilage and fat-free chia flour were diluted with deionized water (1:10/g:ml), mixed for 1 h at 4 °C and centrifuged (14,000 g; 20 min; 4 °C). The supernatant was identified as the albumin fraction. For the extraction of globulins fraction, the resulting pellet from the previous extraction was re-suspended with 0.05 mol/L Tris-HCl + 0.5 mol/L NaCl (pH 8.0) (1:10 w pellet/v of buffer), mixed for 1 h at 4 °C and centrifuged (14,000 g; 20 min; 4 °C); the supernatant was collected. The resulting pellet was diluted with isopropanol 70% (1:10 w/v) mixed for 1 h at 4 °C and centrifuged (14,000 g; 20 min; 4 °C). The obtained supernatant was the source of the prolamin fractions. The resulting pellet was added with 0.1 mol/L $Na_2B_4O_7{\cdot}H_2O$ (pH 10) (1:10 w/v), mixed for 1 h at 4 °C and centrifuged (14,000 g; 20 min; 4 °C). The supernatant was the source of the glutelin fractions. All samples were freeze-dried and stored at − 20 °C until further analysis.

2.5. Simulated gastrointestinal digestion

In order to simulate gastrointestinal digestion, enzymatic hydrolysis with pepsin and pancreatin was conducted according by Megías et al. (2004). The total and fraction proteins (prepared as indicated in 2.3 and 2.4) were suspended in water (1:20 w/v) and a sequential enzyme digestion was carried out with pepsin/substrate 1:20 (w/w), pH 2.0, for 2 h, at 37 °C, under stirring. A pancreatin/substrate 1:20 (w/w), pH 7.5, and *in vitro* digestion was carried out for 2 h, at 37 °C, under stirring. Digestion was stopped by placing the samples in a water bath at 75 °C for 20 min. Samples were centrifuged twice at 20,000 g for 15 min at 4 °C and the supernatant was collected. The digested total protein (DTP) and the digested protein fractions (DPF) were dialyzed using a 100–500 Da molecular weight cut-off membrane (Spectra/Por®, Biotech CE Membrane) and freeze-dried. Samples were stored at − 20 °C until analysis.

2.6. Protein quantification and electrophoretic profile

Determination of the protein concentration in protein concentrate (identified as total protein) and protein fractions from chia seeds, before and after the simulated gastrointestinal digestion, were made in triplicate using DC (detergent compatible) protein assay (500-0112; BioRad, USA) according to the manufacturer's instructions. The absorbance was read at 690 nm and the concentration calculated using bovine serum albumin (BSA) for the construction of the standard curve ($y = 0.0002\times + 0.607$, $R^2 = 0.99$).

The protein profile was evaluated by SDS-PAGE (sodium dodecyl sulphate-polycrylamide gel electrophoresis analysis). The samples were diluted (1:1 v/v) to Laemmli buffer with 5% of β-mercaptoethanol, pH 6.8 (161-0737, C, Hercules, CA, USA), warmed for 1 min and loaded onto a gradient Tris-HCl gel of polyacrylamide (4–20%). Standard MW Dual color (BioRad) (10–250 kDa) was used to calculate the molecular mass of the separated proteins. After running (200 V; 35 min), the gel was washed with distilled water for 10 min and then kept on a shaker with Simply Blue Safe Stain for 1 h and then washed with distilled water overnight. The bands were observed using the GL 4000 Pro Imaging System (Carestrem Health Inc., Rochester, NY, USA). The molecular weight was calculated using the Carestream Program®.

2.7. Degree of hydrolysis

The degree of hydrolysis (DH) was determinate according to Nielsen, Petersen, and Dambmann (2001) with modifications. The *o-*

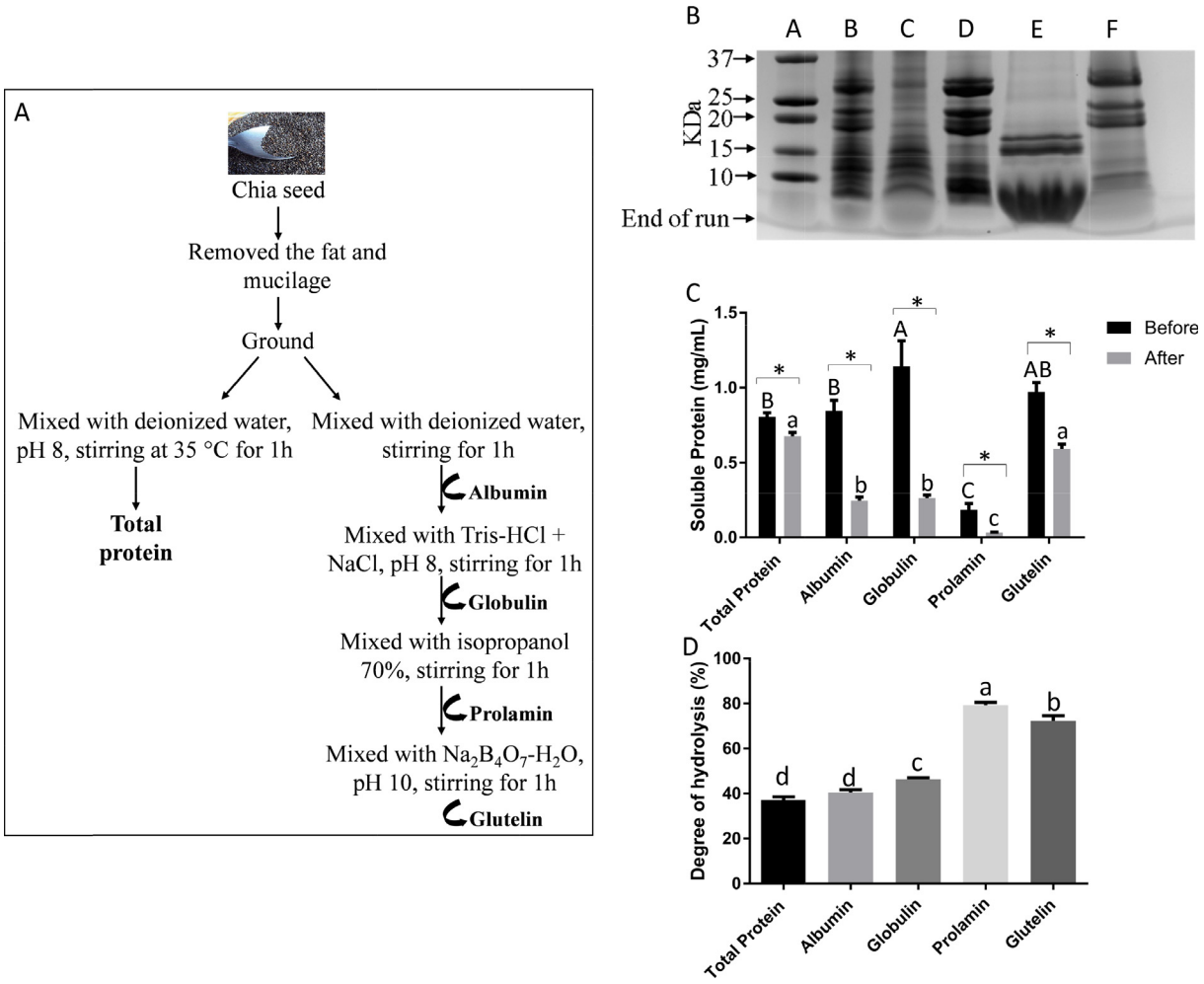


Fig. 1. A: Diagram of the total protein and protein fractions extraction. B: Protein fractions present in chia flour by SDS-PAGE stained with Simply Blue Safe Stain. (A) standard; (B) chia total protein; (C) albumin; (D) globulin; (E) prolamin; (F) glutelin. C: Protein concentration between total protein and fractions of chia seed. Upper and lower-case letters represent, respectively, difference between samples before and after simulated gastrointestinal digestion by the ANOVA test followed by post-hoc Tukey ($p < 0.05$). * Represents the intragroup difference between the soluble proteins before and after the simulated gastrointestinal digestion evaluated by the *t*-Student test. D: Degree of hydrolysis of the digested total protein, albumin, globulin, prolamin and glutelin derived from the chia seed. Dates analyzed by one-way ANOVA and post hoc Tukey ($p < 0.05$).

phthaldialdehyde (OPA) reagent was prepared with 7.620 g di-Natetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) dissolved in 150 ml of deionized water. Then, 160 mg OPA 97% was dissolved in 4 ml ethanol and 176 mg dithiothreitol 99% (DTT) and then dissolved in 200 ml of deionized water. These three solutions were mixed. For the standard solution, 5 mg of serine (Art.7769 Merck, Darmstadt, Germany) were diluted in 50 ml of deionized water. For the sample solution, between 0.1 and 0.5 mg sample was diluted in 1 ml of the deionized water so that there was between 8 and 80% protein in the solution.

In tubes, 600 µL of OPA reagent was added in 80 µL of the standard, sample or water (blank) were mixed for 5 s. Then, 200 µL these solutions were pipetted, in triplicate, in plates a 96-well plate. The absorbance was read at 340 nm. The DH was calculated following the equations:

1) Serine-NH2: $\left(\frac{ODsample - ODblank}{ODstandard - ODblank}\right) * 0.9516 * 0.001 * \left(\frac{100}{X * P}\right)$

where: X = g sample; P = protein % in sample; 0.001 is the sample volume (L).

2) $h = \frac{Serine - NH2 - \beta}{\alpha}$

where: $\beta = 0{,}40$ and $\alpha = 1$.

3) $DH = \left(\frac{h}{hhot}\right) * 100$

where: $h_{hot} = 8.30$

2.8. Identification and characterization of potentially bioactive peptides

The peptides obtained from the DTP and DPF were analyzed according to Mojica, Chen, and de Mejia (2015) by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) using a Q-ToF Ultima mass spectrometer (Waters, Milford, MA, USA), equipped with an Alliance 2795 HPLC system. The gradient mobile phase was A: 95% water, 5% of acetonitrile, and 0.1% of formic acid; B: A: 95% of acetonitrile, 5% of water, and 0.1% of formic acid. The volume of injection was 200 µL/min and PDA detector wavelength at 280 nm. Each peak was analyzed in MassLynx V4.1 software (Waters Corp., Milford, MA, USA) and the sequence of amino acids was identified based on the accurate mass measurements, tandem MS fragmentation using the MassBank database.

The biological activity of peptides with more than 90% of bioactive probability was predicted by using BIOPEP® database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed on February

27, 2018). The isoelectric point, net charge, and hydrophobicity of these peptides were analyzed by PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/index.html, accessed on March 13, 2018). The parental chia protein that contained the peptides after digestion was identified with BLAST® tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on August 15, 2018). The amino acids were presented as one letter nomenclature. Only peptides with more than 90% probability and 100% similarity to *Salvia hispanica* L. parent proteins were selected. For the main table, the peptides should have additionally presented antioxidant characteristics (by BIOPEP® database).

2.9. In silico analysis

The structural mechanism by which peptides from DTP and DPF from chia seed interact with the atherosclerotic marker LOX-1, and the inflammatory markers COX-2, p-65 NF-κB and Toll-Like receptor 4 (TLR4) was evaluated by *in silico* analysis, through molecular docking as described by Alves et al. (2016), using the DockingServer.17 program. Peptides were designed using Instant MarvinSketch (ChemAxon Ltd). Non-polar hydrogen atoms were merged, and rotatable bonds were defined on program AutoDockTools®. Only peptides with antioxidant biological potential (by BIOPEP® database) were selected. Moreover, the pharmacological controls simvastatin (Alves et al., 2016), dexamethasone (Sun et al., 2017), 4-Methyl-1-N-(3-phenylpropyl)benzene-1,2-diamine (JSH23) (Wang et al., 2018), and atorvastatin (Thongnak et al., 2017) were used, respectively for LOX-1, COX-2, p-65 NF-kB, and TLR4.

The crystal structure file of LOX-1, COX-2, p-65 NF-kB, and TLR4 was obtained from the Protein Data Bank (http://www.rcsb.org/) (PDB: 1YXK, 5KIR, 1OY3, 3FXI, respectively). Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Flexible torsions, charges, and grid size were assigned using Autodock Tools. Docking calculations were performed using AutoDock Vina and the binding pose with the lowest binding energy was selected as representative to visualize in the Discovery Studio 2016 Client (Dassault Systemes Biovia Corp®).

The inhibition constant (K_I) was calculated as described by Fan, Johnson, Lila, Yousef, and De Mejia (2013), using the formula:

$$K_I = 2.72^{\left(\frac{EFE*1000}{R*T}\right)}$$

where EFE is the minimum estimated free energies (by DockingServer.17 program), *R* is the gas constant (kcal/mol), and *T* is the absolute temperature.

2.10. Antioxidant, anti-atherosclerotic and anti-inflammatory biochemical assays

2.10.1. Superoxide radical (O_2^-) scavenging activity

The DTP and DPF were diluted in 0.1 M phosphate buffer (PBS), pH 7.4 and analyzed at three concentrations: 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml. Superoxide radicals were generated by the NADH/PMS system according to Ewing and Janero (1995). An aliquot of 25 μL of each DTP and DPF or PBS (blank) was mixed with 200 μL of a solution composed by 0.1 mmol/L of ethylenediaminetetraacetic acid (EDTA), 62 μmol/L of nitroblue tetrazolium chloride (NBT) and 98 μmol/L of nicotinamide adenine dinucleotide (NADH). Then, 25 μL of 33 μmol/L of phenazinemethosulfate (PMS), containing 0.1 mM EDTA, were added to each well. All solutions were prepared in 0.1 M PBS (pH 7.4). Absorbance was read at 550 nm and O_2^- radical scavenging activity was calculated by the following formula:

$$\%\ of\ \text{scavenging} = \left(\frac{Ablank - Asample}{Ablank}\right)$$

A curve with trolox (4.8–300 μg/ml) was made to determined Trolox equivalent (TE) (y = 0.0021x + 0.1272; R^2 = 0.94).

2.10.2. Nitric oxide (NO) activity

Nitric oxide production was determined by the accumulation of nitrite (NO_2), a stable product of the nitric oxide (NO) reaction with oxygen in aqueous solution. Briefly, 50 μL of 20 mM sodium nitroprusside were mixed with 50 μL of each DTP and DPF (as in 2.10.1.) or PBS (blank) for 60 min, at room temperature. All solutions were prepared in 0.1 M PBS (pH 7.4). After incubation, 50 μL of Griess reagent (Sigma®) were added to each well. Then, the absorbance was read at 550 nm. The concentration of NO radical scavenging activity was calculated:

$$\%\ of\ \text{scavenging} = \left(\frac{Ablank - Asample}{Ablank}\right)$$

A curve with Trolox (1.15–300 μg/ml) was made to determine the TE (y = 0.0751ln(x) + 0.0845; R^2 = 0.93) (Tsai, Lin, Lin, & Yang, 2011).

2.10.3. Hydrogen peroxide (H_2O_2) capacity

The ability of the samples to scavenge H_2O_2 was determined according to Ruch, Cheng, and Klaunig (1989). A 117 μL of DTP and DPF (as in 2.10.1) were combined with 30 μL of a H_2O_2 solution (40 mM). PBS was used as blank. Absorbance was measured after 10 min at 230 nm·H_2O_2 radical scavenging activity was calculated by the following formula:

$$\%\ of\ \text{scavenging} = \left(\frac{Ablank - Asample}{Ablank}\right)$$

A curve with Trolox (4.8–300 μg/ml) was made to determine the TE (y = −224.82 × + 176.86; R^2 = 0.90).

2.10.4. DPPH radical scavenging activity

In a test tube, protected from light, 100 μL of each sample (as in 2.10.1) were added to 1.5 ml of methanolic DPPH solution (0.1 mM) (1,1-diphenyl-2-picrylhydrazyl) and stirred by vortex (3000 rpm) for 30 s. After 30 min of incubation, the absorbance of the solution was read at 517 nm (da Silva et al., 2017). DPPH radical scavenging activity was calculated by the following formula:

$$\%\ of\ \text{scavenging} = \left(\frac{Ablank - Asample}{Ablank}\right)$$

The analytical curve was constructed using a 5–205 μg/ml of the Trolox solution to determine TE (y = 0.0016 × - 0.0294; R^2 = 0.94).

2.10.5. Determination of anti-inflammatory and anti-atherosclerotic activity by 5-LOX inhibition assay

Linoleic acid was used as a substrate to determine 5- lipoxygenase (5-LOX) inhibition activity. Then, 250 μL of each DTP and DPF (as in 2.10.1) was mixed with 250 μL of 5-LOX (50U) and incubated during 10 min at 37 °C. The reaction was initiated by the addition of 500 μL of linolenic acid (500 μM) and the absorbance of samples was read after at 234 nm in quartz cuvette. The value for inhibitory (%) of the enzyme activity was calculated as follows:

$$\%\ of\ inhibition = \left(\frac{Ablank - Asample}{Ablank}\right)$$

Ascorbic acid (4.5–17.5 μg/ml) was used as the standard for calculating the ascorbic acid equivalent. DTP, DPF and reagents were diluted in borate buffer (pH = 9) (y = 0.0816x − 0.3044; R^2 = 0.98) (Tsai et al., 2011).

2.10.6. COX-1 and 2 inhibitor screening test

Cyclooxygenase 1 and 2 (COX-1 and 2) inhibitor screening tests were conducted on DTP and DPF (as in 2.10.1) to evaluate the constitutively and anti-inflammatory effects, respectively. The COX-2 (human) and COX-1 (ovine) inhibitor screening assay were performed

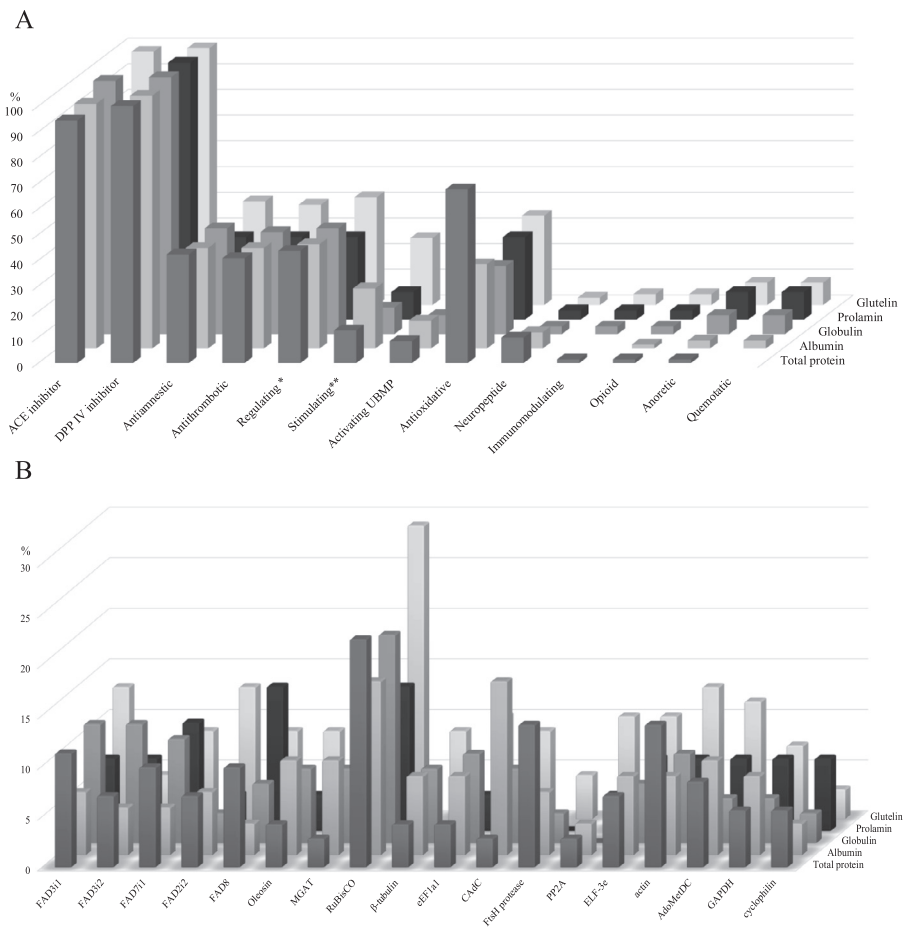


Fig. 2. A: Potential biological activity and B: parental protein identified with peptides found in the digested total protein and fractions from chia seed. ACE inhibitor: angiotensin-converting-enzyme inhibitor; DPP IV inhibitor: dipeptidyl peptidase IV inhibitor; Activating UBMP: Activating ubiquitin-mediated proteolysis. *Peptide regulating the stomach mucosal membrane activity; ** Stimulating vasoactive substance release or glucose uptake stimulating peptide. FAD3i1: fatty acid desaturase 3 isoform 1; FAD3i2: fatty acid desaturase 3 isoform 2; FAD7i1: fatty acid desaturase 7 isoform 3; FAD2i2: fatty acid desaturase 2 isoform 2; FAD8: fatty acid desaturase 8; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; MGAT: monoacylglycerol acyltransferase; eEF1a1: elongation factor 1-alpha; CAdC: clathrin adaptor complex; PP2A: protein phosphatase 2A; ELF-3e: eukaryotic translation initiation factor 3 subunit E; AdoMetDC: S-adenosylmethionine decarboxylase; GAPDH: glyceraldehyde-3-phosphate-dehydrogenase.

using a kit (Cayman Co., St. Louis, MO, USA) according to manufacturer's instructions. This analysis is based in the production of PGF2α by $SnCl_2$ (TinII chloride) reduction of COX-derived PGH_2 in the COX reaction. Both ovine COX-1 and human recombinant COX-2 enzymes were tested separately in this assay. The results were expressed in % of inhibition of COX-2,1 and calculated the IC_{50}.

2.10.7. iNOS inhibitor screening test

This method made with nitric oxide synthase (NOS) inhibitor screening kit (Fluorometric) (Biovision, Milpitas, CA, USA) according to manufacturer's instructions. Inhibition of iNOS was measured by comparing the amount of NO produced in the presence of inhibitor with the control background having no inhibitor in DTP and DPF (as in 2.10.1). Results were expressed as % of inhibition of iNOS and calculation of IC_{50} was made.

2.11. Statistical analysis

Data were analyzed using a *one-way* analysis of variance (ANOVA) and *post-hoc* of Tukey ($\alpha = 0.05$) for independent samples in order to verify the difference between DTP and DPF. Data were analyzed in triplicate and expressed as the mean ± standard deviation of two independent experiments. IC_{50} was calculated using linear regression. All statistical analyses were conducted using GraphPad Prism software, version 7.

3. Results

3.1. Chemical composition of chia seeds

The macronutrient breakdown of protein, carbohydrates, and lipids in chia seed was 21.1%, 2.4% and 27.8%, respectively. Moisture was 7.2% and ash, 4.2%. There was a higher concentration of insoluble fiber (33.3%) than soluble fiber (4.0%).

3.2. Quantification and protein electrophoresis fractions

After the extraction and concentration of total proteins at pH 8, chia protein concentrate flour (called total protein) presented 0.63 g protein/g, with molecular weight between 7.1 and 114.6 kDa. As for the electrophoretic pattern, the protein fractions showed several bands and a majority of low molecular weight bands. Albumin fractions showed the highest variation in molecular weight ranging from 8.0 to 114.6 kDa, whereas globulins and prolamins, molecular weights were concentrated between 5.0 and 69.0 kDa and glutelins ranged from 10.6 to 98.4 kDa (Fig. 1B). In addition, after separation by solubility, the protein fraction of globulins had the highest amount (34.7%), followed by glutelin (31.7%), albumin (25.7%) and prolamin (7.8%) (Fig. 1C).

3.3. Simulated gastrointestinal digestion and degree of hydrolysis

After simulated gastrointestinal digestion, there was a reduction in protein concentration in the DTP and DPF. In addition, the proportion of soluble proteins among DPF was changed; glutelins increased, followed by albumins, globulins, and prolamins (Fig. 1C). Digested prolamin had the highest degree of hydrolysis, followed by digested

glutelin and globulin. Digested albumin and DTP showed the lowest values (Fig. 1D).

3.4. Bioactive peptides

DTP and DPF of the chia seed showed a high amount of peptides. There were about 596, 631, 612, 634 and 707 peptide sequences for DTP and digested albumin, globulin, prolamin and glutelin, respectively. DTP presented the highest proportion of sequences with more than 90% probability for accurate sequence of amino acids (12%), followed by digested globulin and glutelin (11% each), albumin (10%) and prolamin (4%) (Supplementary Table 1). Supplementary Table 1 also presents similarities in peptide sequences with other proteins reported in chia seed.

The average molecular weight was 1036.9, 1328.3, 1283.4, 1286.1, and 1422.0 Da for DTP and digested albumin, globulin, prolamin, and glutelin, respectively. The lowest isoelectric point was 2.87 for digested glutelin, and the highest was 12.20 for DTP. The net charge was ranked between −3 and 3 and hydrophobicity, between 0.23 and 28.71 (Supplementary Table 1).

Most of the peptides showed antioxidant, anti-atherosclerotic, antiamnestic, hypoglycemic and hypotensive activity (Fig. 2A and Supplementary Tables 1 and 2). There were peptide sequences identified in DTP and in digested protein fractions that had similarities with other proteins reported in chia seed, besides storage proteins (Fig. 2B). Among those, fatty acid desaturase (FAD) was the parental protein most identified among the peptides from chia. FAD8, and FAD3i1, were other parental proteins with similar sequences present in DTP. There were some peptides found in the digested protein fractions (storage proteins) that also showed similarities in sequence with other chia proteins. For instance, albumin presented peptide sequences also present in FAD8, and FAD3i1, and FAD7i1 had similar sequences with digested globulin, prolamin, and glutelin. Oleosin was mostly identified in digested albumin, the monoacylglycerol acyltransferase (MGAT), in digested prolamin and ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO), in digested glutelin (Fig. 2B and Supplementary Tables 1 and 2).

3.5. Molecular docking study of peptides inhibiting LOX-1, COX-2, p-65 NF-kB, and TLR4

The minimum estimated free energies (EFE) of the interactions of the peptides with LOX-1, COX-2, p-65 NF-kB, and TLR4 are shown in Table 1. The estimated free energies indicated that compounds with a more negative value are more likely to interact with these receptors. In general, the estimated average free energies were −5.8, −6.1, −5.8, and −7.2 kcal/mol respectively for LOX-1, COX-2, p-65 NF-κB, and TLR4.

These results emphasize the beneficial health effects of chia seed proteins and their contributions to the diet. In order to compare the results from chia peptides, pharmacological controls were used and those showed lower negative values (less interactions) than some of chia peptides. The peptide TGPSPTAGPPAPGGGTH had more interaction with LOX-1 (EFE: −10.6 kcal/mol) and TLR-4 (EFE: −10.3 kcal/mol); and SPKDLALPPGALPPVQ, more interaction with COX-2 (EFE: −10.4 kcal/mol), both peptides produced from digested albumin. The peptide HYGGPPGGCR, from DTP, had more interaction with p65-NF-κB (EFE: −7.1 kcal/mol) (Fig. 3, Supplementary Tables 1 and 3).

3.6. Antioxidant assays

3.6.1. Superoxide scavenging capacity

DTP and DPF revealed that the concentration of 1 mg/ml had the best scavenging ability of the superoxide radical; digested glutelin had the highest scavenging capacity, both in the concentration of 1 mg/ml and 0.5 mg/ml, which was around 58%. Digested prolamin was the protein with the lowest capacity of scavenging in all concentrations evaluated. DTP and digested albumin, and globulin did not differ from each other (Fig. 4A).

3.6.2. Nitric oxide (NO) scavenging capacity

The concentrations of 0.5 mg/ml and 1.0 mg/ml presented the same scavenging capacity of nitric oxide radical in both, DTP and digested albumin. However, for digested globulin, prolamin and glutelin, the concentration of 1.0 mg/ml showed higher scavenging capacity in comparison to the other two concentrations. Digested albumin was the fraction with the best scavenging capacity (1.0 mg/ml, around 35%), whereas, in the concentrations of 0.5 and 0.1 mg/ml, its capacity was similar to that of digested globulin (Fig. 4B).

3.6.3. Hydrogen peroxide scavenging capacity

The concentration of 0.1 mg/ml was the best for scavenging hydrogen peroxide in DTP and DPF (about 80%). Only for digested glutelin the concentration of 0.5 had similar activity to 0.1 mg/ml and was the best for scavenging hydrogen peroxidase. At the concentration of 1.0 mg/ml, digested globulin and prolamin presented the best results compared to DTP and digested albumin and glutelin, which did not differ from each other (Fig. 4C).

3.6.4. DPPH inhibition capacity

At 1 mg/ml the best antioxidant capacity was presented by DTP, digested albumin and glutelin followed by globulin and prolamin. At 0.5 mg/ml digested albumin and globulin presented the best antioxidant capacity, followed by DTP, digested glutelin and prolamin. The digested prolamin (0.5, 1.0 mg/ml) showed the lowest antioxidant capacity and digested globulin (0.1 mg/ml) (Fig. 4D).

3.7. Anti-inflammatory assays

3.7.1. 5-LOX inhibition capacity

Digested globulin, prolamin and glutelin (0.5 mg/ml and 1.0 mg/ml) presented the best inhibition of 5-LOX (about 75%) followed by DTP and digested albumin. At 0.1 mg/ml DTP had the lowest inhibition of the enzyme ($p < 0.05$) (Fig. 4E).

3.7.2. COX 1 and COX 2 inhibition

DTP and digested albumin demonstrated the best inhibition of COX-2, with a low IC_{50}. Digested globulin showed inhibition of COX-2 but not equally for COX-1; digested glutelin had inhibitory preference for COX-1, since the value of COX-1/COX-2 ratio was < 1.0. Digested prolamin had no inhibition of these enzymes (Fig. 4F).

3.7.3. iNOS inhibition

Based on IC_{50}, DTP and digested albumin showed a greater ability to inhibit iNOS, followed by digested globulin and glutelin. Digested prolamin, despite had the highest IC_{50} (Fig. 4F).

4. Discussion

Our objectives were to identify and characterize peptides from chia seed after simulated gastrointestinal digestion of total proteins and isolated protein fractions. In addition, their potential health benefits were determine highlighting the importance of consumption of chia seed as a food. The protein concentration in chia seed was 21.1%. This concentration is higher than that found in other traditional cereals as wheat (14%), corn (14%), rice (8.5%), oats (15.3%), and barley (9.2%) (Ayerza, 2005). Storage proteins are those proteins that supply intermediary nitrogen compounds for biosynthesis at a metabolic active stage (Kačmárová et al., 2016). Globulin, a storage protein, from chia seed was the fraction present in more quantity (34.7%); similarly as reported by other authors (Kačmárová et al., 2016; Sandoval-Oliveros & Paredes-López, 2013). Sandoval-Oliveros and Paredes-López (2013)

Table 1
Estimated free energy binding (EFE), interaction constant (K_I), and chemical interactions among the peptides present in chia seed with the catalytic site of LOX-1, COX-2, p65NF-kB and TLR4.

Sequence of peptides	**LOX-1**			**COX-2**			**NF-kB**			TLR4		
	EFE (kcal/mol)	K_I (μM)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μM)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μM)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μM)	Interacting amino acid residues
Pharmacological*	−5.4	110.09	ALA B: 194; PHE B: 190; MG A: 401; SER A: 160	−10.0	0.05	ARG A: 44; LYS B: 546; ALA B: 543ILE A: 124; PRO B: 542; HIS A: 122; SER A: 126; GLN A: 372	−5.9	47.34	GLY D: 274; ARG D: 275; LEU D: 283	−6.6	14.53	ALA B : 158; VAL B: 134; ASP B: 181; LYS B: 230; THR B: 232; ARG B: 289; ARG B: 234; PHE B: 263; ASP B: 209; SER B: 183
HYGGPPGGCR (total protein)	−6.5	17.20	PHE A:158; TYR A:197; SER A: 160; SER B: 196; TYR B:197; GLN B: 193; ARG B: 248	−8.0	1.37	ASN B: 382; HIS B: 386; GLN B: 454; HIS B: 207; THR B: 212; HIS B: 214; LYS B: 215; ILE B: 274; VAL B: 291; LYS B: 211; LEU B: 294; TYR B: 409; TYR B: 212	**−7.1**	6.25	HIS C: 245; ARG C: 246; GLY D: 274; GLY D: 273; LEU D: 282; LEU D: 283; GLY D: 304; LYS C: 221	−7.7	2.27	LYS B: 130; HIS B: 179; SER B: 207; THR B: 232; LYS B: 230; ARG B: 289; ARG B: 234; ASN B: 339; ARG B: 264
SPKDLALPPGALPPVQ (albumin)	−5.5	92.99	SER B: 199; SER A: 199; ARG A: 248; PHE B: 163; GLY A: 249; ARG A: 231	**−10.4**	0.05	ARG B: 333; SER A: 143; GLY B: 225; ASN B:375; ARG B: 376; PHE B: 142; LEU B: 224; SER B: 143; SER B: 146; ASN A: 144; LEU A: 224; ARG A: 216	−5.7	66.35	PRO D: 285; ARG D: 275; LYS C: 221; GLY D: 304	−8.0	1.37	VAL B: 132; ASN B: 156; SER B: 183; HIS B: 179; LYS B: 230; THR B: 359; SER B: 360; ASN B: 339; SER B: 317; ALA B: 291; VAL B: 259
TGPSPTAGPPAPGGGTH (albumin)	**−10.6**	0.02	SER B: 160; GLN A: 193; PHE B: 158; SER A: 196	−8.6	0.50	SER A: 119; VAL A: 116; TYR A: 115; ALA A: 111; ILE A: 92; ILE A: 112; PHE A: 99; TYR A: 115	3.1	1.87×10^8		**−10.3**	0.03	ARG B: 289; ARG B: 257; GLU B: 287; SER B: 312; GLU B: 336; GLN B: 430; SER B: 407; ASP B: 428; ARG B: 382; ASP B: 379; ASP B: 405

Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. The most potent interaction between peptides and receptor are in bold. *Pharmacological controls: simvastatin, dexamethasone, JHS23 and atorvastatin, respectively for LOX-1 (lipoxygenase-1), COX-2 (cyclooxygenase-2), p65-NFκB (nuclear factor kappa B) and TLR4 (toll-like receptor).

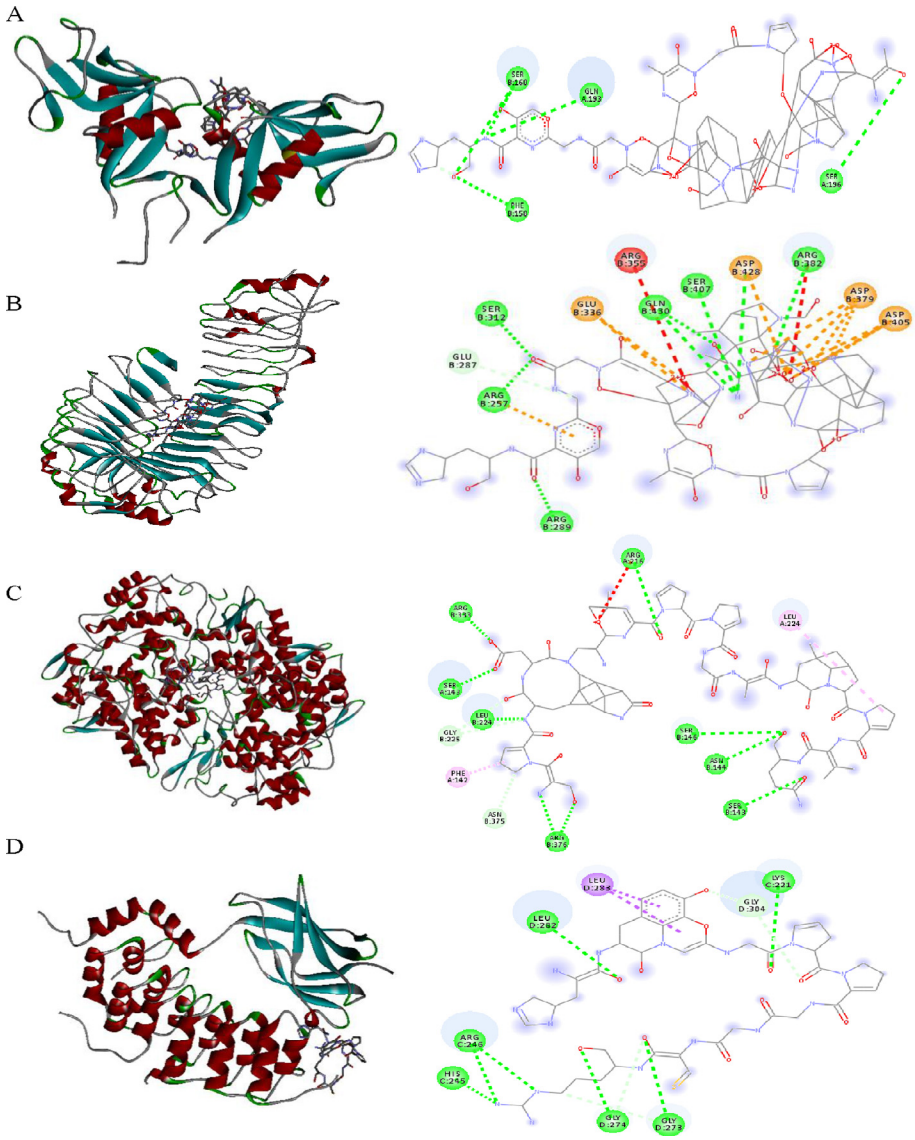


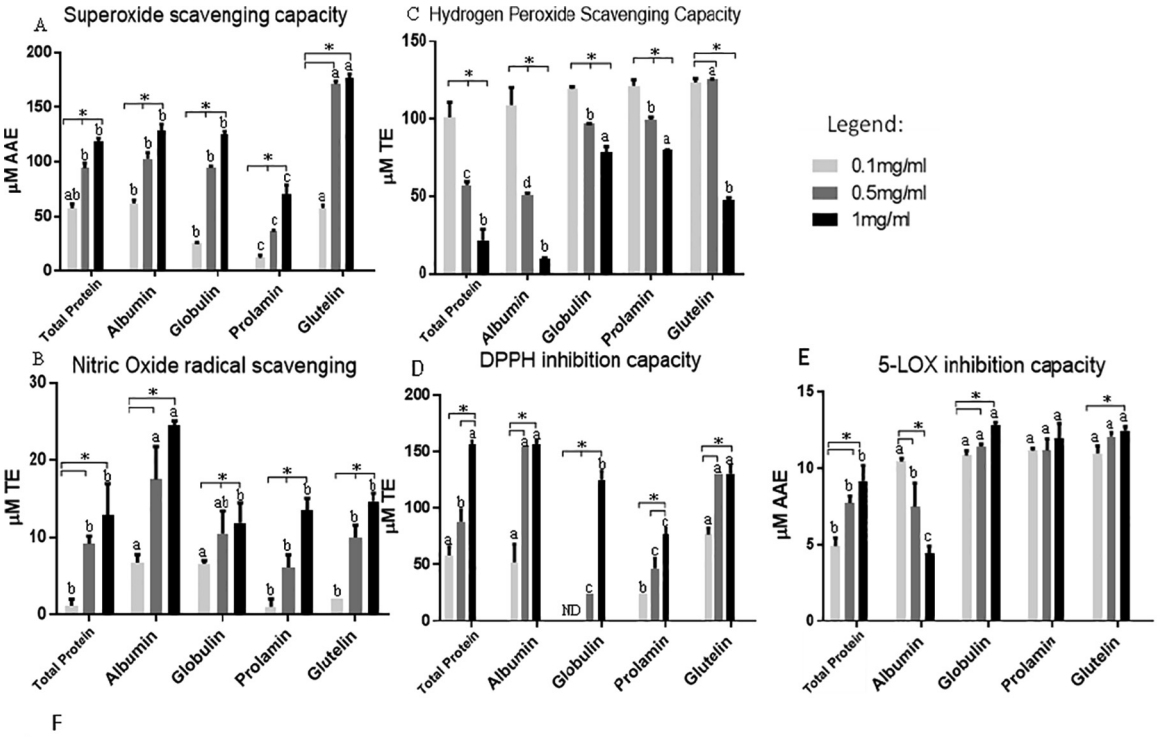
Fig. 3. Molecular docking diagrams exemplifying the interaction of peptides with receptors. A: Interaction between peptide TGPSPTAGPPAPGGGTH and LOX-1 receptor; B: TGPSPTAGPPAPGGGTH and Toll-like receptor 4; C: Interaction between peptide SPKDLALPPGALPPVQ and COX-2; D: Interaction between peptide HYGGPPGGCR and p65-NF-kB. LOX-1 receptor: lipoxygenase-1 receptor; COX-2: cyclooxygenase-2.

also showed that the temperature of the denaturation peak for albumin, globulin, prolamin, and glutelin from chia seeds was 103.6, 104.7, 85.6, and 91.3 °C, respectively. Then, the temperature used in this work (60 °C) was not expected to denature proteins. The combination of pH and temperature used caused the precipitation of proteins, as indicated in our previous research (Alves et al., 2016; Montoya-Rodríguez et al., 2014). As demonstrated by Orona-Tamayo et al. (2015) and Sandoval-Oliveros and Paredes-López (2013), glutelins are soluble at pH 10 and globulins at pH 8.

After the simulated digestion process, glutelin was the more abundant fraction. This is probably because glutelin had a high degree of hydrolysis that exposed its tryptophan and tyrosine-rich amino acid fractions that are the amino acids more detectable on the protein analyses used (Lowry, Resebrough, Farr, & Randall, 1951). Pepsin and pancreatin were used to simulate the physiological process of digestion (Gardner & Jensen, 1984). In both, DTP and DPF, a high degree of hydrolysis was obtained in comparison with the one obtained by other authors that utilized microbial proteases (Coelho et al., 2018; Segura-Campos et al., 2013), and others that used the same enzymes (Orona-Tamayo et al., 2015). Higher degree of protein hydrolysis may directly influence the availability of bioactive peptides generated after digestion (Mojica et al., 2015).

In the simulated gastrointestinal digestion, all proteins produced numerous peptides that, after analysis, showed potential activities as hypotensive, hypoglycemic and antioxidant. The hypotensive effect is due to the role of peptides inhibiting the ACE-enzyme, which converts angiotensin I in angiotensin II. Angiotensin II increases the peripheral vascular resistance and inactivate bradykinin, a vasodilator peptide, inducing a hypertensive action (Cugno, Tedeschi, & Nussberger, 2016). On the other hand, the hypoglycemic effect is mainly due to the inhibition of the enzyme dipeptidyl peptidase-IV (DPP-IV) and by lowering glucose uptake; activities that aid in the control of glucose homeostasis (Muñoz, Luna-Vital, Fornasini, Baldeón, & de Mejia, 2018).

The observed antioxidant activity of peptides can be due to its metal chelation or hydrogen/electron donor activity, allowing the interaction with free radicals, terminating the radical chain reaction or preventing their formation. It is believed that those peptides with < 20 amino acid residues per molecule are the most efficient antioxidants. These small peptides have a better chance of crossing the intestinal barrier and exert their biological effects (Kou, Gao, Zhang, Wang, & Wang, 2013). This



F

Samples	IC_{50} (μg/ml)			
	COX-1	COX-2	COX-1/COX-2 ratio	iNOS
Total Protein	23.3 ± 6.2	13.5 ± 2.6	1.7	0.40 ± 1.7
Albumin	9.8 ± 1.2	13.8 ± 2.0	0.7	0.1 ± 0.7
Globulin	66.7 ± 23.8	27.7 ± 1.5	2.4	7.7 ± 4.0
Prolamin	1030 ± 20.6	1087 ± 13.3	0.9	1120 ± 70.2
Glutelin	9.9 ± 2.6	23.2 ± 15.4	0.4	24.9 ± 1.5

Fig. 4. A: Superoxide scavenging capacity, B: Nitric oxide scavenging, C: Hydrogen Peroxide scavenging, D: DPPH inhibition capacity, E: 5-LOX inhibition capacity of digested total protein and digested protein fractions from chia in three concentrations (0.1, 0.5 and 1.0 mg/ml). Results express in ascorbic acid equivalent (AAE) or Trolox equivalent (TE). Data analyzed by one-way ANOVA and post hoc Tuckey ($p < 0.05$), * indicates the intragroup difference. The different letters are the intergroup difference between the same concentrations of samples. ND: not detected. F: IC_{50} (concentration needed to inhibit 50% activity) COX-1, COX-2 and iNOS inhibition capacity. Data analyzed by linear regression. 5-LOX: 5-lipooxygenase, COX-1: cyclooxygenase-1, COX-2: cyclooxygenase-2, iNOS: inducible nitric oxide synthase, DPPH: 1.1-diphenyl-2-picrylhydrazyl.

characteristic is supported by the results obtained in this study.

The peptides produced from chia seed proteins demonstrated lower hydrophobicity and a large number of glycine and threonine groups. It was demonstrated that peptides with hydrophobicity ≤20 kcal/mol are more effective for penetrating the cell membrane and to exercise effects on the molecule (Mojica, Luna-Vital, & de Mejía, 2017). Also, peptides with hydrophobic amino acids, proline, histidine, tyrosine and/or tryptophan have more antioxidant activity, due to its ionizable groups that block free radicals (Brandelli, Daroit, & Correa, 2015). This demonstrates that the structure of the peptides found may be directly associated with possible beneficial biological functions.

Several peptides from chia proteins interacted with markers of inflammation and atherosclerosis. This interaction was more effective for certain peptides than the pharmacological controls. When a compound shows lower binding energy compared to the pharmacological controls, this demonstrates the higher activity of the compound (Mojica et al., 2017). In particular, TGPSPTAGPPAPGGGTH peptide from digested albumin had the best interaction with LOX-1 and TLR4. The amino acid type and sequence determine the potential interaction between the peptides with the catalytic site of the enzymes that can promote the competitive inhibition of these enzymes (Mojica et al., 2015). The inhibition constant (K_I) is directly related to the binding energy. In that context, it is determine how much of the peptide is needed to inhibit the enzyme (Fan et al., 2013). In general, chia peptides demonstrated a low K_I, which reinforces the promising effects of these compounds in the diet.

A high antioxidant effect of digested protein by high scavenging ability and acting similarly to known potent antioxidants (trolox and

ascorbic acid) was observed. The superoxide and hydrogen peroxide are produced by alterations of the mitochondrial electron transport chain that causes oxidation of cell membranes which is associated with several pathologies (Román, Urra, Porras, Pino, Rosen, Rodríguez, 2017). In our study, digested glutelin showed the best results for superoxide scavenging capacity, and digested albumin and DTP for DPPH. Such findings are in line with results by other researchers (Orona-Tamayo et al., 2015).

In the inflammatory process, the enzyme iNOS is activated and produce NO, a key mediator of inflammation. The mediators of arachidonic acid cascade from COX and LOX pathways are also responsible of inflammation and of several associated diseases (Khodabandehloo et al., 2016). Digested glutelin, globulin, and prolamin were the best protein fractions to inhibit 5-LOX. Peptides from digested albumin were the best to inhibit iNOS activity and for scavenging its metabolite NO, as well as reduce the activity of COX-2.

The pharmacological controls used in this study show therapeutic effects. Statins, such as simvastatin, are drugs that show cholesterol-lowering properties and lipid-independent pleiotropic effects (Hofnagel, Luechtenborg, Weissen-Plenz, & Robenek, 2007). Dexamethasone is a synthetic glucocorticoid with anti-inflammatory and immunosuppressant effects. This compound is used to treat many inflammatory conditions, such as allergic disorders, skin conditions, ulcerative colitis, arthritis, lupus, psoriasis, and breathing disorders (Boumpas, Chrousos, Wilder, Cupps, & Balow, 1993). The aromatic diamine 4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) inhibits nuclear translocation of the NF-κB p65/p50 heterodimer, without affecting IκBα degradation (Shin et al., 2004). Furthermore, the administration of atorvastatin attenuated the activation of toll-like receptor (TLR4) expression and the NF-κB signaling in a lipopolysaccharide-stimulated human cell line (Yilmaz et al., 2006).

In general, digested chia proteins reduced the activity of COX-1 (constitutive), but DTP and digested globulin showed less preference to inhibit this enzyme; this is the same phenomenon showed when using anti-inflammatory drugs (Khodabandehloo et al., 2016). These results demonstrated the promising positive activity of chia peptides produced after digestion of chia proteins provided in the diet.

5. Conclusions

Chia seed showed a high concentration of protein. After simulated gastrointestinal digestion, total protein and specific protein fractions yielded numerous peptides. These peptides, especially from albumin, had promising health benefits as confirmed by *in silico* analyses. Digested total protein showed antioxidant and anti-inflammatory effects, but isolated proteins, mainly digested albumin and glutelin, were more powerful. These results are innovative since, for the first time, the sequence of peptides from different protein fractions of chia seed are provided, and highlight the potential beneficial health effects of chia seed proteins if consumed as part of the diet.

Conflict of interest

The authors do not have any conflict of interest.

Acknowledgements

To the “Conselho Nacional de Ciência e Tecnologia Brasileira (CNPq)” – Brazil for MG’s scholarship [grant number 200739/2017-4] and HSDM Research Productivity’s fellowships. This study was financed in part by the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior”(CAPES)- Brazil [grant number 001]. The main funding was provided by ACES International Joint Research Program, University of Illinois, United States [Research was supported by the USDA-NIFA-HATCH project 1014457, United States].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.036.

References

Alves, N. E. G., Vasconcelos, C. M., Bassinello, P. Z., de Mejía, E. G., & Martino, H. S. D. (2016). Digested protein isolate from fresh and stored Carioca beans reduced markers of atherosclerosis in oxidized LDL-induced THP-1 macrophages. *Journal of Functional Foods, 24*, 97–111. https://doi.org/10.1016/j.jff.2016.03.027.

AOAC (2012). *Official Methods of Analysis of AOAC International: Agricultural Chemicals, Contaminants, Drugs.* Gaithersburg: AOAC International.

Ayerza, R. (2005). *Chia: Rediscovering a Forgotten Crop of the Aztecs.* Arizona: University of Arizona Press.

Boumpas, D. T., Chrousos, G. P., Wilder, R. L., Cupps, T. R., & Balow, J. E. (1993). Glucocorticoid therapy for immune-mediated diseases: Basic and clinical correlates. *Annals of Internal Medicine, 119*(12), 1198–1208. https://doi.org/10.7326/0003-4819-119-12-199312150-00007.

Brandelli, A., Daroit, D. J., & Correa, A. P. F. (2015). Whey as a source of peptides with remarkable biological activities. *Food Research International, 73*, 149–161. https://doi.org/10.1016/j.foodres.2015.01.016.

Capitani, M. I., Spotorno, V., Nolasco, S. M., & Tomás, M. C. (2012). Physicochemical and functional characterization of by-products from chia (Salvia hispanica L.) seeds of Argentina. *LWT – Food Science and Technology, 45*(1), 94–102. https://doi.org/10.1016/j.lwt.2011.07.012.

Chakrabarti, Subhadeep, Jahandideh, Forough, & Wu, Jianping (2014). Food-derived bioactive peptides on inflammation and oxidative stress. *BioMed Research International, 2014*, 1–11. https://doi.org/10.1155/2014/608979.

Cicero, A. F. G., Fogacci, F., & Colletti, A. (2017). Potential role of bioactive peptides in prevention and treatment of chronic diseases: A narrative review. *British Journal of Pharmacology, 174*(11), 1378–1394. https://doi.org/10.1111/bph.13608.

Coelho, M. S., Soares-Freitas, R. A. M., Areas, J. A. G., Gandra, E. A., Salas-Mellado, M., & de las, M. (2018). Peptides from Chia present antibacterial activity and ihibit cholesterol synthesis. *Plant Foods for Human Nutrition, 73*, 101–107. https://doi.org/10.1007/s11130-018-0668-z.

Cugno, M., Tedeschi, A., & Nussberger, J. (2016). Bradykinin in idiopathic non-histaminergic angioedema. *Clinical and Experimental Allergy, 47*(1), 139–140. https://doi.org/10.1111/cea.12869.

da Silva, B. P., Anunciação, P. C., Matyelka, J. C. S., Della Lucia, C. M., Martino, H. S. D., & Pinheiro-Sant’Ana, H. M. (2017). Chemical composition of Brazilian chia seeds grown in different places. *Food Chemistry, 221*, 1709–1716. https://doi.org/10.1016/j.foodchem.2016.10.115.

Ewing, J. F., & Janero, D. R. (1995). Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Analytical Biochemistry, 232*(2), 243–248. https://doi.org/10.1006/abio.1995.0014.

Fan, J., Johnson, M. H., Lila, M. A., Yousef, G., & De Mejia, E. G. (2013). Berry and citrus phenolic compounds inhibit dipeptidyl peptidase IV: Implications in diabetes management. *Evidence-Based Complementary and Alternative Medicine, 2013*, 479505. https://doi.org/10.1155/2013/479505.

Fruton, J. S. (2002). A history of pepsin and related enzymes. *The Quarterly Review of Biology, 77*(2), 127–147. https://doi.org/10.1086/340729.

Gardner, J. D., & Jensen, R. T. (1984). Cholecystokinin receptor antagonists. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 246*(5), G471–G476. https://doi.org/10.1152/ajpgi.1984.246.5.G471.

Hofnagel, O., Luechtenborg, B., Weissen-Plenz, G., & Robenek, H. (2007). Statins and foam cell formation: Impact on LDL oxidation and uptake of oxidized lipoproteins via scavenger receptors. *Biochima et Biophysica Acta, 1771*, 1117–1124.

Ixtaina, V. Y., Nolasco, S. M., & Tom, M. C. (2008). Physical properties of chia (Salvia hispanica L.) seeds. *Industrial Crops and Products, 28*, 286–293. https://doi.org/10.1016/j.indcrop.2008.03.009.

Kačmárová, K., Lavová, B., Socha, P., & Urminská, D. (2016). Characterization of protein fractions and antioxidant activity of chia seeds (Salvia hispanica L.). *Potravinarstvo Slovak Journal of Food Sciences, 10*(1), 78–82. https://doi.org/10.5219/563.

Khodabandehloo, H., Gorgani-Firuzjaee, S., Panahi, G., & Meshkani, R. (2016). Molecular and cellular mechanisms linking inflammation to insulin resistance and β-cell dysfunction. *Translational Research, 167*(1), 228–256.

Kou, X., Gao, J., Xue, Z., Zhang, Z., Wang, H., & Wang, X. (2013). Purification and identification of antioxidant peptides from chickpea (Cicer arietinum L.) albumin hydrolysates. *LWT – Food Science and Technology, 50*(2), 591–598. https://doi.org/10.1016/j.lwt.2012.08.002.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275. https://doi.org/10.1016/0304-3894(92)87011-4.

Megías, C., del Mar Yust, M., Pedroche, J., Lquari, H., Girón-Calle, J., Alaiz, M., … Vioque, J. (2004). Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. *Journal of Agricultural and Food Chemistry, 52*(7), 1928–1932.

Mojica, L., Chen, K., & de Mejía, E. G. (2015). Impact of commercial precooking of common bean (Phaseolus vulgaris) on the generation of peptides, after pepsin-pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase-IV. *Journal of Food Science, 80*(1), H188–H198. https://doi.org/10.1111/1750-3841.12726.

Mojica, L., Luna-Vital, D. A., & González de Mejía, E. (2017). Characterization of peptides from common bean protein isolates and their potential to inhibit markers of type-2

diabetes, hypertension and oxidative stress. *Journal of the Science of Food and Agriculture, 97*(8), 2401–2410. https://doi.org/10.1002/jsfa.8053.

Montoya-Rodríguez, A., de Mejía, E. G., Dia, V. P., Reyes-Moreno, C., & Milán-Carrillo, J. (2014). Extrusion improved the anti-inflammatory effect of amaranth (Amaranthus hypochondriacus) hydrolysates in LPS-induced human THP-1 macrophage-like and mouse RAW 264.7 macrophages by preventing activation of NF-κB signaling. *Molecular Nutrition and Food Research, 58*(5), 1028–1041. https://doi.org/10.1002/mnfr.201300764.

Morettini, M., Storm, F., Sacchetti, M., Cappozzo, A., & Mazzà, C. (2015). Effects of walking on low-grade inflammation and their implications for type 2 diabetes. *Preventive Medicine Reports, 2*, 538–547. https://doi.org/10.1016/j.pmedr.2015.06.012.

Muñoz, E. B., Luna-Vital, D. A., Fornasini, M., Baldeón, M. E., & Gonzalez de Mejia, E. (2018). Gamma-conglutin peptides from Andean lupin legume (Lupinus mutabilis Sweet) enhanced glucose uptake and reduced gluconeogenesis in vitro. *Journal of Functional Foods, 45*, 339–347. https://doi.org/10.1016/j.jff.2018.04.021.

Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science, 66*(5), 642–646.

Orona-Tamayo, D., Valverde, M. E., Nieto-Rendón, B., & Paredes-López, O. (2015). Inhibitory activity of chia (Salvia hispanica L.) protein fractions against angiotensin I-converting enzyme and antioxidant capacity. *LWT – Food Science and Technology, 64*(1), 236–242. https://doi.org/10.1016/j.lwt.2015.05.033.

Román, F., Urra, C., Porras, O., Pino, A. M., Rosen, C. J., & Rodríguez, J. P. (2017). Real-time H_2O_2 measurements in bone marrow mesenchymal stem cells (MSCs) show increased antioxidant capacity in cells from osteoporotic women. *Journal of Cellular Biochemistry, 118*(3), 585–593. https://doi.org/10.1002/jcb.25739.

Ruch, R. J., Cheng, S-j, & Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis, 10*(6), 1003–1008. https://doi.org/10.1093/carcin/10.6.1003.

Sacks, F. M., Lichtenstein, A. H., Wu, J. H. Y., Appel, L. J., Creager, M. A., Kris-Etherton, P. M., ... Van Horn, L. V. (2017). Dietary fats and cardiovascular disease: A presidential advisory from the American Heart Association. *Circulation, 136*(3), e1–e23. https://doi.org/10.1161/CIR.0000000000000510.

Sandoval-Oliveros, M. R., & Paredes-López, O. (2013). Isolation and characterization of proteins from chia seeds (Salvia hispanica L.). *Journal of Agricultural and Food Chemistry, 61*(1), 193–201. https://doi.org/10.1021/jf3034978.

Segura-Campos, M. R., Salazar-Vega, I. M., Chel-Guerrero, L. A., & Betancur-Ancona, D. A. (2013). Biological potential of chia (Salvia hispanica L.) protein hydrolysates and their incorporation into functional foods. *LWT – Food Science and Technology, 50*(2), 723–731. https://doi.org/10.1016/j.lwt.2012.07.017.

Shin, H. M., Kim, M. H., Kim, B. H., Jung, S. H., Kim, Y. S., Park, H. J., ... Kim, Y. (2004). Inhibitory action of novel aromatic diamine compound on lipopoly- saccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Letters, 571*, 50–54.

Sun, H., Cai, W., Wang, X., Liu, Y., Hou, B., Zhu, X., & Qiu, L. (2017). Vaccaria hypaphorine alleviates lipopolysaccharide-induced inflammation via inactivation of NFKB and ERK pathways in Raw 264.7 cells. *BMC Complementary and Alternative Medicine, 17*(1), 120. https://doi.org/10.1186/s12906-017-1635-1.

Thongnak, L., Pongchaidecha, A., Jaikumkao, K., Chatsudthipong, V., Chattipakorn, N., & Lungkaphin, A. (2017). The additive effects of atorvastatin and insulin on renal function and renal organic anion transporter 3 function in diabetic rats. *Scientific Reports, 7*(1), 13532.

Tsai, M. L., Lin, C. C., Lin, W. C., & Yang, C. H. (2011). Antimicrobial, antioxidant, and anti-inflammatory activities of essential oils from five selected herbs. *Bioscience, Biotechnology, and Biochemistry, 75*(10), 1977–1983. https://doi.org/10.1038/s41598-017-13206-5.

Wang, Q., Dong, X., Li, N., Wang, Y., Guan, X., Lin, Y., ... Xu, T. (2018). JSH-23 prevents depressive-like behaviors in mice subjected to chronic mild stress: Effects on inflammation and antioxidant defense in the hippocampus. *Pharmacology Biochemistry and Behavior, 169*, 59–66. https://doi.org/10.1016/j.pbb.2018.04.005.

Yilmaz, A., Reiss, C., Weng, A., Cicha, I., Stumpf, C., Steinkasserer, A., ... Garlichs, C. D. (2006). Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *Journal of Leukocytic Biology, 79*, 529–538.