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Chia (*Salvia hispanica* L.) Seed Total Protein and Protein Fractions Digests Reduce Biomarkers of Inflammation and Atherosclerosis in Macrophages In Vitro

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Scope: The objectives are to evaluate the anti-inflammatory and anti-atherosclerotic effects of digested total protein and digested protein fractions from chia seed in macrophages in vitro.
Methods and results: Total protein and protein fractions (albumin, globulin, glutelin, and prolamin) are isolated from chia seed and digested using simulated gastrointestinal conditions, resulting in digested total protein (DTP) and digested protein fractions (DPF). DTP and DPF are applied (1.0 mg mL^{-1}) in RAW 264.4 macrophages stimulated with LPS (1 μg mL^{-1}) for inflammation or ox-LDL (80 μg mL^{-1}) for atherosclerosis. In the inflammatory process, DTP and DPF reduce p-NF-κB, iNOS, p-JNK, and AP-1. Digested glutelin reduces the secretion of nitric oxide (65.1%), reactive oxygen species (19.7%), prostaglandins (34.6%), TNF-α (24.1%), MCP-1 (18.9%), IL-6 (39.6%), and IL-10 (68.7%). DTP and DPF reduce the NF-κB translocation to nuclei. DTP and digested glutelin reduce iCAM expression (86.4%, 80.8%), LOX-1 (37.3%, 35.7%), iNOS (67.0%, 42.2%), and NF-κB (57.5%, 71.1%). DTP is effective in reducing secretion of nitric oxide (43.4%), lipid accumulation (41.9%), prostaglandins (41.9%), TNF-α (43.3%), MCP-1 (47.6%), and IL-6 (50.5%). Peptides from chia DTP and DPF are also characterized.
Conclusion: DTP and digested glutelin from chia seed reduce expression and secretion of markers related to inflammation and atherosclerosis pathways.

1. Introduction

Inflammation and atherosclerosis are directly associated with the development of cardiovascular disease (CVD),[1] a main cause of mortality worldwide, representing around 17.3 million deaths per year.[2] Chronic inflammation is a complex and multisystem event affecting a wide range of cells, tissues, and organs. Macrophages are the main cells involved in the inflammatory condition, secreting mediators such as cytokines, chemokines, reactive oxygen species (ROS), and adhesion molecules, which are directly linked with the development and progress of chronic inflammation. The main activated intracellular signaling pathways, which lead to inflammation, are nuclear factor-kappa B (NF-κB), mitogen-activated protein (MAP) kinases, and activator protein-1 (AP-1).[1]

Atherosclerosis is a condition characterized by the formation of an atherosclerotic plaque, formed by deposition of lipids, cell infiltration, and cells proliferation on the intima layer of the arteries.[3] The high accumulation of lipids and cells result in vulnerable plaques that reduce the flexibility of arteries and obstruct blood circulation, thereby increasing blood pressure. The plaque can also become unstable and thus rupture, leading to thrombosis, myocardial infarction, or stroke.[4] Inflammation and atherosclerosis are closely related, since inflammation plays a role in all atherogenesis steps, like foam cell accumulation, fibrous plaque formation, acute plaque fissuring, rupture, and thrombosis.[5]

Diet has an impact on non-communicable diseases such as hypertension, obesity, diabetes, and CVD, being able to aggravate or prevent their development, depending on composition.[6] The consumption of protein can provide bioactive peptides, derived from fermentation, enzymatic hydrolysis, chemical hydrolysis, or gastrointestinal digestion of food proteins[7] that could prevent the occurrence of inflammation and subsequent atherosclerosis. An enzymatic digestion of protein made with trypsin, chymotrypsin, and pepsin demonstrated the physiological process of protein hydrolysis and generation of peptides in humans.[8]

Chia seed (*Salvia hispanica* L.) is a rich source of protein (18.9%), greater than in other traditional grains[9,10] and therefore represents a promising source of bioactive peptides. Chia is an herbaceous plant native to Mexico and Central America, which supplies seeds that are noteworthy due their high nutritional and functional value.[11] The main storage protein fractions present in chia are albumin, globulin, glutelin, and prolamin. These storage proteins are responsible for supplying nitrogen necessary for biosynthesis of metabolically active plants.[12] Chia seeds contain

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every essential amino acid for human nutrition. The protein fractions in chia seed contain mostly 11S and 7S proteins with a molecular weight from 15 to 50 kDa, under native conditions.[13] A total of 20 proteins were compiled in chia seed, 12 of those were involved in the regular metabolic processes of the plant cells and 8 were related to production and storage of plant lipids, which can explain the amount of lipids in chia seed.[14]

In human studies, the consumption of chia was related to several beneficial effects such as improvement of insulin resistance,[15] reduction of arterial pressure, prevention of lipid peroxidation, decrease of plasma nitrite concentrations,[16] and C-reactive protein, and increase in adiponectin.[17] The effects of peptides derived from chia protein have been demonstrated by biochemical indicators. They include inhibition of ACE (angiotensin-converting enzyme),[18–20] antioxidant capacity, antibacterial properties, and anti-cholesterolemic effects,[20,21] but there are not studies of the effects of chia peptides at the cellular level.

In sum, despite the existing evidence about the beneficial effects of whole chia seed or its bioactive peptides by biochemical analysis, there is no evidence about the potential action of its bioactive peptides on inflammation and atherosclerosis processes in vitro. Furthermore, the cellular synergistic effect of peptides from the totality of proteins in chia versus peptides from isolated chia protein fractions is unknown.

The objectives were to determine the effect of chia (*S. hispanica* L.) seed digested total protein and digested protein fractions (albumin, globulin, glutelin, and prolamin) on inflammation and atherosclerosis in macrophages in vitro, and their mechanism of action. The hypothesis was that the digested total protein and digested protein fractions from chia seed can reduce the expression of proteins and the secretion of markers related with the development and progression of inflammation and the atherosclerosis process in macrophages.

2. Experimental Section

2.1. Materials

RAW 264.7 cells, from a mouse monocytic-derived cell line, were used as the in vitro cell model. They were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The Dulbecco's modified Eagle medium (DMEM) was purchased from Corning cellgro (Manassas, VA, USA), fetal bovine serum (FBS) and penicillin–streptomycin (100×) were obtained from Gibco Life Technologies (Grand Island, NY, USA). Primary antibodies NF-κB p65 (nuclear factor kappa-light-chain-enhancer of activated B cells, sc-8008), p-NF-κB p65 (Ser536, sc-136548), p-JNK (sc-6254), JNK (sc-7345), MMP-9 (matrix metallopeptidase 9, sc-13520), and RIPA lysis buffer system were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody iNOS (inducible nitric oxide synthase, PA1-036), LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1, MA5-23895), COX-2 (cyclo-oxygenase enzyme-2, MA5-14568), iNOS (710278), GAPDH (glyceraldehyde-3-phosphate dehydrogenase, MA5-15738), Alexa Fluor 488 goat anti-mouse IgG (H+L), and H2DCFDA (2′,7′-dichlorofluorescin diacetate, D339) were obtained from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

2.2. Chia Total Protein Preparation

Chia seeds grown in Rio Grande do Sul/Brazil were prepared as decribed by Orona-Tamayo et al.[18] with modifications. The seeds were immersed in distilled water (1:10, g: mL) for 1 h for mucilage formation and freeze-dried (Labconco Freeze Dryer 4.5; Kansas, MO, USA). The mucilage was manually removed from the seeds with the aid of a sieve (500 µm per 35 mesh). The free-mucilage seeds were ground using a coffee grinder (Mr. Coffee), sieved (500 µm per 35 mesh), and 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) and the resulted flour was left overnight under a hood and then stored at 4 °C until use.

Deionized water was added to the mucilage and fat-free chia flour (1:20, g: mL), the pH was adjusted to 8.0 and placed under constant stirring (35 °C per 1 h). The mixture was centrifuged (5000 × *g*; 15 min; 25 °C) and the supernatant collected, freeze-dried, and stored at −20 °C (Figure S1, Supporting Information).

2.3. Chia Protein Fractions Preparation

The storage protein fractions from chia seeds were isolated according to the Osbore[22] classification using the method reported by Orona-Tamayo[18] and Sandoval-Oliveros & Paredes-López.[13] Briefly, the mucilage-free 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 labeled as the albumin fraction. The resulting pellet was re-suspended with 0.05 mol L^{-1} Tris-hydrochloric acid (HCl) + 0.5 mol L^{-1} sodium chloride (NaCl) (pH 8.0) (1:10, g:mL), mixed, centrifuged, as above, and the supernatant defined as globulin fraction. The precipitate was diluted with isopropanol 70% (1:10, g: mL), processed as above, and the supernatant was then labeled as prolamins fraction. Finally, the resulting pellet was added with 0.1 mol L^{-1} sodium tetraborate decahydrate ($Na_2B_4O_7$-H_2O) (pH 10.0) (1:10, g: mL), processed as above, and the supernatant was then named as glutelin fraction (Figure S1, Supporting Information). All sample were freeze-dried, stored at −20 °C, and used within 7 months.

2.4. Simulated Gastrointestinal Digestion

The simulated gastrointestinal digestion was conducted using the procedure outlined by Megías et al.,[23] with adaptations. The total protein and protein fractions were suspended in deionized water (1:20, g:mL), the pH adjusted to 2.0, and pepsin added in concentration 1:20 (enzyme:protein) and kept under stirring for 2 h, at 37 °C. After the pH was adjusted to 7.5, pancreatin was added (1:20 enzyme:protein) and the digestion was then conducted as above. The simulated digestion was stopped by heating the suspension on a water bath (75 °C, 20 min). The samples were centrifuged twice at 20 000 × *g* for 15 min at

4 °C and the supernatant was collected and dialyzed using a 100–500 Da molecular weight cut-off membrane (Spectra/Por, Biotech CE Membrane) and freeze-dried. The proteins were labeled as digested total protein (DTP) and digested protein fraction (DPF) and stored at −20 °C until analysis.

2.5. Identification, Characterization, and Bioactive Potential of Peptides from Chia

The peptides from DTP and DPF resulting from the simulated gastrointestinal digestion, were analyzed 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: 95% of acetonitrile, 5% of water, and 0.1% of formic acid. The volume of injection was 200 µL min^{-1} and PDA detector wavelength at 280 nm.[24] The results were 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 peptides with more than 90% sequence probability had the biological activity predicted by BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed on February 27, 2018). The parental protein was identified with BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on July 30, 2018). The amino acids were presented in one letter nomenclature.

2.6. Monocyte Treatment with Chia Seed Total Protein and Protein Fractions Digests

The RAW 267.4 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO_2/95% air using a CO_2 Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). A concentration of 2.7×10^4 cells was seed in 96-well plate and tested in concentrations of 0.1, 0.5, and 1.0 mg mL^{-1} of DTP or DPF. The efficiency of the cells growing in the presence of all treatments was assessed by aqueous solution CellTiter 96 one proliferation assay kit- MTS (Promega Corporation, Madison, WI, USA). The best doses (0.1, 0.5, or 1.0 mg mL^{-1}) for DTP and DPF that did not decrease the levels of nitric oxide and did not reduce cell viability were used in all analyses.

To test the anti-inflammatory potential of DTP and DPF, RAW 264.7 macrophages were seeded at 2.5×10^5 in a six-well plate, and the total volume then adjusted to 2 mL with growth medium and incubated for 24 h at 37 °C in 5% CO_2/95%. After incubation, the cells were treated with the lipopolysaccharide (LPS) (1.0 µM) and either DTP or each DPF for 24 h.

For the analysis of anti-atherosclerosis potential, the RAW 264.7 macrophages were seeded at 2×10^5 in a six-well plate, and the total volume adjusted to 2 mL with growth medium and incubated to 24 h at 37 °C in 5% CO_2/95%, followed by another 24 h starve. The cells were treated with oxidized low-density lipoprotein (ox-LDL, 80 µg mL^{-1}) for 48 h with/without DTP or each DPF.

In both experiments, a negative control (NC) group did not receive any treatment on culture media and a positive group (PC) was treated only with either ox-LDL or LPS. As a pharmacological control (FC) group, the cells were treated with dexamethasone (1 µM) and LPS (inflammation) or simvastatin (0.1 µM) and ox-LDL (atherosclerosis).

After treatment for 48 h (atherosclerosis)/24 h (inflammation), the growth medium and cell lysates were collected and frozen at −80 °C until analysis. All experiments were performed in duplicate.

2.7. Effect of Chia Seed Total Protein and Protein Fractions Digests on Nitric Oxide Production

The nitric oxide (NO) production was determined by the accumulation of nitrite (NO_2), a stable product of NO reaction with oxygen in aqueous solution. The accumulation of nitrite in the culture supernatant was measured by the Griess reaction, as described by Green et al.,[25] with modifications. Dosing of NO_2^- was performed in a 96-well microplate. The reaction was inhibited by adding 100 µL of the culture supernatant to the same volume of the Griess reagent (Sigma) and incubating at room temperature for 10 min. The absorbance was determined at 540 nm in a microplate reader (BioTek, Winnoski, USA). The concentration of NO_2^- was established from a standard curve of sodium nitrite ($NaNO_2$) (0.4–100 µM) established for each experiment ($y = 0.0087x + 0.0027$, $R^2 = 0.99$).

2.8. Influence of Chia Seed Total Protein and Protein Fractions Digests on Reactive Oxygen Species

To determine the ability of the protein digests to inhibit the production of ROS, 2.5×10^4 cells were seeded in dark 96-well plates in triplicate. After 24 h, the cells were treated with samples and controls as described in Section 2.6. *N*-acetyl-cysteine (15 µM) was used as standard control. The cells were followed by 48 h of incubation (anti-atherosclerotic effect) or 24 h (anti-inflammatory effect). One hour prior to completion of the treatment, the media was removed and DCFDA in culture media (50 µM per total volume) was loaded in all wells. After this period, the plate was transferred to the microplate reader without washing and read with excitation wavelength at 485 nm and emission wavelength at 535 nm. Results were expressed as fluorescence intensity.

2.9. Impact of Chia Seed Total Protein and Protein Fractions Digests on Prostaglandin-2, TNF-α, MCP-1, and Cytokines Secretion

Commercial kits were used to analyze prostaglandin-2 (PGE-2, 500141) (Cayman Chemical), tumor necrosis factor alpha (TNF-α, DY008), monocytes chemoattractant protein-1 (MCP-1, DY479-05), interleukin-10 (IL-10, DY417-05), IL-12 (DY419-05), and IL-6 (DY406-05) (R&D Systems), following the

manufacturer's instructions. The cell culture supernatant was diluted 1:50 (v/v, sample:buffer) for TNF-α and PGE-2, 1:10 for MCP-1 and 1:25 for cytokines. The amount of PGE-2 ($y = -0.2766x + 0.3636$, $R^2 = 0.99$), TNF-α ($y = 0.7991x - 2.0792$, $R^2 = 0.99$), MCP-1 ($y = 0.7074x - 1.5536$, $R^2 = 0.98$), IL-6 ($y = 0.7681x - 2.4798$, $R^2 = 0.99$); IL-10 ($y = 0.7159x - 1.2982$, $R^2 = 0.99$), IL-12 ($y = 0.7433x - 1.8984$, $R^2 = 0.99$) were calculated using $\log_{10}$, including their respective standard curves that was run at the same time as the treatments. Absorbance was determined at 450 nm and results were expressed in pg mL^{-1}.

2.10. Influence of Chia Seed Total Protein and Protein Fractions Digests on the Expression of Proteins Related to Inflammation and Atherosclerosis Pathways

Cell lysates were used for western blot to measure the expression of proteins related to inflammation and the atherosclerosis process in the cells. Briefly, after treatments, the cell culture supernatant was collected and immediately frozen at −80 °C. The cells were lysed with RIPA lysis buffer, sonicated, and added with Laemmli buffer (Bio-Rad) containing 5% β-mercaptoethanol. Protein concentration was quantified using RC-DC Assay (Bio-Rad) and 20 µg protein was loaded in 4–20% Tris-HCl gels (Bio-Rad) for protein separation. Then, proteins were transferred to a PVDF membrane (polyvinylidene difluoride membrane, Hybond-P, Millipore, Billerica, MA, USA) and incubated with respective primary antibodies (1:500) (COX-2, iNOS, p-p65-NF-κB, p65-NF-κB, p-JNK, JNK, LOX-1, MMP-9, or ICAM-1) at 4 °C overnight. The membranes were incubated with secondary antibody for 2 h (if required) and the proteins bands visualized with a GL 4000 Pro Imaging system (Carestream Health Inc., Rochester, NY, USA). The intensity of the bands of each protein was normalized using GAPDH protein present in each well, as a protein loading control, and all analyses were performed at least in duplicate. The calculation was performed by dividing the intensity of the protein in question by the intensity of GAPDH, in each well. This value was expressed in comparison to the positive control, which was considered 100%.[3]

2.11. Effect of Chia Seed Total Protein and Protein Fractions Digests on Nuclear Translocation of NF-κB p65 in the Inflammatory Process

Immunofluorescence and confocal laser-scanning microscopy were used to evaluate the nuclear translocation of NF-κB p65 in the inflammatory process and the effects of the digested proteins. RAW 264.7 cells were seeded (3×10^5) onto ibiTreat µ-slide eight-well chambers. The macrophages were treated according to the conditions indicated in Section 2.6 for the inflammatory process. After 24 h of treatment, the cells were fixed by 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences, Hatfield, PA, USA) and permeabilized with 0.5% Triton X-100. The cells were blocked with Image-iT FX Signal Enhancer (Invitrogen), followed by incubation with NF-κB p65 primary antibody (1:50) overnight at 37 °C. The cells were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:200) and cured with Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The slides were stored at 4 °C in the dark until analysis.

Images were acquired by Zeiss LSM 880 laser-scanning confocal microscope (Carl Zeiss AG, Germany) using a Plan-Apochromat 63×/1.4 Oil DIC M27 objective and Laser at 488 nm. Fluorescence intensity was determined in the nucleus and normalized to DAPI staining using the Zeiss Pro program. The values were expressed in comparison to the positive control, which was considered 100%.

2.12. Influence of Chia Seed Total Protein and Protein Fractions Digests in the Formation of Foam Cells in the Atherosclerotic Process

The analysis of foam cells formation was performed as described by Xu et al.,[26] with modifications. Briefly, a concentration of 2.7×10^4 cells was seeded in a 96-well plate and were treated according to Section 2.6 for atherosclerosis process; the cells were fixed in 10% formalin for 10 min. Then, the cells were rinsed in PBS, followed by 60% isopropanol for 15 s and stained with Oil Red-O working solution at 37 °C for 10 min in darkness. After, the cells were destined with 60% isopropanol for 15 s and washed with PBS three times. Finally, the Oil-Red O was diluted with 100% isopropanol, incubated about 10 min and transferred to 96-well plates, and read at 510 nm.

2.13. Potential Inhibitory Interactions of iCAM and CCR2 by Peptides from Total Protein and Protein Fractions Digests: In Silico Analyses

Based on the characterization of peptides present in each digest, the interactions of single peptides from total protein and protein fractions from chia seed with intracellular adhesion molecules (iCAM) and C-C chemokine receptor (CCR2) were evaluated by in silico analysis. Only peptides with antioxidant activity determined by the biological potential (BIOPEP database) and with 100% parental protein identification from *S. hispanica* L. (BLAST tool) were selected. Peptides were designed using Instant MarvinSketch (ChemAxon Ltd). The crystal structure file of iCAM, CCR2 was obtained from the Protein Data Bank (PDB: 1IAM and 5T1A, respectively). Flexible torsions, charges, and the grid size were assigned by AutoDock Tools[27] and the docking calculations were performed using AutoDock Vina.[28] The binding pose with the lowest binding energy (highest binding affinity) was selected as a representative image to visualize in the Discovery Studio 2016 Client (Dassault Systemes Biovia Corp.).

2.14. Statistical Analysis

Results are presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey test. Differences were considered significant at $p < 0.05$. The statistical analysis was performed using GraphPad Prism 7. The analysis were performed in triplicate in at least two independent experiments.

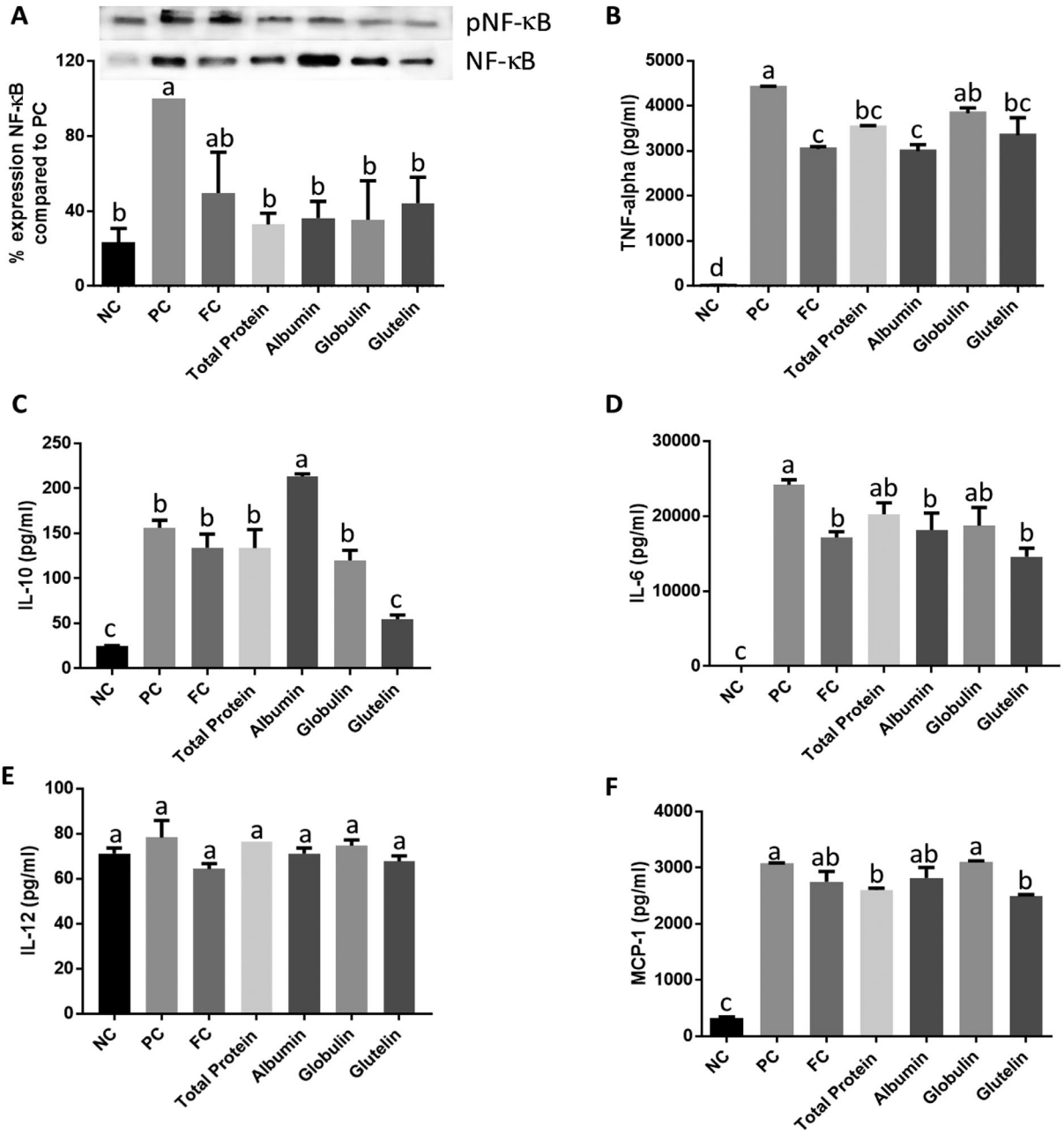

Figure 1. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of A) NF-κB by Western blotting, and secretion of B) TNF-α, C) IL-10, D) IL-6, E) IL-12, and F) MCP-1 by ELISA analysis, of LPS-stimulated RAW 264.7 macrophages. The net intensity of markers was normalized by net intensity of GAPDH (37 kDa). All experiments were performed in triplicate from at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain LPS (1 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

3. Results

3.1. Chia Seed Total Protein and Protein Fractions Digests Reduced Nitric Oxide Excretion in the Inflammatory Process and Had No Effects on Cell Viability

All treatments tested, 0.1, 0.5, and 1.0 mg mL^{-1} of DTP and DPF, promoted cell viability similarly as in cells without treatment (negative control-NC) (Figure S2A, Supporting Information), averaging more than 100% for both. The nitric oxide excretion was analyzed and for DTP, digested albumin, globulin, and glutelin, the 1.0 mg mL^{-1} of digested protein was the best dose that reduced this inflammation marker (Figure S2B, Supporting Information). This was then the concentration used in the following experiments.

For the atherosclerosis process, the same dose used in the inflammation processes, 1.0 mg mL^{-1}, showed cell viability similar or higher than NC and was used in the following experiments

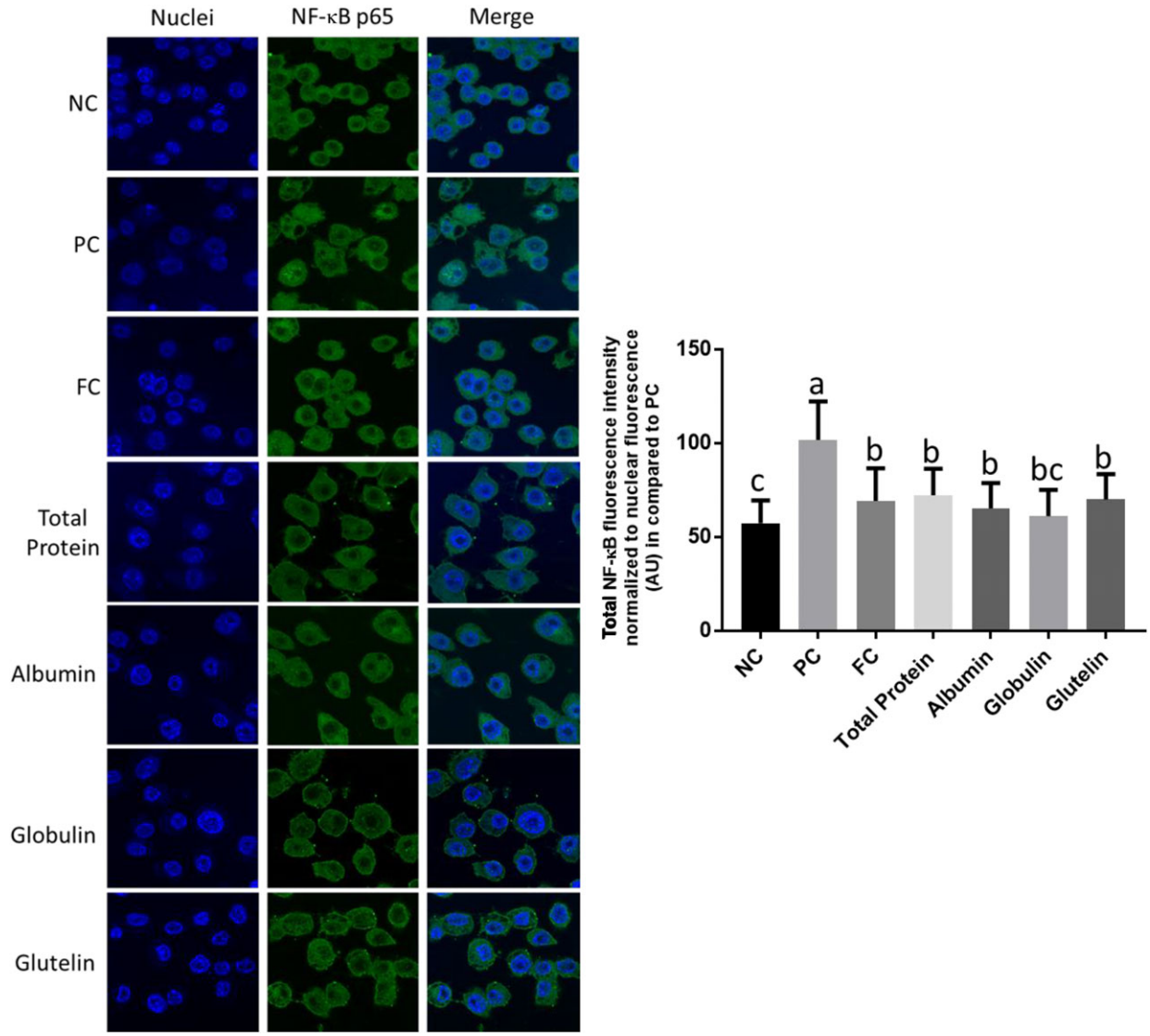

Figure 2. Confocal laser scanning microscopy depicting 2D immunocytochemical localization of NF-κB (green) normalized by nuclei (blue) in RAW264.7 macrophages after 24 h of treatment with digested total protein and digested albumin, globulin, and glutelin from chia seeds. Ten independent fields of view from two independent cellular replicates were merged together per treatment group. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain LPS (1 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

(Figure S3, Supporting Information). Table S1, Supporting Information presents the peptides discovered in chia seed total protein and protein fractions digests.

3.2. Effects of Chia Seed Total Protein and Protein Fractions Digests on the Inflammatory Process

3.2.1. Chia Seed Total Protein and Protein Fractions Digests Inhibited the Expression of Proteins Related with NF-κB Pathway, Reduced Its Translocation to Nuclei, and Reduced the Secretion of Inflammation-Mediators

DTP and DPF decreased the activity of NF-κB by a decline of phosphorylated proteins (**Figure 1**A). Confirming results presented in Figure 1A, we observed a reduction in the translocation of NF-κB to nuclei by all digested proteins, and the digested globulin was similar to NC (**Figure 2**).

DTP and digested albumin and glutelin reduced TNF-α (Figure 1B). Digested glutelin produced the lowest IL-10 value (Figure 1C). Moreover, digested albumin and glutelin decreased IL-6 secretion in comparison to PC (Figure 1D). A low secretion of IL-12 was observed by all treatments including PC (Figure 1E). In addition, DTP and digested glutelin reduced MCP-1 secretion in comparison to PC (Figure 1F).

DTP and DPF lowered the expression of iNOS in the cells, especially digested glutelin that was similar to NC (**Figure 3**A). All digested proteins inhibited NO secretion, especially digested glutelin, and ROS production (Figure 3B,C).

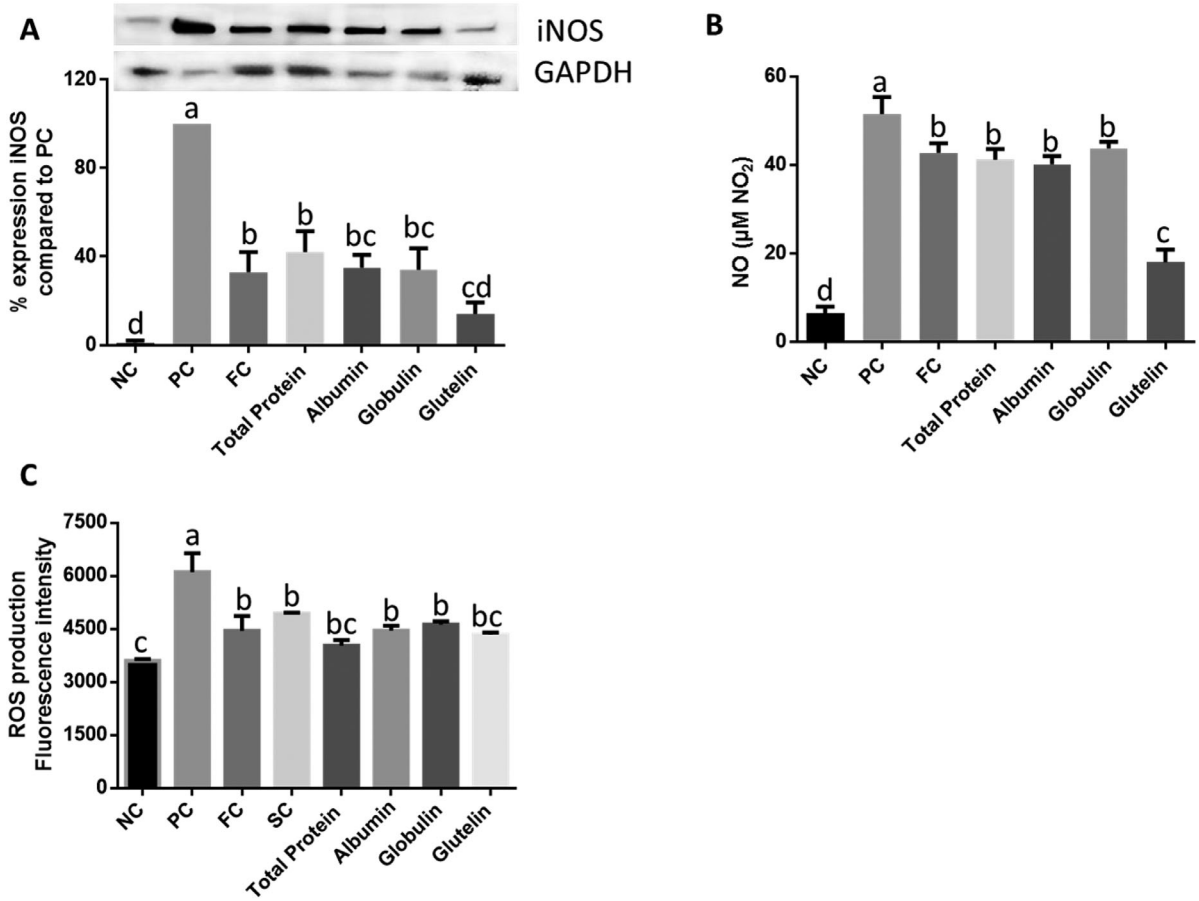

Figure 3. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of A) iNOS by western blotting, B) secretion of NO, and C) ROS production of LPS-stimulated RAW 264.7 macrophages. The net intensity of markers was normalized by net intensity of GAPDH (37 kDa). All experiments were performed in triplicate from at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain LPS (1 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control; SC, standard control.

3.2.2. Chia Seed Total Protein and Protein Fractions Digests Inhibited Expression of Proteins Related to AP-1 Pathway, but Only Glutelin Reduced the Secretion of PGE-2

DTP and DPF reduced the expression of phosphorylated JNK (**Figure 4**A) as well as AP-1 expression (Figure 4C). However, only DTP reduced the expression of COX-2 and was similar to FC (Figure 4B). The digested glutelin was the only sample that reduced PGE-2 secretion in comparison to PC and was similar to FC (Figure 4D).

3.3. Effect of Chia Seed Total Protein and Protein Fractions Digests on the Atherosclerosis Process

3.3.1. Chia Seed Total Protein and Protein Fractions Digests Decreased Foam Cell Formation by Reduction of LOX-1 Receptor and ICAM Expression

All digested proteins (**Figure 5**A) reduced the expression of iCAM ligand on macrophages treated with ox-LDL. Furthermore, the treatment with digested proteins reduced lipid accumulation inside macrophages, which indicates a reduction in foam cells formation (Figure 5B). The LOX-1 expression was reduced by DTP and DPF (Figure 5C).

However, ROS production was the same among DTP, DPF, and PC, and different ($p < 0.05$) than NC (Figure 5D).

3.3.2. Chia Seed Total Protein and Protein Fractions Digests Decreased the Expression of Some Inflammatory Markers Related to Atherosclerosis

All the digested proteins reduced the expression of NF-κB (**Figure 6**A) and iNOS (Figure 6B) on macrophages treated with ox-LDL. No secretion was reduced by DTP and digested albumin and globulin (Figure 6C). The MMP-9 expression was not induced by the amount of ox-LDL used and every treatment, including the NC, had the same value of expression (Figure 6D).

Associated with this, all digested proteins reduced TNF-α secretion (**Figure 7**A), glutelin having the lowest value. DTP and

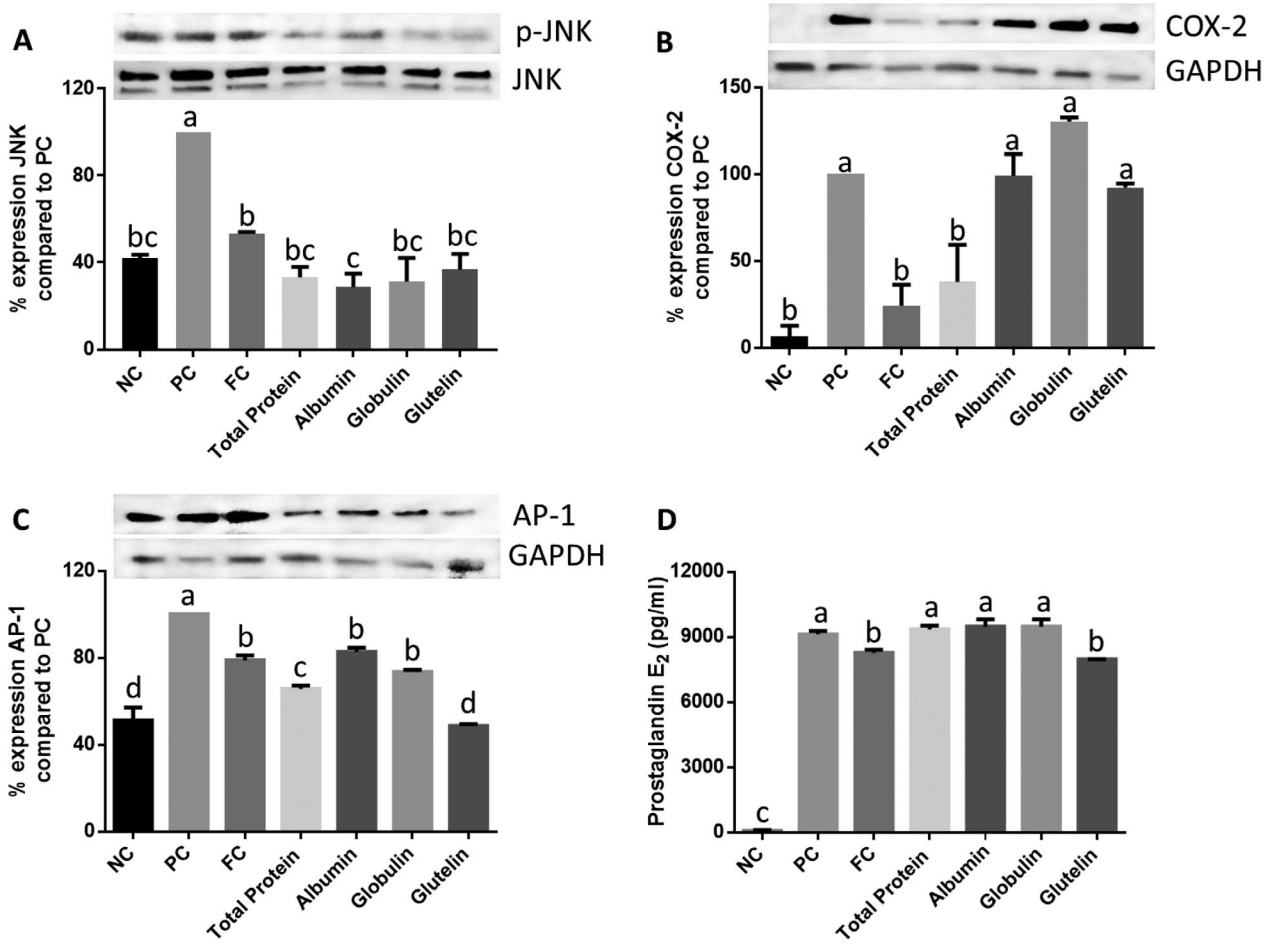

Figure 4. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression A) of p-JNK, B) COX-2, and C) AP-1 by western blotting, and secretion of D) PGE-2 by ELISA analysis of LPS-stimulated RAW 264.7 macrophages. The net intensity of markers was normalized by net intensity of GAPDH (37 kDa). All experiments were performed in triplicate from at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain LPS (1 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

digested glutelin had the lowest values compared to PC in PGE-2 and MCP-1 secretion (Figure 7B,C, respectively), but digested albumin and globulin had no effects in these markers. In addition, in the atherosclerosis process, the production of IL-10 and IL-12 were low and every group showed the same values (Figure 7D,E, respectively). However, the secretion of IL-6 was reduced in all samples, showing results similar to NC ($p > 0.05$, Figure 7F).

3.3.3. Peptides from Chia Seed Total Protein and Protein Fractions Digests Had Interaction with Receptors Associated with Atherosclerosis by In Silico Analysis

The minimum estimated free energies (EFE) of the interactions of the peptides with CCR2 and ICAM1 are shown in Table S1, Supporting Information. The estimated free energies indicated that compounds with a more negative value are more likely to inhibit these receptors. The ICAM1 receptor showed the highest interaction with peptide FAFFEFFELLFAFFT from digested glutelin (EFE, −8.4 kcal mol^{-1}) (**Figure 8**A). This interaction was strongest in comparison with simvastatin (EFE, −4.8 kcal mol^{-1}). Peptide LPGPPATF from digested albumin and glutelin had the highest interaction with CCR2 (EFE: −8.1 kcal mol^{-1}) (Figure 8B); however, the pharmacological control, simvastatin, showed slightly better interaction value than this peptide (EFE: −8.4 kcal mol^{-1}).

4. Discussion

Inflammation is a condition related to a host defense against pathogens that may cause injury to the body, such as bacteria. However, when the mechanisms are dysregulated may cause human diseases as atherosclerosis, a key condition for cardiovascular diseases.[29,30] When proteins are digested by the gastrointestinal track in humans, the specific enzymes, mainly pepsin, pancreatin, and chymotrypsin, cut the bonds between specific amino acids, forming peptides and/or free amino acids which are absorbed on the small intestine and the peptides get into circulation.[31] Therefore, once protein fractions from

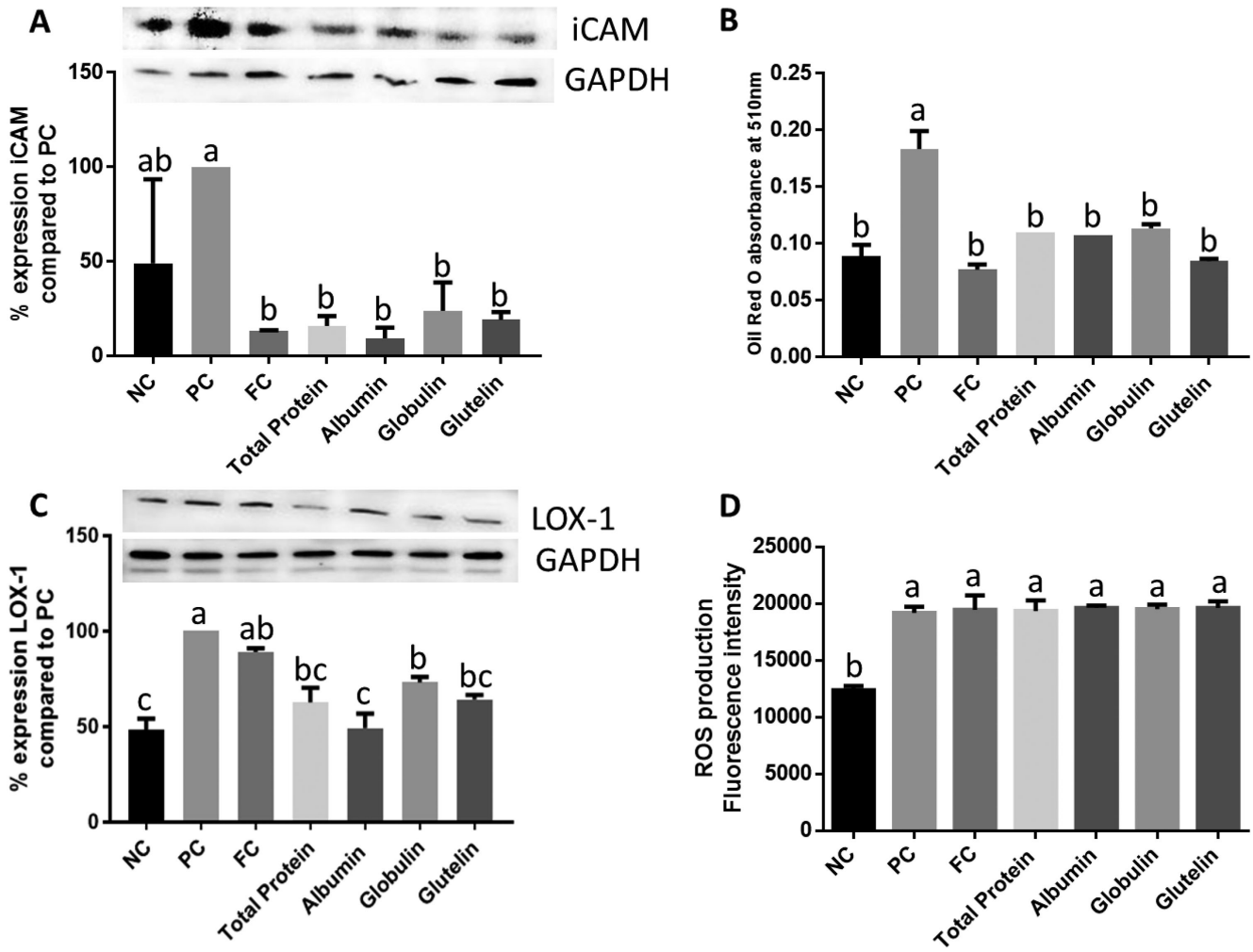

Figure 5. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of A) iCAM by western blotting, B) lipids accumulation by Oil-Red O, C) LOX-1 by western blotting, and D) ROS production in ox-LDL stimulated RAW 264.7 macrophages. The net intensity of markers (A) and (C) was normalized by net intensity of GAPDH (37 kDa). All experiments were performed in at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain ox-LDL (80 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

chia seed were isolated each one had a specific amino acid composition that, after gastrointestinal digestion, generated a variety of peptides with different amino acid composition and sequences; this leads to specific physicochemical properties and different biological effects in the organism.[14,32] Thus, because of this, each digested protein tested in this study showed distinct effects on the inflammation and atherosclerosis processes.

Macrophages are the major cells related to the inflammatory process. These cells are activated when stimulus, for example, LPS, binds to cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4) on cell membrane.[33] Such a ligation activates downstream proteins, as MyD88 (myeloid differentiation protein), IRAK (IL-1 receptor kinase), and TRAF6 (TNF receptor kinase), that activate IKK (kinase transcription factor inhibitor NF-κB). The activation of IKK promotes the phosphorylation and consequent degradation of NF-κB transcription factor inhibitor (IKB-α), allowing translocation of NF-κB from the cytosol to the nucleus.[34,35] In this study, we observed a block of this pathway, confirmed by reduction of NF-κB activation, as well of its translocation to nuclei. Digested globulin and glutelin showed effect as confirmed by the reduction of NF-κB activation, and its translocation to nuclei was reduced. These results are similar to results with amaranth hydrolysates which reduced the expression of p65 NF-κB in THP-1 and RAW 264.7 cells[36] and with whey protein hydrolysates in RAW 264.7 cells.[37]

The activation and translocation of NF-κB to nuclei promotes activation of genes encoding proteins involved in the inflammatory response, as iNOS. This enzyme converts L-arginine to L-citrulline and nitric oxide (NO). The NO may promote tissue injury at the inflammatory site and DNA damage.[38] Once NF-κB had its activation reduced, the expression of iNOS and consequently NO secretion were reduced by DTP and DPF treatment on inflamed macrophages. Similar results were observed with hydrolysates from strawberry–banana soymilk, mixed berry soymilk, vanilla soymilk,[39] and lunasin, a peptide from soy, on macrophages.[6] Using only biochemical analyses, the digests

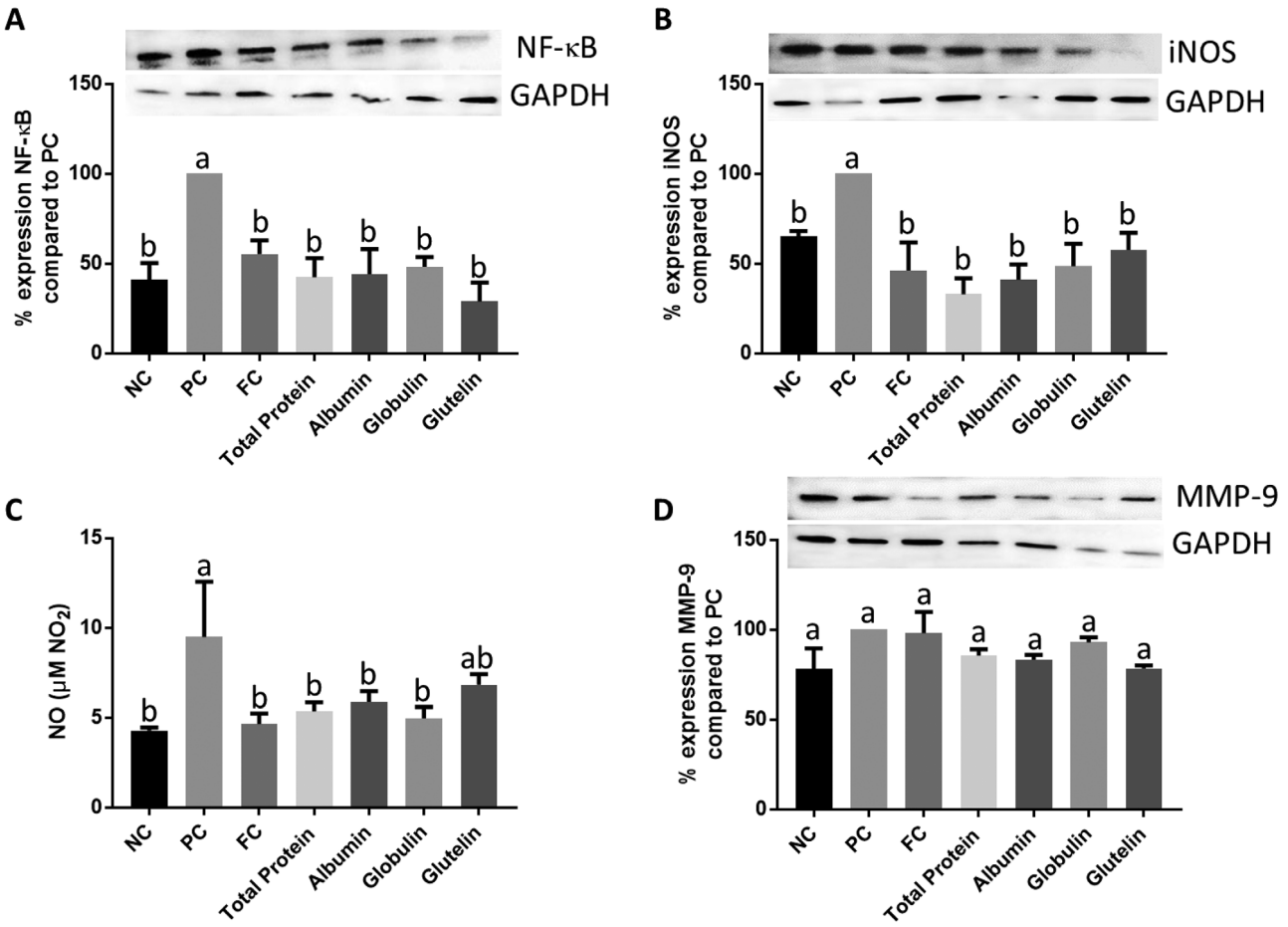

Figure 6. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of A) NF-κB, B) iNOS, C) NO secretion by ox-LDL stimulated RAW 264.7 macrophages and D) MMP-9 by western blotting. The net intensity of markers (A), (B), and (D) was normalized by net intensity of GAPDH (37 kDa). All experiments were performed in triplicate from at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain ox-LDL (80 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

from chia albumin, globulin, prolamin, and glutelin showed scavenging capacity and inhibition of 5-LOX, COX-1-2, and inducible nitric oxide synthase (iNOS) enzymes; prolamin showed poor results.[32]

Moreover, the translocation of NF-κB induced the expression of inflammatory cytokines, as TNF-α and IL-6 that can negatively activate immune response, giving rise to a number of diseases as asthma, multiple sclerosis, and rheumatoid arthritis.[40] DTP and digested albumin and glutelin reduced the secretion of cytokines, mainly TNF-α. This one is the major inflammatory mediator secreted by macrophages when stimulated by LPS in vitro and in vivo,[33] unlike IL-12.[41] On the another hand, IL-10 is a cytokine with anti-inflammatory effects by switching the metabolic program induced by inflammatory stimuli in macrophages.[42] Bean protein hydrolysates also showed effects reducing the secretion of TNF-α by inflamed macrophages[43]; similar results were obtained with amaranth hydrolysates,[44] whey protein hydrolysate,[37,45] and ovomucin hydrolysates.[46] The IL-10 cytokine was increased by digested albumin. Despite globulin decreasing phosphorylation and translocation of NF-κB, its secretion of cytokines was similar to PC, possibly because the mechanisms for regulating their secretion, as vesicles secreted by Golgi complex,[41] were upregulated by globulin.

The MCP-1 is classified as a chemotactic cytokine, a key mediator of monocyte chemotaxis, attracting other monocytes to the inflammation site.[47] DTP and digested glutelin decreased MCP-1 secretion. LPS and TNF-α are two of the main inducers of MCP-1 expression[47] and may have contributed to overexpression of this cytokine in cells treated with digested albumin and globulin, which were not effective. The results found in this study were better in comparison to amaranth hydrolysates' effects on inflamed THP-1 macrophages, which increased the MCP-1 cytokine.[44]

COX-2 is an inducible enzyme that catalyzes the transformation of arachidonic acid to prostaglandin H2, a precursor of other biologically active inflammation mediators, such as PGE-2, prostacyclin, and thromboxane A2.[48] Despite NF-κB and AP-1 reduced their expression by digested samples, only DTP was effective in reducing COX-2 expression, while digested glutelin decreased PGE-2 secretion. Other proteins related with these

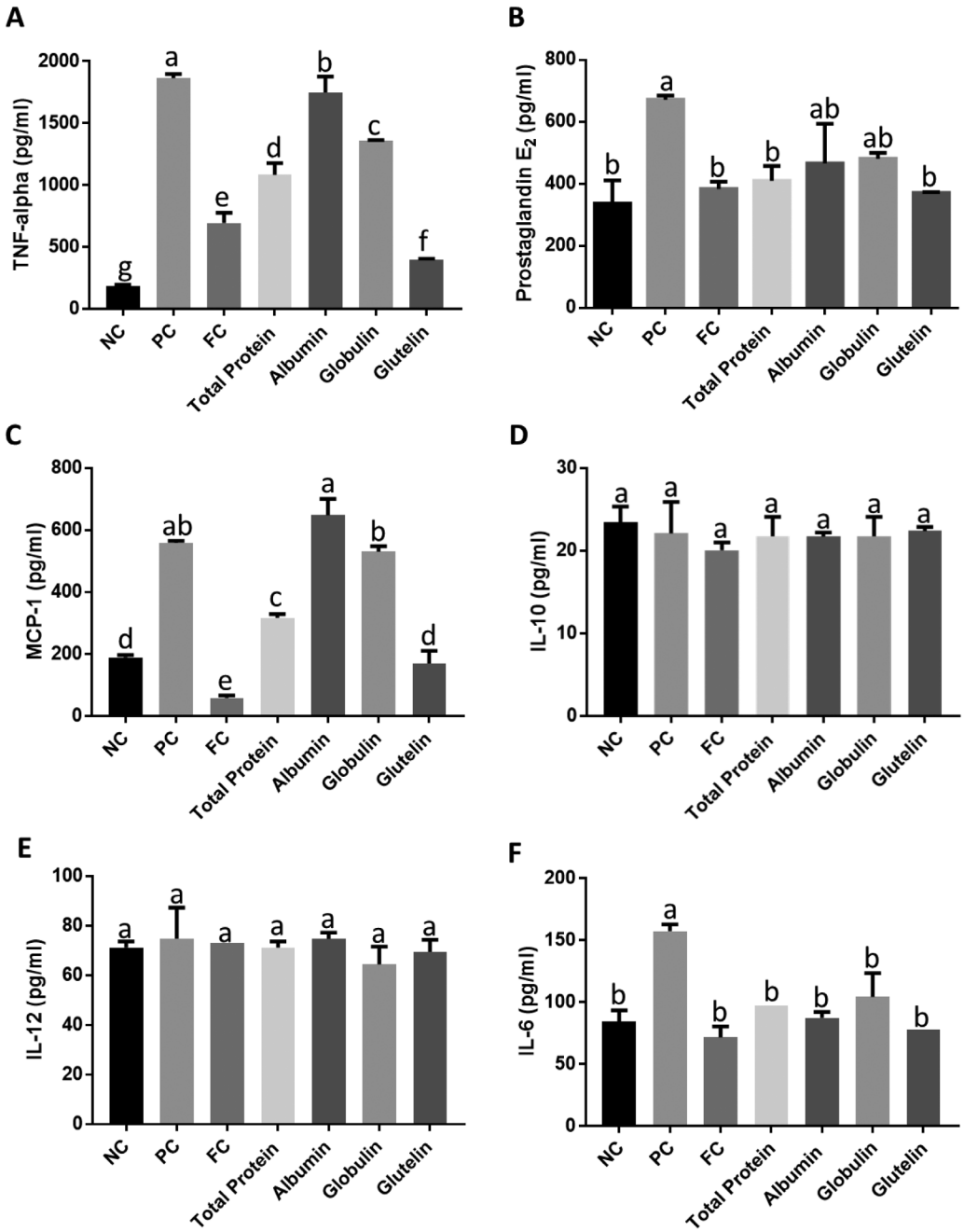

Figure 7. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on secretion A) TNF-α, B) PGE-2, C) MCP-1, D) IL-10, E) IL-12, and F) IL-6 by ELISA analysis by ox-LDL stimulated RAW 264.7 macrophages. All experiments were performed in triplicate from at least two independent trials. Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tukey-test). All treatments contain ox-LDL (80 μM) except the negative control (NC) treated only with media. NC, negative control; PC, positive control; FC, pharmacological control.

pathway, as p38 mitogen-activated protein kinase (p38MAPK)[49] and cAMP-PKA-AKAP- pathway[50] may have been modulated by other digested proteins. The fact that DTP reduced COX-2 expression agrees with our previous findings that this protein digest reduced biochemically the activation of COX-2 without deactivating COX-1 (constitutive protein).[32] Successfully, in this study, digested albumin and glutelin also reduced COX-2 activity.

On the other hand, the inflammatory process is closely related to oxidative stress, since inflammation and EROs stimulate each other, causing a vicious circle.[51] Peptides with proline, histidine, tyrosine, and/or tryptophan, the hydrophobic amino acids, have more ionizable groups that block free radicals and, thus, increase the antioxidant activity.[52] The antioxidant activity was also observed in peptides with less than 20 amino acid

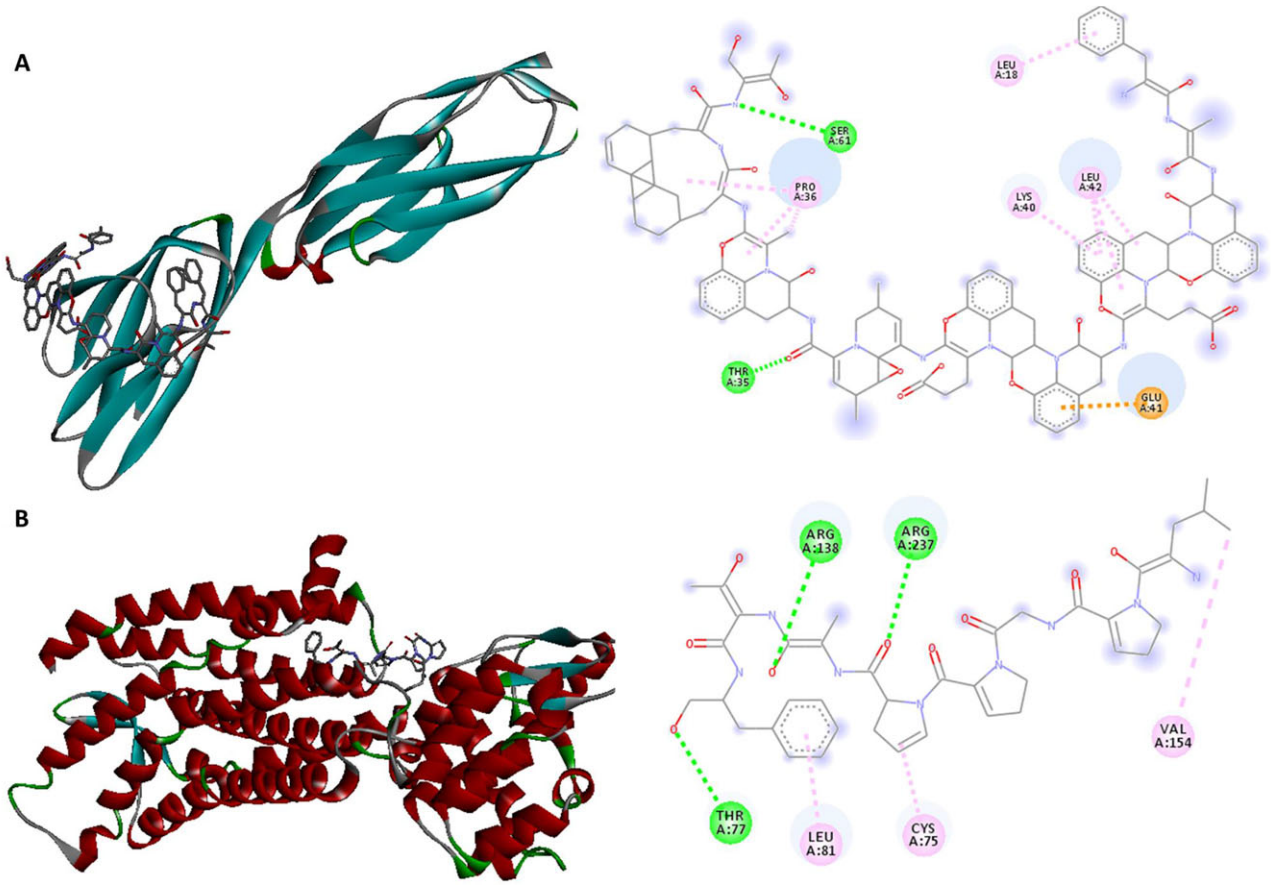

Figure 8. The in silico interaction of the peptide FAFFEFFELLFAFFT, found in digested glutelin, with ICAM (A) and peptide LPGPPATF, found in the digested albumin with CCR2 (B). These peptides showed the most potent interaction, by lower estimated free energies (EFE), analyzed by AutoDock Vina and visualized by Discovery Studio 2016 Client.

residues per molecule, because these small peptides have a better ability to cross the intestinal barrier and exert their biological effects.[53] Finally, peptides with hydrophobicity ≤20 kcal mol^{-1} are more effective for penetrating the cell membrane and to exercise effects on the molecule.[54] Interestingly, most of the peptides found in DTP and digested protein fractions from chia seed showed these characteristics,[14,32] which may explain the benefits found in the present study.

We observed that digested proteins, mainly DTP and glutelin, reduced ROS production. The oxidative stress is related to the inflammatory process by promotion of IkBα-degradation, which allows the activation and translocation of NF-κB.[55] Then, the reduction of ROS may have been the key-point to the beneficial effects observed by the samples. Our results are similar to previous studies that used digested proteins from other food sources as beans,[43] extruded amaranth,[36] and strawberry–banana soymilk, mixed berry soymilk, and vanilla soymilk and found a reduction of inflammation.[39] Thus, this study reinforces the anti-inflammatory effects of food peptides regarding modulation of transcription and expression of markers related with inflammation.

Inflammation and oxidative stress are key factors at all stages of development of atherosclerosis.[56] The origin of the atherosclerotic plaque is initiated and sustained by the combined endothelial dysfunction caused by chronic exposure to factors that promote vascular inflammation, such as hyperlipidemia, hypertension, smoking, and diabetes.[57] This endothelial dysfunction increases the permeability of the artery, allowing the entry of low-density plasma lipoproteins (LDL) that accumulate in the subendothelial space and undergoes oxidation (ox-LDL) by free radicals, as ROS.[56] The DTP and DPF were not effective reducing ROS production, maybe because LDL was already oxidized in the cells, increasing ROS and the digested proteins were not effective to reduce this condition, unlike hydrolyzed bean proteins.[58]

In this condition, the epithelium expresses adhesion molecules, called vascular adhesion molecules (VCAM) that binds with ICAM, expressed by monocytes, and facilitates the migration of monocytes into the arteries wall. These monocytes differentiated into macrophages and phagocyte ox-LDL. We observed a reduction of expression of ICAM and LOX-1 by macrophages treated with DTP and DPF, resulting in less formation of foam cells, similar with unprocessed and extruded amaranth hydrolysate.[59]

These foam cells secrete MCP-1, a chemokine that binds to the CCR2 and attracts new monocytes, increasing the atherosclerosis plaque.[4] Also, the accumulation of modified LDL by macrophages activates cytokine production, like TNF-α, IL-6, IL-1β, and IL-12, and active the enzymes iNOS and COX-2 to

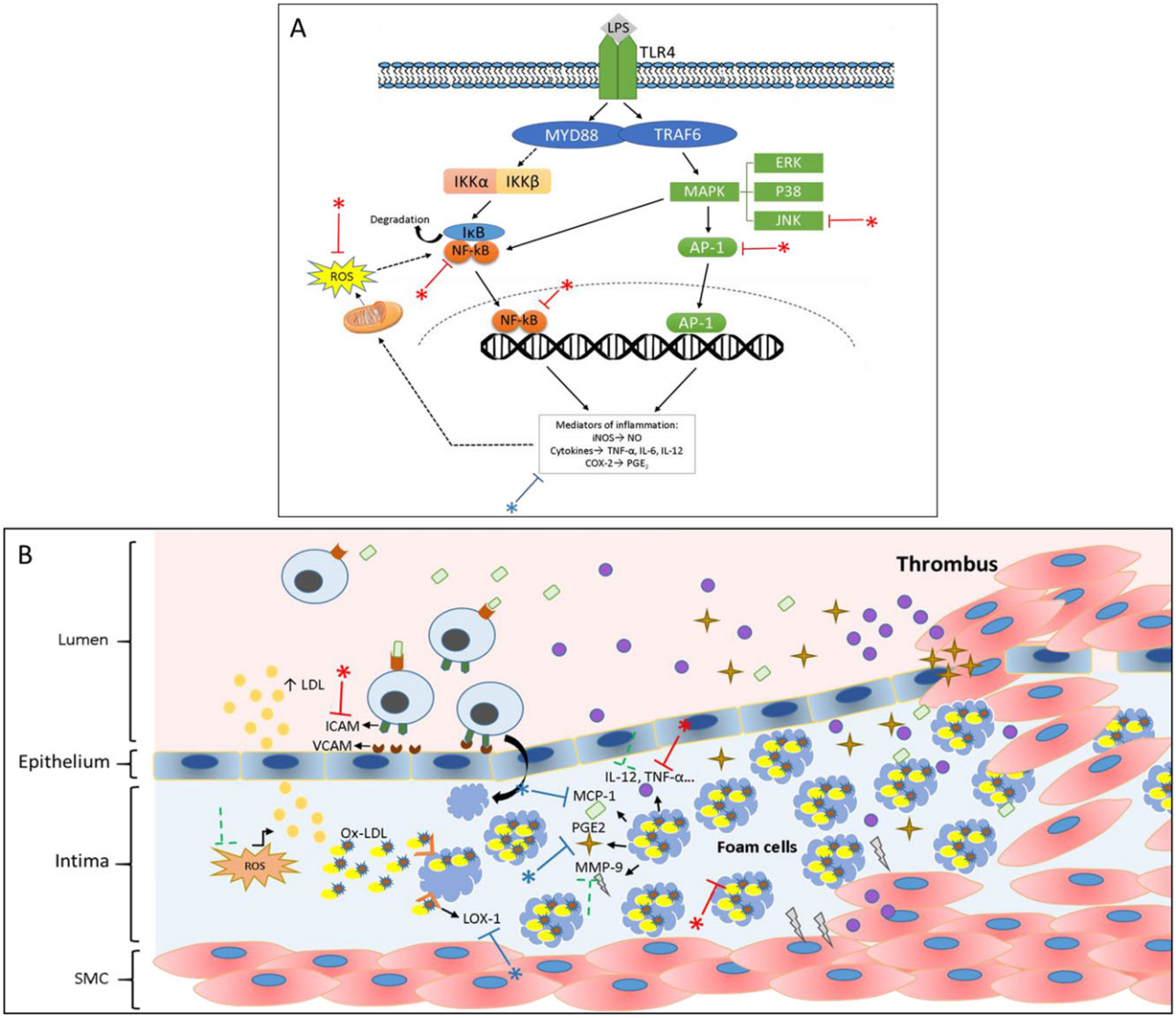

Figure 9. The proposal effects of digested total protein and digested albumin, globulin, and glutelin from chia seeds on A) inflammation pathway and B) atherosclerosis processes. The **red** symbols mean that every sample had effect on marker. The **blue** symbols mean that some samples had effect on marker. The **green** symbols mean that samples had no effect on marker. A) The digested total protein and digested albumin reduced the expression of NF-κB, and iNOS. The last one also had reduced expression by digested globulin and glutelin. The translocation of NF-κB to nuclei was inhibited by all digested proteins. The JNK and AP-1 expression and ROS production were reduced by all digested proteins, but COX-2 expression just by digested total protein. The mediators of inflammation secretion were reduced especially by digested glutelin. B) All digested proteins reduced the expression of markers related with the macrophage adherence, lipid accumulation, and related with the inflammation, but any reduced MMP-9 expression. The mediators of inflammation secretion were reduced especially by digested total protein.

produce NO and PGE-2, respectively. These markers promote the influx and activation of other inflammatory cells, as T-lymphocytes, and mediate their retention in the plaque that increases the inflammatory process around it.[60,61] Besides that, the NO induces high production of peroxynitrite and consequent cell toxicity.[62]

DTP and digested glutelin were the most effective to reduce the expression of NF-κB and iNOS, and the secretion of TNF-α, PGE-2, MCP-1, and IL-6, which are the inflammatory markers in the atherosclerosis process. These results showed the effectiveness of digested protein from chia seed, like others studies with bean hydrolysates[43] and extruded amaranth hydrolysate.[36,44] These results, associated with the reduction of lipid accumulation, as demonstrated by Oil-Red analysis, reduced the chances of plaque formation. This plaque can obstruct blood circulation and increase blood pressure, leading to artery rupture and vascular complications as thrombosis, myocardial infarction, or stroke.[63]

Figure 9 shows a summary of the potential mechanistic effects of chia seed digested total protein and digested protein fraction on inflammation (Figure 9A) and atherosclerosis (Figure 9B) pathways. On inflammation, DTP reduced all protein expression related to the pathways of NF-κB and AP-1. Digested glutelin

reduced the secretion of markers related with lipid absorption and inflammation. DTP also reduced the secretion of all markers analyzed.

In summary, the results obtained are innovative to explain the benefits of chia seed proteins on inflammation and the atherosclerosis process in macrophages. As we found, the mix of all peptides from DPF was more powerful than the isolated protein fractions. This study contributes to the evidence on the potential action of bioactive peptides from chia on inflammation and atherosclerosis processes using in silico and in vitro models. In vivo studies are needed and underway in our laboratory, to validate the physiological relevance of these results.

Taken together, our results support the concept that chia seed digested proteins, albumin, globulin, and glutelin, showed beneficial effects reducing the levels of markers related to induction of the processes of inflammation and atherosclerosis in macrophages. DTP showed the best results in both induced pathogeneses, indicating that the mix of all peptides from DPF was more effective than isolated proteins fractions. The digested proteins were effective in reducing the expression of proteins related to inflammation and atherosclerosis pathways and consequently lowering the secretion of these markers by the cells. These results suggest a promising effect of DTP and DPF from chia seeds in the prevention of CVD by modulating the inflammatory and atherosclerosis processes. These results are innovative and highlight the potential health benefits of chia seed proteins. In addition, for the first time, the characterization of peptides from chia seed total protein and protein fractions (albumin, globulin, and glutelin) digests are presented.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

atherosclerosis, bioactive peptides, digestion, inflammation, *Salvia hispanica* L

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