

MARIANA GRANCIERI

EFFECT OF DIGESTED TOTAL PROTEIN, PROTEIN FRACTIONS, AND PURE PEPTIDES FROM CHIA SEEDS (*Salvia Hispanica L.*) ON INFLAMMATION, ATHEROSCLEROSIS AND ADIPOGENESIS *IN SILICO* AND *IN VITRO*

Thesis submitted to the Universidade Federal de Viçosa, as part of the requirements of Program in Science of Nutrition to obtaining the title of *Doctor Scientiae*.

Adviser: Hércia Stampini Duarte Martino

Co-adviser: Neuza Maria Brunoro Costa

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Assent:


Mariana Granieri
Author


Hércia Stampini Duarte Martino
Adviser

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ABSTRACT

GRANCIERI, Mariana, D.Sc., Universidade Federal de Viçosa, January, 2020. **Effect of digested total protein, protein fractions, and pure peptides from chia seeds (*Salvia hispanica* L.) on inflammation, atherosclerosis and adipogenesis *in silico* and *in vitro*.** Adviser: Hércia Stampini Duarte Martino. Co-Adviser: Neuza Maria Brunoro Costa.

Cardiovascular diseases (CVD) are the leading cause of death in the world. Some pathologies are associated with the development of CVD, such as low-grade inflammation, atherosclerosis and obesity. Chia seed (*Salvia hispanica* L.) is a food that has shown promising results in experimental and clinical studies. These seeds are a rich source of total proteins and storage proteins that, after gastrointestinal digestion, can generate bioactive peptides with benefits in specific physiological functions. The general objective of this study was to investigate the effects of total digested protein (DTP), digested protein fractions (DPF) and pure chia seed peptides (*Salvia hispânica* L.) on inflammation, atherosclerosis and adipogenesis *in silico* and *in vitro*. **Manuscript 1** was a review study that aimed to identify the composition and beneficial effects of chia seeds (*Salvia hispanica* L.), their proteins, peptides and their potential impact on health. The Scopus and PubMed databases were used to identify studies that investigated the effects of chia seed consumption on humans. In addition, chia proteins were identified using the Uniprot database and their bioactive peptides were evaluated for their potential biological effects through the BIOPEP® database. As a result, it was observed that the chia seed has proteins with promising bioactive peptides, especially with antioxidant, hypoglycemic and hypotensive effects, which can be correlated to the beneficial effects resulting from the consumption of this seed in studies with humans. However, there was a lack of studies that investigate these effects of chia protein in chronic diseases. Therefore, in **Manuscript 2**, it aimed to identify and characterize the DTP and DPF peptides of chia seeds (*Salvia hispanica* L.) and to determine their potential antioxidant, anti-inflammatory and anti-atherosclerotic effect. Chia seed grown in Brazil was used, which was ground to obtain the flour and, from this, the mucilage and lipids were removed. Such mucilage and lipid-free flour was used to extract total protein by alkaline precipitation, as well as protein fractions by solvent solubility. Then, the proteins, total and fractions, underwent gastrointestinal digestion simulated with pepsin and pancreatin and their bioactive peptides were identified by Ultra Efficiency Liquid Chromatography, coupled to the mass spectrometer (UPLC-ESI-MS). The biological potential, parental proteins and physicochemical properties were characterized using the databases BIOPEP®, BLAST tool® and PedDraw®, respectively. In addition, the

antioxidant and anti-inflammatory effects were evaluated using biochemical and *in silico* analyzes. DTP and digested albumin, globulin and glutelin showed the ability to eliminate superoxide, hydrogen peroxide, nitric oxide and DPPH and inhibition of the enzymes 5-LOX, COX-1-2 and iNOS in biochemical analyzes. In addition, the peptides from the samples mentioned above had interaction with the NF- κ B, LOX-1, TLR4 and COX-1 markers in *in silico* analyzes. In **Manuscript 3**, the objective was to determine the effect of DTP and DPF (albumin, globulin and glutelin) from chia seeds (*Salvia hispanica* L.) on inflammation and atherosclerosis in macrophages and the mechanisms of action. Macrophages RAW.264.7 had inflammation and atherosclerosis induced by the addition of lipopolysaccharide (1 μ M) and oxidized low density lipoprotein (80 μ M), respectively. It was observed that in the inflammatory process, DTP and DPF reduced the expression and translocation of NF- κ B to the nucleus and, consequently, the expression of p-NF- κ B, iNOS, p-JNK and AP-1 was also reduced. Digested glutelin reduced the secretion of nitric oxide (-65.1%), reactive oxygen species (-19.7%), prostaglandins (-34.6%), TNF- α (-24.1%), among others. As an anti-atherosclerotic, DTP and digested glutelin reduced the expression of iCAM (-86.4%, -80.8%), LOX-1 (-37.3%, -35.7%), iNOS (-67, 0%, -42.2%) and NF- κ B (-57.5%, -71.1%). DTP was effective in reducing nitric oxide secretion (-43.4%), accumulation of lipids in macrophages (-41.9%) and prostaglandin secretion (-41.9%), TNF- α (-43, 3%), MCP-1 (-47.6%) and IL-6 (-50.5%). In addition, in **Manuscript 4**, the objective was to evaluate the *in vitro* effect of DTP and DPF (albumin and glutelin) and pure peptides from chia seed (*Salvia hispanica* L.) on adipogenesis, as well as on the prevention and inhibition of induced inflammation in 3T3-L1 adipocytes. The DTP, DPF samples, and two pure peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) were tested for the effects of preventing adipogenesis. In addition, 3T3-L1 cells were induced to mature adipocytes and were exposed to conditioned media (CM) of inflamed macrophages and the efficacy of DTP and DPF in preventing inflammation was tested (samples were added together with CM) inhibition (samples were added after adding CM). All treatments prevented adipogenesis, reducing the expression of PPAR γ by more than 50% and, to a lesser extent, LPL, FAS, SREBP1, lipase and triglyceride activity. Inflammation induced by conditioned means was reduced mainly during prevention. The expression of NF- κ B, iNOS and COX-2 and the secretions of nitric oxide, PGE2 and TNF α were reduced in all treatments. In general, the DPF fractions and digested proteins: albumin, globulin and glutelin showed promising effects on inflammation, atherosclerosis and adipogenesis, reducing expression and markers related to these pathways. In addition, DTP, as

a set of proteins, presented more potent effect compared to the isolated proteins. Thus, chia seed proteins can be a tool in reducing the risk of developing cardiovascular disease markers.

Keywords: Adipocyte. Hydrolyzates. Macrophages. Peptides. Protein. Inflammation.

RESUMO

GRANCIERI, Mariana, D.Sc., Universidade Federal de Viçosa, janeiro de 2020. **Efeito da proteína total digerida, frações proteicas e peptídeos puros da semente de chia (*Salvia hispanica* L.) na inflamação, aterosclerose e adipogênese *in silico* e *in vitro*.** Orientadora: Hércia Stampini Duarte Martino. Coorientadora: Neuza Maria Brunoro Costa.

As doenças cardiovasculares (DCV) são a principal causa de morte no mundo. Algumas patologias estão associadas ao desenvolvimento de DCV, como inflamação de baixo grau, aterosclerose e obesidade. A semente de chia (*Salvia hispanica* L.) é um alimento que vem apresentando resultados promissores em estudos experimentais e clínicos. Essas sementes são rica fonte de proteínas totais e proteínas de reserva que, após digestão gastrointestinal, podem gerar peptídeos bioativos com benefícios em funções fisiológicas específicas. O objetivo geral deste estudo foi investigar os efeitos da proteína total digerida (DTP), frações proteicas digeridas (DPF) e peptídeos puros da semente de chia (*Salvia hispânica* L.) na inflamação, aterosclerose e adipogênese *in silico* e *in vitro*. O **Manuscrito 1** consistiu de um estudo de revisão que teve como objetivo identificar a composição e os efeitos benéficos das sementes de chia (*Salvia hispanica* L.), suas proteínas, peptídeos e seu potencial impacto na saúde. Foram identificados, por meio de busca nas bases de dados Scopus e PubMed, trabalhos que investigaram os efeitos do consumo da semente de chia em humanos. Também, identificou-se as proteínas de chia utilizando a base de dados Uniprot e os peptídeos bioativos destas foram avaliados quanto aos seus potenciais efeitos biológicos por meio da base de dados BIOPEP®. Como resultados, observou-se que a semente de chia possui proteínas com promissores peptídeos bioativos, especialmente com efeitos antioxidantes, hipoglicêmicos e hipotensivos, os quais podem correlacionar-se aos efeitos benéficos decorrentes do consumo desta semente nos estudos com humanos. Porém, observou-se a falta de estudos que investiguem esses efeitos da proteína da chia nas doenças crônicas. Portanto, no **Manuscrito 2**, teve como objetivo identificar e caracterizar os peptídeos da DTP e das DPF de sementes de chia (*Salvia hispanica* L.) e determinar seu potencial efeito antioxidante, anti-inflamatório e anti-aterosclerótico. Foi utilizada semente de chia cultivada no Brasil, a qual foi moída para a obtenção da farinha e, desta, removeu-se a mucilagem e os lipídeos. Tal farinha livre de mucilagem e lipídeos foi utilizada para extração da proteína total por precipitação alcalina, bem como das frações proteicas por solubilidade em solventes. Em seguida, as proteínas, total e frações, sofreram digestão gastrointestinal simulada com pepsina e pancreatina e seus peptídeos bioativos foram identificados por Cromatografia Líquida de Ultra Eficiência,

acoplado ao espectrômetro de massas (UPLC-ESI-MS). O potencial biológico, as proteínas parentais e as propriedades físico-químicas foram caracterizadas utilizando os bancos de dados BIOPEP®, BLAST tool® e PedDraw®, respectivamente. Além disso, avaliou-se os efeitos antioxidantes e anti-inflamatórios utilizando análises bioquímicas e *in silico*. O DTP e a albumina digerida, globulina e glutelina mostraram capacidade de eliminação de superóxido, peróxido de hidrogênio, óxido nítrico e DPPH e inibição das enzimas 5-LOX, COX-1-2 e iNOS nas análises bioquímicas. Além disso, os peptídeos das amostras citadas acima tiveram interação com os marcadores NF-κB, LOX-1, TLR4 e COX-1 nas análises *in silico*. No **Manuscrito 3**, teve-se o objetivo de determinar o efeito da DTP e das DPF (albumina, globulina e glutelina) de sementes de chia (*Salvia hispanica* L.) na inflamação e aterosclerose em macrófagos e os mecanismos de ação. Macrófagos RAW.264.7 tiveram a inflamação e a aterosclerose induzidas pela adição de lipopolissacarídeo (1μM) e lipoproteína de baixa densidade oxidada (80 μM), respectivamente. Observou-se que no processo inflamatório o DTP e as DPF reduziram a expressão e a translocação de NF-κB para o núcleo e, conseqüentemente, a expressão de p-NF-κB, iNOS, p-JNK e AP-1 também foi reduzida. A glutelina digerida reduziu a secreção de óxido nítrico (-65,1%), espécies reativas de oxigênio (-19,7%), prostaglandinas (-34,6%), TNF-α (-24,1%), dentre outros. Como anti-aterosclerótico, DTP e glutelina digerida reduziram a expressão de iCAM (-86,4%, -80,8%), LOX-1 (-37,3%, -35,7%), iNOS (-67,0%, -42,2%) e NF-κB (-57,5%, -71,1%). O DTP foi eficaz na redução da secreção de óxido nítrico (-43,4%), acúmulo de lipídios em macrófagos (-41,9%) e secreção de prostaglandinas (-41,9%), TNF-α (-43,3%), MCP-1 (-47,6 %) e IL-6 (-50,5%). Também, no **Manuscrito 4** objetivou-se avaliar o efeito *in vitro* da DTP e das DPF (albumina e glutelina) e peptídeos puros da semente de chia (*Salvia hispanica* L.) na adipogênese, bem como na prevenção e inibição da inflamação induzida em adipócitos 3T3-L1. Além das amostras de DTP e DPF, dois peptídeos puros NSPGPHDVALDQ (Pep1) e RMVLPEYELLYE (Pep2) foram testados para os efeitos de prevenção da adipogênese. Além disso, células 3T3-L1 foram induzidas a adipócitos maduros e foram expostas a meios condicionados (CM) de macrófagos inflamados e testou-se a eficácia da DTP e das DPF na prevenção da inflamação (amostras foram adicionadas junto ao CM), quanto na inibição desta (amostras foram adicionadas após adição de CM). Todos os tratamentos com DTP, DPF e peptídeos puros impediram a adipogênese, reduzindo mais de 50% a expressão de PPARγ e, em menor grau, LPL, FAS, SREBP1, atividade de lipase e triglicerídeos. A inflamação induzida por meios condicionados foi reduzida principalmente durante a prevenção. A

expressão de NF- κ B, iNOS e COX-2 e as secreções de óxido nítrico, PGE2 e TNF α foram reduzidas em todos os tratamentos. Em geral, as frações de DPF e proteínas digeridas: albumina, globulina e glutelina apresentaram efeitos promissores na inflamação, aterosclerose e adipogênese, reduzindo a expressão e os marcadores relacionados a essas vias. Além disso, o DTP, como um conjunto de proteínas, apresentou efeito mais potente do que as proteínas isoladas. Assim, as proteínas da semente de chia podem ser uma ferramenta na redução do risco do desenvolvimento dos marcadores de doenças cardiovasculares.

Palavras-chave: Adipócitos. Hidrolisados. Macrófagos. Peptídeos. Proteínas. Inflamação.

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LIST OF ABBREVIATIONS

°C: Degree Celsius
μM: micromolar
5-LOX: 5-lipoxygenase
AAR: Amino acid residues
ACE inhibitor: angiotensin-converting-enzyme inhibitor
ACE-enzyme: angiotensin-converting enzyme
Activating UBMP: Activating ubiquitin-mediated proteolysis
AdoMetDC: S-adenosylmethionine decarboxylase
ALA: alpha-linolenic acid
ANOVA: analysis of variance
AP-1: activator protein-1
ATCC: American Type Culture Collection
AUC: area under the curve
BSA: bovine serum albumin
CAdC: clathrin adaptor complex
CCR2: C-C chemokine receptor
cm: centimeters
CM: conditioned media
COX-1: cyclooxygenase 1
COX-2: cyclooxygenase
CVD: cardiovascular diseases
DAPI: 4', 6-diamidino-2-phenylindole
DBP: diastolic blood pressure
DH: degree of hydrolysis
DMEM: Dulbecco's modified Eagle medium
DPF: protein fractions
DPPH: 1.1-diphenyl-2-picrylhydrazyl
DPP-IV: dipeptidyl peptidase-IV
DTP: digested total protein
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid

eEF1a1: elongation factor 1-alpha
EFE: minimum estimated free energies
ELF-3e: eukaryotic translation initiation factor 3 subunit E
EPA: docosahexaenoic acid
ERK: extracellular signal-regulated kinases
FAD2i2: fatty acid desaturase 2 isoform 2
FAD3i1: fatty acid desaturase 3 isoform 1
FAD3i2: fatty acid desaturase 3 isoform 2
FAD7i1: fatty acid desaturase 7 isoform 3
FAD8: fatty acid desaturase 8
FAS: fatty acid synthase
FBS: fetal bovine serum
FC: pharmacological control
g: gram
GABA: γ -aminobutyric acid
GAPDH: glyceraldehyde-3-phosphate-dehydrogenase
h: hour
H2DCFDA: 2',7'-dichlorofluorescein diacetate
H₂O₂: Hydrogen peroxide
HDL-c: high-density lipoprotein cholesterol
HFF: high fat and fructose diet
HPB: Hydrophobicity
HPLC-ESI-MS: high-performance liquid chromatography-electrospray ionization-mass spectrometry
iAUC: incremental area under the curve
IC₅₀: half maximal inhibitory concentration
ICAM-1: intercellular adhesion molecule-1
IKK: inhibitor of κ B kinase
IL: interleukin
iNOS: inducible nitric oxide synthases
IP: isoelectric point
I κ B: inhibitor of NF- κ B
JNK: c-Jun N-terminal kinases

kDa: kilo Dalton
K_i: inhibition constant
LDL: low-density lipoproteins
LOX-1: lectin-like-oxidized LDL-receptor-1
LPL: lipoprotein lipase
LPS: lipopolysaccharide
M: molar
MAPK: mitogen activated protein kinases
MCP-1: monocyte chemoattractant protein-1
MDA: malondialdehyde
mg: milligram
MGAT: monoacylglycerol acyltransferase
min: minute
mL: milliliter
mm: millimeter
MM: Molecular mass
MMP: mean blood pressure
MMP-9: metalloproteinases-9
NADH: nicotinamide adenine dinucleotide
NaNO₂: sodium nitrite
NC: negative control
NCS: newborn calf serum
NO: nitric oxide
O₂⁻: Superoxide radical
omega-3 (or n-3): alpha-linolenic
omega-6 (or n-6): alpha-linoleic acids
OPA: *o*-phthaldialdehyde
ox-LDL: oxidized-LDL
p38: kinase protein p38
PBS: phosphate buffer
PC: positive control
PDB: Protein Data Bank
PER: protein efficiency ratio

pg: picogram

PGE₂: prostaglandins

PMS: phenazinemethosulfate

PP2A: protein phosphatase 2A

PPAR- γ : peroxisome-proliferator-activated receptors gamma

ROS: reactive oxygen species

RT: Retention time

RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

SBP: systolic blood pressure

SD: standard deviation

SDS-PAGE: sodium dodecyl sulphate-polycrylamide gel electrophoresis

SREBP-1: sterol regulatory element-binding protein-1

TC: total cholesterol

TE: Trolox equivalent

TLR: toll like receptor

TNF- α : Tumor necrosis factor-alpha

TRAF6: TNF receptor kinase

v: volume

VCAM-1: vascular adhesion molecule-1

VLDL-c: very low-density lipoprotein cholesterol

w: weight

* Amino acid nomenclature: C, cys; cysteine; H, his; histidine; I, ile; isoleucine; M, met; methionine; S, ser; serine; V, val; valine; A, ala; alanine; G, gly; glycine; L, leu; leucine; P, pro; proline; T, thr; threonine; F, phe; phenylalanine; R, arg; arginine; Y, tyr; tyrosine; W, trp; tryptophan; D, asp; aspartic acid; N, asn; asparagine; B, asx; either of D or N; E, glu; glutamic acid; Q, gin; glutamine; Z, glx; either of E or Q; K, lys; lysine; X, undetermined amino acid.

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1. GENERAL INTRODUCTION

The cardiovascular diseases (CVD) are the main cause of death and premature incapacity worldwide, representing around 17.3 million deaths per year (SACKS et al., 2017). Cardiovascular diseases are disorders of the heart and vessels, and include coronary heart disease and stroke, and they are a class of chronic non-infectious diseases (ZHAO et al., 2017). The main risk factors are a high blood pressure, hyperlipidemia, diabetes, overweight and obesity, metabolic syndrome, smoking, excessive alcohol consumption, imbalanced diet, and a sedentary lifestyle (TANG et al., 2017). These conditions increase the oxidative stress, this is an unbalanced condition between reactive oxygen species (ROS) production and breakdown (AGITA; THAHA, 2017).

Reactive oxygen species activates transcription factors such as nuclear factor kappa-B (NF- κ B) and activator protein 1 (AP-1) that modulate gene expression, including adhesion molecules and chemokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF- α). These cytokines can bind to the cell surface receptors, as toll-like receptor (TLR), inducing a signaling cascades that converge on the activation of the inhibitor of κ B kinase (IKK) complex that phosphoryl and consequently degrades the inhibitor of NF- κ B (I κ B) molecules that release NF- κ B to nuclei (BAKER; HAYDEN; GHOSH, 2012). Activation of TLR also can activate the mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinases (ERK), kinase protein p38 (p38) and c-Jun N-terminal kinases (JNK) that can activate activator protein-1 (AP-1) (CARGNELLO; ROUX, 2011). The activates NF- κ B and AP-1, translocate to the nucleus and promote the transcription of target genes, such as inducible nitric oxide synthases (iNOS) and cyclooxygenase (COX-2), which will activate nitric oxide (NO) and prostaglandins (PGE₂), respectively, and cytokines, which promote the inflammatory process (KHODABANDEHLOO et al., 2016).

The high inflammation and ROS production increase the permeability of the endothelium, allowing the entrance of low-density lipoproteins (LDL) to intima arteria. These LDLs can be oxidized by ROS becoming oxidized-LDL (ox-LDL) that increase the endothelium dysfunction accompanied by upregulation of adhesion molecules and chemokines, as monocyte chemoattractant protein-1 (MCP-1), which promotes migration of immune cells (monocytes, neutrophils, lymphocytes). The adhesion molecules as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) present on the vascular surface are responsible to promote slow rolling and arrest of circulating monocytes to

the vascular wall (BAKER; HAYDEN; GHOSH, 2012). The monocytes are transformed into macrophages and express the lectin-like-oxidized LDL-receptor-1 (LOX-1) that binds to the ox-LDLs, allowing its entry into macrophages. Then, the macrophages rich in ox-LDL become in foam cells that can accumulate into arteria, generating a thrombus that reduces the size of arteria lumen and increases the blood pressure. Furthermore, the foam cells release growth factors, as metalloproteinases-9 (MMP-9), and cytokines that will increase the inflammatory process and stimulate vascular smooth muscle cells migration into the intima arteria where they divide and produce extracellular matrix such as collagen and contribute to the formation of fibrous cap (FATKHULLINA; PESHKOVA; KOLTSOVA, 2016; TORRES et al., 2015). The destabilization and rupture of atherosclerotic plaques may may migrate to other vasels, leading to local ischemia (LEE et al., 2017).

Another morbidity associate to CVD development is the obesity. In this condition, the adipose tissue undergoes an expansion which may ultimately compromise its function (KOLIAKI; LIATIS; KOKKINOS, 2018). This expansion is mainly controlled by peroxisome-proliferator-activated receptors gamma (PPAR- γ), considered as the ‘master regulator’ of adipogenesis (LUNA-VITAL; WEISS; GONZALEZ DE MEJIA, 2017; ZEBISCH et al., 2012). The activation of PPAR- γ promotes adipocyte differentiation by fibroblasts and the lipid accumulation into adipocytes. Adipocytes are not only a lipid storage but also secretory cells that produce proinflammatory cytokines and adipokines that drive to a chronic low-grade inflammatory state (MARTINO et al., 2016). This process may lead to a activation of NF- κ B and downstream pro-inflammatory genes involving TNF- α , IL-6 and MCP-1 and others (GHIGLIOTTI et al., 2014). Furthermore, the adipogenesis stimulate the monocytes into adipose tissue and activate their differentiation to macrophages that produce the pro-inflammatory cytokines and chemokines, such as TNF- α , IL-6, IL-1 β , resistin, leptin, and others markers that will increase the inflammation process (DEBOER, 2013; KOLIAKI; LIATIS; KOKKINOS, 2018; LUNA-VITAL; WEISS; GONZALEZ DE MEJIA, 2017)

Considering these morbidities and the severity of CVD, strategies should be developed to reduce the risk factors and control this disease. The consumption of plant proteins has been inversely related to the risk of cardiovascular diseases (TANG et al., 2017). One of these plant protein source is the chia seed (*Salvia hispanica* L.) (DA SILVA et al., 2017). Chia 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).

Chia consumption has increased over the years worldwide, mainly due to its beneficial effects on markers related to obesity, diabetes, cancer, dyslipidemia and, cardiovascular diseases (JIN et al., 2012; NIEMAN et al., 2012; POUDYAL et al., 2013; TOSCANO et al., 2014; VUKSAN, et al., 2017a, b). These benefits are mostly due to high concentration of essential fatty acids, dietary fiber, phenolic compounds and proteins present in chia (AYERZA, COATES, 2011; SILVA et al., 2017).

The chia seed present about 20% of protein (DA SILVA et al., 2017) and the main storage protein fractions present in chia are prolamin, glutelin, albumin, and globulin, being the last two found in higher concentrations (KAČMÁROVÁ et al., 2016; ORONA-TAMAYO et al., 2015). After the gastrointestinal digestion, these proteins can originate peptides that according to their composition and amino acid sequence, may exert antimicrobial, antihypertensive, hypocholesterolemic, antithrombotic, antioxidants, and immunomodulatory effects, among others (CICERO, FOGACCI, & COLLETTI, 2017; SEGURA CAMPOS et al., 2013 a,b).

Research has shown that digested protein of chia inhibited the ACE-enzyme (angiotensin-converting enzyme) (ORONA-TAMAYO et al., 2015; SEGURA-CAMPOS et al., 2013; SEGURA CAMPOS et al., 2013b), reducing the blood pressure, high antioxidant, and antibacterial effects (COELHO et al., 2018; SEGURA-CAMPOS et al., 2013a) and showed inhibition of cholesterol synthesis (COELHO et al., 2018). Although there are many studies showing the promising results of the chia seeds, none of them have identified the bioactive peptides from this seed and from its storage proteins and their effects.

This study elucidated, for the first time, which are the peptides formed through simulated gastrointestinal digestion from the total protein and protein fractions from chia seeds and their physicochemical and biological potential properties. Furthermore, this study shows the effects of those digested proteins and pure peptides on inflammation, atherosclerosis, and adipogenesis process *in silico* and *in vitro*. In addition, pure peptides were *in vitro* tested to their anti-adipogenic effects.

2 OBJECTIVES

2.1 General objective

To investigate the effects of the digested total protein, protein fractions, and pure peptides from chia seed (*Salvia hispanica* L.) on inflammation, atherosclerosis, and adipogenesis process *in silico* and *in vitro*.

2.2 Specifics objectives

- To identify in the literature which are the proteins and peptides presents in chia seeds and biological effects on organism;
- To determine the chemical composition of chia seeds;
- To isolate, identify, and quantify the total and storage proteins from chia seeds;
- To identify and characterize the bioactive peptides from digested total protein and protein fractions from chia seeds;
- To determine the biochemical anti-inflammatory and antioxidant potential of the digested total protein and protein fractions;
- To analyze the interaction of peptides from chia seeds with markers of inflammation, atherosclerosis, and adipogenesis *in silico*;
- To identify the anti-inflammatory, anti-atherosclerotic, and anti-adipogenic potential of the digested total protein and protein fractions from chia seeds *in vitro*;
- To evaluate the anti-adipogenic effect of the pure peptides derived from chia seeds *in vitro*;
- To test the effect of the digested total protein and protein fractions from chia seeds to prevent and inhibit the induced.

3 HYPOTESIS

The digested total protein, digested protein fractions and pure peptides from chia seed (*Salvia hispanica* L.) will reduce the inflammation, atherosclerosis, and adipogenesis markers *in vitro*. Futhermore, digested total protein will present better results in comparison with digested protein fractions because the combination of these peptides probably is more powerful than fractions.

4 GENERAL METODOLOGY

4.1 Sample characterization and study design

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. 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).

This study can be organized into 5 stages as show the **Figure 1**: 1) Literature review to determine the auspicious effects of proteins from chia seeds; 2) Extraction, simulated gastrointestinal digestion of total protein and protein fractions, and identification and characterization of resulted peptides; 3) *In silico* analyses to identify the interactions between the peptides from chia seed and markers of inflammation, atherosclerosis, and adipogenesis; 4) Biochemically analysis to determine the effects of of digested total protein and digested protein fractions from chia seed on scavenging and inhibition of markers of oxidative stress, inflammation, and atherosclerosis; 5) *In vitro* analysis to verify the effects of digested total protein, digested protein fractions and pure peptides from chia seed on inflammation, atherosclerosis, and adipogenesis.

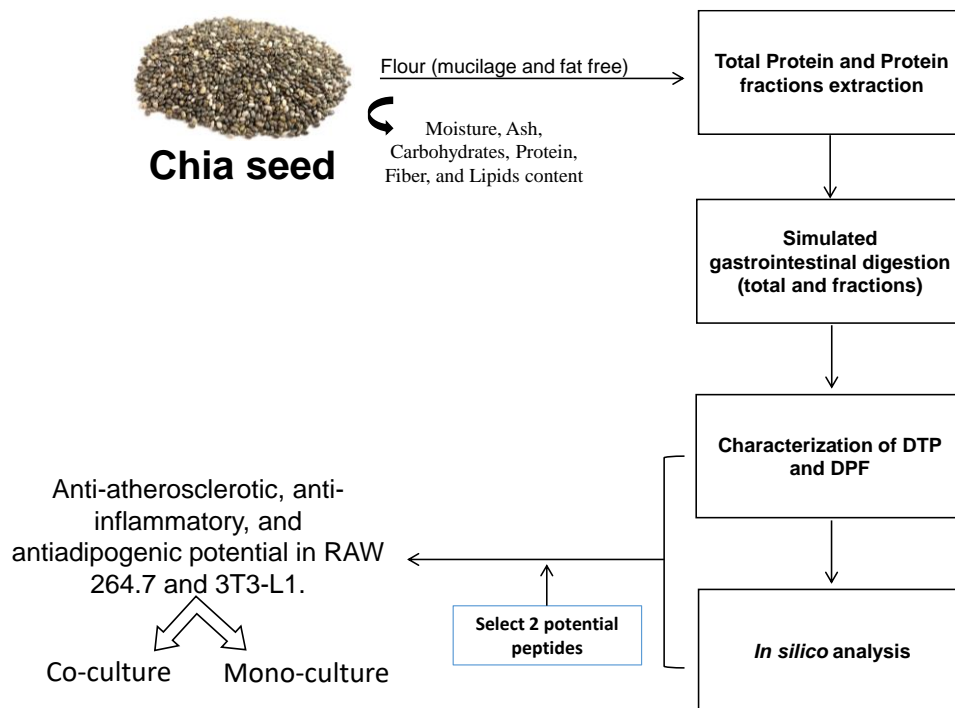


Figure 1. Study design

4.2 Chemical composition of chia seeds

The flour were obtained by seed grinding. Moisture was determined using an air 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 x 6.25 (AOAC, 2012; CAPITANI et al., 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: $[100 - (\% \text{ moisture} + \% \text{ lipids} + \% \text{ proteins} + \% \text{ total dietary fiber} + \% \text{ ash})]$. All chemical analyses were performed in triplicate. Only this step of study were performed in a Brazilian laboratory (Experimental Nutrition Laboratory - Nutrition and Health Department/UFV, Brazil).

4.3 Chia flour preparation

Chia seeds were processed according to Orona-Tamayo et al. (2015), with modifications (**Figure 2**). Briefly, the seeds were immersed in distilled water in a proportion 1:20 (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 by sieving (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.

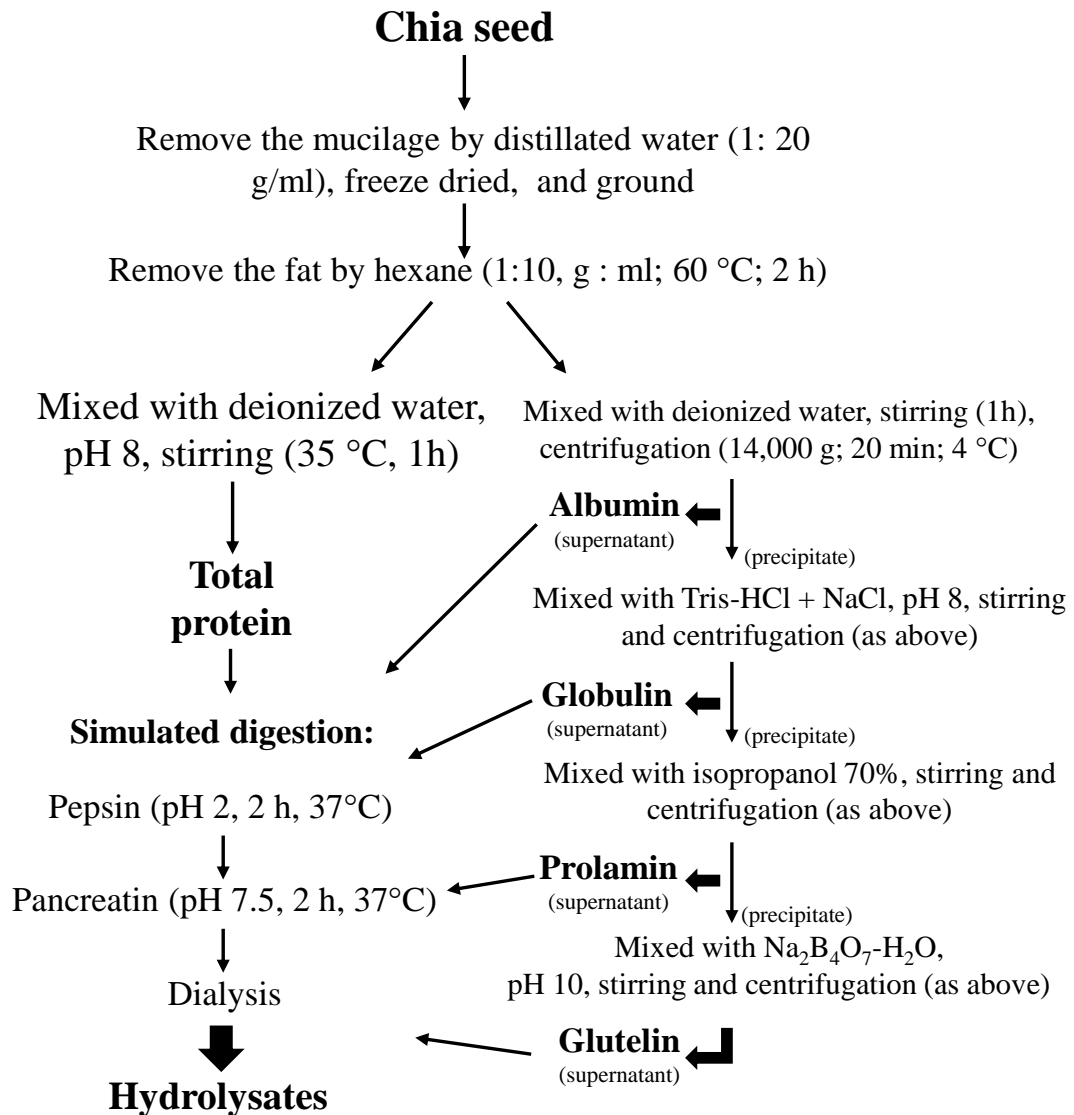


Figure 2. Diagram of the total protein and protein fractions extraction.

4.4 Extraction of total proteins

The mucilage and fat-free flour were mixed with deionized water (1:20 w/v), the pH adjusted to 8 with 0.1M sodium hydroxide and placed under constant stirring at 35 °C for 1 h. The mixture was centrifuged (5000g; 15 min; 25 °C), the precipitate was discarded and the supernatant (concentrated protein) was freeze-dried and stored at -20 °C (ALVES et al., 2016a; MONTOYA-RODRÍGUEZ et al., 2014a) (**Figure 2**). This extract were 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 fractions of proteins.

4.5 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 & 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 resuspended 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 Na₂B₄O₇•H₂O (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 (**Figure 2**). All samples were freeze-dried and stored at -20 °C until further analysis.

4.6 Simulated gastrointestinal digestion

In order to simulate gastrointestinal digestion, enzymatic hydrolysis with pepsin and pancreatin was conducted according by Mejias et al. (2004). The total and fraction proteins (prepared as indicated in 2.3 and 2.4) were suspended in distilled 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 (**Figure 2**).

4.7 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 performed 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 ($y = 0.0002x + 0.607$, $R^2 = 0.99$).

The protein profile was evaluated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide 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 (200V; 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 (Carestream Health Inc., Rochester, NY, USA). The molecular weight was calculated using the Carestream Program[®].

4.8 Degree of hydrolysis

The degree of hydrolysis (DH) was determined according to Nielsen, Petersen, & Dambmann, (2001) with modifications. The *o*-phthalaldehyde (OPA) reagent was prepared with 7.620 g di-Natetaborate 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 0.1–0.5 mg sample was diluted in 1 ml of the deionized water so that there was between 8-80% protein in the solution.

In tubes, 600 μ l of OPA reagent was added in 80 μ l of the standard, sample or water (blank) were mixed for 5 sec. Then, 200 μ l 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:

$$1) \quad \text{Serine-NH}_2: \left(\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \right) * 0.9516 * 0.001 * \left(\frac{100}{X * P} \right)$$

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

$$2) \quad h = \frac{\text{Serine-NH}_2 - \beta}{\alpha}$$

Where: $\beta = 0,40$ and $\alpha = 1$.

$$3) \quad DH = \left(\frac{h}{h_{hot}} \right) * 100$$

Where: $h_{hot} = 8.30$

4.9 Identification and characterization of potential bioactive peptides

The peptides obtained from the DTP and DPF were analyzed according to Mojica, Chen, & de Mejia (2015) by ultra-performance liquid chromatography–electrospray ionization–mass spectrometry (UPLC–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 μ L/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).

Peptides NSPGPHDVALDQ and RMVLPEYELLYE from glutelin fraction were selected by its high relative abundance on chromatogram (34.6% and 92.2%, respectively), antioxidant activity (predicted by BIOPEP database), low hydrophobicity and the presence of antioxidant and hydrophobic amino acids. The peptide NSPGPHDVALDQ was called as Pep1 and RMVLPEYELLYE as Pep2.

4.10 Antioxidant, anti-atherosclerotic and anti-inflammatory activity of DTP and DPF

4.10.1 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 & Janero (1995). An aliquot of 25 μ L of each DTP and DPF or PBS (blank) was mixed with 200 μ L of a solution composed by 0.1 mmol/L of ethylenediaminetetraacetic acid (EDTA), 62 μ mol/L of nitroblue tetrazolium chloride (NBT) and 98 μ mol/L of nicotinamide adenine dinucleotide (NADH). Then, 25 μ L of 33 μ mol/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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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

4.10.2 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 μ L of 20 mM sodium nitroprusside were mixed with 50 μ L of each DTP and DPF or PBS (blank) for 60 min, at room temperature. All solutions were prepared in 0.1 M PBS (pH 7.4). After incubation, 50 μ L 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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

A curve with Trolox (1.15-300 μ g/ml) was made to determine the TE ($y=0.0751\ln(x) + 0.0845$; $R^2 = 0.93$) (TSAI et al., 2011).

4.10.3 Hydrogen peroxide (H_2O_2) capacity

The ability of the samples to scavenge H_2O_2 was determined according to Ruch, Cheng, & Klaunig (1989). A 117 μ l of DTP and DPF were combined with 30 μ l of a H_2O_2

solution (40 mM). PBS was used as blank. Absorbance was measured after 10 min at 230 nm. H₂O₂ radical scavenging activity was calculated by the following formula:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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

4.10.4 DPPH radical scavenging activity

In a test tube, protected from light, 100 µL of each sample 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 sec. 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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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

4.10.5 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 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:

$$\% \text{ of inhibition} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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).

4.10.6 COX-1 and 2 inhibitor screening test

COX-1 and 2 (cyclooxygenase 1 and 2) inhibitor screening tests were conducted on DTP and DPF to evaluate the constitutively and anti-inflammatory effects, respectively. The COX-2 (human) and COX-1 (ovine) inhibitor screening assay were performed using a kit (Cayman Co., St. Louis, MO, USA) according to manufacturer's instructions. This analysis is based in the production of PGF₂ α by SnCl₂ (Tin(II) chloride) reduction of COX-derived PGH₂ 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 IC₅₀.

4.10.7 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. Results were expressed as % of inhibition of iNOS and calculation of IC₅₀ was made.

4.11 Anti-inflammatory effect of DTP and DPF *in vitro*

4.11.1 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% CO₂/95% air using a CO₂ Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). A concentration of 2.7x10⁴ cells was seed in 96 well plate and tested in concentrations of 0.1, 0.5, and 1.0 mg/ml 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) 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 effect of DTP and DPF, RAW 264.7 macrophages were seeded at 2.5 x 10⁵ in a six-well plate, and the total volume then adjusted to 2 ml with growth medium and incubated to 24 h at 37 °C in 5% CO₂/95%. After incubation, the cells were treated with the lipopolysaccharide (LPS) (1.0 μ M) and either DTP or each DPF to 24 h.

It were run a negative control (NC) group did not receive any treatment on culture media and a positive group (PC) treated with only LPS. As a pharmacological control (FC) group, the cells were treated with dexamethasone (1 μ M) and LPS.

After treatment for 24 h the growth medium and cell lysates were collected and frozen at -80 °C until analysis. All experiments were performed in duplicate.

4.11.2 Effect of chia seed total protein and protein fractions digests on nitric oxide production by cells

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., (GREEN et al., 1982) with modifications. Dosing of NO_2^- was performed in a 96-well microplate. The reaction was inhibited by adding 100 μ L 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 μ M) established for each experiment.

4.11.3 Influence of chia seed total protein and protein fractions digests on reactive oxygen species by cells

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 4.11.1. N-acetyl-cysteine (15 μ M) was used as standard control. One hour prior to completion of the treatment (as section 4.11.1), the media was removed and DCFDA in culture media (50 μ M/ 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.

4.11.4 Impact of chia seed total protein and protein fractions digests on prostaglandin-2, TNF-alpha, 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 for inflammation experiment. The amount of PGE-2, TNF- α , MCP-1, IL-6; IL-10, IL-12 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.

4.11.5 Influence of chia seed total protein and protein fractions digests on the expression of proteins related to inflammation pathways

Cell lysates were used for western blot to measure the expression of proteins related to inflammation, atherosclerosis and adipogenesis 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 μ g 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- κ B, p65-NF- κ B, p-JNK, JNK or c-JUN) 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). Band intensity was normalized using GAPDH antibody. All analysis were performed at least in duplicate and the results expressed in % of expression compared to a positive control.

4.11.6 Effect of chia seed total protein and protein fractions digests on nuclear translocation of NF- κ B p65 in the inflammatory process

Immunofluorescence and confocal laser-scanning microscopy were used to evaluate the nuclear translocation of NF- κ B 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 8-well chambers. The macrophages were treated according to the conditions indicated in Section 4.10.1 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- κ B 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 \times /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. Results were expressed in % of expression compared to a positive control.

4.12 Anti-atherosclerotic effect of DTP and DPF *in vitro*

4.12.1 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% CO₂/95% air using a CO₂ Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). A concentration of 2.7 \times 10⁴ cells was seeded in 96 well plate and tested in concentrations of 0.1, 0.5, and 1.0 mg/ml 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) for DTP and DPF that did not decrease the levels of nitric oxide and did not reduce cell viability were used in all analyses.

For the analysis of anti- atherosclerosis potential, the RAW 264.7 macrophages were seeded at 2 x 10⁵ 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% CO₂/95%, followed by another 24 h starve. The cells were treated with oxidized low-density lipoprotein (ox-LDL, 80 μ g/mL) for 48 h with/without DTP or each DPF.

It were run a negative control (NC) group did not receive any treatment on culture media and a positive group (PC) treated with only ox-LDL. As a pharmacological control (FC) group, the cells were treated with simvastatin (0.1 μ M) and ox-LDL.

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

4.12.2 Effect of chia seed total protein and protein fractions digests on nitric oxide production by cells

The accumulation of nitrite in the culture supernatant was measured by the Griess reaction, as described by Green et al., (GREEN et al., 1982) 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.

4.12.3 Influence of chia seed total protein and protein fractions digests on reactive oxygen species by cells

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 48 h, the cells were treated with samples and controls as described in Section 4.12.2. N-acetyl-cysteine (15 μM) was used as standard control. One hour prior to completion of the treatment (as section 4.10), the media was removed and DCFDA in culture media (50 μM / 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.

4.12.4 Impact of chia seed total protein and protein fractions digests on prostaglandin-2, TNF-alpha, 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 for atherosclerotic experiments. The amount of PGE-2, TNF- α , MCP-1, IL-6; IL-10, IL-12 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.

4.12.5 Influence of chia seed total protein and protein fractions digests on the expression of proteins related to atherosclerosis pathway

Cell lysates were used for western blot to measure the expression of proteins related to inflammation, atherosclerosis and adipogenesis 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 μ g 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, p65-NF- κ B, 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). Band intensity was normalized using GAPDH antibody. All analysis were performed at least in duplicate and the results expressed in % of expression compared to a positive control.

4.12.6 Influence of chia seed total protein and protein fractions digests in the formation of foam cells

The analysis of foam cells formation was performed as described by Xu et al., (2010) with modifications. Briefly, a concentration of 2.7×10^4 cells was seeded in a 96 well plate and were treated according to Section 4.12.1 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 destained with 60% isopropanol for 15 s and washed with PBS 3 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.

4.13 Anti-adipogenic effect of DTP, DPF, and pure peptides *in vitro*

4.13.1 Adipocyte treatment with chia seed total protein and protein fractions digests

3T3-L1 preadipocytes were grown in DMEM supplemented with 10% NCS (v/v) and 1% penicillin/streptomycin (v/v). The cells were seeded at a density of 6,000 viable cells/cm² and differentiated in adipocytes according to by Zebisch, Voigt, Wabitsch, & Brandsch (2012). Briefly, 3T3-L1 cells were seeded (Day 1) and differentiation stimulated by incubation after 48 h with DMEM containing 10% FBS (v/v), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 µM dexamethasone, 2 µM rosiglitazone and 1 µg/mL insulin (Day 3). After 48 h, the medium was replaced with DMEM containing 10% (v/v) FBS and 1 µg/mL insulin (Day 5). At day 7, 8, and 10 the media were replaced with 10% FBS-DMEM when 80-90% of the cells exhibited mature adipocyte phenotype. The cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere.

The media from inflamed RAW 267.4 macrophages (as explained above) were designated as conditioned media (CM) and used to inflame the mature adipocytes. The efficiency of the cells growing in the presence of 1 mg/mL of DTP, digested albumin, digested glutelin, and 100 µM of each pure peptide was assessed by aqueous solution CellTiter 96 one proliferation assay kit- MTS (Promega Corporation, Madison, WI, USA).

A total of four experiments were conducted in order to analyze the effects of DTP, digested albumin and glutelin, or pure peptides on adipogenesis process and inflammation on adipose tissue:

I- Effects of DTP or digested albumin or glutelin in 3T3-L1 adipocytes during the differentiation process: 3T3-L1 cells were seeded and differentiated (as above) and the DTP or digested albumin or glutelin at 1 mg/ml were added to the culture media during the differentiation process (days 3, 5, 7, 8, and 10).

II- Effects of pure peptides in 3T3-L1 adipocytes during the differentiation process: 3T3-L1 cells were seeded and differentiated as described above. The pure peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) at 100 µM were added to the culture media during the differentiation process (days 3, 5, 7, 8, and 10).

III- Effects of DTP or digested albumin or glutelin in prevention of inflammation in mature adipocytes stimulated with conditioned media from inflamed macrophages: 3T3-L1 cells were seeded and differentiated (as above). At 10th and 11th days of differentiation, CM replaced 50% of grown media from inflamed macrophages together with DTP or digested albumin or glutelin at 1 mg/ml for 24 h.

IV- Effects of DTP or digested albumin or glutelin on inhibition of inflammation in mature adipocytes stimulated with conditioned media from inflamed macrophages:

3T3-L1 cells were seeded and differentiated as described above. At 10th and 11th days of differentiation, CM from inflamed macrophages replaced 50% of grown media, and at days 12th and 13th were added in addition to the grow media plus CM, DTP or digested albumin or glutelin at 1 mg/ml.

After each treatment, the growth medium and cell lysates were collected and frozen at -80 °C until analysis. All experiments were performed at least in duplicate.

4.13.2 Effect of chia seed total protein, protein fractions digests, and pure peptides on nitric oxide production by cells

The accumulation of nitrite in the culture supernatant was measured by the Griess reaction, as described by Green et al., (GREEN et al., 1982) with modifications. Dosing of NO₂⁻ 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 NO₂⁻ was established from a standard curve of sodium nitrite (NaNO₂) (0.4-100 µM) established for each experiment.

4.14 Influence of chia seed total protein, protein fractions digests, and pure peptides on reactive oxygen species by cells

To determine the ability of the protein digests to inhibit the production of ROS, 2.5x10⁴, 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 4.13.1. N-acetyl-cysteine (15 µM) was used as standard control. One hour prior to completion of the treatment (as section 4.13.1), the media was removed and DCFDA in culture media (50 µM/ 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.

4.14.1 Impact of chia seed total protein and protein fractions digests on prostaglandin-2, TNF-alpha, 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 1:5 (v/v, sample: buffer) for experiments I and II and 1:25 (v/v, sample: buffer) for experiments III and IV. The amount of PGE-2, TNF- α , MCP-1, IL-6; IL-10, IL-12 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.

4.14.2 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, atherosclerosis and adipogenesis 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 μ g 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, p65-NF- κ B, PPAR γ , FAS, LPL, or SREBP1) 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). Band intensity was normalized using GAPDH antibody. All analysis were performed at least in duplicate and the results expressed in % of expression compared to a positive control.

4.14.3 Effect of digested total protein, digested albumin, glutelin or pure peptides on cellular lipid accumulation

The Oil RedO lipid staining was used to determine the differentiation parameter (LUNA-VITAL; WEISS; GONZALEZ DE MEJIA, 2017). The 3T3-L1 cells were seeded in 12-well plates and induced to differentiation (4.13.1 section). At the last day of each experiment, the cells were fixed with 10% formalin, washed with 60% isopropanol and a working solution of Oil Red O was added to each well (0.35% w/v Oil Red O in isopropanol overnight). Then, pictures were taken, and Oil Red O staining was eluted with 100% isopropanol for detection at 510 nm in a plate reader.

4.14.4 Effects of digested total protein, digested albumin, glutelin or pure peptides on inhibition of lipase activity and triglyceride content

3T3-L1 cells were cultured and treated as in sections 4.13.1. On the last day of each experiment, the cells were collected with RIPA buffer and at -80 °C. Inhibition of lipase activity was determined using a lipase activity assay kit (Cayman Chemical Item No. 700640), following manufacturer's instructions. Results were expressed as nmol/min/ml. Triglyceride content was analyzed using a Triglyceride Colorimetric Assay Kit (Cayman Chemical Item No. 10010303) according to manufacturer's instructions. Results were expressed as mg/dl.

4.15 *In silico* analysis

The structural mechanism by which peptides from DTP and DPF from chia seed interact with the atherosclerotic marker LOX-1, intracellular adhesion molecules (iCAM) and C-C chemokine receptor (CCR2), the inflammatory markers COX-2, p-65 NF- κ B and Toll-Like receptor 4 (TLR), and adipogenic markers PPAR γ , FAS, and monoacylglycerol lipase (MAGL) were evaluated by *in silico* analysis, through molecular docking as described by (ALVES et al., 2016a), 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., 2016a), dexamethasone (SUN et al., 2017), JSH23 (4-Methyl-1-N-(3-phenylpropyl) benzene-1,2-diamine) (WANG et al., 2018), and atorvastatin (Thongnak, Pongchaidecha, Jaikumkao, Chatsudthipong, Chattipakorn & Lungkaphin, 2017) were used, respectively for LOX-1, COX-2, p-65 NF- κ B, and TLR4.

The crystal structure file of LOX-1, iCAM, CCR2, COX-2, NF- κ B, TLR, PPAR γ , FAS, and MAGL were obtained from the Protein Data Bank (<http://www.rcsb.org/>) (PDB: 1YXK, 1IAM, 5T1A, 5KIR, 1OY3, 3FXI, 5DSH, 2PX6, and 3PE6, 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, & 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.

4.16 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 treatments. Data were analyzed in triplicate and expressed as the mean \pm standard deviation of two experiments. IC_{50} was calculated using linear regression. All statistical analyses were conducted using GraphPad Prism software, version 7.

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6. RESULTS

6.1 Manuscript 1



Chia Seed (*Salvia hispanica* L.) as a Source of Proteins and Bioactive Peptides with Health Benefits: A Review

Mariana Grancieri, Hercia Stampini Duarte Martino, and Elvira Gonzalez de Mejia 

Abstract: The consumption of chia seed (*Salvia hispanica* L.) has increased in recent years due its high content of omega-3 fatty acids and dietary fiber. This seed also has a high concentration of proteins and essential amino acids, becoming a promising source of bioactive peptides. The objective of this review was to identify the composition and the beneficial effects of chia seeds (*S. hispanica* L.), their proteins, peptides, and their potential impact on human health. The UniProt database was used to identify the chia proteins and their amino acid sequences. The BIOPEP database was used to analyze the peptides's bioactive potential. A total of 20 proteins were cataloged in chia seed, 12 of those were involved in the regular metabolic processes of the plant cells. However, eight proteins were specifically related to production and storage of plant lipids, thus explaining the high concentration of lipids in chia seeds (around 30%), especially omega-3 fatty acids (around 20%). The analyses of amino acid sequences showed peptides with bioactive potential, including dipeptidyl peptidase-IV inhibitors, angiotensin-converting enzyme inhibitors, and antioxidant capacity. These results correlated with the main health benefits of whole chia seed in humans such as antioxidant capacity, and hypotensive, hypoglycemic, and anticholesterolemic effects. Such relation can be associated with chia protein and peptide compositions and therefore needs further investigation *in vitro* and *in vivo*.

Keywords: antioxidant, bioactive peptides, chia seed, linolenic acid, linoleic acid, protein

Abstract

The consumption of chia seed (*Salvia hispanica* L.) has increased in recent years due its high content of omega-3 fatty acids and dietary fiber. This seed also has a high concentration of proteins and essential amino acids, becoming a promising source of bioactive peptides. The objective of this review was to identify the composition and the beneficial effects of chia seeds (*Salvia hispanica* L.), their proteins, peptides, and their potential impact on human health. The UniProt database was used to identify the chia proteins and their amino acid sequences. The BIOPEP database was used to analyze the peptides's bioactive potential. A total of 20 proteins were cataloged in chia seed, 12 of those were involved in the regular metabolic processes of the plant cells. However, eight proteins were specifically related to production and storage of plant lipids, thus explaining the high concentration of lipids in chia seeds (around 30%), especially omega-3 fatty acids (around 20%). The analyses of amino acid sequences showed peptides with bioactive potential, including dipeptidyl peptidase-IV inhibitors, angiotensin-converting enzyme inhibitors, and antioxidant capacity. These results correlated with the main health benefits of whole chia seed in humans such as antioxidant capacity, and hypotensive, hypoglycemic, and anticholesterolemic effects. Such relation can be associated with chia protein and peptide compositions and therefore needs further investigation *in vitro* and *in vivo*.

Keywords: chia seed, protein, bioactive peptides, antioxidant, linolenic acid, linoleic acid.

Introduction

History, classification, and botanical description of chia

Chia (*Salvia hispanica* L.) is an herbaceous plant that belongs to the order Lamiales, family Lamiaceae, subfamily Nepetoideae and genus *Salvia* (“Arctos Specimen Database”, 2018). The *Salvia* genus is considered the most numerous in the family Lamiaceae. It consists of approximately 900 species widely distributed throughout several regions of the world, including Southern Africa, Central America, North America, South America, and South-East Asia (TAKANO, 2017). The chia plant is native to northern Guatemala and southern Mexico and today is cultivated in Australia, Bolivia, Colombia, Guatemala, Peru, Argentina, and Mexico, the latter being the world's largest producer (BUSILACCHI et al., 2013).

Pre-Columbian populations consumed chia in the 16th century to provide energy, endurance, and strength. During the battles and expeditions, Aztec soldiers consumed chia to meet their nutritional needs. The oil extracted from the seeds has been used to produce cosmetics. In addition, chia seed was an offering to the gods in religious ceremonies or used as a form of payment of taxes (AYERZA, 2005). Other parts of the plant, such as the branches, leaves and roots, were used less commonly to fight respiratory infections (SOSA, 2016).

The chia plant is about 1 m tall and has simple leaves, which measure 4 to 8 cm long and 3 to 5 cm wide, oval-elliptical shape, pubescent, and with acute apex. A chia seed is quasi-oval, with a length between 1 and 2 mm, a diameter between 0.8 and 1.3 and a width between 0.8 and 1.4 mm. It has a smooth and shiny peel and coloring that can be black, brown, gray, black-spotted, or white. The mucilage is present inside the epidermal cells of mature chia seeds and when they come into contact with water it immediately expands rupturing the primary cell layer that protrudes from these epidermal cells thus surrounding the

seed, which increases its size and imparts a characteristic gel appearance to chia (**Figure 1**) (MUÑOZ et al., 2012).

The largest cultivation of seeds of this genus occurs in mountainous regions from temperate to subtropical (CAPITANI et al., 2012). The chemical composition and nutritional value of chia seed may vary according to climatic conditions, geographic location, nutrients, and soil conditions, as well as year of cultivation (Ayerza & Coates, 2009; Ayerza, 2009; da Silva et al., 2017). For example, the composition of fatty acids varies according to climate and the altitude of the plant; the colder and higher the region, the higher the content of omega-3 unsaturated fatty acids (AYERZA H; COATES, 2011).

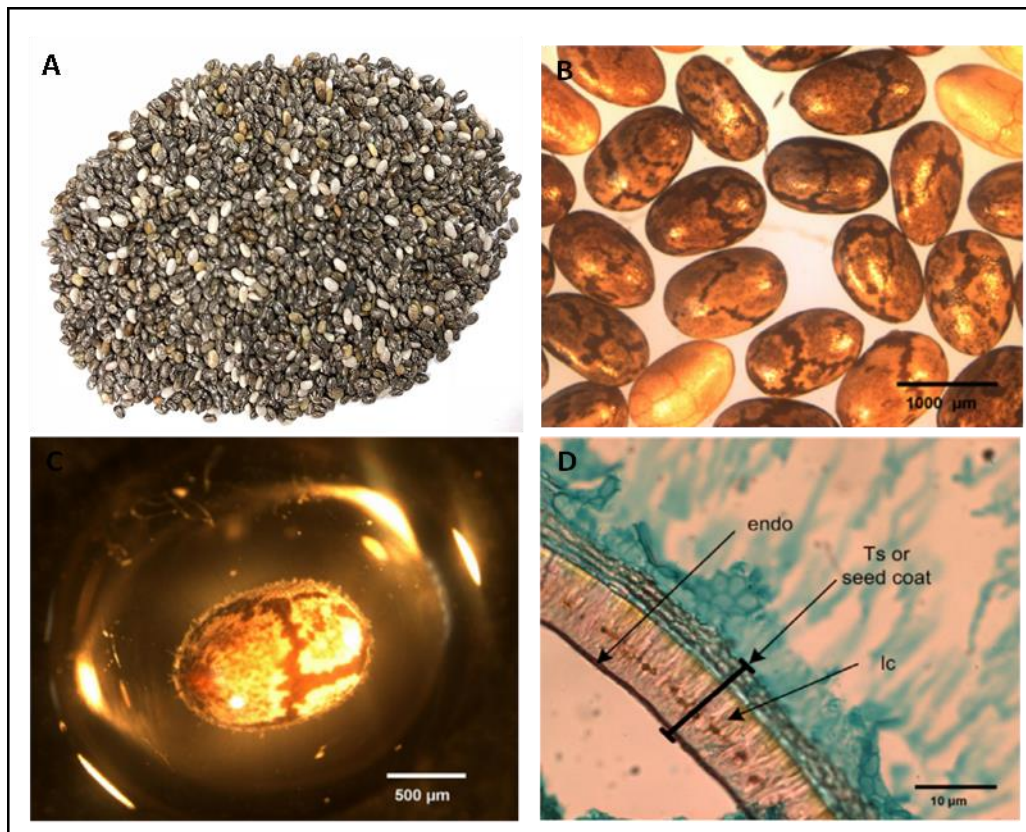


Figure 1. Chia seed. A: Seeds in full-size; B: Whole dry seed (approximate image); C: Whole seed hydrated with mucilaginous capsule around; D: Inside the seed: the three layers of rectangular cells forming the seed shell are observed; endo: endocarp layer; lc: sclereid layer. Source: Adapted from Muñoz et al. (2012) with permission from Elsevier.

Chia seed uses

The consumption of chia has been increasing over the years, given its health benefits related to chronic diseases such as obesity, cardiovascular diseases, diabetes, and cancer (Ixtaina, Nolasco, & Tom, 2008; Vázquez-Ovando, Rosado-Rubio, Chel-Guerrero, & Betancur-Ancona, 2010; Poudyal, Panchal, Ward, & Brown, 2013). These benefits result mainly from the high concentrations in chia seeds of essential fatty acids, dietary fibers, proteins, antioxidants, vitamins, carotenoids, and minerals (Reyes-Caudillo, Tecante, & Valdivia-López, 2008; Ayerza & Coates, 2011). Today, the chia seed is consumed whole or in the form of flour, alone (*in natura*), added to other foods, such as yogurts, salads, and fruits (Cahill, 2004; Vuksan et al., 2007), in preparations such as breads, cakes, granola bars, beverages, and others (**Figure 2**).

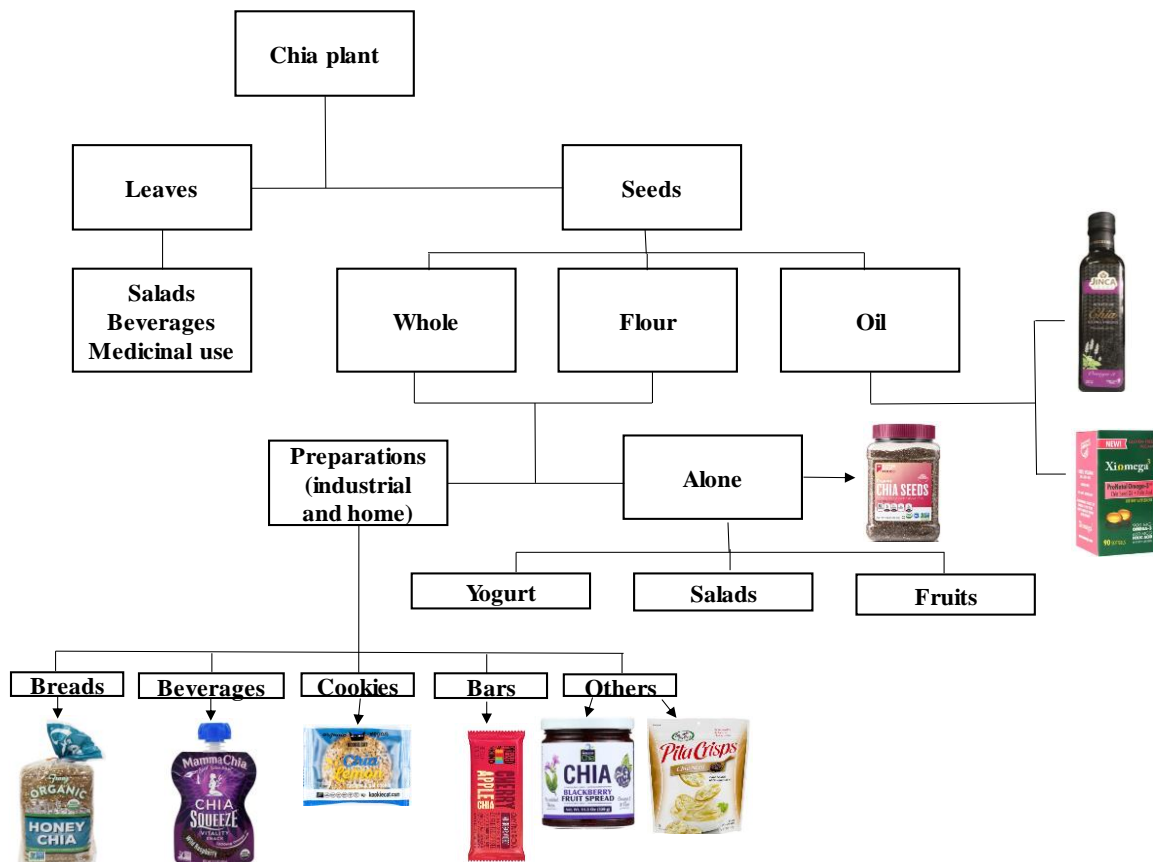


Figure 2. Uses of chia plant and some products obtained from chia seed

In general, the incorporation of chia in foods improve their physicochemical and sensory characteristics, especially their nutritional properties. The incorporation of chia seeds in tortillas reduces the rate of enzymatic hydrolysis of starch and the glycemic index (RENDÓN-VILLALOBOS et al., 2012). The chia seeds in bakery products increase their concentrations of proteins, unsaturated fatty acids, antioxidants, and dietary fiber (Iglesias-Puig & Haros, 2013; Segura-Campos, Salazar-Vega, Chel-Guerrero, & Betancur-Ancona, 2013). The gum present in chia seed has the ability to hold water and oil as well as having emulsifier and stabilizer potential (Segura-Campos et al., 2014). Furthermore, chia seed when added to wheat bread increases its antioxidant activity, nutritional content, textural properties (higher moisture content and lower hardness), color, and sensory profiles with 3.7 points in global acceptability score (1-5) (Sayed-Ahmad et al., 2018). Similarly, the use of 10% of chia flour in gluten-free bread received an overall acceptability scores of 8.1 on a 10 cm scale, and increased the levels of lipid, protein and dietary fiber in comparison with white gluten-free bread (Sandri, Santos, Fratelli, & Capriles, 2017). The use of 30% chia seed flour (w/w) in a gluten-free noodle formulation increased the content of protein, fat, antioxidant activity and total phenolic compounds in comparison to the control sample. Phytic acid and phytate phosphorus increased 889.39 mg/100 g and 250.81 mg/ 100 g, respectively. In this study, the content of Ca, P, K, Mg, Fe and Zn increased in noodles containing chia seed and there was a decrease of surface smoothness, appearance and chewiness score of raw and cooked noodle samples (Levent, 2017).

The protein-rich fraction obtained from the seeds has shown high thermal stability, between 70.4-125.0 °C, and good water-holding (4.06 g/g) and oil-holding (4.04 g/g) capacities (OLIVOS-LUGO; VALDIVIA-LÓPEZ; TECANTE, 2010). This high stability is associated with hydrophobic interactions between amino acids (OLIVOS-LUGO; VALDIVIA-LÓPEZ; TECANTE, 2010). The protein-rich fraction has high emulsifying

activity independent of pH, but at pH 8 and 10 the emulsion stability has been shown to be the highest, about 92%. These protein fractions also had good foam stability and viscosity (Vázquez-Ovando, Betancur-Ancona, & Chel-Guerrero, 2013). Furthermore, due to the ability of the fractions to form a gel, chia proteins are promising for food processing by providing consistency and thickening to various foods (BORNEO; AGUIRRE; LEÓN, 2010). These characteristics indicate that the proteins from chia seed are promising food additives that can help improve food quality and extend the shelf-life of foods (VALDIVIA-LÓPEZ; TECANTE, 2015).

Chia seed oil is also commercially exploited because of its rich content of essential fatty acids, such as alpha-linolenic (omega-3 or n-3) and alpha-linoleic acids (omega-6 or n-6) (MOHD ALI et al., 2012). The residual content of the oil extraction process is a source of dietary fiber (36.97 – 39.94 g/100 g) and polyphenolic compounds [chlorogenic acid (0.05-0.102 g/100 g), caffeic acid (0.01-0.003 g/100 g), phenolic glycoside-Q (0.25-0.31 g/100 g), and phenolic glycoside-K (0.40-0.50 g/100 g)] with antioxidant activity. In this way, chia seed can be used as a source of natural antioxidants with commercial applications (REYES-CAUDILLO; TECANTE; VALDIVIA-LÓPEZ, 2008).

The objective of this review was to identify the composition and the beneficial effects of chia seeds (*Salvia hispanica* L.), their proteins, peptides, and their potential impact on health. As a descriptive review, the information presented is related mainly to protein and bioactive peptides from chia seed only. Scopus and PubMed databases were used to locate publications with the descriptors: “chia seed”; “*Salvia hispanica*”; “chia seed” AND either “composition”, “health benefits”, “cholesterol”, “oxidative stress”, “hypertension”, “glycemia”, “protein”, “bioactive peptides”, “amino acids”, or “simulated digestion”; “*Salvia hispanica*” AND either “composition”, “health benefits”, “cholesterol”, “oxidative stress”, “hypertension”, “glycemia”, “protein”, “bioactive peptides”, “amino acids”, or “simulated

digestion”. Furthermore, Uniprot database was used to identify proteins using “*Salvia hispanica* L.” as a keyword. BIOPEP database was used to identify the bioactive potential of amino acid sequences from each identified protein. In the topic “Benefits of chia seed”, research on chia seed from human studies were included. Studies about chia oil were excluded.

Bioactive Compounds of Chia

Although the nutritional composition of chia depends on the cultivation conditions, it has, in general, a good nutritional value and promising bioactive compounds for human health (**Table 1**). Chia seed has low amounts of carbohydrates (3.4%) and high protein (18.9%) and lipid (31.2%) contents. There are high contents of alpha-linolenic (omega-3 or n-3) (19.5%) and alpha-linoleic acids (omega-6 or n-6) (around 5.2%), both essential nutrients since the human organism cannot synthesize them. The proportion n-6/n-3 in Brazilian chia seeds has been reported as 1:3 (DA SILVA et al., 2017). This high concentration of n-3 is associated with reduced risk of coronary artery disease, hypertension, type 2 diabetes, rheumatoid arthritis, autoimmune diseases, and cancer (MEYER; GROOT, 2017).

Table 1. Composition of chia seeds

Components	Content in Brazilian chia seeds ^A	Content in Mexican chia seeds ^B
Total dietary fiber (g/100 g)	33.37 ± 0.26	41.41 ± 0.2
Soluble dietary fiber (g/100 g)	2.89 ± 0.09	6.84 ± 0.9
Insoluble dietary fiber (g/100 g)	30.47 ± 0.35	34.90 ± 0.9
Lipids (g/100 g)	32.16 ± 0.29	35.13 ± 0.04
18:2 (n-6) (g/100 g)	5.69 ± 0.42	58.07
18:3 (n-3) (g/100 g)	20.37 ± 1.38	68.52 ± 0.02
Proteins (g/100 g)	18.18 ± 1.20	24.11 ± 0.43
Moisture (g/100 g)	7.14 ± 0.26	6.82 ± 0.13
Carbohydrates (g/100 g)	4.59 ± 0.34	1.51 ± 0.08
Total phenolic compounds (mg GAE)	0.97 - 0.99	0.757
Tannins (mg GAE/g)	14.93 - 19.08	n.d
Phytates (g 100 g ⁻¹)	0.96 - 1.16	n.d
Carotenoids (µg 100 ⁻¹)	57.01	n.d
Flavones (µg 100 ⁻¹)	6.07 - 16.03	n.d
Flavanones (µg 100 ⁻¹)	4.39 - 9.34	n.d
Vitamin E (µg 100 ⁻¹)	8169.50 - 8237.64	n.d

^AAdapted from Silva et al. (2017); ^BAdapted from Segura-Campos et al. (2014) and Reyes-

Caudillo, Tecante, Valdivia-Lopez (2008). n.d: not determined.

In addition, chia seed is rich in vitamins such as riboflavin (0.17 mg / 100 g), niacin (8.83 mg / 100 g), and thiamine (0.62 mg / 100 g) at levels above those of other seeds (MUÑOZ et al., 2012). Chia also has high concentrations of calcium (455 mg / 100 g), phosphorus (585 mg / 100 g), potassium (585 mg / 100 g), magnesium (340 mg / 100 g), iron (8.54 mg / 100 g), and zinc (3.70 mg / 100 g) (DA SILVA et al., 2017). The concentration of calcium in chia seeds is higher than that found in milk, as well as the concentration of iron, which is higher than found in good sources of this mineral such as liver (ULLAH et al., 2016).

Despite the high concentration of minerals in chia seed, its consumption by Wistar rats for 35 days revealed lower calcium balance and lower calcium absorption and retention rates in comparison with the group of animals that received calcium carbonate (control group) (DA

SILVA et al., 2019). However, male Sprague-Dawley rats fed a longer term (13 month) with 10% chia seed, versus a conventional isocaloric diet, showed higher bone mineral content and improved morphology of hepatocytes and gut tissue (MONTES CHAÑI et al., 2018). This study provided new data suggesting the potential benefits associated with the long-term intake of chia seed. Furthermore, the consumption of chia showed an iron bioavailability similar to ferrous sulfate (control group) (DA SILVA et al., 2016a).

Another characteristic of chia seed is its high concentration of antioxidant compounds, mainly phenolic acids and flavonoids. It is now known that rosmarinic acid is the phenolic compound present in the greatest amount (0.927 mg/g), followed by protocatechuic acid (0.747 mg/g), caffeic acid (0.027 mg/g), and gallic acid (0.012 mg/g) (MARTÍNEZ-CRUZ; PAREDES-LÓPEZ, 2014). Flavonoids are present, in great number, as flavones and flavanones. Tannins and phytates are present in small quantity, but other antioxidant compounds such as carotenoids and vitamin E appear in high amounts (DA SILVA et al., 2017; OLIVEIRA-ALVES et al., 2017) (**Table 1**).

Chia seed is also a source of dietary fiber (35%) (DA SILVA et al., 2017) in higher levels than other seeds, such as amaranth (7.3%), quinoa (7.0%) and corn (8.3%) (SRICHUWONG et al., 2017). Insoluble fiber is also present in greater quantity in chia (**Table 1**). It is primarily composed of lignin, cellulose, and hemicellulose, whereas mucilage is the main type of soluble fiber of the seed (REYES-CAUDILLO; TECANTE; VALDIVIA-LÓPEZ, 2008). This mucilage has high capacity for water absorption and can absorb about 27 times its own weight (MUÑOZ et al., 2012).

Benefits of Chia Seeds

Despite the existence of some chia protein studies that include bioactive peptides, most investigations have been focused on research regarding the whole seed. As described in

Table 2, several beneficial effects to living organisms, including humans, have been reported when consuming chia seed.

Table 2. Healthy benefits of chia seeds in humans.

Population	Age (years)	Dose	Duration (weeks)	Effects	Reference
Antioxidant capacity					
29 Hypertensives and overweight	35-65	35 g/day	12	↓ plasma MDA ↓ plasma nitrite	(TOSCANO et al., 2014)
76 Overweight	20-70	50 g/day	12	No effects	(NIEMAN et al., 2009)
Anticholesteremic					
10 postmenopausal women	53-60	25 g/day	7	↑ ALA ↑ EPA	(JIN et al., 2012)
29 overweight and obesity patients	35-65	35 g/day	12	↓ TC ↓ VLDL-c ↑ HDL-c	(Toscano et al., 2015)
76 Overweight	20-70	50 g/day	12	No effects	(NIEMAN et al., 2009)
62 postmenopausal women	53-60	25 g/day	10	↑ ALA ↑ EPA	(NIEMAN et al., 2012)
Antihypertensive					
29 Hypertensives and overweight	35-65	35 g/day	12	↓ MBP ↓ DBP ↓ SBP	(TOSCANO et al., 2014)
76 Overweight	20-70	50 g/day	12	No effects	(NIEMAN et al., 2009)

20 patients with type-2 Diabetics	18-75	37 g/day	12	↓ DBP ↓ SBP	(Vuksan et al., 2007)
Anthropometrics					
77 overweight or obese patients with type 2 diabetes	35-75	30 g/1000kcal	24	↓ body weight ↓ waist circumference ↑ adiponectin	(Vuksan et al., 2017)
29 overweight and obesity patients	35-65	35 g/day	12	↓ body weight ↓ % body fat	(TOSCANO et al., 2015)
76 Overweight	20-70	50 g/day	12	No effects	(NIEMAN et al., 2009)
29 Hypertensives and overweight	35-65	35 g/day	12	No effects	(TOSCANO et al., 2014)
62 postmenopausal women	53-60	25 g/day	10	No effects	(NIEMAN et al., 2012)
20 patients with type-2 Diabetics	18-75	37 ± 4 g/day	12	No effects	(Vuksan et al., 2007)
Hypoglycemic					
13 health patients	-	7, 15 or 24 g	acute	↓ iAUC	(HO et al., 2013)
11 health patients	-	7, 15 or 24 g	acute	↓ iAUC ↓ blood glucose	(Vuksan et al., 2010)
15 healthy participants	23.9 ± 3	25 g	acute	↓ AUC ↓ glucose peak ↑ time to glucose peak	(Vuksan et al., 2017)

62 postmenopausal women	53-60	25 g/day	10	No effects	(NIEMAN et al., 2012)
29 overweight and obesity patients	35-65	35 g/day	12	No effects	(TOSCANO et al., 2015)
76 Overweight	20-70	50 g/day	12	No effects	(NIEMAN et al., 2009)
77 overweight/obese with type 2 diabetes	35-75	30 g/1000kcal	24	No effects	(VUKSAN et al., 2017b)

MDA: malondialdehyde; ALA: alpha-linolenic acid; EPA: docosahexaenoic acid; TC: total cholesterol; VLDL-c: very low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; MBP: mean blood pressure; DBP: diastolic blood pressure; SBP: systolic blood pressure; iAUC: incremental area under the curve; AUC: area under the curve.

Antioxidant capacity

Chia seeds have numerous antioxidant compounds, such as vitamins, polyphenols, and peptides. These compounds can inhibit the activation of the NF- κ B transcription factor *in vitro*, thus reducing the inflammatory and carcinogenic processes (AGGARWAL; SHISHODIA, 2006; ELLULU, 2017; RAHMAN; BISWAS; KIRKHAM, 2006) and protecting against the attack of reactive oxygen species or nitrogen (ROS) (KAMPA et al., 2002). These antioxidant actions can protect the organism from pathologies, like neurological diseases, inflammation, immunodeficiency, ischemic heart disease, strokes, Alzheimer's and Parkinson's diseases, and cancer (MARCINEK; KREJPCIO, 2017).

It has been demonstrated that rats fed a high-fat diet including chia seeds for 6 or 12 weeks experienced a decrease of thiol levels and plasma catalase and glutathione peroxidase activities, while liver levels of the glutathione reductase became enhanced (Marineli, Lenquiste, Moraes, & Maróstica, 2015a). Rats that received a long-term sucrose-rich diet and were fed chia seeds, returned to the same activities of antioxidant enzymes catalase, superoxide dismutase, and glutathione reductase as control values (de Souza Ferreira, de Sousa Fomes, da Silva, & Rosa, 2015). In addition, increases in superoxide dismutase and

IL-10 plasma concentrations were observed when Wistar rats consumed chia seed flour plus a high-fat diet for 35 days in comparison with a control group (calcium carbonate) (Silva et al., 2018). In healthy humans who had received chia seeds (12 weeks) a better plasma antioxidant activity was observed compared to hypertensive (TOSCANO et al., 2014) or overweight patients (NIEMAN et al., 2009).

It has been demonstrated that germinated chia showed increased protein quality, as measured by protein efficiency ratio (PER). The amount of the γ -aminobutyric acid (GABA), total phenolic content, and antioxidant activity increased even more in the flour of germinated seeds (GÓMEZ-FAVELA et al., 2017), as well as in normal chia flour (DA SILVA et al., 2017; MARTÍNEZ-CRUZ; PAREDES-LÓPEZ, 2014; SARGI et al., 2013). The albumin and globulin fractions showed a high antiradical activity against DPPH and prolamin as well as globulin ability to chelate ferrous ions (ORONA-TAMAYO et al., 2015).

Anticholesterolemic

High concentrations of blood serum HDL-cholesterol (HDL-c) are directly associated with the development of cardiovascular disease (CVD) in humans (RASHEED; CUMMINS, 2018). The consumption of chia seed has showed promise for reducing the levels of serum cholesterol, since it has high concentrations of dietary fiber and unsaturated omega-3 fatty acids (DA SILVA et al., 2017). Most recently, it has been demonstrated that chia proteins and chia bioactive peptides can block key markers of cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (COELHO et al., 2018).

In a clinical study, 10 postmenopausal women who ingested 25 g / day of milled chia seed over a 7-week period showed increased plasma levels of alpha-linolenic acid (ALA) and docosahexaenoic acid (EPA) by 138% and 30%, compared to baseline levels, respectively (JIN et al., 2012). Also, the consumption of 35 g of chia flour / day by overweight and obese

adults resulted in a reduction in total cholesterol and very low-density lipoprotein cholesterol (VLDL-c), and an increase in HDL-c (TOSCANO et al., 2015). Furthermore, 62 postmenopausal women had increased plasma levels of ALA and EPA after ingestion of 25 g / day milled chia for 10 weeks (NIEMAN et al., 2012).

In animal studies, chia consumption has resulted in better lipid redistribution associated with cardioprotection and hepatoprotection. This was observed in rats receiving a hyperlipidic and hyperglycemic diet, who also presented an inhibition of enzyme stearyl-CoA 9-desaturase index in liver and heart (POUDYAL et al., 2012a). Another investigation with rats also found improvement in dyslipidemia and insulin resistance induced by the consumption of a sucrose-rich diet (62.5%) after ingestion of chia seeds (CHICCO et al., 2009). Wistar rats fed both conventional and thermally treated chia, had lower glucose concentrations, triacylglycerides, LDL-c, VLDL-c, and increased levels of HDL-c, hypertrophy of intestinal muscle layers, and good protein digestibility (DA SILVA et al., 2016b). Another study evaluated the effect of chia seed on rats fed a sucrose-rich diet in the long-term with consequent adipose tissue dysfunction. The authors observed that chia reduced epididymal fat and normalized dyslipidemia and insulin sensitivity induced by sucrose (de Souza Ferreira et al., 2015). In a separate study, a sucrose-rich diet and containing chia seed fed to Wistar rats for either 3 or 5 weeks reduced the epididymal fat, normalized dyslipidemia and insulin sensitivity (Rossi et al., 2013). In this study, the consumption of chia by the animals prevented (3 months) or normalized (5 months) dyslipidemia, liver TAG, FAS, ACC (acetyl-CoA carboxylase) and G-6-PDH (glucose-6-phosphate dehydrogenase) activities and PPAR α and SREBP-1 proteins levels. Also, chia seed increased fatty acid oxidase (FAO) and CPT-1 activities (carnitine-palmitoyl-transferase-1).

In a pregnant rats study in which corn oil was replaced by chia seed in a sucrose-rich diet, the offspring showed lower liver steatosis, hypertriglyceridemia and

hypercholesterolemia. Furthermore, CPT-1 and ACC enzyme activities and free fatty acid were reduced in the plasma of offspring from progenitors fed the chia seed containing diet (Fortino et al., 2017). Similarly, male rats that received chia seed instead of corn oil in the sucrose-rich diet improved heart lipotoxicity, increased FAT/CD36 (fatty acid transporter) proteins levels and M-CPT1 (muscle-type carnitine palmitoyltransferase 1) activity. In addition, there was a reduction in the PPAR α proteins and plasma fatty acids (FAs) levels. Authors suggested that the normalization of dyslipidemia by chia was due to the prevention of translocation of FAT/CD36 that reduced the influx of FAs, decreasing elevation of M-CPT1 activity and lipid storage, thus improving glucose oxidation in cardiac muscles (Creus et al., 2016). Furthermore, the consumption of chia seed flour for 35 days reduced TC, LDL-c, and VLDL-c, but increased PPAR- α protein levels in Wistar rats fed with a high-fat diet (Silva et al., 2018).

Antihypertensive

Together with dyslipidemias, hypertension or high blood pressure (BP) is one of the most important risk factors for CVDs (Fowokan et al., 2018). Some studies have shown a promising potential of chia seeds to reduce BP. In hypertensive adults, the consumption of 35 g / day of chia flour for three months reduced BP, lipid peroxidation, and plasma nitrite concentrations. These effects were attributed to a large amount of n-3 fatty acids in chia that exerted antioxidant and antiinflammatory effects (TOSCANO et al., 2014). Also, patients with type-2 diabetes had a reduction of systolic and diastolic pressure after consumption of 35 g / day of chia for a period of 12 weeks (VUKSAN et al., 2007). However, some studies have not found a change in BP after treatment with chia seeds in humans (Vuksan et al., 2009; Toscano, Toscano, Tavares, Oliveira, & Silva, 2015).

In a study with pregnant rats in which corn oil was replaced by chia seed in a sucrose-rich diet, the offspring showed a lower incidence of hypertension, liver steatosis, hypertriglyceridemia, and hypercholesterolemia. These results were associated with a high concentration of the omega-3 in chia seeds (FORTINO et al., 2017). Similarly, male rats that received chia seeds instead of corn oil in the sucrose-rich diet had, among other effects, normalization of blood pressure after 3 months of treatment (CREUS et al., 2016). Male rats that received a high-fat and high-fructose diet, for 8 weeks plus 8 weeks with 5% of the diet with chia seeds as treatment, showed several benefits regarding markers of CVDs. In addition, there were improvements in insulin sensitivity and glucose tolerance, reduced visceral adiposity, decreased hepatic steatosis and reduced cardiac and hepatic inflammation and fibrosis, but there was no change in blood pressure (POUDYAL et al., 2012a).

Furthermore, chia seeds have shown a hypotensive effect by enzymatic analysis. The chia protein hydrolysate has the same blocking activity of angiotensin-converting enzyme I (ACE-I) as done by synthetic ACE-I inhibitors. The authors of this study observed that the hydrophobic residues of chia protein had a similar action to that of synthetic ACE-I inhibitors, likely because they block the production of angiotensin II (SEGURA CAMPOS et al., 2013b). In addition, the C-terminal amino acids were believed to be responsible for the higher inhibitory ACE activity (SEGURA-CAMPOS et al., 2013).

Anthropometrics

Due to the composition of chia, high in dietary fiber and low in carbohydrates, this seed has demonstrated the ability to increase satiety and reduce the desire to eat (AYAZ et al., 2017; VUKSAN et al., 2017a). These observations can be associated with other results that have demonstrated that the consumption of 30 g /1000 kcal of chia seed during six months by overweight and diabetic adults resulted in weight loss, reduction of waist circumference and

C-reactive protein and increase in adiponectin (VUKSAN et al., 2017a). Moreover, overweight and obese adults who received 35 g / day for 12 weeks had reductions in weight and percent fat (TOSCANO et al., 2015).

However, in one other study, overweight adults who consumed 25 g / day of chia did not have a reduction in their body mass index, waist circumference, or insulin resistance (NIEMAN et al., 2009). Also, the consumption of 35 g / day of chia seed by overweight adults for 12 weeks did not promote any significant change in body mass index or waist circumference (TOSCANO et al., 2014). Similarly, individuals with type 2 diabetes who consumed 37 g / day chia did not have changes in body weight (Vuksan et al., 2007). Furthermore, 62 postmenopausal women had no changes in their body composition after consumption of 25 g / day milled chia for 10 weeks (NIEMAN et al., 2012).

In a Wistar rat experiment in which soybean oil and cellulose contained in a standard basal diet were replaced by chia flour and heat-treated or untreated chia seeds, experienced weight loss as compared to the group of animals consuming the standard control diet (DA SILVA et al., 2016b). However, in another investigation, chia flour did not reduce the weight of Wistar rats fed with a high-fat diet (HFD) (DA SILVA et al., 2016a; MARINELI et al., 2015a, 2015b). It has also been reported that chia seed consumption for 13 months increased the body weight in rats as compared to a control group (Poudyal et al., 2012; Montes Chañi et al., 2018). Although chia seeds increased the weight in rats fed a HFD, it also reduced the visceral adiposity index and decreased the retroperitoneal and omental fat depositions (POUDYAL et al., 2012a).

Hypoglycemic

High levels of glycemia in blood can activate pathways related to overproduction of reactive oxygen species that induce a biochemical cascade resulting in increased inflammation

and endothelial dysfunction. These conditions are associated with the development of diseases such as diabetes and cardiovascular problems (Nazarian-Samani, Lorigooini, & Rafieian-Kopaei, 2018).

In an acute study, healthy adults who consumed 25 g of ground chia, together with a glucose challenge, had a reduced blood glucose area under the curve (AUC) over 120 min and a reduction of peak glucose and increased time to peak compared with the control (Vuksan et al., 2017). Also, ground and whole chia were incorporated in bakery products (7, 15, and 24 g) and consumed by healthy individuals after 10-12 h of fasting, and there were reduced blood glucose incremental areas under the curve (iAUC) as compared to the control (HO et al., 2013) and postprandial glycemia (VUKSAN et al., 2010). However, individuals with type 2 diabetes who consumed 30 g / 100 kcal chia for 6 months did not experience changes in glycated hemoglobin or fasting glucose (Vuksan et al., 2017). Similar results were observed in overweight or obese individuals who ingested, for 12 weeks, 35 g / day chia flour (TOSCANO et al., 2015) or 25 g / day chia seeds (NIEMAN et al., 2009). Furthermore, consumption of 25 g / day of chia seed by postmenopausal women over 10 weeks did not show any effects on serum glucose (NIEMAN et al., 2012). These results demonstrate a positive action of chia in acute studies, but not in chronic studies in humans.

In animal studies, both, chia seed and chia flour (heat-treated and untreated), reduced plasma glucose in normal Wistar rats after 14 days of treatment (DA SILVA et al., 2016b). In one of these studies, a group of animals received a HFD together with chia flour for 12 weeks (prevention group). The other group was fed initially only a high-fat and high-fructose (HFF) diet for 6 weeks, followed by an additional 6 weeks with HFF diet containing chia seed (treatment group). On the final day of the study, the consumption of chia seeds had improved glucose and insulin tolerance for both groups, prevention and treatment (Marineli et al., 2015b). These results may be associated with the expression of HSP70, HSP25 (heat shock

proteins) and peroxisome proliferator-activated receptor-g coactivator-1a (PGC-1a) in skeletal muscle. Both of these proteins protect against insulin intolerance, increase control of energy homeostasis and glucose metabolism (Marineli et al., 2015b). Similar results were observed in Wistar rats fed with a high-fat and high-carbohydrate diet and 5% of chia seeds during 8 weeks in comparison with a control group (POUDYAL et al., 2012a).

Proteins from chia (*Salvia hispanica* L.)

Nutritional quality

Proteins from animal sources are of good quality, but costly, and in some individuals can cause allergies or intolerances, such as egg or milk proteins. Plant proteins can be a good source of essential amino acids, complementing or even replacing animal sources (Sandoval-Oliveros & Paredes-López, 2013; Montoya-Rodríguez, Gómez-Favela, Reyes-Moreno, Milán-Carrillo, & Gonzalez de Mejia, 2015). The chia seed contains around 19% protein, and therefore it is considered a good source of this nutrient (**Table 1**). The protein concentration is greater than that found in other traditional grains, such as wheat (14%), barley (9.2%), oats (15.3%), corn (14%) and rice (8.5%) (MONROY-TORRES et al., 2008).

Moreover, chia protein has a good digestibility (78.9%), similar to that of casein (88.6%) (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013) and beans (77.5%), and higher than maize (66.6%), rice (59.4%), and wheat (52.7%) proteins (BETANCUR-ANCONA; GALLEGOS-TINTORÉ; CHEL-GUERRERO, 2004), but less than amaranth (90%) (GROBELNIK-MLAKAR et al., 2009). The digestibility value of chia protein is a general indicator of the nutritional quality of its proteins, and it may be associated with their chemical structures which make them more or less susceptible to proteolytic enzymes (LÓPEZ et al., 2018).

Chia seeds contain all essential amino acids for human nutrition: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine, and valine (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013). Among all amino acids, glutamine is in highest concentration and histidine is the least present. The chemical score of chia seeds is 100% satisfactory for the sulfur amino acids and the coverage for the remaining essential amino acids ranges from 52 to 76%. The content of essential amino acids in seed flour varies from 66 to 126%. Lysine is the limiting amino acid with the lowest coverage of requirement (**Table 3**) (FAO/WHO/UNU, 2008; SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013).

Table 3. Amino acid composition of chia seed and contribution of essential amino acids with required patterns for infants and adults.

Amino acid	Content in the seed flour (mg/ g raw protein)	Contribution of essential amino acids (%)			
		Infants (0.5-1 year)		Adults (>18 years)	
		RP	% CR	RP	%CR
Aspartate	47.3 ± 0.9				
Glutamine	70.8 ± 1.1				
Serine	26.2 ± 0.3				
Glycine	22.8 ± 0.7				
Arginine	42.3 ± 0.4				
Alanine	26.8 ± 0.3				
Proline	19.9 ± 0.7				
Histidine	13.7 ± 0.1	20	69	15	91
Threonine	18.0 ± 0.2	31	58	23	78
Valine	25.5 ± 0.4	43	66	39	73
Methionine + cysteine	27.8 ± 0.5	28	99	22	126
Isoleucine	24.2 ± 0.4	32	76	30	81
Leucine	41.5 ± 0.6	66	63	59	70
Phenylalanine + tyrosine	38.8 ± 0.5	52	75	38	102
Lysine	29.9 ± 0.5	57	52	45	66

RP= requirement patterns (mg/g raw protein) (FAO/WHO/UNU, 2008); CR= coverage of requirement for that specific essential amino acid in percentage according of age. Source: Adapted from Sandoval-Oliveros, Paredes-López, 2013.

Seed proteins can be classified based on different criteria such as function and differential solvent solubility, among others. The storage proteins are those proteins which are present to supply intermediary nitrogen compounds for biosynthesis at a metabolically active stage of seed development (LÓPEZ et al., 2018). The main storage proteins present in chia are prolamins, glutelins, albumins, and globulins, the latter two are found in greater quantity than the others (Sandoval-Oliveros & Paredes-López, 2013; Orona-Tamayo, Valverde, Nieto-Rendón, & Paredes-López, 2015; Kačmárová, Lavová, Socha, & Urmínská, 2016).

The amount of protein may vary according to the botanical source, plant variety, preparation of the meal, extraction method, and other factors (Vázquez-Ovando et al., 2010). The protein fractions in chia seed have been shown to contain mostly 11S and 7S proteins whose molecular sizes range from 15 to 50 kDa, under native conditions. The presence of the globulins 7S and 11S in ingredients may confer nutritional and physiological characteristics to foods that are dependent on their structural sequence and physicochemical properties (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013).

The denaturation temperatures of crude albumins, globulins, prolamins, and glutelins are 103, 105, 86, and 91 °C, respectively, thus indicating an excellent thermal stability for albumins and globulins (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013). The stability at high temperatures can be an indicator of hydrophobic bonds between the amino acids that make up the proteins, since hydrophobic interactions are entropy-driven and endothermic; they are stabilized at a high temperature (60-70 °C), but destabilized at a low temperature (OLIVOS-LUGO; VALDIVIA-LÓPEZ; TECANTE, 2010). Moreover, the denaturation peak temperature (T_d) has been reported as single and high (97 °C), but the thermal stability of chia proteins was improved (108.6 °C) when it interacted with chia seed gum by complex coacervation (TIMILSENA et al., 2016).

Chia protein isolates showed a good water-holding capacity, which is the amount of water withheld by the hydrated protein after having applied an external force. Isolates also have an excellent oil-holding capacity, which is the union of fat by means of the lateral nonpolar protein chains. These characteristics indicate the presence of hydrophobic amino acids and demonstrate the possibility of using chia in emulsions and bakery products (OLIVOS-LUGO; VALDIVIA-LÓPEZ; TECANTE, 2010).

Identified proteins from chia seeds

Twenty proteins from chia seeds have been identified in the literature based on their amino acids sequences (www.uniprot.org). Twelve of these proteins are responsible for the metabolic functions needed for the existence of the seed: as metabolism, cell division, and pathways. Eight proteins are related to lipid production and storage (**Table 4**). The main functions of these proteins are described below.

Table 4. Proteins from chia seed (*Salvia hispanica*) and its function.

Protein	ID	Function	Sequence	AAR	MW
Ribulose biphosphate carboxylase large chain	Q36769	The enzyme that converts carbon dioxide from the biosphere into organic carbon in the rate-limiting step of the Calvin cycle and photorespiration, depending on its affinity for carbon dioxide or molecular oxygen (ANDERSSON; BACKLUND, 2008).	MSPQTETKASVGFKAGVKEYKLYYTPEYE TKDTDILAAF RVTPQPGVPPEEAGAAVAAE SSTGTWTTVWTDGLTSLDRYKGRCYHIEPV PGEKDQYICYVAYPLDLFEEGSVTNMFTSIVGN VFGFKALRALRLEDLRIPVAYVKTFQGGPHGIQ AERDKLNKYGRPLLGCTIKPKLGLSAKNYGRA VYECLRGGLDFTKDDENVNSQPFMRWRDRFL FCAEAIYKSQAETGEIKGHYLNATAGTCEEMM KRAIFARELGVPIVMHDYLTGGFTANTSLAHY CRDNGLLLHIHRAMHAVIDRQKNHGMHFRVL AKALRLSGGDHIHSGTVVVGKLEGERDITLGFV DLLRDDFVEKERSRGIYFTQDWVSLPGVIPVAS GGIHVWHMPALTEIFGDDSVLQFGGGTLGHP WGNAPGAVANRVAVEACVLARNEGRDLAAE GNAIIREACKWSPELAAACE VWKEIKFEFP AMD	473	52,390
Oleosin	A0A0F6PN28	It is responsible by triacylglycerol stored, protecting, and stabilization in seeds. Oleosins contain the amphipathic N- and C- terminal domains exposed to the cytosol, and a hydrophobic central domain formed by a long chain of non-polar amino acids (HUANG, 2017; VALDIVIA-LÓPEZ et al., 2017).	MADQHYGQFQ SRPHHLQQHH PRSHQMVKAA TAVTAGGSLL VLSGLTLAAT VIALTIATPL LVIFSPVLVP AALAVFALAG GFLASGGFGV AALSVLSWIY KYMTGKHPVG ADQLDTARTK LAGKARDMKD RVDHNVSVAQ SS	142	14,894

Fatty acid desaturase 3 isoform 2	A0A1Z1EC60	Catalyze the introducing of a third double bond at the delta-15/ omega-3 carbon position of linoleic acid in the endoplasmic reticulum, for producing α -linolenic acid (ALA), also called, omega-3, in plants (XUE et al., 2018).	MAVSSGADAE HHGHAQYEHL GKRAADKFDP AAPPPFKIAD IRAAIPPHCW VKDPLRSLSY VAWDFVVAAL LAAAAFFDS WIFWPIYWAA QGTMFVALFV LGHDCGHGSF SDNTTLNNVV GHVLHSSILV PYHGWRISHR THHQNHGHVE NDESWVPLTE NLYKQLDFST KFLRYKIPFP MFAYPLYLWY RSPGKSGSHF NPYSLFFKPN ERDLVITSTI CWAAMVACLL YASTIVGPTM LFKLYGVPYL IFVVWLDTVT YLHHHGVDKK LPWYRSKEWS YLRGGLTTVD QDYGIFNKIH HDIGTHVHH LFPQIPHYHL VEATREAKRV LGNYYREPRK SGAVPFHLVP TLLKSLSRDH YVSDNGDIVY YQTDGELFSS KEI	386	43,983
Fatty acid desaturase 3 isoform 1	A0A1Z1EC53	As above	MAVSSGARLS ESGAEGGEPY AGQCEHLEGI GKRAADKFDP AAPPPFKIAD IRAAIPPHCW VKDPLRSLSY VAWDLIAVAA LAAAAFFDS WIFWPIYWAA QGTMFVALFV LGHDCGHGSF SDSTTLNNVV GHILHSSILV PYHGWRISHR THHQNHGHVE KDESWVPLPE NLYKQLDFST KFLRYKIPFP MFAYPLYLWY RSPGKTGSHF NPSSLFFKPN ERDLVITSTV CWAAMVAFLL YASTIVGPTM LFKLYGVPYL IFVVWLDTVT YLHHHGVDKK LPWYRSKEWS YLRGGLTTVD QDYGIFNKIH HDIGTHVIHH LFPQIPHYHL VEATREAKRV LGNYYREPRK SGPVPFHLIP TLLKSLSRDH YVSDNGDIVY YQTDSQLFSS KEI	393	44,873

Fatty acid desaturase 7 isoform 1	A0A1Z1EC64	Catalyze the introducing of a third double bond at the delta-15/ omega-3 carbon position of linoleic acid in the plastids, for producing α -linolenic acid (ALA), also called, omega-3, in plants (XUE et al., 2018).	MASWVLSGCG LKPLPRIYPM PRTVSSPNPS KLRISTADFS SDSSSLCSVG RGRNWGLNVS APLRFQEVGE EENEERESEV VNGFGGGDGF DPGAPPPFKL ADIRAAIPKH CWVKNPWKSM SYVVRDVAVV FGLAAAAAYL NNWAVWPLYW FAQGTMFWAL FVLGHDCGHG SFSNDPKLNS VAGHLLHSSI LVPYHGWRIS HRTHHQNHGH VENDESWHPL SEKIYKQLDF VTKKLRFTLP FPMLAYPIYL WSRSPGKKGS HFHPDSDLFV PNERKDVITS TVCWTAMVAI LAGLSFVMGP LQLLKLYGIP YFGFVAWLDL VTYLHHHGHE DKLPWYRGKE WSYLRGGLTT LDRDYGWINN IHHDIGTHVI HHLFPQIPHY HLIATEAAK PVLGKYYKEP QKSGPLPLYL LGVLAKSMKK DHYVSDTGD VYYQTDPKLN	440	49,789
Fatty acid desaturase 8	A0A1Z1EC52	As above	MASFVISGCG LKPLPRIYPK PRSVQNSFST SNLRISRPNQ FSSSSIGINQ KRNWGLGVSA PLRIQPLEEE NEEFDPAAPP PFKLSDIKAA IPKHCWVKDP WRSVGYVVRD VVAVLGMAAA AAYFNWIVW PLYWFAQSTM FWALFVLGHD CGHGSFSNNP KLNSVFGHFL HSSILVPYHG WRISHRTHHQ NHGHVENDES WHPMPEKIYN SLDSMAKKLR FTLPPMLAY PIYLWTRSPG KKGSHYHPDS DLFVPAERKD VITSTVCWTA MAALLVGLSF VMGPIQLLKL YGIPYLGFA WLDTVTYLHH HGHEKLPWY RGKEWSYLRG GLTTLDRDYG LNNIHHDIG THVIHHLFPQ IPHYNLIEAT EAAKGVLGKY YREPKKSGPL PLHLLGDLVR SLKKDHVSD TGDVVYYQTD PQLNGGQKS	429	48,721

Fatty acid desaturase 2 isoform 2	A0A1Z1EC55	It is a bi-functional hydroxylase/desaturase and tri-functional acetylenase responsible for inserting a double bond at the delta-12 position of oleic acid, producing linoleic acid (omega-6). The FAD2 is a multifunctional enzyme that act in the biological membrane systems, signaling, energy storage, thermal adaptation and resistance to biotic and abiotic stresses in plants (SHARMA; CHAUHAN, 2012).	MGAGGRMSVP PAEKAAKSDI VQRPHTKPP FTLGDIKKAI PPHCFKRSIP RSFSYVVYDL VFASLFYYVA TNYIHQLPHP LSYPAWILYG ICQGCILTGV WVIAHECGHH AFSDYQWLDD TVGLILHSFL LVPFFSWKYS HRRHHSNTGS LERDEVFVPK VKTGVSAAK YMNNPPGRLI TLVVQLTLGW PLYLMFNVS G RPYDRFACHF DPNSPIYS DR ERAQIFISDA GILAVTYGLY RLSVAKGLAW VLCVYGGPLL VVNGFLVLIT FLQHTHPSLP HYDSSEWDWL RGALSTVDRD YGILNTVFHN ITDTHVAHHL FSTMPHYHAM EATKVIKPI L GKYYQFDGTP VFKAMFREVK ECIYVEPDEG EENKGVFWYN NKL	383	43,717
Fatty acid desaturase 2 isoform 1	A0A1Z1EC46	As above	MGAGGRMSVP PADKKA KSDV IQRVPHAKPP FTLGEIKKAI PPHCFKRSIP RSFSYVVYDL IIASLFYYVA TNYIHQLPQP LSYLAWTLYG ICQGCILTGV WVLAHECGHH AFSDYQWLDD TVGLILHSFL LVPYFSWKYS HRRHHSNTGS LERDEVFVPK VKSGVSWTAK YMNNPPGRVI TLIVQLTLGW PLYLMFNVS G RPYDRFACHF DPKSPIYS DR ERAQIFISDA GILAVLYGLY RMSVAKGLAW VLCVYGGPLL VVNGFLVLIT FLQHTHPALP HYDSSEWDWL RGALATVDRD YGILNTIFHN ITDTHVAHHL FSTMPHYHAM EATKAIKPI L GKYYQLDETP VFKAMFREVK ECIYVEPDEG EENKGVFWYN NKL	383	43,788
Monoacylglycerol acyltransferase	AJW67342.1	This enzyme converted monoacylglycerol (MAG) to diacylglycerol (DAG) by transferred an acyl moiety from acyl-CoA to MAG. Then, DAG is then acylated by DAG acyltransferase (DGAT) to produce Triacylglycerol (TAG) (Sreedhar, Priya, Sunny, Ram, & Malathi, 2015; Vijayaraj et al., 2012).	MSPENPSNFW GDTPEEEYYA SQGVRNSKSY FDSPHGRLFT QSFLPLDPTR PVKASVFMTH GYGSDSSWMF QKFCISYAAW GYAVFAADML GHGRSDGIRC YMGDLPKVA A ASLAFFRSVR VSDEYKDLPA FLVGESMGGL ATLLMYFQSEKDLWTGLIFS APLFVIPESM MPSKVHLFAY GMLFGLADTW AAMPDNKMVG KAIKDPEK LK VIASNPMRYT GKPRVGT MRE LLRQTEYAQN NFDKVTIPFF TAHGTS DGLA EWSGSQMLYD KASSEDKTLK LYEGMYHSLI QGEPDENANL VLADMRAWID ERVERYGKKN	320	36,009

Eukaryotic translation initiation factor 3 subunit E	A0A2R4LNR4	It is required for steps in the initiation of protein synthesis and is required for disassembly and recycling of post-termination ribosomal complexes. Also, the eIF-3 is involved in pathway of cell proliferation (CONSORTIUM, 2017)	<p>MASKYDLTPR IAPNDRHLV FPLLEFLQER GLYPEEDILK AKIELLNHTN MVDYAMDIHK SLYHSDDVPQ DMIDRRAEVV GRLKALEDGA APLIGFLQNP NAVQELRADK QYNLQMLKDR YQIGPEQIDA LYDYAKFQFE CGNYSGAADY LYQYRALCTN SDKSLSALWG KLAAEVLMQN WDIALEELNR LKEIIDSKNF SSPLNQVQSR IWLMHWLFI FFNHDNGRTQ IIDLFNQDKY LNAIQTNAPH LLRYLATAFI VNKRRRPQFK EFIKVIQQEQ YSHEDPITEF LACIYVNYDF DGAQKKMKEC EEVILNDPFL GKRIEENFT TVPLRDEFLE PSYTNVYEQL IDHTKALSTR TYKIVHQLLE NAPGQTARCRIHQRIDMG VLADKLNLY EEAERWIVNL IRTSKLEAKI DSKLGTIIME NARLFIFETY</p>	438	51,240
S-adenosylmethionine decarboxylase proenzyme	A0A2R4LNQ8	It is a key rate-limiting enzyme in the polyamine biosynthesis is required for plant growth, development, and protection in response to stress (GUPTA et al., 2017).	<p>MDMPVSAIGF EGYEKRLEIS FVEPGVFADP DGYGLRALTK AQLDEILDPA QCTIVASLKN DDVDSYVLSE SSLFVYSYKI ILKTCGTTKL LLSIPPILRL ADGLGLTVSS VRYSRGSFIF PGAQPFPHRS FNEEVAVLDD HFSKLGLMSE AYVMGDADEH EKWHVYSAYL EPSSDVEPVY TLEMCMTNLD QKKASVFFKN QSSSATIMTD ASGIRNILPE SEICDFDFDP CGYSMNSIEG GAVSTIHVTP EDGFSYASFE TGGYDFEKVD LTQLVERVLA CFNPAKFSVA VRASIAGKEL DSAFKLDIAK YGCAGRRCEV LGDGGSVIYC NFTSATGCGS PRSTLHLCWS ESEDEEIEKK</p>	360	39,453

Tubulin beta chain	A0A2R4LNR5	It is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain (CONSORTIUM, 2017)	MREILHIQGG QCGNQIGSKF WEVICDEHGV DPTGRYKGDG SESDTQLERI NVYFNEASGG RYVPRAVLMD LEPGTMD SIR SGYPYQQIFRP DNFVFGQSGA GNNWAKGHYT EGAELIDSVL DVVRKEAENC DCLQGFQVCH SLGGGTGSGM GTLISKIRE EYPDRMMLTF SVFPSPKVS TVVETYNATL SVHQLVENAD ECMVLDNEAL YDICFRTLKL STPSFGDLNH LISATMSGVT CCLRFPQQLN SCLRKLAVNL IPFRLHFFM VGFAPLTSRG QHYISLTVP ELTQQMWDSK NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMLNVQKN NVKSSVCDIP PTGLKMSSTF VGNSTSIQEM FRRVSEQFTA MFRKAFHLW YTGEGMDEME FTEAESNMND LVAEQYQYQD ATADEEEDYE EDGAEGEYED SSYFVEWIPN	450	50,485
Peptidyl-prolyl cis-trans isomerase	A0A2R4LNR0	It is the ubiquitous enzymes found in all kingdoms of life, responsible by catalyses a rate-limiting step in protein folding by <i>cis-trans</i> isomerization of proline imidic peptide bonds (CONSORTIUM, 2017; THONGNAK et al., 2017).	MSGNHMISIV IAMVCIGVFR GSITAIATVP ELGSARVVFQ TNYGDIEFGF YHSVAPKTVE HIFKLVRLGG YNTNHHFRVD KGFVAQVADV GGRTAPMNE VQRLEAEKTV VGEFSDVKHV RGILSMGRYS DPDSAQSSFS ILLGDAPHL GQYAFGKVT KGDETLKME EVPTRKEGIF VMPTERITIF STYYYDTETE SCEDDRLELK RRILASAVEI EKQRMKCFP	229	25,507
Serine/threonine -protein phosphatase	A0A2R4LNQ7	It acts in phosphorylation and dephosphorylation of target proteins related to cellular function (PARK et al., 2011).	MPGHGDLDRQ IEQLMECKPL SEAEVKILCD QARAILVEEW NVQPVKCPVT VCGDIHGQFY DLIELFRIGG NAPDTNYLFM GDYVDRGYYS VETVLLVAL KVRDRITIR LRGNHESRQI TQVYGFYDEC LRKYGNANVW KFFTDLFDYL PLTALIESQV FCLHGGSPS LDTLDNIRAL DRMQEVPHEG PMCDLLWSDP DDRCGWGISP RGAGYTFGQD IAAQFNHTNG LTLISRAHQL VMEGFNWCQE KVVVTVFSAP NYCYRCGNMA AILEIGEHEM QNFLQFDPAP RQIEPDTTRK TPDYFL	306	35,001

Elongation factor 1-alpha	A0A2R4LNQ6	<p>During protein biosynthesis, this protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes.</p> <p>Also, this protein can be part of messenger ribonucleoprotein particle (mRNP) complexes, part of the valyl-tRNA synthetase complex, bind to actin, to be associated with the endoplasmic reticulum or the mitotic apparatus, and to be involved in protein degradation or ribosome association (CONSORTIUM, 2017).</p>	<p>MGKEKIHISI VVIGHVDSGK STTTGHLLIYK LGGIDKRVIE RFEKEAAEMN KRSFKYAWVL DKLKAERERG ITIDIALWKF ETTKYYCTVI DAPGHRDFIK NMITGTSQAD CAVLIIDSTT GGFEAGISKD GQTREHALLA FTLGVKQMIC CCNKMDATTP KYSKARYDEI IKEVSSYLKK VGYNPEKIPF VPISGFEGDNMIERSTNLDW YKGPTLLEAL DAVQEPKRPS DKPLRLPLQD VYKIGGIGTV PVGRVETGVI KPGMVVTFGP TGLTTEVKSVMHHEALQEA LPGDNVGFNV KNVAVKDLKR GFVASNSKDD PAKEAANFTS QVIIMNHPGQ IINGYAPVLD CHTSHIAVKF SELMTKIDRR SGKELEKEPK FLKNGDAGMV KMIPTKPMVV ETFSQYPLG RFAVRDMRQT VAVGVIKSVE KKDPGAKVT KAAAKKGAK</p>	449	49,408
Glyceraldehyde-3-phosphate dehydrogenase	A0A2R4LNR9	<p>This protein is involved on glycolysis pathway by synthesizes of pyruvate from D-glyceraldehyde 3-phosphate. Also, GAPDH had roles in plant development, abiotic stress and immune responses in plants (ZENG et al., 2018).</p>	<p>MAKIKIGING FGRIGRLVAR VALQRDDVEL VAVNDPFITV DYMTYMFKYD SVHGQWKHHE LKVKDEKTLFGEKPVTVFG FRNPEEIPWA STGAEYIVES TGVFTDKDKA AAHLKGGAKK VVISAPSKDA PMFVVGVNEK SYTPDLDIVS NASCTTNCLA PLAKVINDRF GIVEGLMTTV HSITATQKTVDGPSAKDWRG GRAASFNIIP SSTGAAKAVG KVLPALNGKL TGMAFRVPTV DVSVDLTVR LEKEATYDEI KAALKEESEG NLKGILGYTE DDVSTDFVG DNRSSIFDAK AGIALSKNFV KLVSWYDNEW GYSTRVVDLI KHIHSTQ</p>	337	36,706

Actin	A0A2R4LNQ3	This protein is part of cellular cytoskeleton and acts in cell division and expansion, vesicle trafficking, organelle movement, and cell growth (PAEZ-GARCIA et al., 2018).	MADAEDIQPL VCDNGTGMVK AGFAGDDAPR AVFPSIVGRP RHTGVMVGMG QKDAYVGDEA QSKRGILTLK YPIEHGIVSN WDDMEKIWHH TFYNELRVAP EEHPILLTEA PLNPKANREK MTQIMFETFN TPAMYVAIQA VLSLYASGRT TGIVLDSGDG VSHTVPIYEG YALPHAILRL DLAGRDLTDS LMKILTERGY MFTTTAEREI VRDIKEKLAY IALDYEQELE TAKTSSAVEK NYELPDGQVI TIGAERFRCP EVLFQPSMIG MEAAGIHETT YNSIMKCDVD IRKDLYGNIV LSGGSTMFPG IADRMSKEIT ALAPSSMKIK VVAPPERKYS VWIGGSILAS LSTFQQMWIA KAEYDESGPS IVHRKCF	377	41,738
Clathrin adaptor complex	A0A2R4LNR7	This is a complex of proteins that regulates of movement of proteins and lipids between the cellular membranes and have a role in the signaling and homeostasis, defining the interactions of cells with their surroundings (JACKSON et al., 2010).	MPLAASAIYF LNLRGDVLIN RLYRDDVGGN MVDAFRVHIM QTKELGTCPV RQIGGCSFFY MRISNVYIVI VVSSNANVAC AFKFFVEAVT LFKSYFGGSF DEDAIRNNFV LIYELLDEIM DFGYPQNLSP EILKLYITQE GVRSPFSSKT ADKPVPNATL QVTGAVGWRR EGLVYKKNEV FLDIVESVNL LMSSKGSVLR CDVTGKILMK CFLSGMPDLK LGLNDKIGLE KESQLKSRPA KSGKTIELDD VTFHQCVNLT RFNSEKTVSF VPPDGEFELM KYRITEGVNL PFRVLPTIKE LGRTRMEVNV KVKS VFGAKM FALGVVIKIP VPKQTAKTSF QVTSGKAKYS PSIDCLVWKI RKFPGQTEPT LSAEVELIST ITEKKSWSTRP PIQMEFQVPM FTASGLRVRF LKVWEKSGYN TVEWVRYITK AGSYEVRC	438	49,311
Alpha-tubulin	A0A2R4LNS8	It is a cytosolic proteins related with the microtubules, which are essential for cell expansion and division (CHU et al., 2018).	MISNNTAVAE VFSRIDHKFD LMYSKRAFVH WYVVGEGMEEG EFSEAREDLA ALEKDYEEVG AEGVDDEDDE GEDY	74	8,488

FtsH protease	A0A2R4LNS4	<p>It is an inner membrane-embedded zinc-dependent metalloproteases, involved in degradation and assembly of protein complexes in the photosynthetic electron-transport pathways, and act on chloroplasts manutention (KATO; SAKAMOTO, 2018).</p>	<p>FDRNIVVPPNP DVEGRRQILE SHMSKVLKGE DVDIEIIARG TPGFSGAELA NLVNVA AIKA AMDGAKAVSM ADLEHAKDKI VMGSEKSAV ISDESRRNTA YHEGGHALVA MFTDGALPVH KATIVPRGNA LGMVSQLPDK DQTSVSRKQM LARLDVCMGG RVAEELIFGE SEVTSGASSD LESATRMARS MVTRYGMSKQ LGFVSHDYND NGRSMSTETR LLIEQEVKDL LEKAYNNAKT ILTTHSKELH ALANELLDKE TLTGAQVKAL LENVKAQNTQ QKQKQIVT</p>	289	31,473
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*Amino acid nomenclature: C, cys; cysteine; H, his; histidine; I, ile; isoleucine; M, met; methionine; S, ser; serine; V, val; valine; A, ala; alanine; G, gly; glycine; L, leu; leucine; P, pro; proline; T, thr; threonine; F, phe; phenylalanine; R, arg; arginine; Y, tyr; tyrosine; W, trp; tryptophan; D, asp; aspartic acid; N, asn; asparagine; B, asx; either of D or N; E, glu; glutamic acid; Q, gin; glutamine; Z, glx; either of E or Q; K, lys; lysine; X, undetermined amino acid. Protein sequence was obtained from UniProt database (<http://www.uniprot.org>). AAR = Amino acid residues; MM= Molecular mass (Da).

Fatty acid desaturases. The desaturase enzymes are responsible of dehydrogenation reactions, they introduce a double bond between defined carbons of fatty acyl chains. These enzymes can be soluble and are found in the plant plastid acting as acyl-acyl carrier protein desaturases (Sharma & Chauhan, 2012; Dar, Choudhury, Kancharla, & Arumugam, 2017).

The seed-specific delta-12 fatty acid desaturase 2 (FAD2) is a bi-functional hydroxylase/desaturase and tri-functional acetylenase responsible for inserting a double bond at the delta-12 position of oleic acid, thereby producing linoleic acid (omega-6). The FAD2 is a multifunctional enzyme that acts in the biological membrane systems: signaling, energy storage, thermal adaptation, and resistance to biotic and abiotic stresses in plants (SHARMA; CHAUHAN, 2012). The fatty acid desaturases 3, 7, and 8 (FAD3, FAD7, FAD8) are key enzymes responsible for producing α -linolenic acid (ALA), also called omega-3 in plants. The FAD3 catalyzes the introduction of a third double bond at the delta-15/ omega-3 carbon position of linoleic acid in the endoplasmic reticulum, and the FAD7 and FAD8 in plastids (XUE et al., 2018). Fatty acid desaturases 2 (FAD2) and 3 (FAD3) are the main enzymes responsible for the delta-12 and delta-15 desaturation in plants (RADOVANOVIC et al., 2014). The FAD 2, 3, 7, and 8 are different by nucleotide and amino acid sequences of the conserved region (DEHGHAN NAYERI; YARIZADE, 2014).

Monoacylglycerol acyltransferase (MGAT). The lipids produced by FAD are organized into molecules as triacylglycerol (TAG) formed by several enzymes. First, the glycerol-3-phosphate is acylated by G3P acyltransferase (GPAT) in two free hydroxyl positions to produce lysophosphatidic acid (LPA). This one is acylated by LPA acyltransferase (LPAT), producing phosphatidic acid (PA). Both, LPA and PA, can be dephosphorylated to monoacylglycerol (MAG) and diacylglycerol (DAG). MAG is converted to DAG by MAG acyltransferase (MGAT) that transfers an acyl moiety from acyl-

CoA to MAG. Finally, DAG is then acylated by DAG acyltransferase (DGAT) to produce TAG (SREEDHAR et al., 2015; VIJAYARAJ et al., 2012).

The MGAT can be soluble, as above, or associated with oleosin. In this case, MGAT has a role to form TAG that will be stored in oleosin (SREEDHAR et al., 2015). Because of this, MGAT can have a role in preserving unsaturated fatty acids in plants, such as *Ricinus communis*, *Brassica napus*, and maize (BAUD; LEPINIEC, 2010). While DAG is a signaling molecule and an intermediate for the synthesis of neutral and membrane lipids, the MGAT pathway may operate for storage purposes. Also, it has been demonstrated that MGAT activity is more evident with unsaturated than saturated fatty acids (VIJAYARAJ et al., 2012).

Oleosin. In plants, more specifically in seeds, the triacylglycerol (TAG) is typically stored in lipid droplets that are stabilized by associated proteins, as oleosins (WINICHAYAKUL et al., 2013). Oleosins contain the amphipathic N- and C- terminal domains exposed to cytosol, and a hydrophobic central domain formed by a long chain of non-polar amino acids. This structure stabilizes the lipids inside the cells, protecting, for example, against phospholipases and desiccation (HUANG, 2017).

Chia seeds have a high concentration of lipids, on average 31%, and the main type of fatty acid found in chia seed is the omega-3 (around 20%), followed by omega-6 (about 6%) (DA SILVA et al., 2017). This seed is one of the most efficient omega-3 sources for enriching foods, its proportion of omega-3:omega-6 is the highest among crop sources (SEGURA-CAMPOS et al., 2014). The high concentration of omega-3 fatty acid desaturases (FAD3, FAD7, and FAD8) and the presence of oleosin and MGAT may be responsible for the elevated production and storage of omega-3 in chia seed.

Ribulose biphosphate carboxylase large chain. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is a bifunctional multimeric plant metabolic enzyme that converts carbon dioxide from the biosphere into organic carbon in a rate-limiting step of the

Calvin cycle and photorespiration, depending on its affinity for carbon dioxide or molecular oxygen (ANDERSSON; BACKLUND, 2008). The RuBisCO structure is a heterohexadecamer with eight large subunits with high molecular weight, and eight small subunits with low molecular weight. In chia, only large chains have been detected (**Table 4**) (UDENIGWE et al., 2017). This enzyme is made up of about 50% soluble proteins in the plant leaf and can be found in autotrophs, including bacteria and algae; RuBisCO is the most abundant protein on earth (ANDERSSON; BACKLUND, 2008).

RuBisCO is a good source of bioactive peptides that have demonstrated beneficial effects for health promotion both *in vitro* and *in vivo* (UDENIGWE et al., 2017). Rubiscolin, a δ -opioid peptide with sequence YPLDLF shows a memory-consolidating effect, since it is blocked by naltrindole and raclopride, antagonists of the δ -opioid receptor and dopamine D2-receptor, respectively (YOSHIKAWA, 2015). Rubiscolin also shows anorexigenic effects (KANEKO et al., 2014).

Eukaryotic translation initiation factor 3 subunit E. This protein is required for some steps in the initiation of protein synthesis and it is required for the disassembly and the recycling of post-termination ribosomal complexes. Also, the “eukaryotic translation initiation factor 3 subunit E” is involved in the pathway of cell proliferation (CONSORTIUM, 2017)

S-adenosylmethionine decarboxylase proenzyme. It is a key rate-limiting enzyme in the polyamine biosynthesis required for plant growth, development, and protection in response to stress (GUPTA et al., 2017).

Alpha and beta tubulin chain. This protein is the major constituent of microtubules, responsible of several cell functions, such as mitosis, cell expansion and division, and movement of organelles and vesicles. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain (Chu et al., 2018).

Peptidyl-prolyl cis-trans isomerase. This ubiquitous enzyme is found in all kingdoms of life, responsible for catalyzing a rate-limiting step in the protein-folding by *cis-trans* isomerization of proline peptide bonds (CONSORTIUM, 2017; THONGNAK et al., 2017).

Serine/threonine-protein phosphatase. In plants, many cellular functions are controlled by the phosphorylation and dephosphorylation of target proteins by the serine/threonine-protein phosphatase family. These proteins are involved in a variety of biological processes, such as transcriptional control, cell cycle regulation, and signal transduction (PARK et al., 2011).

Elongation factor 1-alpha. During protein biosynthesis, this protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes. Also, this protein can be part of messenger ribonucleoprotein particle (mRNP) complexes, part of the valyl-tRNA synthetase complex, bind to actin, to be associated with the endoplasmic reticulum or the mitotic apparatus, and to be involved in protein degradation or ribosome association (CONSORTIUM, 2017).

Glyceraldehyde-3-phosphate dehydrogenase. This protein is involved in the glycolysis pathway by synthesis of pyruvate from D-glyceraldehyde 3-phosphate. Also, GAPDH has roles in plant development, abiotic stress, and immune responses in plants (ZENG et al., 2018).

Actin. This protein is part of the cellular cytoskeleton and acts in cell division and expansion, vesicle trafficking, organelle movement, and cell growth (PAEZ-GARCIA et al., 2018).

Clathrin adaptor complex. This is a complex of proteins that regulates of the movement of proteins and lipids between the cellular membranes and has a role in the signaling and homeostasis, defining the interactions of cells with their surroundings (JACKSON et al., 2010).

FtsH protease. It is an inner membrane-embedded zinc-dependent metalloprotease, involved in the degradation and assembly of protein complexes in the photosynthetic electron-transport pathways, and it acts on chloroplast maintenance (KATO; SAKAMOTO, 2018).

Analysis of Bioactive Peptides from Chia Seed Proteins

Bioactive peptides are inactive within the sequence of the parent protein from plants, animals, or marine foods. However, after fermentation, enzymatic, chemical hydrolysis, or gastrointestinal digestion, peptides can be released (MEISEL, 1997; UDENIGWE; ALUKO, 2012). On the other hand, peptides formed by gastrointestinal digestion, may act as regulatory compounds with hormone-like activity, as well as hypotensive, hypocholesteremic, anticancer, immunomodulatory agents, among others (CICERO; FOGACCI; COLLETTI, 2017). Thus, peptides represent potential health-enhancing nutraceuticals for food and pharmaceutical applications (MEISEL, 1997).

For this review, all protein sequences, including the protein related to lipid and general metabolism in chia seed had their profile of active peptides evaluated using the database BIOPEP (<http://www.uwm.edu.pl/biochemia>). Each protein showed many effects and, as an example, **Figure 3** shows the bioactivity effect map of FAD3i1 (MONTOYA-RODRÍGUEZ et al., 2015). The results presented in **Figure 4** were calculated as follows:

$$\% \text{ occurrence of frequency} = \frac{\text{n}^\circ \text{ amino acids of each bioactive effects}}{\text{total n}^\circ \text{ of amino acids with bioactive effects}}$$

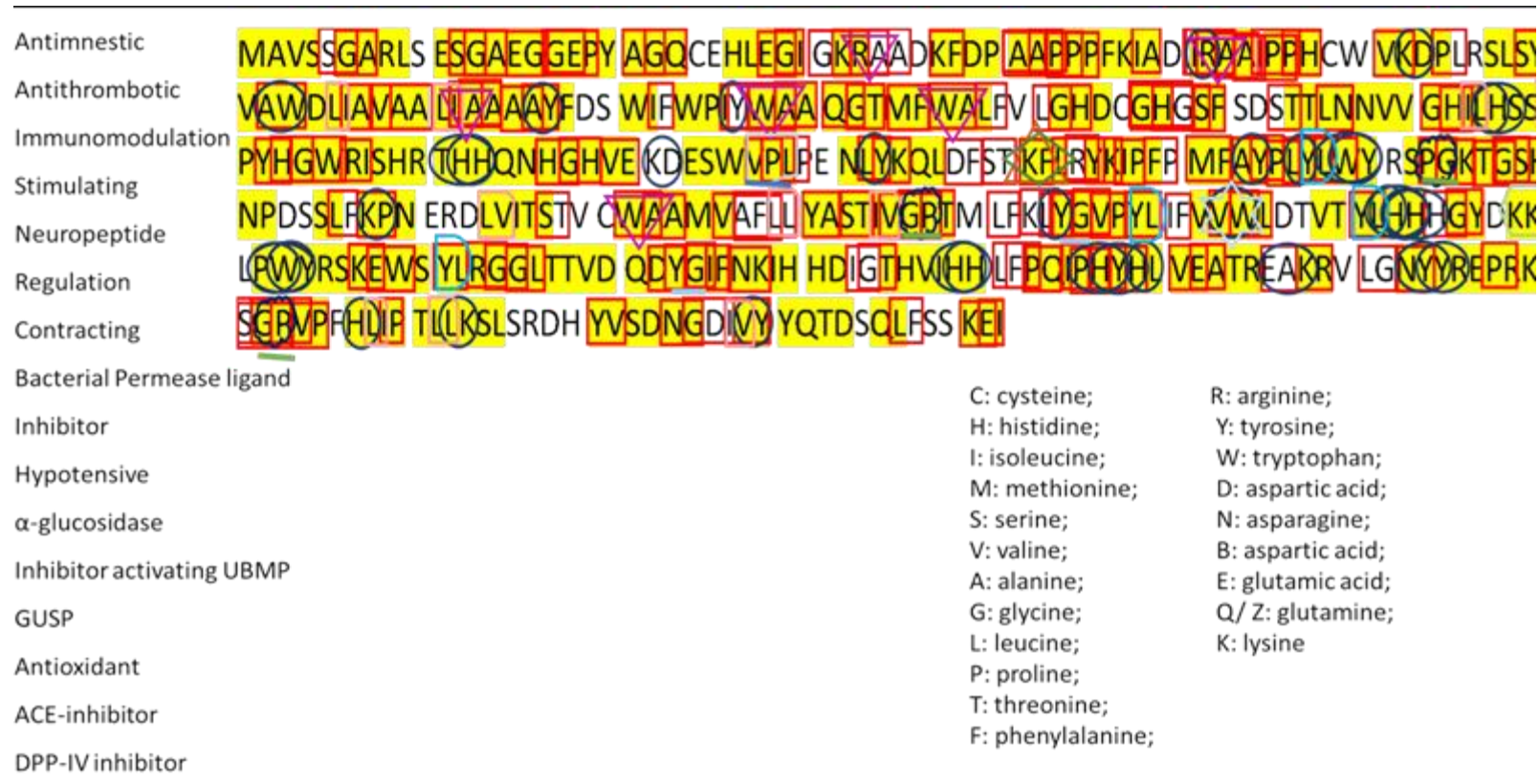


Figure 3. Bioactivity effect map of FAD3i

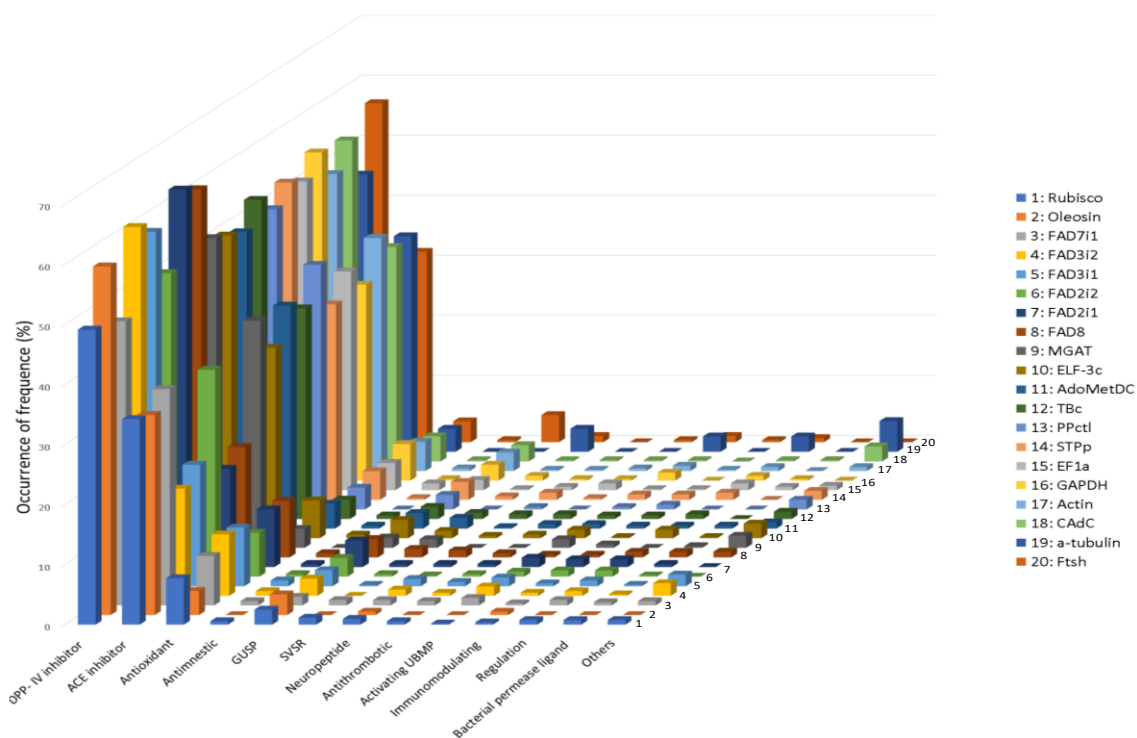


Figure 4. Bioactive potential of peptides sequenced found in chia protein. Sequences identified in BLAST tool ® Program and analyzed in BIOPEP® database. DPP IV inhibitor: dipeptidyl peptidase IV inhibitor; ACE inhibitor: angiotensin-converting-enzyme inhibitor; GUSP: Glucose uptake stimulating peptide; SVSR: Stimulating vasoactive substance release; Activating UBMP: Activating ubiquitin-mediated proteolysis; Regulation: peptide regulating the stomach mucosal membrane activity; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; FAD7i1: fatty acid desaturase 7 isoform 1; FAD3i2: fatty acid desaturase 3 isoform 2; FAD3i1: fatty acid desaturase 3 isoform 1; FAD2i2: fatty acid desaturase 2 isoform 2; FAD2i1: fatty acid desaturase 2 isoform 1; FAD8: fatty acid desaturase 8; MGAT: monoacylglycerol acyltransferase; ELF-3e: eukaryotic translation initiation factor 3 subunit E; AdoMetDC: S-adenosylmethionine decarboxylase; TBc: Tubulin beta chain; PPcti; Peptidyl-prolyl cis-trans isomerase; STPp; Serine/threonine-protein phosphatase; eEF1a: elongation factor 1-alpha; GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; CAdC: clathrin adaptor complex.

The peptides found demonstrated mainly hypoglycemic and hypotensive activity, since most of them presented dipeptidyl peptidase IV (DPP IV inhibitor) and angiotensin-converting-enzyme (ACE) inhibitor activities, respectively (**Figure 4, Table 5 and 6**).

Table 5. Identification and characterization of peptides from general metabolic protein of chia seed.

Bioactive Potential	RuBisco	ELF-3e	AdoMetDC	TBc	PPctI	STPp	EF1a	GAPH	Actin	CAdC	a-tubulin	Ftsh
Antimnestic	PG, GP	VPL, PG, GP	PG	PG, GP	-	PG	PG, GP	GP	PG, GP	PG	-	PG
Antithrombotic	GP, PG	GP, PG, NQDK	PG, DEE	GP, PG, DEE	-	PG	GP, PG	GP	GP, PG	PG	-	-
Immunomodulating	YG	EAE	YG	YG, EAE	YG, EAE	YG, EAE	KRP	-	YG	-	-	YG
Stimulation	EEE, SSS, EE	VPL, EE	SSS, EE, SE	EEE, EE, SE	-	EE, SE	SE	EE, SE	EE	SE	EE, SE	EE, SE
Neuropeptide	GQ	GQ, YL	YL	GQ, YL	GQ	GQ, YL	GQ, YL	GQ	GQ	GQ	-	-
Regulation	DY, GP, PG	GFL, GLY, DY, GP, PG	PG	DY, GP, PG	-	DY, PG	GP, PG	LGY, DY, GP	DY, PG, GP	PG	DY	DY, PG
Contracting	PLRP	-	-	-	-	-	-	-	-	-	-	-
Bacterial permease ligand	KK	KK	KK	-	-	-	KK	KK	-	KK	-	-
Inhibitor	EF	IR, KF, EF	IR, KF	IR, KF	EF	IR, KF	KF	-	IR	IR, KF, EF	KF, EF	-
Hypotensive	EF	IR, KF, EF	IR, KF	IR, KF	EF	IR, KF	KF	-	IR	IR, KF, EF	KF, EF	-
α -glucosidase inhibitor	VW	-	-	-	-	VW	-	-	VW	VW	-	-
activating UBMP	LA, WA	RA, LA	RA, LA	RA, LA, WA	LA	RA	LA	RA, LA, WA	LA	LA	RA, LA	LA
GUSP	VL, LV, IV, IL, LI, LL	VL, LV, IV, IL, LI, II, LL	VL, LV, IV, IL, LL	VL, LV, IL, LI, LL	LV, IV, IL, LL	LV, IL, LI, LL	VL, LI, II, LL	VL, LV, IV, IL, LI, II, LL	VL, IV, IL, LL	VL, LV, IV, IL, LI, LL	-	VL, LV, IV, IL, LI, II, LL
Antioxidant	LHH, LH, HL, HH, AY, LY, IY, WY, IHH, HYH, THH, YYQ, VYY, LHL, LWT, PHY, PWY, KD, PW, LKP, LK, KP,	HL, LY, IY, EL, YDY, YQY, LWG, RHL, RWI, EAK, KVI, KD, RW, IR, LK, TY, VY, LW, LLR, GAA, WG, LAC	LH, HL, AY, IY, EL, YSY, LHL, PHR, YVL, IR, LK, VY	LH, EL, LHF, LHI, LHW, IR, LK, TY, VY, MM	HL, EL, YYD, YYY, TYY, PHL, LK, TY	LH, GYY, AH, EL, YYS, YCY, LHG, LWS, PHE, IR, KP, VY, VW, LW, WG, FC	HL, HH, IY, EL, MHH, WY, HIH, KYY, YYC, KHH, PWA, KVI, KD, KP, VY, AW, LW, QYP	HL, HH, AH, EL, WY, HIH, KYY, YYC, KHH, PWA, KVI, KD, KP, VY, AW, LW, GAA	HH, AY, LY, IY, EL, WHH, PHA, RHT, MY, KD, IR, LK, TERGY, WDDMEK, LDY, VW, YVGD	LY, IY, EL, IR, LK, KP, VY, VW, CLV	WY, MY, KD	LH, AY, EL, LARL, KD, LK

TY,

ACE inhibitor

IYP, PLP,	RL, IR, RY, LY,	NILP, ILP,	RL, IR,	VMP,	IR, RY,	RL,	VLP, RL,	RL, IR, FQP,	VLP, RL,	VF, MY,	RL,
LKL, AAP,	IY, LW, VY,	RL, IR, RY,	LKL, RY,	RL, FGK,	VF, LW,	AKK,	AKK,	ALPHA,	IR, LKL,	AF, LA,	RY, MF,
AKK, LY,	FP, PR, LAA,	IY, VF, KW,	VF, MF,	RY, VF,	VW, VY,	RY, IY,	VF, MF,	YALPHA,	RY, LY,	KR, RA,	VAA,
IY, VF,	YL, LF, FNQ,	VY, FP, GGY,	FAP, RF,	TAP,	GY, LSP,	LW, RF,	RF, GY,	GRP, LY, IY,	IY, VF,	VG, GM,	PR, YG,
LW, VW,	YP, GP, PL, IW,	IPP, GY, PR,	VY, HY,	GDAP,	IRA, YL,	VY, GY,	GP, PL,	VF, MF, MY,	VW, RF,	GV, GE,	AY, VK,
YW, VY,	LYP, YQY,	YL, LF, YG,	FP, PR,	FP, VAP,	LF, YG,	YL, YP,	VK, IA,	VW, RF, FP,	VY, FP,	EG, EA,	IA, LA,
HY, FP,	PSY, IA, LKA,	AY, YGL, IA,	YL, YG,	LVR,	FY,	PLG,	IP, AF,	VAP, GY, PR,	VPP,	KF, AR,	VP, AA,
LVR, GY,	RW, RP, AF,	IP, AF, LA,	YP, GP,	YG, VK,	TQVY,	LPG, GP,	AP, LA,	LNP, LF, YG,	GY,	EV, ME,	GF, IF,
PR, YL,	AP, LA, KR,	KR, RA, YA,	PL, VFPS,	IA, AP,	PL, VK,	PL, AW,	VP, RA,	FY, LAY, AY,	LAA,	EK, HK,	GM, GA,
LF, YG,	VP, RA, YA,	GF, IF, IG, GI,	VK, IP,	LA, VP,	IA, GW,	VK, IA,	AA, FR,	YP, GP, PL,	LSP, LF,	AV,	GH, GR,
LAY, AY,	AA, GF, IF, IG,	GA, GL, AG,	RP, AF,	YA, GF,	AP, VP,	LKA, IP,	IF, VG,	IW, IWH,	FY, YP,	LEK,	KG, FG,
LA, KR,	GA, GL, HL,	HL, GR, DA,	AP, LA,	FR, IF,	RA, AA,	RP, AP,	IG, GI,	VFPS, VK, IA,	VPK, PL,	DY, YV,	GS, MG,
VP, AA,	GR, DA, MG,	GS, GV, MG,	VP, RA,	VG, IG,	GF, FR,	LA, KR,	GM, GA,	LAP, RP, AP,	VK, GW,	YE	GE, GG,
GF, VG,	GQ, GK, GT,	GK, GT, GG,	AA, GF,	GI,	IG, GI,	VP, YA,	GL, AG,	LA, KR, VP,	IP, RP,		SG, LG,
IG, GI,	WG, SG, LG,	SG, LG, GD,	FR, IF,	HLGR,	GA, GL,	AA, GF,	HL, GR,	YA, AA, GF,	AF, LA,		TG, EG,
GM, GL,	EG, EA, NG,	TG, EG, PG,	VG, IG,	KG, FG,	AG, GH,	VG, IG,	KG, FG,	FR, VG, IG,	VP, AA,		NG, PG,
GH, HL,	PG, LRY, QK,	IAK, VR,	GM, GA,	DA, GS,	FG, GQ,	GI, GM,	DA, GV,	GI, GM, GA,	FR, VG,		IFG, QK,
KG, FG,	DG, NY, NF,	VAV, QK,	GV, MG,	WG, HG,	WG, HG,	GA, GL,	GQ, GK,	AG, GR, DA,	IG, GM,		DG, RR,
GS, GV,	SY, KY, KF,	DG, NF, SY,	GR, KG,	GQ, GK,	GE, GG,	AG, GH,	HG, GE,	GS, GV, MG,	GA, GL,		AR, KA,
MG, GQ,	KL, YK, NK,	SF, KF, KL,	FG, GS,	GE, GG,	GD, EG,	HL, GR,	GG, LG,	GQ, GT, HG,	AG, GR,		EI, IE,
GK, WG,	RR, AR, KA,	YK, RR, KA,	GV, GQ,	SG, LG,	EA, NG,	KG, FG,	TG, EG,	GG, SG, GD,	KG, FG,		EV, VE,
HG, GG,	IAP, EI, IE, EV,	LVE, VIY,	GK, GT,	GD, EG,	PG, VR,	DA, GV,	EA, NG,	TG, EG, EA,	DA, GS,		TE, TQ,
SG, LG,	LQ, LN, TQ,	CF, EI, IE,	HG, GE,	EA, VR,	PAP, NY,	MG, GQ,	VR,	NG, PG, VR,	GV, GQ,		EK, KE,
GF, TG,	PQ, ME, KE,	EV, VE, TQ,	GG, QG,	IFG, NY,	NF, KY,	GK, GT,	VAV,	QK, DG, NY,	GK, GT,		AI, AV,
EA, NG,	PH, HK, AI,	PP, EK, KE,	SG, LG,	SF, KL,	KF, AR,	GG, SG,	TTN,	KY, KL, KA,	GE, GG,		ALP,
PG, VR,	LEF, AV, LNY,	PH, AI, VRY,	GD, TG,	RR, AR,	LVE, KP,	LG, GD,	QK, DG,	CF, EY,	SG, LG,		LEK,
VAV,	LEE, GLY, DY,	AV, ASL,	EG, EA,	CF, EI,	EI, IE,	TG, EG,	NF, SY,	VWIG, EI, IE,	GD, TG,		VTR,
GHF, QK,	TP, DF, DM,	FNE, AQL,	PG, VR,	IE, EV,	EV, VE,	EA, NG,	SF, KY,	EV, VE, TE,	EG, EA,		DY, TP,
SY, SF,	FQ, YV, YE, IL,	DGL, AVL,	YVP,	VE, TE,	LQ, TQ,	PG, VR,	KL, AR,	LN, TQ, PP,	PG, VR,		IL, YH,
KY, KL,	YH, WL, ST,	TP, DF, DM,	LTF, DG,	PT, ME,	AH, EW,	VAV,	KA, EY,	ME, EK, KE,	LIY, DG,		AEL,
KA, IPY,	YN, LR, RRR	YV, YE, IL,	NF, SY,	EK, KE,	ME, PH,	KYY,	KP, EI,	HP, PH, IQP,	NF, SY,		RG, ST,
KP, RIY,		RG, ST, LR	SF, KF,	PH, AI,	EP, TF,	IYK,	VE, TE,	TF, AI, AV,	SF, KY,		YN
IE, VE,			KL, YK,	AV,	AI, VRY,	LIY, NF,	LQ, LN,	ALP, YVA,	KF, KL,		
LN, PPPQ,			NK, RR,	GKV,	DY, TP,	SY, SF,	PT, TQ,	FYN, DY, TP,	YK, RR,		
EW, EK,			KA, LVE,	VVF, FQ,	YV, IL,	KY, KF,	AH, EW,	DM, FQ, YV,	KA, CF,		
KE, HP,			CF, EY,	IL, YH,	RG, GHG,	KL, YK,	EK, KE,	YE, IL, MW,	KP, FAL,		
PH, IQP,			EI, EV,	TLS, RG,	LFR, LR	NK, RR,	AV,	SGP, RG,	EI, IE,		
FVP, AV,			VE, TE,	ST, YN		AR, KA,	LEK,	GTG, ST, YN,	EV, VE,		
LEE, GSH,			LQ, LN,			KP, EI,	VSW,	LR, LDY	TE, LN,		
LPF, AVL,			PT, TQ,			IE, EV,	DY, TP,		PT, TQ,		
DY, YV,			EW, ME,			VE, TE,	DF, IL,		PP, PQ,		
IL, YH,			KE, TF,			LQ, PT,	WA, RG,		EW, ME,		
WA, WL,			AV, FNE,			PP, EK,	ST		EK, KE,		
MGP, SGP,			AFL,			KE, HP,			TF, AI,		
RG, ST,			AVL, DY,			TF, AV,			VRSP,		
YN, GHG,			TP, FQ,			TKY,			VRY,		

	LR, LGV			YV, YE, IL, MM, AEL, WA, MW, SGP, RG, GTG, ST, YN, LR			AVL, LEK, TP, DF, DM, KGP, RG, ST, YN, LR, LGV			LVY, AV, DF, FQ, YE, IL, RG, ST, YN, AGS			
DPP- IV inhibitor	GP, PP, MP, VA, MA, KA, LA, FA, AP, PA, LP, VP, LL, VV, GQ, IP, SP, FP, RP, KP, HP, YP, WA, EP, NP, TA, QP, FL, WV, HL, EK, AL, SL, GL, VR, WRI, AA, PL, WR, WL, WI, WY, WT, WS, LW, WF, IQP, VGL, WG, AE, AS, AT, AV, AY, DP, DR, ES, EW, FN, GF, GG, GH, GI, GV, GY, HD, HE, HF, HH, HR, HS, HV, HY, IH, IL, IQ, KE, KG, KH, KI, KK, KR, KS, KY, LH, LI, LN, LT,	GP, MA, KA, LA, AP, VP, LL, VV, VPL, GQ, APG, TP, SP, FP, RP, YP, GA, IA, RA, NP, TA, FL, HL, AL, SL, GL, AA, , PL, WL, WI, WS, LW, YT, WG, AD, AE, AF, AS, AT, AV, DN, DP, DR, EG, EI, ET, EV, FN, FQ, GF, HD, HE, HS, HT, HW, IH, II, IL, IM, IQ, IR, IW, KE, KF, KI, KK, KR, KS, KV, KY, LI, LM, LN, LT, LV, ME, MG, MH, MI, MK, ML, MQ, MV, NA, ND, NF, NG, NH, NL, NQ, NR, NV, NY, PF, PG, PH, PI, PN, PQ, PS, QD, QE, QF, QI, QL, QN, QQ, QS, QT, QV, QY, RH, RI, RL, RR, RW, SH, SK, SY, TE, TI, TK, TN, TQ, TR, TS, TV, TY, VD, VH, VI, VL, VN, VQ, VY, WD, YA, YD, YE, YH,	PP, MP, VA, KA, LA, FA, PA, LP, LL, IP, TP, FP, GA, IA, RA, EP, NP, QP, HL, EK, AL, SL, GL, VR, WS, AD, AF, AG, AS, AT, AV, AY, DP, EG, EH, EI, ES, EV, FN, GF, GG, GI, GV, GY, HF, HR, HV, IH, IL, IM, IR, KE, KF, KI, KK, KR, KT, KV, KW, LH, LM, LT, LV, MG, MN, NE, NF, NL, PF, PG, PH, PI, PS, PV, QL, QS, RG, RL, RN, RR, SF, SI, SK, SV, SY, TD, TG, TI, TK, TL, TN, TQ, TS, TT, TV, VD, VE, VF, VI, VL, VM, VS, VT, VY, WH, YA, YD, YE, YG, YK, YL, YS, YV	GP, VA, KA, LA, FA, AP, VP, LL, VV, GQ, FP, IA, TA, HL, EK, VR, YP, GA, RA, WA, AS, AT, EP, TA, FL, AL, DR, EG, SL, GL, VR, AA, PL, WI, MW, WE, YT, AD, AE, AF, AS, AT, AV, DN, DP, DR, EG, EH, EI, ES, ET, EV, EW, EY, FN, FQ, FR, GE, GF, GG, GH, GV, HF, HI, HW, HY, IL, IQ, IR, KE, KF, KG, KI, KS, KV, LH, LI, LM, LN, LT, LV, ME, MF, ML, MM, MN, MR, MV, NA, ND, NE, NF, NH, NL, NM,	MP, VA, LA, AP, VP, LL, VV, GQ, FP, IA, TA, HL, EK, VR, AD, AE, AS, AT, AV, DP, DR, EG, EI, ET, EV, FQ, FR, GE, GF, GG, GI, GV, HF, HI, HS, HV, IL, KE, KG, KH, KT, KV, LV, ME, MG, MI, MK, MN, MV, NE, NH, NT, NY, PH, PK, PM, PT, QS, QV, QY, RG, RI, RK, RL, RM, RR, SF, SI, SK, SV, TE, TI, TL, TN, TR, TV, TY, VD, VE, VF, VG, VK, VM, VQ, VT, YA, YD, YG,	MP, VA, MA, AP, PA, VP, LL, VV, GQ, TP, SP, KP, GA, IA, RA, EP, TA, QP, FL, AL, GL, VR, AA, PL, WC, WS, LW, YT, WG, AE, AG, AH, DN, DP, DR, EG, EH, EI, ES, ET, EV, EW, FN, FR, GE, GF, GG, GH, GI, GW, GY, HE, HT, IH, IL, IR, KF, KI, KV, KY, LH, LI, LM, LT, LV, ME, MQ, NA, NF, NG, NH, NM, NV, NW, NY, PG, PH, PM, PS, PV, QA, QD, QE, QF, QI, QL, QN, QV,	GP, VA, MA, KA, LA, FA, AP, PA, LP, VP, VP, LL, VV, GQ, IP, TP, LL, VV, VV, HA, GQ, TP, FP, RP, HP, YP, GA, KP, GA, IA, RA, QP, EK, AL, SL, VR, AA, PL, WI, MW, EK, AL, GL, VR, WV, HL, EK, AL, GL, VR, WR, WK, LPL, AA, PL, AE, AF, AG, AH, AS, AT, AV, DN, DP, DR, AE, AG, AS, AT, AV, DN, FR, GE, GG, GI, GV, GY, HE, HH, HI, HS, IH, II, IL, IN, KE, KG, KH, KI, KK, KT, KV, IH, II, KY, LI, LM, LN, LT, LV, LI, LM, KI, KK, KR, KS, KV, KY, LI, LM, LT, MG, MH, MI,	GP, PP, VA, MA, KA, LA, FA, AP, PA, LP, VP, LL, VP, LL, VV, HA, GQ, TP, FP, RP, HP, YP, GA, IA, NP, TA, QP, EK, AL, WA, NP, TA, HL, EK, AL, IQA, AD, AE, GL, VR, AG, AS, AV, AY, DN, DR, EG, EH, EI, ES, ET, EV, EY, FN, FQ, FR, GF, GG, GI, GV, GY, HE, HH, HR, HT, IH, IL, IM, IQ, IR, IW, KE, KI, KR, KT, KY, LM, LN, LT, ME, MF, MG, MI, MK, MV, MY, NE, NG, NR, NY, PG, PH, PI, PK, PS, QA, QE, QI, QQ, QS, QV, RG, RH, RK, RL, RM, SH, SI, SK, TD, TE, TF, TG, TI, TL, TM, TQ, TS, TT, TV, VD, VE, VF, VG, VH, VI, VK, VL, VM, VS, VW, WD, WH, YA, YD,	PP, MP, VA, KA, LA, FA, PA, LP, VP, LL, VP, LL, VV, GQ, IP, SP, FP, RP, KP, YP, LM, ME, MI, MY, NN, NT, RI, SK, AL, GL, VR, WRR, AA, PL, WR, WK, WT, WE, AD, AE, AF, AG, AS, AT, AV, EG, EI, ES, EV, EW, FN, FQ, FR, GE, GG, GV, GW, GY, HI, IL, IM, IN, IQ, IR, KE, KF, KG, KI, KK, KS, KT, KV, KY, LI, LM, LN, LT, LV, ME, MK, MR, MV, NA, ND, NE, NF, NL,	VA, LA, RA, TA, EK, AL, LP, VP, WY, AE, LL, VV, AF, AV, HA, TP, GA, IA, NP, TA, EK, AL, AA, AD, AE, AS, AT, AV, AY, DQ, DR, EG, EH, EI, ES, ET, EV, GE, GF, GG, GH, HD, HE, HS, II, IL, KE, KG, KI, KS, KT, KV, LH, LI, LT, LV, MF, MG, MV, NA, ND, NE, NG, NL, NN, NT, NV, PG, PN, PV, QE, QI, QL, QN, QQ, QT, QV, RG, RK, RL, RM, RN, RR, SH, SK, SV, TD, TE, TG, TH, TI, TL, TQ,			

LV, MG,	YK, YL, YN,	NN, NQ,	YH, YN,	RG, RI,	MN, MR,	PV, PW,	YE, YG, YN,	NN, NV,	TR, TS,
ML, ND,	YQ, YR, YS,	NV, NW,	YS, YY	RK, RM,	MV, NF,	QW, RG,	YS, YV	PF, PG,	TT,
NE, NG,	YV	PF, PG,		TD, TF,	NG, NH,	RI, RL,		PI,	VD, VE,
NH, NL,		PK, PN,		TI, TL,	NL, NM,	RN, SF,		PK, PM,	VH, VK,
NN, NQ,		PS, PT,		TN, TQ,	NV, PF,	SI, SK,		PN, PQ,	VL, VM,
NW, PF,		PY, QD,		TR, TT,	PG, PI,	SV, SW,		PS, PT,	VN, VS,
PG, PH, PI,		QE, QF,		TV, VD,	PK, PM,	SY, TD,		PV, QE,	VT, YG,
PK, PM,		QG, QH,		VE, VF,	PS, PT,	TE, TG,		QI, QL,	YH, YN
PN, PQ,		QI, QL,		VK, VM,	PV, QA,	TL, TN,		QN, QT,	
PW, PY,		QN, QQ,		VQ, VT,	QD, QE,	TQ, TR,		QV, RG,	
QL, QN,		QS, QV,		VW, VY,	QT, QV,	TT, TV,		RI, RK,	
QS, QT,		QY, RG,		YD, YF,	QY, RG,	TY, VD,		RL, RM,	
RG, RI,		RH, RI,		YG, YL,	RL, RR,	VE, VF,		RN, RR,	
RK, RN,		RK, RL,		YR, YS,	SF, SH,	VG, VH,		SF, SI,	
SF, SH, SI,		RM, RR,		YV, YY	SI, SK,	VI, VK,		SK, SV,	
SV, SW,		SF, SI,			SV, SY,	VL, VN,		SW, SY,	
SY, TD,		SK, SV,			TE, TF,	VS, VT,		TE, TF,	
TG, TH,		SY, TE,			TG, TI,	YD, YI,		TG, TI,	
TL, TM,		TF, TG,			TK, TL,	YM, YS		TK, TL,	
TR, TS,		TK, TL,			TN, TR,			TQ, TR,	
TT, TV,		TM, TQ,			TS, TT,			TS, TV,	
TY, VE,		TS, TV,			TV, VD,			VD, VE,	
VF, VG,		TY, VD,			VE, VG,			VF, VG,	
VI, VK,		VE, VF,			VI, VK,			VH, VI,	
VL, VM,		VG, VH,			VL, VQ,			VK, VL,	
VQ, VS,		VI, VK,			VS, VT,			VN, VS,	
VT, VW,		VL, VN,			VY, YA,			VT, VW,	
VY, WH,		VQ, VS,			YD, YK,			VY, YE,	
YF, YG,		VT, VY,			YL, YN,			YF, YI,	
YH, YL,		WD, YD,			YS, YY			YK, YN,	
YN, YQ,		YE, YF,						YR, YS	
YR, YV,		YG, YI,							
YW, YY		YK, YL,							
		YN, YQ,							
		YV							

Sequences identified in BLAST tool ® Program and analyzed in BIOPEP® database. ACE inhibitor: angiotensin-converting-enzyme inhibitor; DPP IV inhibitor: dipeptidyl peptidase IV inhibitor; Activating UBMP: Activating ubiquitin-mediated proteolysis; GUSP: Glucose uptake stimulating peptide; SVSR: Stimulating vasoactive substance release; Regulation: peptide regulating the stomach mucosal membrane activity. RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; eEF1a: elongation factor 1-alpha; CAdC: clathrin adaptor complex; ELF-3e: eukaryotic translation initiation factor 3 subunit E; AdoMetDC: S-adenosylmethionine decarboxylase; GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; TBc: Tubulin beta chain; PPcti; Peptidyl-prolyl cis-trans isomerase; STPp; Serine/threonine-protein phosphatase.

Table 6. Identification and characterization of peptides from protein related with lipids metabolic in chia seed.

Biologic activity	Oleosin	FAD2i1	FAD2i2	FAD3i1	FAD3i2	FAD7i1	FAD8	MGAT
Antimnestic	-	PG, GP	PG, GP	VPL, PG, GP	VPL, PG, GP	PG, GP	PG, GP	
Antithrombotic	-	GP, PG	GP, PG	GP, PG	GP, PG	GP, PG	GP, PG	
Immunomodulating	YG	YG, YGG	YG, YGG	YG	YG	YG	YG	YG
Stimulating	-	EE, SE	EE, SE	VPL	VPL	EE, SSS, SE	EEE, EE, SSS	EEE, EE, SE
Neuropeptide	GQ	YL	YL	GQ, YL	YL	YL	GQ, YL	
Regulation	-	GLY, DY, GP, PG	GFL, GLY, DY, GP,	GP, PG	GP, PG	DY, GP, PG	GP, PG	
Contracting	-		-	-		PLPR	PLPR	
Bacterial permease ligand	-	KK	KK	KK	KK	KK	KK	KK
Inhibitor	-	-	-	PPPF, IR, KF	PPPF, IR, KF	PPPF, IR	EF	IR, KF
Hypotensive	-	-	-	IR KF	IR, KF	IR	EF	LW, FT, LR, IR, KF
α -glucosidase inhibitor	-	-	-	VW	WW	VW	VW	
activating UBMP	-	RA, LA	RA, LA, WA	RA, LA, WA	RA, LA, WA	RA, LA, WA	LA, WA	RA, LA
GUSP	VL, LV, LL	VL, LV, IV, IL, LI, II	VL, LV, IL, LI, LL	LV, IV, IL, LI, LL	VL, LV, IV, IL, LL	VL, LV, LI, LL	VL, LV, IL, LI, LL	

Antioxidant	PHH, HL, HH, IY, QHH, KD KYY, YYV, YYQ, FYY, RHH, LHS, PHA, PHY, KAI, KP, VY, AW, YQLD, YQL, YVE,	LH, HL, HH, IKK, LY, IY, AH, WY, AHH, HYH, GHH, GHH, KYY, YYV, YYQ, FYY, RHH, LHS, PHT, PHY, KAI, KVI, KP, TY, VY, AW, YVE	LH, HL, HH, IKK, LY, IY, AH, WY, AHH, HYH, GHH, KYY, YYV, YYQ, FYY, RHH, LHS, PHT, PHY, KAI, KVI, KP, TY, VY, AW, YVE	LHH, LH, HL, HH, AY, LY, IY, WY, IHH, HYH, THH, YYR, NYY, HHH, LHS, LWY, PHY, PWH, GGE, EAK, KD, PW, IR, LK, KP, VY, AW, VW	LHH, LH, HL, HH, VHH, AY, LY, IY, EL, WY, HYH, THH, YYR, NYY, HHH, LHS, LWY, PHY, PWY, EAK, KD, PW, IR, LK, KP, VY, AW, VW, LW	LHH, LH, HL, HH, AY, ADF, LY, IY, WY, IHH, HFH, THH, KYY, YYK, YYQ, VYY, HHH, LHS, PHY, PWY, KD, WPL, PW, IR, LKP, LK, KP, TY	LHH, LH, HL, HH, AY, LY, IY, WY, IHH, HYH, THH, YYQ, VYY, LHL, LWT, PHY, PWY, KD, PW, LKP, LK, KP, TY, VY, VW, LW, WG	HLAY, LY, AH, EYY, YYA, LWT, PHG, MY, KAI, KD, IR, LK, KP, TW, AW, LW, LLR, FC
ACE inhibitor	IY, HY, PR, LAA, YG, LQQ, HHL, PL, VK, IA, RP, LA, VP, AA, GF, IF, VG, GL, AG, HL, FG, GS, GV, GQ, GK, GG, SG, TG, KY, AR, KA, FAL, LQ, HP, PH, LSW, AV, TP, DM, FQ	LY, IY, VF, MF, LVL, RF, VY, HY, YL, LF, YG, FY, YGL, VPK, HHL, VFK, GPL, GP, PL, IKP, AW, VK, IA, GW, IP, RP, AF, LA, KR, VP, RA, GF, FR, IF, VG, GI, GA, GL, AG, GH, HL, GR, KG, DA, GS, GV, MG, GK, GE, GG, QG, SG, LG, TG, EG, EA, NG, PG, NPP, YGG, KYY, NKL,	RL, IKP, LY, IY, VF, MF, LVL, RF, VY, HY, YL, LF, YG, FY, YP, YGL, VPK, HHL, VFK, GPL, GP, PL, AW, VK, IA, GW, IP, RP, AF, LA, KR, VP, RA, AA, GF, FR, IF, VG, GI, GA, GL, AF, AP, LA, KR, KG, DA, GS, GV, MG, GK, GT, IF, VG, IG, GI, GG, QG, SG, LG,	PLP, RL, IR, AAP, RY, LY, IY, MF, LW, VW, YW, VY, HY, FP, VAA, IPP, GY, PR, LAA, IRA, YL, LF, YG, AY, AIP, YP, GP, PL, IVY, LF, YG, AY, AIP, LAA, IRA, YL, YP, GP, PL, IVY, AW, VK, IA, GW, IP, AF, AP, LA, KR, VP, RA, YA, AA, IF, VG, IG, GI, GA, GL, GH, HL, DA, GS, GV, GK, GT, HG, GE, GG, QG, SG, LG, GD, EA,	IR, VP, AAP, RY, LY, IY, VF, MF, LW, VW, YW, VY, HY, FP, VAA, IPP, GY, PR, LAA, IRA, YL, LF, YG, AY, AIP, YP, GP, PL, IVY, AW, VK, IA, GW, IP, AF, AP, LA, KR, VP, RA, YA, AA, IF, VG, IG, GI, GA, GL, GH, HL, DA, GS, GV, GK, GT, HG, GE, GG, QG, SG, LG, GD, EA,	IYP, PLP, IR, LKL, LY, IY, MF, VW, YW, RF, VY, HY, FP, PR, LAA, IRA, YL, LF, YG, LAY, AY, AIP, YP, HHL, HLL, GPL, GP, PL, AW, LKP, VK, GW, IP, AP, LA, VP, RA, AA, GF, VG, IG, GI, GA, GL, AG, GH, HL, GR, KG, FG, GS, GV, MG, GK, GT, WG, HG,	IYP, PLP, LKL, AAP, AKK, LY, IY, VF, LW, VW, YW, VY, HY, FP, LVR, GY, PR, YL, LF, YG, LAY, AY, YP, HHL, HLL, GPL, GP, PL, LKP, VK, IP, RP, AP, LA, KR, VP, AA, GF, VG, IG, GI, GM, GL, GH, HL, KG, FG, GS, GV, MG, GQ, GK, WG, HG, GG, SG, LG, GD, TG,	RL, IR, RY, LY, VF, MF, MY, LW, GKP, VAA, GY, PR, LF, YG, AY, PL, DLP, AW, GEP, VK, IA, IP, AF, AP, LA, RA, YA, AA, FR, IF, VG, GI, GM, GL, GH, HL, GR, FG, GS, GV, MG, GK, GT, HG, GE, GG, QG, SG,

NY, SY, SF, KY, KL,	GD, TG, EG, EA,	HL, GS, GV, GQ,	NG, PG, LRY, AIPP,	GE, GG, QG, SG,	EA, NG, PG, VR,	GD, TG, EG,
NK, RR, KA, CF, KP,	NG, PG, NPP,	GK, GT, HG, GE,	DG, NY, SY, SF, KF,	LG, GD, TG, EA,	VAV, GHF, QK, SY,	VR, QK, DG,
EI, EV, VE, LQ, LN,	YGG, KYY,	GG, QG, SG, LG,	KL, YK, NK, KP, EI,	NG, PG, VR,	SF, KY, KL, KA,	NF, SY, SF, KF,
AH, PP, PQ, EW, HP,	NKL, DG, NY,	GD, TG, EG, EA,	VE, TE, LN, PT, PP,	VAV, KYY, IYK,	IPY, KP, RIY, IE,	KL, YK, NK,
PH, AI, RPY, FVP,	SY, SF, KY, KL,	NG, PG, LRY,	PQ, EW, KE, PH, AI,	QK, DG, SY, SF,	VE, LN, PP, PQ,	KA, EY, KP, VE,
AV, ASL, ALP, GLY,	NK, RR, KA, CF,	AIPP, NY, SY,	AV, GSH, VGP, DY,	KY, KL, YK, KP,	EW, EK, KE, HP,	TE, PT, AH, EW,
YVA, IVQ, VLY,	KP, EV, VE, LQ,	SF, KF, KL, YK,	DF, YV, YE, IL, YH,	RIY, IE, EV, VE,	PH, IQP, FVP, AV,	EK, PH, AI, AV,
AVL,	LN, AH, PP, EW,	NK, AR, KP, EI,	WA, WL, PPP, RG,	TE, LQ, LN, PP,	LEE, GSH, LPF,	ASL, FDK,
VSW, DY, TP, YV,	EK, HP, PH,	VE, LN, PT, PP,	ST, GHG, LR	PQ, EK, KE, HP,	AVL, DY, YV, IL,	DGL, TP, DM,
IL, YH, WL, RG, ST,	VIKP, AI, RPY,	PQ, EW, KE, PH,		PH, AI, VWP, AV,	YH, WA, WL, MGP,	FQ, YE, YH,
YN, FKR	FVP, AV, ASL,	VAF, AI, AFLL,		DY, DF, FQ, YV,	SGP, RG, ST, YN,	WM, GHG, LR,
	GLY, YVA,	GSH, AFL, VGP,		YH, WA, WL,	GHG, LR, LGV	
	VSW, DY, TP,	DY, DF, YV, IL,		MGP, SGP, PPP,		
	YV, IL, YH, WA,	YH, WA, WL,		RG, ST, GHG,		
	WL, RG	SGP, PPP, RG,		FGG, AVV, LR,		
		ST, GHG		LGV		

DPP- IV inhibitor	VA, MA,	LA, GP, PP, MP, VA,	GP, PP, MP, VA,	GP, PP, VA, MA,	GP, PP, VA, MA,	GP, PP, VA, MA,	GP, PP, MP, VA,	MP, VA, KA,
	KA, LA, FA,	KA, LA, FA, PA, LP,	KA, LA, FA, PA,	LA, FA, AP, LP,	LA, FA, AP, LP, VP,	LA, FA, AP, LP,	MA, KA, LA, FA,	LA, FA, AP, PA,
	VP, LL, GQ,	VP, LL, VV, HA, IP,	LP, VP, LL, VV,	VP, LL, VV,	LL, VV, HA, VPL,	VP, LL, VV, IP,	AP, PA, LP, VP, LL,	LP, LL, IP, TP,
	TP, SP, RP,	TP, SP, RP, KP, HP,	HA, IP, TP, SP,	VPL, GQ, IP,	IP, WP, SP, FP, KP,	WP, SP, FP, KP,	VV, GQ, IP, SP, FP,	SP, KP, IA, RA,
	HP, IA, TA,	GA, IA, RA, EP, NP,	RP, KP, HP, YP,	WP, SP, FP, KP,	YP, GA, IA, RA,	HP, YP, GA, RA,	RP, KP, HP, YP,	EP, NP, TA, FL,
	FL, HL, AL,	TA, QP, FL, WV, HL,	GA, IA, RA, WA,	YP, GA, IA, RA,	WA, EP, NP, FL,	WA, EP, NP, TA,	WA, EP, NP, TA,	HL, EK, SL, GL,
	SL, GL, AA,	AL, SL, GL, PL, PPG,	EP, NP, FL, WV,	WA, EP, NP, FL,	WV, HL, AL, SL,	WV, HL, EK, AL,	QP, FL, WV, HL,	VR, LPL, AA,
	PL, ALAV,	WK, WL, WY, WT,	HL, EK, AL, SL,	WV, HL, AL, SL,	GL, WRI, AA, PL,	SL, GL, VR, WRI,	EK, AL, SL, GL,	PL, WI, WM,
	WI, AD, AG,	VGL, AW, AD, AF,	GL, AA, PL,	GL, WRI, AA,	WR, WL, WI, WY,	LPL, AA, PL, WR,	VR, WRS, WRI, AA,	WT, WS, LW,
	AS, AT, AV,	AG, AH, AS, AT,	PPG, WK, WL,	PL, WR, WL, WI,	WS, LW, AW, AD,	WK, WL, WI,	PL, WR, WL, WI,	AW, YT, AD,
	DQ, FQ, GF,	AV, DP, DR, EG, EI,	WI, WY, VGL,	WY, WS, LW,	AE, AF, AS, AT, AV,	WY, WT, WS,	WY, WT, WS, LW,	AF, AH, AS, AT,
	GG, GV, HH,	ET, EV, EW, FN, FR,	AW, AE, AF,	AW, AD, AE,	AY, DN, DP, EH, EI,	AW, WG, AD,	WF, IQP, VGL, WG,	AV, AY, DN,
	HY, KH, KY,	GE, GF, GG, GH, GI,	AG, AH, AS, AT,	AF, AG, AS, AT,	ES, EW, FN, GG,	AG, AS, AT, AV,	AE, AS, AT, AV,	DP, EG, ES, EW,
	LT, LV, MK,	GV, GW, HE, HF,	AV, DP, DR, EG,	AV, AY, DN, DP,	GH, GI, GV, GW,	AY, DP, DR, ES,	AY, DP, DR, ES,	EY, FQ, FR, GE,
	MV, NV, PH,	HH, HR, HS, HT, HV,	EV, EW, FN, GF,	EG, EH, EI, ES,	GY, HD, HF, HH,	EV, FQ, GE, GF,	EW, FN, GF, GG,	GG, GH, GI, GV,
	PV, QF, QH,	HY, IH, IL, IQ,	GG, GH, GI, GV,	FN, GE, GG, GH,	HR, HS, HV, HY, IH,	GG, GH, GI, GV,	GH, GI, GV, GY,	GY, HS, IR, KF,
	QL, QQ, SH,	KG, KK, KR, KS,	GW, HE, HF,	GI, GV, GW, GY,	IL, IR, KE, KF, KI,	GW, HD, HE, HF,	HD, HE, HF, HH,	KK, KS, KT,
	SV, SW, TG,	KY, LH, LI, LM, LN,	HH, HR, HS, HT,	HD, HF, HH, HI,	KK, KR, LH, LN,	HH, HR, HS, HV,	HR, HS, HV, HY,	KV, LI, LM, LV,
	TI, TK, TL,	LT, LV, MF, MG,	HV, HY, IH, IL,	HR, HS, HV, HY,	LT, LV, MF, MV,	HY, IH, IN, IR,	IH, IL, IN, IQ, KE,	MF, MG, ML,
	VD, VF, VG,	MN, NG, NN, NT,	KG, KK, KR, KS,	IH, IL, IR, KE,	ND, NG, NH, NL,	KE, KG, KH, KI,	KG, KH, KI, KK,	MR, MV, MY,
	VI, VK, VL,	NV, NY, PG, PH, PI,	KT, KV, KY, LH,	KF, KI, KK, KR,	NN, NT, NV, NY,	KK, KS, KY, LH,	KR, KS, KY, LH, LI,	NA, NF, NL, PF,
	VS, VT, YG,	PK, PQ, PY, QG, QH,	LI, LM, LN, LT,	KS, KT, LH, LI,	PF, PG, PH, PI, PN,	LI, LN, LT, LV,	LN, LT, LV, MG,	PH, PK, PM, PS,
	YM	QI, QL, QW, RG, RH,	LV, MF, MG,	LN, LT, LV, MF,	PQ, PT, PW, PY, QD,	MF, MG, MK,	ML, ND, NE, NG,	PT, PV, QG, QN,
		RM, RR, SF, SI, SV,	MN, NG, NN,	MV, NG, NH,	QG, QI, QL, QN,	ML, MV, ND, NE,	NH, NL, NN, NQ,	QS, QT, RL, RN,
		SW, SY, TD, TG, TH,	NT, NV, NY, PF,	NL, NN, NV, NY,	QT, QY, RG, RI, RK,	NG, NH, NN, NV,	NW, PF, PG, PH, PI,	SF, SK, SV, SW,

TI, TK, TL, TM, TN,	PG, PH, PI, PK,	PF, PG, PH, PI,	SF, SH, SI, SK, SW,	NW, PF, PG, PH,	PK, PM, PN, PQ,	SY, TE, TG, TH,
TV, VD, VE, VF, VG,	PN, PS, PY, QF,	PN, PQ, PT, PV,	SY, TD, TE, TH, TI,	PI, PK, PM, PN,	PW, PY, QL, QN,	TI, TL, TM, TR,
VI, VK, VL, VN, VQ,	QG, QH, QI, QL,	PW, PY, QD, QG,	TL, TM, TR, TS, TT,	PQ, PS, PV, PW,	QS, QT, RG, RI, RK,	TS, TW, VE, VF,
VS, VY, WD, YD,	QW, RG, RH,	QI, QL, QN, QT,	TV, VD, VE, VF,	PY, QE, QG, QI,	RN, SF, SH, SI, SV,	VG, VH, VI, VK,
YF, YG, YH, YI, YL,	RL, RM, RR, SF,	RG, RI, RK, RL,	VG, VH, VI, VK,	QL, QN, QT, RG,	SW, SY, TD, TG,	VL, VS, VT, YA,
YM, YN, YQ, YS,	SI, SV, SW, SY,	SF, SH, SI, SK,	VL, VS, VT, VW,	RI, RK, RN, SF,	TH, TL, TM, TR,	YD, YE, YF,
YV, YY, LPQ	TD, TG, TH, TK,	SW, SY, TD, TG,	VY, WD, YA, YD,	SI, SV, SW, SY,	TS, TT, TV, TY, VE,	YG, YH, YK,
	TL, TM, TN, TV,	TH, TI, TL, TM,	YE, YG, YH, YK,	TD, TE, TG, TH,	VF, VG, VI, VK,	YM, YY
	TY, VD, VE, VF,	TR, TS, TT, ,VD,	YL, YQ, YR, YS,	TK, TL, TM, TS,	VL, VM, VQ, VS,	
	VG, VI, VK, VL,	VE, VG, VI, VK,	YV, YW, YY	TT, TV, TY, VE,	VT, VW, VY, WH,	
	VN, VQ, VS, VT,	VS, VT, VW,		VG, VI, VK, VL,	YF, YG, YH, YL,	
	VY, WD, YD,	VY, WD, YA,		VM, VN, VS, VT,	YN, YQ, YR, YV,	
	YG, YH, YI, YL	YD, YF, YG, YH,		VW, VY, WH,	YW, YY	
		YK, YL		YF, YG, YH, YK,		
				YL, YQ, YR, YV		

Sequences identified in BLAST tool ® Program and analyzed in BIOPEP® database. ACE inhibitor: angiotensin-converting-enzyme inhibitor; DPP IV inhibitor: dipeptidyl peptidase IV inhibitor; Activating UBMP: Activating ubiquitin-mediated proteolysis; GUSP: Glucose uptake stimulating peptide; SVSR: Stimulating vasoactive substance release; Regulation: peptide regulating the stomach mucosal membrane activity. 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; MGAT: monoacylglycerol acyltransferase.

The antioxidant effect and the glucose uptake stimulating peptide (GUSP) had a high occurrence of frequency (5.9 and 2.7%, respectively). The other effects, for example, antimnestic, stimulating vasoactive substance release, neuropeptides, and immunomodulating had a frequency of occurrence of less than 1% (**Figure 4**).

The number of studies using isolated peptides from chia are limited but show beneficial promising health effects. A protein hydrolysate from chia seed, produced by enzymatic hydrolysis, with alcalase and/or Flavourzyme, demonstrated antibacterial activity, reducing the velocity of enzymatic reaction of and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (Coelho, Soares-Freitas, Areas, Gandra, & Salas-Mellado, 2018). The chia protein hydrolysate has also shown ACE-inhibitory and antioxidant activities (Segura-Campos et al., 2013; Segura Campos, Peralta González, Chel Guerrero, Betancur Ancona, & Betancur Ancona, 2013; Chim-Chi, Gallegos-Tintoré, Jiménez-Martínez, Dávila-Ortiz, & Chel-Guerrero, 2018). In another study, peptides with molecular weight < 15 kDa were produced from a chia seed co-product, generated during oil production, by digestion with papain. Peptides showed a potent radical scavenging effect against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals in comparison with non-digested samples (Cotabarren et al., 2019).

The simulated digestion of chia to produce protein hydrolysates and, consequently, peptides, can be performed with such enzymes as alcalase and Flavourzyme. These are commercial enzymes obtained from bacteria, for example, *Bacillus amyloliquefaciens*. They act by interacting with peptide bonds through a serine residue at the active site (OTTESEN; SVENDSEN, 1970). However, digestion with the enzymes trypsin, chymotrypsin, and pepsin demonstrates a hydrolytic physiological process, since the human gastrointestinal system produces such enzymes. This allows to evaluate the production of the bioactive peptides after

normal consumption of food proteins (UDENIGWE; ALUKO, 2012). These enzymes, under optimum pH and temperature conditions, break the bonds between specific amino acids and release peptides and free amino acids that can be absorbed (GARDNER, 1984).

The processing conditions, as hydrolysis time, degree of hydrolysis of the proteins, kind of enzyme, enzyme–substrate ratios, and pretreatment of the protein prior to hydrolysis can influence the bioactive properties of the peptides. Peptide properties can also be influenced by net charge, hydrophobicity and the size of the peptide, which are factors that affect their absorption across the enterocytes (UDENIGWE; ALUKO, 2012).

Chia protein fractions have been also separated and hydrolyzed with pepsin and pancreatin. Each of the resulting fractions, after gastrointestinal digestion, presented different compositions of bioactive peptides with different physiological actions (ORONA-TAMAYO et al., 2015). In this study, the highest antiradical activity against 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and angiotensin-converting enzyme (ACE) was by peptides from albumin and globulin. Moreover, prolamin and globulin fractions showed the most potent ability to chelate ferrous ion. These results highlight the antioxidative and antihypertensive potential of peptides from chia (ORONA-TAMAYO et al., 2015).

Conclusions

This review shows that all proteins identified in chia seeds (*Salvia hispanica* L.) and their peptide sequences have auspicious biological potentials, mainly antioxidative, antihypertensive, and hypoglycemic properties. Among other bioactive compounds that may exert biological functions, these peptides can be responsible for the positive effects found in research studies in humans that consumed the whole chia seed; although, many results are still inconclusive. New investigations that focus on chia proteins and their bioactive peptides are

necessary to demonstrate specifically the mechanisms of action that contribute to the observed health benefits.

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Author Contributions

Mariana Grancieri: compiled data and prepared the initial draft

Hercia Stampini Duarte Martino: critical analysis

Elvira de Mejia: overall concept, editing of the manuscript, and critical analysis

All authors critically revised the manuscript and gave their attention, conclusions, and final approval for submission.

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6.2 Manuscript 2

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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

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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.

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

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.

Keywords: chia seeds, peptides, simulated gastrointestinal digestion, inflammation, antioxidant, atherosclerosis

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 et al., 2014). In the inflammatory process, the receptors present in immune cells, as TLR (toll-like receptor) and LOX (lipoxygenase) can be activated, sending signals for activation of cellular molecules, such as NF- κ B (nuclear factor kappa B)(MORETTINI et al., 2015). This promotes the activation of the enzyme iNOS (inducible nitric oxide synthases) and COX-2 (cyclooxygenase), which will produce NO (nitric oxide) and PGE₂ (prostaglandins), respectively, which will propagate the inflammatory process (KHODABANDEHLOO et al., 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 et al., 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 release 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 et al., 2016; ORONA-TAMAYO et al., 2015). Research has shown that digested protein of chia inhibited the ACE-enzyme (angiotensin-converting enzyme) (ORONA-TAMAYO et al., 2015; SEGURA-CAMPOS et al., 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 x 6.25 (AOAC, 2012; CAPITANI et al., 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: $[100 - (\% \text{ moisture} + \% \text{ lipids} + \% \text{ proteins} + \% \text{ total dietary fiber} + \% \text{ ash})]$. All chemical analyses were performed in triplicate.

2.2 Sample preparation

Chia seeds were processed according to Orona-Tamayo et al. (2015), with modifications (**Figure 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.

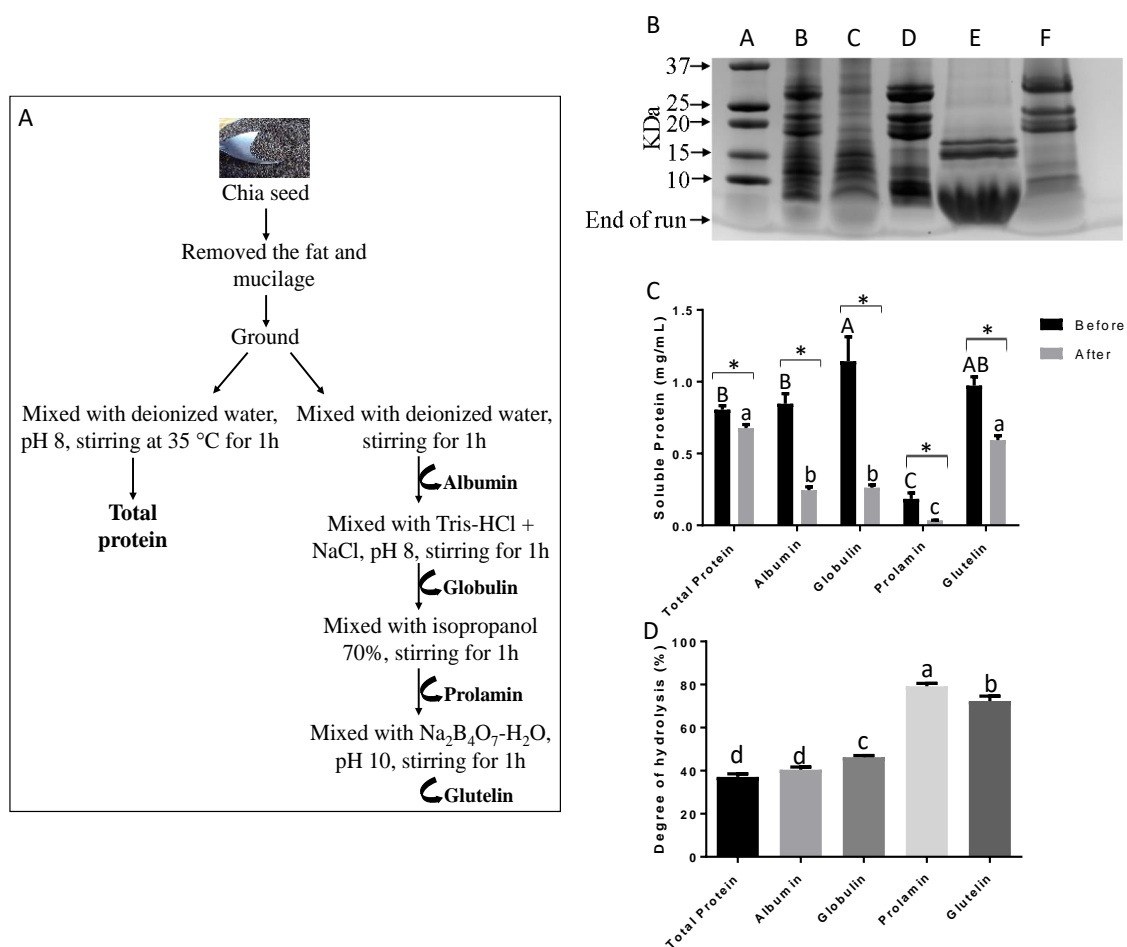


Figure 1. Prepare and characteristics of chia seeds. A: Diagram of the total protein and protein fractions extraction. B: Protein fractions present in chia flour by SDS-PAGE stained with Simply Blue Safe Stain. (A) standard; (B) chia protein extract; (C) albumin; (D) globulin; (E) prolamin; (F) glutelin. 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 ($p < 0.05$).

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 0.1M sodium hydroxide and placed under constant stirring at 35 °C for 1 h.

The mixture was centrifuged (5000g; 15 min; 25 °C), the precipitate was discarded and the supernatant (concentrated protein) was freeze-dried and stored at -20 °C (ALVES et al., 2016a; MONTOYA-RODRÍGUEZ et al., 2014a). 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 fractions of proteins.

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 & 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 resuspended 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 Na₂B₄O₇·H₂O (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 Mejias 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 ($y = 0.0002x + 0.607$, $R^2 = 0.99$).

The protein profile was evaluated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide 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 (200V; 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 (Carestream 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 determined according to Nielsen, Petersen, & Dambmann, (2001) with modifications. The *o*-phthalaldehyde (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 0.1–0.5 mg sample was diluted in 1 ml of the deionized water so that there was between 8-80% protein in the solution.

In tubes, 600 μ l of OPA reagent was added in 80 μ l of the standard, sample or water (blank) were mixed for 5 sec. Then, 200 μ l 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:

$$4) \quad \text{Serine-NH}_2: \left(\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \right) * 0.9516 * 0.001 * \left(\frac{100}{X * P} \right)$$

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

$$5) \quad h = \frac{\text{Serine-NH}_2 - \beta}{\alpha}$$

Where: $\beta = 0,40$ and $\alpha = 1$.

$$6) \quad \text{DH} = \left(\frac{h}{h_{\text{hot}}} \right) * 100$$

Where: $h_{\text{hot}} = 8.30$

2.8 Identification and characterization of potentially bioactive peptides

The peptides obtained from the DTP and DPF were analyzed according to Mojica, Chen, & 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 $\mu\text{L}/\text{min}$ 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- κ B and Toll-Like receptor 4 (TLR) was evaluated by *in silico* analysis, through molecular

docking as described by (ALVES et al., 2016a), 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., 2016a), dexamethasone (SUN et al., 2017), JSH23 (4-Methyl-1-N-(3-phenylpropyl)benzene-1,2-diamine) (WANG et al., 2018), and atorvastatin (Thongnak, Pongchaidecha, Jaikumkao, Chatsudthipong, Chattipakorn & Lungkaphin, 2017) were used, respectively for LOX-1, COX-2, p-65 NF-kB, and TLR4.

The crystal structure file of LOX-1, COX-2, NF-kB, and TLR 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, & De Mejia (2013), using the formula:

$$K_I = 2.72 \left(\frac{EFE * 10000}{R * T} \right)$$

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 & Janero (1995). An aliquot of 25 μ L of each DTP and DPF or PBS (blank) was mixed with 200 μ L of a solution composed by 0.1 mmol/L of ethylenediaminetetraacetic acid (EDTA), 62 μ mol/L of nitroblue tetrazolium chloride (NBT) and 98 μ mol/L of nicotinamide adenine dinucleotide (NADH). Then, 25 μ L of 33 μ mol/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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

A curve with trolox (4.8-300 μ g/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 μ L of 20 mM sodium nitroprusside were mixed with 50 μ L 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 μ L 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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

A curve with Trolox (1.15-300 μ g/ml) was made to determine the TE ($y=0.0751\ln(x) + 0.0845$; $R^2 = 0.93$)(TSAI et al., 2011).

2.10.3 Hydrogen peroxide (H₂O₂) capacity

The ability of the samples to scavenge H₂O₂ was determined according to Ruch, Cheng, & Klaunig (1989). A 117 µl of DTP and DPF (as in 2.10.1) were combined with 30 µl of a H₂O₂ solution (40 mM). PBS was used as blank. Absorbance was measured after 10 min at 230 nm. H₂O₂ radical scavenging activity was calculated by the following formula:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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

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 sec. 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:

$$\% \text{ of scavenging} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

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

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 μ L of linolenic acid (500 μ M) 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:

$$\% \text{ of inhibition} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right)$$

Ascorbic acid (4.5–17.5 μ g/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

COX-1 and 2 (cyclooxygenase 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 using a kit (Cayman Co., St. Louis, MO, USA) according to manufacturer's instructions. This analysis is based in the production of PGF 2α by SnCl $_2$ (Tin(II) 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 \pm 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 - 114.6 kDa, whereas globulins and prolamins, molecular weights were concentrated between 5.0 – 69.0 kDa and glutelins ranged from 10.6 - 98.4 kDa (**Figure 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%) (**Figure 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 (**Figure 1C**). Digested prolamins had the highest degree of hydrolysis, followed by digested glutelin and globulin. Digested albumin and DTP showed the lowest values (**Figure 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, prolamins 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 prolamins (4%) (**Supplementary Table 1**). **Supplementary Table 1** also presents similarities in peptide sequences in 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, prolamins, 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 material Table 1**).

Most of the peptides showed antioxidant, anti-atherosclerotic, anti-amnesic, hypoglycemic and hypotensive activity (**Figure 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 (**Figure 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 fractions that also show similarities in sequence with other chia proteins. For instance, albumin presented peptides 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 (**Figure 2B and Supplementary Tables 1 and 2**).

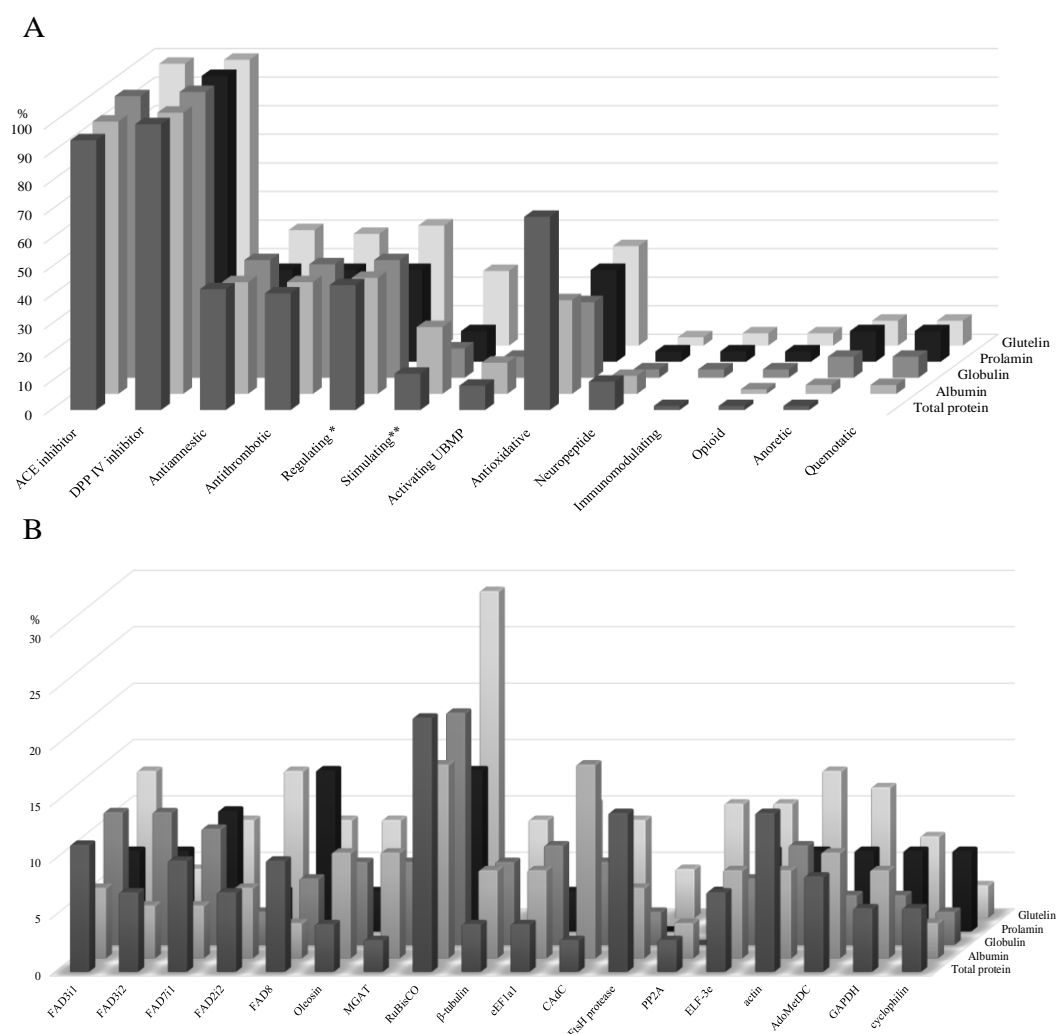


Figure 2. A: Potential biological activity and B: parental protein identified with peptides found in the digested total protein and fractions from chia seed. ACE inhibitor: angiotensin-

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.

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

The minimum estimated free energies (EFE) of the interactions of the peptides with LOX-1, COX-2, p-65 NF-κB, 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 interaction) 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 HYGPPGGCR, from DTP, had more interaction with p65-NF-κB (EFE: -7.1 kcal/mol) (**Figure 3, Supplementary Tables 1 and 3**).

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

Sequence of peptides	LOX -1			COX-2			NF-kB			TLR4		
	EFE (kcal/mol)	K_I (μ M)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μ M)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μ M)	Interacting amino acid residues	EFE (kcal/mol)	K_I (μ M)	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: 543; ILE A: 124; PRO B: 542; HIS A: 122; SER A: 126	-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;
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;	-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 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
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;	-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

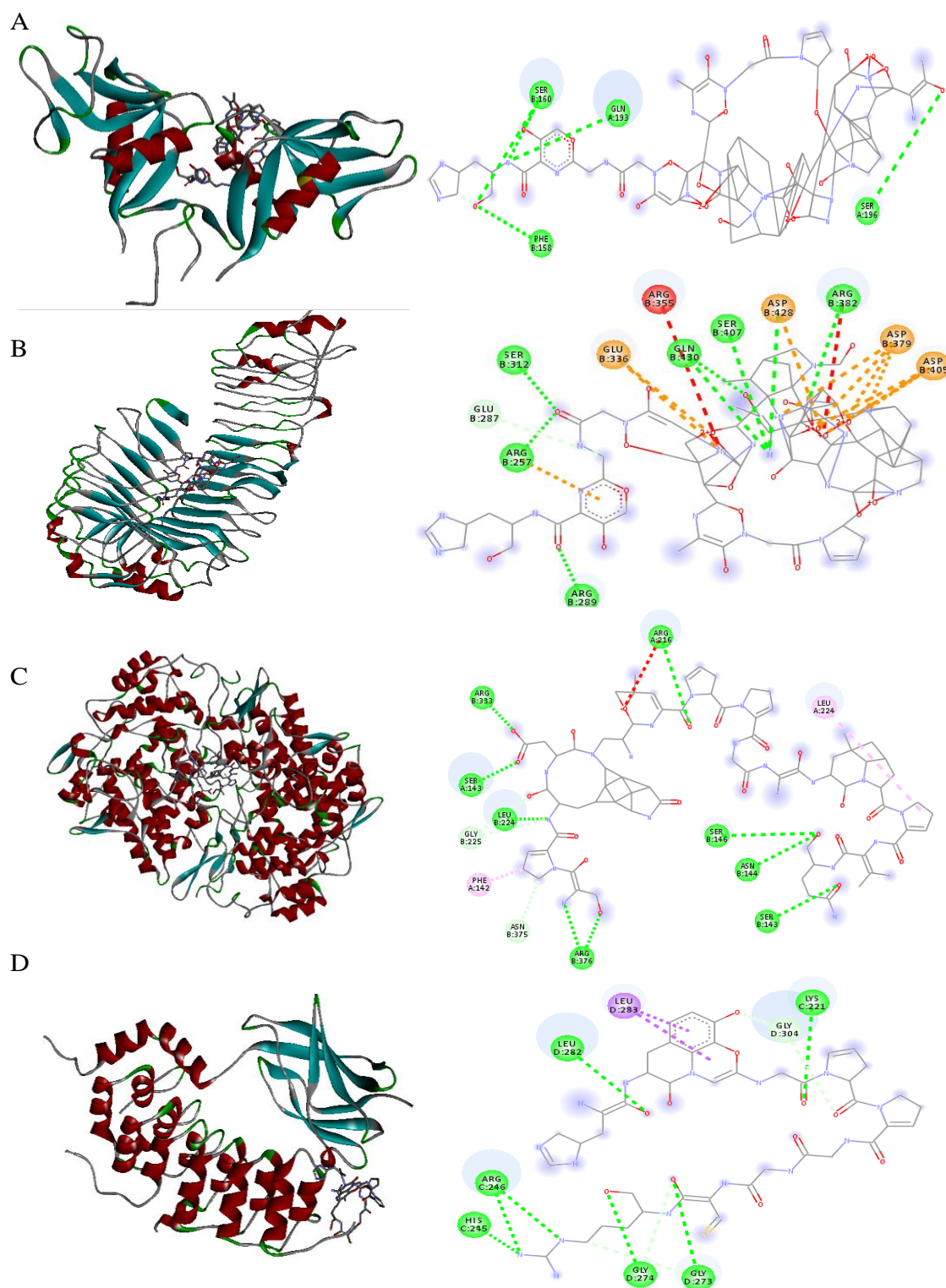


Figure 3. Molecular docking diagrams exemplifying the interaction of peptides with receptors. A: Interaction between peptide TGPSPTAGPPAPGGGTH and LOX-1 receptor; B: TGPSPTAGPPAPGGGTH and Toll-like receptor 4; C: Interaction between peptide SPKDLALPPGALPPVQ and COX-2; D: Interaction between peptide HYGGPPGGCR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

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 (**Figure 4A**).

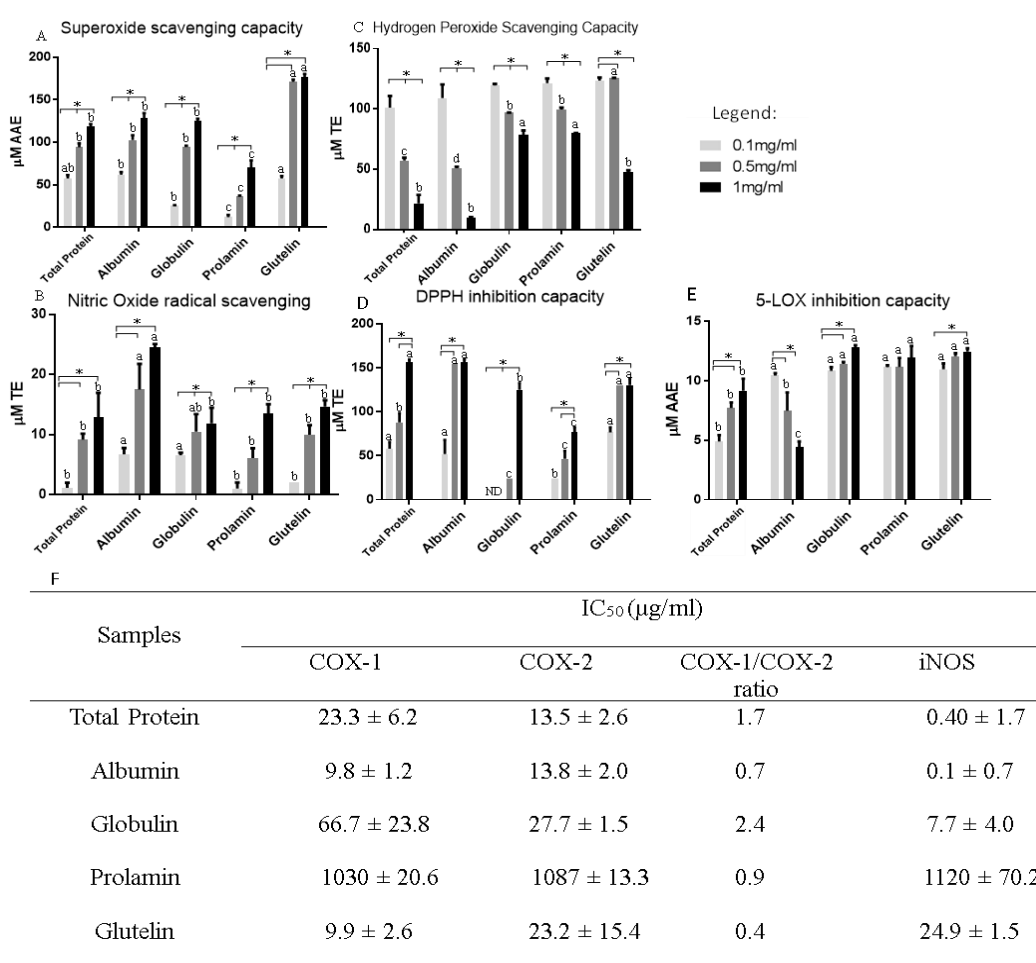


Figure 4. Biochemistry effects of digested protein from chia seeds. A: Superoxide scavenging capacity, B: Nitric oxide scavenging, C: Hydrogen Peroxide scavenging, D: DPPH inhibition capacity, E: 5-LOX inhibition capacity of digested total protein and digested protein fractions from chia in three concentrations (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-lipoxygenase, COX-1: cyclooxygenase-1, COX-2: cyclooxygenase-2, iNOS: inducible nitric oxide synthase, DPPH: 1.1-diphenyl-2-picrylhydrazyl.

3.6.2. Nitric oxide (NO) scavenging capacity

The concentrations of 0.5 mg/ml and 1 mg/ml presented the same ($p < 0.05$) scavenging capacity of nitric oxide radical in both, DTP and digested albumin. However, for digested globulin, prolamin and glutelin, the concentration of 1 mg/ml showed higher scavenging capacity in comparison to the other two concentrations. Digested albumin was the fraction with the best scavenging capacity (1 mg/ml, around 35%), whereas, in the concentration of 0.5 and 0.1 mg/ml, its capacity was similar to that of digested globulin (**Figure 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 ($p > 0.05$) to 0.1 mg/ml and was the best for scavenging hydrogen peroxidase. At the concentration of 1 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 (**Figure 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 (1.0, 0.5 mg/ml) showed the lowest antioxidant capacity and digested globulin (0.1 mg/ml) (**Figure 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 ($p < 0.05$, about 75%) followed by DTP and digested albumin. At 0.1 mg/ml DTP had the lowest inhibition of the enzyme ($p < 0.05$) (**Figure 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 less than 1.0. Digested prolamin had no inhibition of these enzymes (**Figure 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} (**Figure 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 & Paredes-López (2013) also showed that the peak of denaturation temperature 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 & Paredes-López (2013), glutelins are soluble at pH 10 and globulin 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 acid 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, J. D.; 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; CHEN; DE MEJÍA, 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, 2017). On the other hand, the hypoglycemic effect is mainly due to the inhibition of the enzyme DPP-IV (dipeptidyl peptidase-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 less than 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 et al., 2013). This 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; LUNA-VITAL; GONZÁLEZ DE MEJÍA, 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; CHEN; DE MEJÍA, 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, URRÁ, 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 et al., 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 et al., 1993). The aromatic diamine 4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) inhibits nuclear translocation of the NF- κ B p65/p50 heterodimer, without affecting I κ B α degradation (SHIN et al., 2004). Furthermore, the administration of atorvastatin attenuated the activation of toll-like receptor (TLR4) expression and the NF- κ B 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 fractions of chia proteins are provided, and highlight the potential beneficial health effects of chia seed proteins consumed as part of the diet.

Conflict of interest

The authors do not have any conflict of interest.

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Supplementary material.

Table 1. Main bioactive peptides from hydrolysates of the total extract and protein fractions of chia.

Peptide	RT	Molecular mass (Da)	IP	Net Charge	Hydrophobicity	Parental Protein	Activity	Sequence
					Total Protein			
NPGGGAPSGH	2.22	849.37	7.69	0	16.92	RuBisCO	antiamnestic ACE inhibitor	PG AP, GA, GH, GG, SG, PG
						FAD8	antithrombotic regulating	PG PG
							DPP-IV inhibitor	AP, GA, NP,GG, GH, PG, PS
HYGGPPGGCR	3.12	999.43	8.62	1	16.19	FAD2i2	antiamnestic ACE inhibitor	PG, GP HY, YG, GP, GG, PG, YGG, GPP,PP
							antithrombotic immunomodulating	GP, PG YG, YGG
						FAD2i1	regulating antioxidative	GP, PG GPP
LHNGGPGGEY	4.93	999.43	7.69	0	17.49	RuBisCO	DPP-IV inhibitor antiamnestic ACE inhibitor	GP,PP, PPG, GG, HY, PG, YG GPGG, PG, GP GP, GE, GG, NG, PG, EY
							antithrombotic regulating	GP, PG, GPGG GP, PG, GPGG
							antioxidative DPP-IV inhibitor	LH, LHN,GGE GP, EY, GE, GG, LH, NG, PG
DVNPGGPAPHPWLSVNDL	5.06	1980.96	3.91	-2	17.66	Actin	antiamnestic	PG, GP
							ACE inhibitor	GPA, GP, AP, GG, PG, PAP, PP, HP, PH, VNP, WL
						FtsH protease	antithrombotic regulating antioxidative	GP, PG GP, PG PWL, PW
TGPPRPALVFPHAVVP	7.13	1653.96	11.56	1	10.8	ELF-3e	DPP-IV inhibitor	GP, PP, AP, PA, HP, GPA, NP, WL, GG, ND, PG, PH, PW,SV
						FAD3i2	antiamnestic	GP
						Actin	ACE inhibitor	VF, FP, PR, GP, RP, VP, TG, GPP, PP, PH, AV, TGP, AVV
NSSAQYSDPFLALH	7.31	1548.71	4.98	-1	13.09	RuBisCO	antithrombotic	GP
						GAPDH	stimulating regulating	LV GP
						FtsH protease	antioxidative inhibitor	PHA, GPP PPRP
						Cyclophilin	DPP-IV inhibitor ACE inhibitor	GP, PP, PA, VP, VV, HA, FP, RP, AL, AV, LV, PH, TG, VR LA

						ELF-3e	antioxidative	LH
						FtsH protease	Activating UBMP	LA
						GAPDH	DPP-IV inhibitor	LA, FL, AL, DP, LH, PF, QY, YS
						FAD7i1	ACE inhibitor	VF, LAA, YL, LA, AA, GL, FG, EG
						RuBisCO	neuropeptide	YL
						FAD3i2	antioxidative	LH, TY
						FAD3i1	Activating UBMP	LA
LPVFGLAAEGNVVTYLH	10.04	1798.95	5.06	-1	10.85	FAD8	DPP-IV inhibitor	LA, LP, VV, GL, AA, AE, EG, LH, NV, PV, TY, VF, VT, YL
						MGAT		
						FAD2i2		
							antiamnestic	PG
							ACE inhibitor	FP, GF, VG, GA, GV, WG, GG, LG, PG, PP, TP
						FAD8	antithrombotic	PG
TPPGVGGFPWGGAMLGAQ	10.12	1698.91	5.29	0	11.06	FAD2i2	regulating	PG
						FAD2i1	antioxidative	PWG, PW, WG
							DPP-IV inhibitor	PP, TP, FP, GA, PPG, WG, GF, GG, GV, ML, PG, PW, VG
						Cyclophilin	antiamnestic	GP
						GAPDH	ACE inhibitor	LGP, GP, AP, LG, GPP, PP
							antithrombotic	GP
APSPPVLGPP	25.17	930.52	5.65	0	9	FtsH protease	stimulating	VL
							regulating	GP
						FAD7i1	antioxidative	GPP
							DPP-IV inhibitor	GP, PP, SP, VLGP, PS, PV, VL

Cont.

Albumin								
						antiamnestic	GP	
						ACE inhibitor	GPA, GP, GA, AG, TG, GPP, PT, PP, HP, AGP, GAGP	
HPTGPAGAGPPMSL	0.8	1389.66	7.89	0	14.28	MGAT	antithrombotic	GP
							regulating	GP
							antioxidative	GPP
							DPP-IV inhibitor	GP, PP, PA, HP, GPA, GA, SL, AG, PM, PT, TG, TH
						MGAT	antiamnestic	PG
							ACE inhibitor	LPG, DLP, AA, GA, AG, GS, GD, PG
						RuBisCO	antithrombotic	PG
RGSSSPMAGAAGDLPGSVT	0.89	1716.8	6.46	0	19.44		stimulating	SSS
						FAD3i2	regulating	PG
						FAD3i1	antioxidative	GAA
							DPP-IV inhibitor	MA, LP, SP, GA, AA, AG, PG, PM, RG, SV, VT
							antiamnestic	PG
YLGHAHPGTAN	1.92	999.47	7.37	0	12.81	FAD2i2	ACE inhibitor	YL, GA, GT, LG, PG, AH, HP

							antithrombotic	PG
							neuropeptide	YL
						FAD2i1	regulating	PG
							antioxidative	AH, GAH
						CAdC	ACE inhibitor	VLP, PT, AQL
							stimulating	VL
RMVLPTYFSAQLE	4.51	1553.78	6.68	0	9.41	ELF-3e	antioxidative	TY
						FtsH protease	DPP-IV inhibitor	LP, MV, PT, QL, RM, TY, VL, YF
						FtsH protease	anti-amnesic	LPPV, PG
							ACE inhibitor	LPP, ALPP, LALPP, LA, GA, PG, PP, ALP
						CAdC	antithrombotic	PG
SPKDLALPPGALPPVQ	5.02	1598.9	6.47	0	14.21		regulating	PG
							antioxidative	KD
						GAPDH	Activating UBMP	LA
						Oleosin	DPP-IV inhibitor	PP, LA, LP, SP, GA, AL, PPG, PG, PK, PV, VQ
RHMDLPALVFT	5.88	1573.78	6.68	0	9.41	CAdC	ACE inhibitor	VF, DLP

							stimulating	LV
						GAPDH	antioxidative	RHM
							DPP-IV inhibitor	PA, LP, AL, LV, RH, VF
							anti-amnesic	GP
						CAdC	ACE inhibitor	GP, GA, EG, DG, GPP, TE, PP
GPPDGPCTEGA	6.18	999.39	2.99	-2	19.77		antithrombotic	GP
							regulating	GP
						β -tubulin	antioxidative	GPP
							DPP-IV inhibitor	GP, PP, GA, EG, TE
							anti-amnesic	VPL, GP
							ACE inhibitor	LW, GLP, GP, PL, VP, FR, TG, LN, PT, TGP
							antithrombotic	GP
TVPLLWFRQDPTGPLNL	6.4	1966.66	6.74	0	7.78	Oleolin	stimulating	VPL, LL
							regulating	GP
							antioxidative	LWF, LW
							DPP-IV inhibitor	GP, VP, LL, VPL, PL, LW, WF, DP, FR, LN, NL, PT

						RuBisCO	anti-amnesic	PG, GP
							ACE inhibitor	GP, AP, AG, GT, GG, TG, PG, PAP, GPP, PT, PP
TGPSPTAGPPAPGGGTH	9.6	1457.68	7.69	0	18.89	MGAT	antithrombotic	GP, PG
							regulating	GP, PG
						Oleosin	antioxidative	GPP
							inhibitor	PPAP, GP, PP, AP, PA, APG, SP, TA, AG, GG, PG, PS
							ACE inhibitor	LA, GK, SG, VE, GKV, AEL
							stimulating	EE
SGKVEELAELPDL	17.17	1598.72	3.58	-3	23.27	Actin	antioxidative	EL
							Activating UBMP	LA
							DPP-IV inhibitor	LA
							DPP-IV inhibitor	LP, AE, KV, VE
						FAD8	ACE inhibitor	VLP, LKL, KL, AV, AVL
							stimulating	SSS
AVLPDLTSSSLKLDK	27.9	1698.97	6.60	0	16.34	FAD7i1	stimulating	VL, LL
						FAD3i2	antioxidative	LK

							DPP-IV inhibitor	LP, LL, SL, AV, LT, TS, VL
							antiamnestic	PGP, PG, GP
						RuBisCO	ACE inhibitor	LPG, GP, PG, GPP, PP, TF
							antithrombotic	GP, PGP, PG
							regulating	GP, PG, PGP
LPGPPATF	30.88	798.42	5.52	0	7.26	eEF1a1	antioxidative	GPP
							chemotactic	PGP
						FtsH protease	anorectic	PGP
							DPP-IV inhibitor	GP, PP, PA, LP, AT, PG, TF, TT

Cont.

Globulin

						FAD3i2	antiamnestic ACE inhibitor antithrombotic neuropeptide	PGP, PG, GP VF, FP, LAA, GP, PL, LA, AA, PG, KP, GPP, TE, PT, PP GP, PGP, PG KPT
KPTEPGPPVFPLAAADL	1.88	1718.92	4.00	1	16.90	FAD3i1	regulating antioxidative chemotactic	GP, PG, PGP KP, GPP PGP
						RuBisCO	Activating UBMP	LA
						FAD7i1	DPP-IV inhibitor	GP, PP, LA, FP, KP, EP, AA, PL, AD, PG, PT, PV, TE, VF
						FAD3i2	ACE inhibitor	AP, GA, GL, GS, GG, SG, NG, PT, PP
NGGGLKSGSGAPPT	2.14	1198.59	9.82	1	18.00	RuBisCO	antioxidative	LK
						MGAT	DPP-IV inhibitor	PP, AP, GA, GL, GG, KS, NG, PT

TLPHEPGGVAGPATNFPQ	4.72	1788.87	5.06	$\bar{1}$	17.57	FAD7i1	antiamnestic ACE inhibitor antithrombotic regulating antioxidative DPP-IV inhibitor	PG, GP GPA, FP, GP, AG, GV, GG, PG, NF, PQ, PH, AGP GP, PG GP, PG PHE GP, VA, PA, LP, FP, GPA, EP, AG, AT, GG, GV, HE, NF, PG, PH, PQ, TL, TN
						FAD3i2	antiamnestic ACE inhibitor	PG FP, AA, GA, AG, GR, GG, TG, PG, SF, PT, PP
NPGAAGALMVSFPPTGGR	5.2	1698.84	10.73	1	13.70	FAD3i1	antithrombotic regulating antioxidative	PG PG GAA
						RuBisCO	DPP-IV inhibitor	PP, GA, FP, NP, AL, AA, AG, GG, LM, MV, PG, PT, SF
						FtsH protease	antiamnestic ACE inhibitor	PG AVP, VRP, FP, VPP, RP, VP, GA, PG, VR, PP, PH, AV
EPAVPPVPHVRPFFGALP	6.8	1873.06	8.32	0	14.46	Actin	antithrombotic regulating antioxidative	PG PG PHV
						eEF1a1	DPP-IV inhibitor	PP, PA, LP, VP, FP, RP, GA, EP, AL, VR, AV, HV, PF
						β -tubulin	antiamnestic ACE inhibitor antithrombotic regulating antioxidative	PG, GP PGL, GLP, GP, GL, SG, PG, GPP, PP, SGP GP, PG GP, PG GPP
SGPPDPGLPN	7.06	949.44	3.05	$\bar{1}$	14.46	FAD7i1	DPP-IV inhibitor	GP, PP, LP, GL, DP, PG, PN
						FAD8	antiamnestic ACE inhibitor antithrombotic regulating antioxidative	PG,GP GGY, GY, GP, LA, AG, GG, GD, EG, PG, PP, AGP, YV GP, PG
						FAD2i2	antiamnestic ACE inhibitor antithrombotic stimulating regulating antioxidative	VL GP, PG YVL
EGGYVLAGPSPVQPPGDH	8.78	1775.84	3.99	$\bar{2}$	21.51	RuBisCO	DPP-IV inhibitor	GP, PP, LA, SP, QP, PPG, AG, EG, GG, GY, PG, PS, PV, VL, VQ, YV
							ACE inhibitor antioxidative	AG, GT, PP TY
SPPAGTACTY	21.97	966.41	5.11	0	10.56	RuBisCO	DPP-IV inhibitor	PP, PA, SP, TA, AG, TY
RKTRARLMLLATELQ	24.07	1799.5	12.2	3	16.36	AdoMetDC	ACE inhibitor stimulating	RL, LA, RA, AR, TE, LQ LL

							antioxidative	EL
						GAPDH	Activating UBMP	RA, LA
							DPP-IV inhibitor	LA, LL, RA, AT, KT, LM, ML, RK, RL, TE, TR
						FAD3i2	ACE inhibitor	GGY, AY, GY, YP PLG, PL, GG, LG, PH, TP
TPHAYPLGGGY	25.3	1131.53	7.57	0	12.04	FAD3i1	opioid	YPLG, PLG
						RuBisCO	antioxidative	AY, PHA
							DPP-IV inhibitor	HA, TP, YP, PL, AY, GG, GY, PH
							antiamnestic	PG
							ACE inhibitor	GW, GF, AG, GG, PG
QPGWPVAGGF	30.95	1014.49	5.38	0	8.64	Oleosin	antithrombotic	PG
							antioxidative	AGGF
							DPP-IV inhibitor	VA, WP, QP, AG, GF, GG, GW, PG, PV
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							antiamnestic	GP
							ACE inhibitor	VF, GP, GPV, AA, HG, PH
							antithrombotic	GP
							regulating	GP
							antioxidative	PHG
							DPP-IV inhibitor	GP, FA, NP, AA, DP, PH, PK, PV VF, GPV
							antiamnestic	PG
							ACE inhibitor	LY, PLG, GL, LG, PG, SF, LEE
							antithrombotic	PG
							stimulating	EE
							regulating	PG
							antioxidative	LY
							DPP-IV inhibitor	EP, FL, GL, PG, SF, TS
							ACE inhibitor	RY, YP, GR, KP
							neuropeptide	KPS
							antioxidative	KP
							DPP-IV inhibitor	PA, KP, YP, AL, PS
							ACE inhibitor	AAP, AP, PR, AA, GA, GG, SG, PP
							antioxidative	GAA
							DPP-IV inhibitor	PP, AP, GA, AA, GG, VS
							antiamnestic	PGP, PG, GP
							ACE inhibitor	GPL, GP, PL, PG, LQ, PT, PH, TP
							antithrombotic	GP, PGP, PG

						regulating antioxidative chemotactic DPP-IV inhibitor	GP, PG, PGP PHW PGP GP, TP, PL, DP, HD, HW, PG, PH, PT
Glutelin							
KKLELTEYLYEDKK	0.75	1798.56	6.78	0	28.71	FAD3i1 ACE inhibitor neuropeptide antioxidative	LY, YL, KL, EY, TE, YE YL
						FAD8 DPP-IV inhibitor antiamnestic	LY, EL, YLY EY, KK, LT, TE, YE, YL
NSPGPHDVALDQ	1.32	1248.57	3.91	-2	19.81	RuBisCO ACE inhibitor antithrombotic regulating	PGP, PG, GP GP, PG, PH GP, PG, PGP
						Oleosin antioxidative chemotactic DPP-IV inhibitor	PHD PGP GP, VA, SP, AL, DQ, HD, PG, PH
KDSGHPNGGLFAESSP	1.45	1598.73	5.19	-1	23.80	RuBisCO ACE inhibitor opioid regulating	LF, GL, GH, GG, SG, NG, HP GLF GLF
						FAD3i2 antioxidative DPP-IV inhibitor	KD FA, SP, HP, GL, AE, ES, GG, GH, NG, PN
TGPCNGAAPPAAGSVDCL	2.05	1599.69	3.00	-1	17.22	FAD3i2 antiamnestic ACE inhibitor	GP AAP, GP, AP, AA, GA, AG, GS, TG, NG, PP, TGP, AGS
						FAD3i1 antithrombotic regulating	GP GP
TDQLGWFLGAAAFPAGQ	2.26	1748.84	3.04	-1	10.91	FAD8 antioxidative DPP-IV inhibitor	GAA GP, PP, AP, PA, GA, AA, AG, NG, SV, TG, VD
						Cyclophilin ACE inhibitor neuropeptide antioxidative	FP, AFP, GW, AF, AA, GA, AG, GQ, LG GQ GAA
RGSSPAPSGSELVL	2.34	1355.7	6.51	0	15.3	DPP-IV inhibitor stimulating antioxidative	PA, GQ, FP, GA, FL, AA, WF, AF, AG, DQ, GW, QL, TD VL, LV, SE EL
						Actin PP2A DPP-IV inhibitor	AP, PA, SP, LV, PS, RG, VL PG, GP
QAGAGGPGAAGH	3.49	949.43	7.69	0	18.89	FAD2i2 antiamnestic ACE inhibitor antithrombotic	GP, AA, GA, AG, GH, GG, PG GP, PG

						FAD2i1	regulating antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor antithrombotic	GP, PG GAA GP, GA, AA, GPGA, AG, GG, GH, PG, QA PGP, PG, GP LGP, GP, GL, GS, SG, LG, NG, PG, PP, PH, TP GP, PGP, PG
TPPNGLGPSGSPGPHAQ	3.88	1569.75	7.59	0	17.57	RuBisCO	regulating antioxidative DPP-IV inhibitor ACE inhibitor stimulating	GP, PG, PGP PHA GP, PP, HA, TP, SP, GL, NG, PG, PH, PN, PS VLP, LY, EY, YE VL, LL
RMVLPEYELLYE	4.53	1553.77	3.78	-2	14.44	RuBisCO	immunostimulating antioxidative DPP-IV inhibitor ACE inhibitor stimulating	LLY LY, EL LP, LL, EY, MV, RM, VL, YE VLP, RL, AA, GM, GA, MG, GT, TG, GTG VL
RLTGTGMGAASSVLP	5.42	1416.73	11.56	1	12.09	FAD3i1	antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor antithrombotic	GAA LP, GA, AA, AS, LT, MG, RL, SV, TG, VL GP LSP, GP, PL, DLP, AG, AGP
RHMDLPLSPSSAGP	5.98	1463.71	8.32	0	15.96	FAD2i1	antithrombotic regulating antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor	GP GP RHM GP, LP, SP, LPL, PL, AG, PS, RH PG FP, DA, GV, PG, TNP, TTN, AR, PP, DF
						Oleosin	antithrombotic regulating antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor	PG PG ADF, EL PP, PA, FP, NP, AD, GV, PG, TN, TT, VD PG WY
TTNPGVDADFPAREL	8.78	1698.81	3.67	-2	21.12	RuBisCO	antithrombotic regulating antioxidative DPP-IV inhibitor antithrombotic antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor	GA, WY, AF, DP, FQ, PG, TD, YD PG LSP, LSPA, AP, GF, PG, AH PG
TDWYDPGAFQ	6.07	1198.49	2.87	-2	13.48	ELF-3e	antithrombotic antioxidative DPP-IV inhibitor antiamnestic ACE inhibitor antithrombotic	PG WY GA, WY, AF, DP, FQ, PG, TD, YD PG LSP, LSPA, AP, GF, PG, AH PG
HAPGFLSPAHR	7.36	1188.61	10.91	1	14.3	AdoMetDC	immunomodulating regulating	GFL GFL, PG

KAEPSVLPTAH	7.49	1148.61	7.55	0	16.94	CAdC	antioxidative	AH
							DPP-IV inhibitor	AP, PA, HA, APG, SP, FL, AH, GF, HR, PG
QPADPNQFVYAPDTH	10.03	1698.76	3.91	-2	18.69	GAPDH	ACE inhibitor	VLP, KA, PT, AH
							stimulating	VL
						FAD8	antioxidative	AH
							DPP-IV inhibitor	KA, LP, EP, TA, AE, AH, PS, PT, SV, VL
						FAD3i1	ACE inhibitor	VY, AP, YA, VYAP
						FAD3i2	antioxidative	VY
FAFFEFFELLFAFFT	28.9	1921.23	2.97	-2	0.23	FAD7i1	DPP-IV inhibitor	AP, PA, QP, AD, DP, NQ, PN, QF, TH, VY, YA
							ACE inhibitor	LF, AF, LLF
						FAD8	stimulating	LL
							antioxidative	EL
						eEF1a1	inhibitor	EF
							hypotensive	EF
							DPP-IV inhibitor	LL
							DPP-IV inhibitor	AF
						FtsH protease	ACE inhibitor	LPG, GP, PG, GPP, PP, TF
							antithrombotic	GP, PGP, PG
regulating	GP, PGP, PG							
antioxidative	GPP							
FtsH protease	chemotactic	PGP						
	DPP-IV inhibitor	GP, PP, PA, LP, AT, PG, TF						

Peptides sequenced by HPLC ESI-MS/MS with intensity at least 90%. Only sequences of main proteins of *Salvia hispanica* L. (100%) (BLAST tool ®) and with antioxidative peptides (BIOPEP® database) are presented in the table. Physicochemical properties were determined using PepDraw. RT: Retention time; IP: isoelectric point; HPB: Hydrophobicity; ACE inhibitor: angiotensin-converting-enzyme inhibitor; DPP IV inhibitor: dipeptidyl peptidase IV inhibitor; Activating UBMP: Activating ubiquitin-mediated proteolysis. Regulation: peptide regulating the stomach mucosal membrane activity. Stimulation: 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. The amino acids are presented in one letter nomenclature.

Table 2. Potential bioactive and the parental protein of peptides found in digested protein of extract and protein fractions from the chia seed.

Activity/Parental protein	Samples (%)				
	Extract	Albumin	Globulin	Prolamin	Glutelin
DPP IV inhibitor	100.00	98.40	100.00	100.00	100.00
ACE inhibitor	94.40	95.30	98.53	82.10	98.60
Antioxidative	67.60	32.80	26.47	32.10	34.80
Antiamnestic	42.30	39.10	41.18	32.10	40.40
Antithrombotic	40.80	39.10	39.71	32.10	39.10
Regulating *	43.70	40.60	41.18	32.10	42.00
Stimulating**	12.70	23.40	10.29	10.70	26.10
Activating UBMP	8.50	10.90	7.35	7.10	2.90
Neuropeptide	9.90	6.30	2.94	3.60	2.90
Immunomodulating	1.40		2.94	3.60	4.30
Opioid	1.40	1.60	2.94	3.60	4.30
Anorectic	1.40	3.10	7.35	10.70	8.70
Quemotatic		3.10	7.35	10.70	8.70
FAD8	14.08	9.38	11.76	17.86	17.39
FAD3i1	11.26	14.06	13.24	7.14	13.04
FAD3i2	5.63	10.94	14.71	7.14	10.14
FAD7i1	9.86	9.38	19.12	17.86	17.39
FAD2i2	4.23	7.81	5.88	3.57	13.04
Oleosin	2.82	14.06	8.82	7.14	13.04
MGAT	4.23	10.94	10.29	14.29	4.35
RuBisCO	4.23	10.94	19.12	10.71	20.29
β -tubulin	4.23	7.81	7.35	0.00	8.70
eEF1a1	4.23	7.81	8.82	3.57	10.14
CAdC	2.82	17.19	7.35	3.57	8.70
FtsH protease	14.08	6.25	2.94	0.00	4.35
PP2A	2.82	3.13	0.00	3.57	10.14
ELF-3e	7.04	7.81	5.88	7.14	10.14
actin	14.08	7.81	8.82	7.14	13.04
AdoMetDC	8.45	9.38	4.41	7.14	11.59
GAPDH	5.63	7.81	4.41	7.14	7.25
cyclophilin	5.63	3.13	2.94	7.14	2.90

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.

Table 3. Estimated free energy binding and chemical interactions among the peptides present in chia seeds and the catalytic site of the LOX-1, COX-2, p65NF-κB and TLR4.

Peptides	LOX-1			COX-2			NF-κB			TLR-4		
	EFC	K _i (μM)	Interacting amino acid residues	EFC	KI (μM)	Interacting amino acid residues	EFC	KI (μM)	Interacting amino acid residues	EFC	KI (μM)	Interacting amino acid residues
	Total protein											
HYGGPPGGCR	-6.5	17.2	PHE A:158; TYR A:197; SER A:160; SER B:196; TYR B:197; GLN B:193; ARG B:248	-8.0	1.4	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	6.3	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	2.3	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
SPPHGAGLGGGMT	-5.8	56.1	ASP A: 147; ASP B: 189; ARG B: 248; LEU A: 157; TYR B: 197; SER A: 160; SER B: 196; GLN B: 192; GLN B: 193	-6.3	24.1	LYS A: 369; ASN A: 368; ILE A: 112; TYR A: 115; THR A: 118; LYS A: 83; LYS A:79	-5.6	78.5	VAL C: 244; ARG C: 246; LYS C: 221; GLN C: 241; GLU D: 302; ASP D: 303; GLY D: 304; ARG D: 275	-5.8	56.0	THR B: 359; PHE B: 377; ASP B: 379; THR B: 357; GLU B: 336; ARG B: 289; ARG B: 257; ARG B: 234; VAL B: 316; ARG B: 264; TYR B: 292; TYR B: 451; LYS B: 477; HIS B: 426; ARG B: 355; PHE B: 377; HIS B: 334; TYR B: 403; THR B: 357; ARG B: 381; ARG B: 289; SER B: 381; SER B: 360; GLN B: 430
LPVFLAAEGNVVTYLH	3.1	7.5x10 ⁶		-6.0	39.9	ASN A: 368; GLN A: 370; TYR A: 115; LYS A: 369; TRP A: 100; ILE A: 112; LEU A: 108; LYS A: 79; ALA A: 111; PHE A: 107	3.5	3.7x10 ⁸		-7.1	6.3	
TPPGVGGFPWGGAMLGAQ	-5.8	56.1	ALA A:250; VAL A:251; ARG A:229; GLY A:249; ALA A:186; GLN A:193; SER B:160; SER A:196; PHE B:163	-9.9	0.1	SER B: 143; PHE B: 142; GLN A: 374; LEU A: 224; LEU A: 145; LEU B: 145; LEU B: 224; ASN B: 222; GLU B: 290; THR B: 237; ASN A: 144; TRP A: 139; SER A: 143; GLY B: 536; VAL B: 538; GLY B: 225; ASN B: 587; VAL B: 228	-6.8	10.4	GLN C: 241; GLU C: 222; GLN C: 220; ARG C: 246; LYS C: 221; ARG C: 246; GLY D: 246; GLY D: 304; ASP B: 303; ARG D: 275	-8.6	0.5	SER B: 183; ASP B:209; ARG B: 257; ARG B: 355; HIS B: 426; GLN B: 430

APSPVVLGPP	-6.0	39.9	SER B:160; LEU B:157; GLN A:193; LEU B:175; HIS A:151; ALA A:186; ASP A:189; SER A:196; GLY B:161	-8.7	0.4	LYS A: 79; THR A: 62; ARG A: 44; ALA B: 543; PRO B: 542; PHE B: 367; LYS A: 369; MET A: 471; HIS A: 122;	-5.8	56.1	GLY D: 304; LYS C: 221; GLU C: 222; GLN C: 241; PRO D: 301; ARG D: 275; LEU D: 282.	-7.6	2.7	SER B: 183; ASP B: 181; ASP B: 60; PHE B: 63; MET B: 41; HIS B: 159; VAL B: 132; ILE B: 168; THR B: 110
NPGGGAPSGH	-5.5	92.9	A: 147; PHE B: 190; GLN B: 193; PHE A: 158	-3.8	1638.9	PRO B: 160; PRO B: 162; LYS B: 459; ASP B: 157	-5.4	110.1	ARG D: 275; ASP C: 243; GLU D: 250; HIS C: 245; GLY D: 273; CYS D: 240	-4.6	424.8	ASP B: 181; HIS B: 179; THR B: 232; LEU B: 180
LHNGGPGGEY	-7.2	5.3	ILE B: 149; ALA B: 194; GLN B: 193; SER A: 160; HIS B: 151; ASP B: 189; GLN B: 192; SER B: 196; PHE A: 261; PHE A: 200	-4.3	704.8	LYS B: 33; PRO B: 160; LYS B: 169; CYS B: 159; THR B: 161; LYS B: 459	-8.1	1.2	LEU D: 282; PRO D: 301; GLY D: 279; ARG D: 275; ARG C: 246; VAL C: 244; GLN C: 247; HIS C: 245; GLN C: 241; LYS C: 221	-6.4	20.3	ALA B: 291; VAL B: 316; SER B: 207; LYS B: 230; THR B: 232; ARG B: 289; HIS B: 334; ARG B: 355; THR B: 357; VAL B: 338; THR B: 359; SER B: 360
DVNPGGPAPHPWLSVNDL	-5.3	130.3	GLN A: 193; LYS B: 171; ALA A: 250; ARG A: 248; GLY B: 161; SER A: 196	-4.6	424.8	PRO B: 160; LYS B: 22; LYS B: 168; LYS B: 169	-7.9	1.2	HIS C: 245; GLY D: 304; ARG C: 246; GLN C: 247; ASP D: 303; GLU D: 302; ARG D: 275; LEU D: 282; THR D: 276; LEU D: 283; GLN C: 241; GLU C: 225; ILE C: 224; GLU C: 222	-9.1	0.2	LEU B: 180; SER B: 207; ASP B: 181; PHE B: 263; TYR B: 292; ARG B: 264; LYS B: 230; LYS B: 130; THR B: 106; ARG B: 289; THR B: 357; ARG B: 355; HIS B: 426; TYR B: 403; GLU B: 287; HIS B: 334; THR B: 359; ARG B: 257
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Prolamin															
KPSCGRYPALSAN	-4.7	358.8	ARG A: 248; SER B: 162; PHE B: 261; SER A: 196; SER B: 160; GLN A: 193	-5.3	130.3	LYS A: 83; PRO A: 84; TRP A: 100; TYR A: 115; ALA A: 111; THR A: 118; GLN A: 370; ASN A: 368; LYS A: 369	-6	39.9	ARG C: 246; VAL C: 244; GLU D: 302; ASP D: 303; ARG D: 275; LYS C: 221; PRO D: 285; PRO C: 275; GLN C: 220	-6.8	10.3	THR B: 232; LYS B: 230; ARG B: 234; ARG B: 257; ARG B: 289; GLU B: 336; SER B: 312; THR B: 359; ASP B: 379; TYR B: 403; SER B: 317; ARG B: 264 ASP B: 209; THR B: 232; LEU B: 208; HIS B: 179; GLU B: 154			
VSGGAAPPR	-6.4	20.4	GLN B: 193; SER B: 196; ARG B: 248; PHE B: 190; PHE A: 158; SER A: 160	-5.9	47.3	LYS A: 369; TYR A: 115; LYS A: 79; PRO A: 84; LYS A: 83; ILE A: 89 ARG B: 216; ALA A: 239; LEU A: 238; SER B: 146; LEU B: 224; LEU B: 145; ASN B: 144; ASP B: 229; LEU A: 145; TRP A: 139; SER A: 143; GLY B: 227; ASN B: 375; GLN B: 374; ARG B: 376; GLU A: 140	-6.1	33.7	ARG C: 246; ASP D: 303; ARG D: 275; LYS C: 221; PRO D: 285	-6.6	14.5	LYS B: 153; GLU B: 178; ASN B: 205; HIS B: 229; ARG B: 257; ARG B: 289; THR B: 359; VAL B: 338; VAL B: 316; SER B: 360			
HDPGPLQRDPTPHW	-5.3	130.3	ASP B: 189; HIS B: 151; PHE B: 190; ALA B: 194; GLN B: 193; ARG B: 248	-9.6	0.1	GLN B: 170; LYS B: 169; LYS B: 33; PRO B: 160; PRO B: 162; CYS B: 159; LYS B: 459	-7.2	5.2	PRO D: 285; LEU D: 283; GLN C: 241; GLU C: 222; LYS C: 221; GLY D: 304; GLN C: 247; ARG D: 275; ARG C: 246; HIS C: 245; PHE C: 239; ARG C: 236; SER C: 238 ARG C: 246; ARG D: 275; GLY D: 273; ASP D: 303; THR D: 276; LYS C: 221; LEU D: 282; GLN C: 241; PRO D: 285; ARG C: 236; PRO C: 275	-6.0	39.9	TYR B: 292; ARG B: 264; PHE B: 263; ARG B: 234; THR B: 232; ASP B: 209; VAL B: 316; GLU B: 336; VAL B: 338; HIS B: 334; ARG B: 257; LYS B: 230; ASP B: 181 HIS B: 179; ASP B: 209; ASP B: 181; PHE B: 263; THR B: 232; ARG B: 234; ARG B: 289; LYS B: 230; VAL B: 132; SER B: 86			
DPHGPVFAANPK	-6.2	28.5	SER A: 262; CYS A: 264; GLY A: 161; SER A: 160; ASP A: 147; TYR B: 197; GLN B: 193; HIS B: 151; SER B: 196; ARG B: 248	-5.0	216.2	SER A: 196; GLY B: 161; SER B: 162; LEU A: 188; ASP A: 189; ALA A: 186	-4.5	502.8	CYS B: 159; PRO B: 162; PRO B: 160; LYS B: 169; GLN B: 170	-7.6	2.3				
LEEPGLGTSFLY	-6.6	14.5													
Glutelin															

KKLELTEYLYEDKK	-3.3	3811.2	ARG A: 248; HIS A: 151; GLY A: 152	-6.3	24.1	LYS A: 83; TYR A: 115; LYS A: 369; ILE A: 112; LYS A: 79; ASN A: 368	-4.2	834.4	GLN C: 222; LYS C: 221; GLY D: 279; ARG D: 275; GLY D: 304; ARG D: 270	-6.7	12.4	ARG B: 264; ARG B: 234; PHE B: 263; ARG B: 355; ARG B: 289; THR B: 357; ARG B: 382; SER B: 381; GLU B: 336; GLU B: 287; LYS B: 230; THR B: 232
NSPGPHDVALDQ	-6.0	39.9	SER A: 196; ARG A: 248; SER A: 199; ASP B: 147; TYR A: 197; ALA A: 194; GLN A: 193; LEU B: 157; GLN A: 192; CYS B: 264; LYS B: 171	0.2	1401		-6.9	8.7	ARG D: 275; ARG C: 246; HIS C: 245; GLY D: 273; GLU C: 222; LEU D: 282; LEU D: 283; GLN C: 241; PRO D: 285	-7.0	7.4	GLN B: 430; TYR B: 403; ARG B: 382; THR B: 359; PHE B: 377; VAL B: 338; GLU B: 336; ARG B: 289; ARG B: 257; LYS B: 230; THR B: 232; HIS : 179; LEU B: 155
KDSGHPNGGLFAESSP	-5.2	154.3	GLY B: 161; SER B: 162; SER A: 198; ARG A: 248; GLN A: 192; SER B: 160; SER B: 159; GLN A: 193; ASP B: 147; HIS A: 151	-5.7	66.3	LYS A: 369; GLN A: 370; SER A: 119; ILE A: 112; TRP A: 100; PHE A: 99; VAL A: 103; ILE A: 78; LEU A: 75; PRO A: 84; LYS A: 79; TYR A: 115; HIS A: 122; THR A: 118	-5.7	66.3	ARG C: 246; ARG D: 275; GLU D: 302; GLY D: 304; GLY D: 273; LYS C: 221; LEU D: 283; GLN C: 241; PRO D: 285; ARG D: 284; ILE C: 224	-7.6	2.7	LYS B: 130; LYS B: 230; ARG B: 257; ARG B: 289; ARG B: 355; TYR B: 403; HIS B: 334; PHE B: 377; VAL B: 316; THR B: 359; ARG B: 382; SER B: 360
TGPCNGAAPPAAGSV DCL	-5.1	182.6	ARG A: 248; SER A: 199; PHE B: 200; GLY A: 249; SER B: 160; SER B: 159; PHE B: 163; LEU B: 175; LEU B: 157; GLN A: 193; ASP A: 189	1.2	7.5x10 ⁶	0	-5.7	66.3	ARG C: 246; ARG D: 275; GLY D: 304; LYS C: 221; GLU D: 302; GLN C: 220; SER C: 276; PRO D: 285; GLN C: 241	-6.9	8.7	ARG B: 234; SER B: 211; SER B: 183; ASN B: 156; HIS B: 179; LYS B: 230; ARG B: 289; VAL B: 338; THR B: 359; ARG B: 382; GLU B: 336; PHE B: 377
QAGAGGPGAAGH	-6.3	24.1	ARG A: 248; SER A: 196; SER B: 160; GLY B: 161; SER B: 159; GLN A: 193; TYR A: 197; ASP B: 147	-5.9	47.3	ASN A: 368; GLN A: 370; TYR A: 118; SER A: 119; LYS A: 79; LYS A: 83	-5.9	47.3	LYS C: 221; GLN C: 241; LEU D: 283; GLU C: 222; ARG C: 246; ARG D: 275; GLY D: 274; ARG D: 270	-6.6	14.5	GLU B: 154; HIS B: 179; THR B: 232; ASP B: 209; SER B: 183; SER B: 184; PHE B: 263; THR B: 110; GLN B: 39
TPPNGLGSPGSPGPHAQ	-5.8	56.0	LEU A: 188; SER A: 199; ARG A: 248; SER B: 159; SER B: 160; SER A: 196; PHE B: 261; GLN A: 192	-6.4	20.3	TYR A: 115; LYS A: 83; LYS A: 79; TYR A: 115; GLN A: 370; LYS A: 369; ASN A: 368; ILE A: 112; ALA A: 111; ASN B: 368; THR A: 118	-6.3	24.1	LEU D: 282; PRO D: 301; LEU D: 283; ARG D: 284; PRO D: 285; GLN C: 241; PRO D: 287; ASP D: 303; GLY D: 304; ARG D: 275; ARG C: 246	-7.3	4.4	HIS B: 229; ASP B: 209; VAL B: 259; SER B: 207; ARG B: 289; HIS B: 179; THR B: 359; GLU B: 336; HIS B: 334; THR B: 357; TYR B: 403; ASP B: 405; SER B: 407; ARG B: 382

RMVLPPEYELLYE	-6.7	12.2	ARG A: 248; PHE B: 200; SER B: 162; PHE B: 261; SER A: 196; ASP A: 189; SER B: 160; LEU B: 175; LEU B: 157; TRP B: 148; ASP B: 147; ASP A: 187; HIS A: 151; TYR A: 156; ASN A: 154	-6.9	8.7	LYS B: 369; GLN B: 370; LYS A: 369; SER A: 114; ALA A: 111; THR A: 118; LEU A: 108; SER A: 119; TYR A: 115; PRO A: 84; VAL A: 89; TRP A: 100; ILE A: 92; PHE A: 99; VAL A: 103; ILE A: 112.	-6.4	20.3	ARG D: 275; LYS C: 221; GLN C: 241; GLU C: 222; ARG C: 246	-8.0	1.3	VAL B: 475; TYR B: 451; SER B: 407; ARG B: 382; TYR B: 403; ARG B: 355; ASP B: 379; THR B: 357; THR B: 359; VAL B: 316; ARG B: 289; SER B: 314; SER B: 312
RLTGTGMGAASSVLP	-5.8	56.1	SER A: 196; ARG A: 248; TYR A: 252; ARG A: 231; ASP A: 189; GLN A: 193; PHE B: 163; LYS B: 171; SER B: 160; CYS B: 264; LEU B: 157	-6.3	24.1	GLN A: 370; THR A: 118; LYS A: 369; ASN A: 368; SER A: 114; TYR A: 114; ILE A: 112; ILE A: 92; PRO A: 84	-5.9	47.3	GLN C: 247; ARG C: 246; ARG D: 275; LEU D: 282; ARG D: 284; PRO D: 285; PRO C: 275	-7.4	3.7	LYS B: 230; THR B: 232; VAL B: 259; ALA B: 291; HIS B: 334; ASP B: 379; TYR B: 403; THR B: 359; SER B: 360; THR B: 357; ARG B: 382; ARG B: 234; ARG B: 289; TYR B: 292; ARG B: 264; VAL B: 338; GLU B: 336; THR B: 357; ARG B: 355; PHE B: 377; TYR B: 403; TYR B: 451; HIS B: 426; ARG B: 289; GLN B: 430; ARG B: 234; ASP B: 405; LEU B: 180; HIS B: 179; SER B: 207; LYS B: 230; THR B: 232; SER B: 183; HIS B: 179; ASP B: 181; ASP B: 209; SER B: 211; ARG B: 234; ARG B: 289; GLU B: 336; HIS B: 334; TYR B: 403; GLN B: 430; ASP B: 453; LYS B: 477; TYR B: 451
RHMDLPLSPSSAGP	-6.8	10.4	LYS B: 171; SER B: 160; PHE B: 158; GLN A: 193; ALA A: 194; ASP B: 147; TYR A: 197; ARG A: 248; GLN A: 192; ARG A: 231; ALA A: 250; LEU A: 188	-6.1	33.8	PHE A: 107; LYS A: 369; GLN A: 370; HIS A: 122; THR A: 118; TYR A: 115; LYS A: 79; PRO A: 84	-5.6	78.5	ARG D: 284; LEU D: 283; GLN C: 241; GLY D: 304; GLU C: 222; LYS C: 221; ARG D: 275	-7.2	5.3	THR B: 232; SER B: 183; HIS B: 179; ASP B: 181; ASP B: 209; SER B: 211; ARG B: 234; ARG B: 289; GLU B: 336; HIS B: 334; TYR B: 403; GLN B: 430; ASP B: 453; LYS B: 477; TYR B: 451
TTNPGVDADFPAREL	-5.7	66.3	LEU B: 175; LEU B: 157; ASP B: 147; SER B: 160; ARG A: 248; ASP A: 189; LEU A: 188; GLN A: 193	0.1	1.2x10 ⁶	PHE A: 96; TYR A: 115; ILE A: 112; ALA A: 111; ASN A: 368;	-5.7	66.3	GLN C: 241; LEU D: 283; LYS C: 221; ASP D: 303; VAL C: 244; HIS C: 245; PRO D: 301; ARG C: 246; ARG D: 275	-7.2	5.3	THR B: 232; LYS B: 230; ARG B: 234; ARG B: 264; SER B: 317; VAL B: 338; ASP B: 379; SER B: 381; ARG B: 382; THR B: 359; SER B: 360; GLU B: 336; ARG B: 355; ASP B: 405
QPADPNQFVYAPDTH	-5.3	130.3	SER B: 160; GLN A: 193; HIS A: 151; ARG A: 248; GLN A: 192; SER A: 196; PHE B: 200	-7.1	6.2	HIS A: 122; THR A: 118; GLN A: 370; SER A: 114; LYS A: 369; PHE A: 107; ALA A: 111; TYR A: 115; TRP A: 100; ILE A: 92	-4.7	358.8	PRO D: 285; ARG D: 284; LEU D: 282; GLN C: 241; LEU D: 283; GLY D: 304; ARG C: 246; ARG D: 275	-7.9	1.6	THR B: 232; LYS B: 230; ARG B: 234; ARG B: 264; SER B: 317; VAL B: 338; ASP B: 379; SER B: 381; ARG B: 382; THR B: 359; SER B: 360; GLU B: 336; ARG B: 355; ASP B: 405
FAFFFEFFELLFAFFT	-5.9	47.3	GLU A: 153; ASN A: 154; HIS A: 151;	-7.7	2.3	GLN B: 54; ASP B: 53; LYS B: 33	-4.7	358.8	LEU D: 282; LEU D: 283; PRO D: 285;	-9.7	0.1	THR B: 37; ASN B: 58; VAL B: 32; VAL B: 82;

			ALA A: 186; ARG A: 248; SER B: 199					PRO D: 287; LYS C: 221; ARG C: 246			VAL B: 132; ASP B: 209; VAL B: 316; ARG B: 234; ARG B: 264; PHE B: 263; LYS B: 362 HIS B: 179; LEU B: 155; GLU B: 154; ARG B: 234; ASP B: 209; ARG B: 289; VAL B: 316; ALA B: 291; SER B: 317; GLU B: 336; THR B: 357; TYR B: 403; ASP B: 405; ARG B: 355	
TDQLGWFLGAAAFAGQ	-4.4	595.3	LEU A: 188; GLN A: 193; HIS A: 151; PHE A: 190; SER B: 160; ASP B: 147; LYS B: 171	-3.1	5341.6	LYS B: 168; LYS B: 169; PRO B: 162; LYS B: 459	-6.1	33.8	THR D: 239; HIS C: 245; GLN C: 247; VAL C: 244; ALA C: 242; LYS C: 221; ARG C: 246; ARG D: 275; GLY D: 304; LEU D: 283; ASP D: 303	-6.9	8.7	ARG B: 234; THR B: 232; ARG B: 257; ARG B: 289; ARG B: 335; GLU B: 336; THR B: 357; ARG B: 382; SER B: 381; ASP B: 379; THR B: 359; SER B: 207; VAL B: 316; VAL B: 338
RGSSPAPSGSELVL	-5.5	93.0	HIS B: 151; ALA B: 186; GLN A: 146; TYR B: 197; GLN B: 193; SER A: 160; GLN B: 192; SER B: 196; ARG B: 248	-4.3	704.7	LYS B: 459; ASN B: 34; LYS B: 33; PHE B: 52; LYS B: 169	-6.5	17.2	ARG D: 270; VAL C: 244; ALA C: 242; ARG D: 275; ARG D: 284; ARG C: 246; LYS C: 221; GLN C: 241; PRO D: 285; PHE C: 239; GLU C: 222	-6.7	12.3	ASP B: 405; ARG B: 382; THR B: 359; TYR B: 403; ASP B: 379; VAL B: 338; HIS B: 334; ARG B: 234; ARG B: 289; VAL B: 316; ARG B: 257
TDWYDPGAFQ	-7.1	6.2	SER A: 160; GLN B: 193; ASP A: 147; LEU A: 157; LEU A: 175; TYR B: 197	-5	216.3	ASP B: 157; PRO B: 162; LYS B: 169; PRO B: 160	-7.8	1.9	CYS D: 240; VAL C: 251; MET D: 271; THR D: 239; HIS C: 245; ARG C: 246; LYS C: 221; LEU D: 283; ASP D: 303	-8.1	1.1	SER B: 207; HIS B: 179; LYS B: 230; ARG B: 234; THR B: 232; ARG B: 289; VAL B: 316; VAL B: 338; SER B: 381; ARG B: 382; ASP B: 379; SER B: 360 VAL B: 338; VAL B: 316; ARG B: 234; LYS B: 230; THR B: 232; HIS B: 179; VAL B: 132
HAPGFLSPAHR	-6.2	28.5	SER B: 159; ASP B: 147; GLN A: 193; SER B: 160; PHE B: 200	-4.4	595.3	LYS B: 33; LYS B: 169	-7.7	2.3	LEU C: 215; HIS C: 245; CYS D: 240; ARG C: 246; MET D: 271; LYS C: 221; ARG D: 275; GLU D: 302	-7.6	2.6	
LPGPPATF	-6.2	28.5	ASP A: 147; ALA B: 194; GLN B: 192; ARG B: 248; PHE A: 200	-4.4	595.3	LYS B: 169; LYS B: 33; PRO B: 160; ASP B: 157; PRO B: 162	-8.2	1.0	CYS D: 240; ASP C: 243; HIS C: 245; ARG C: 246; GLY D: 279; ARG D: 275	-6.8	10.4	

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. The most potent interaction between peptides and receptor is in bold.

6.3 Manuscript 3

RESEARCH ARTICLE

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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

Mariana Grancieri, Hercia Stampini Duarte Martino, and Elvira Gonzalez de Mejia*

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 ($1 \mu\text{g mL}^{-1}$) for inflammation or ox-LDL ($80 \mu\text{g mL}^{-1}$) for atherosclerosis. In the inflammatory process, DTP and DPF reduce p-NF- κ B, 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- κ B 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- κ B (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.

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- κ B), 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 fis-

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

ABSTRACT

Scope: The objectives were 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) were isolated from chia seed and digested using simulated gastrointestinal conditions, resulting in digested total protein (DTP) and digested protein fractions (DPF). DTP and DPF were applied (1.0 mg/ml) in RAW 264.4 macrophages stimulated with LPS (1 µg/ml) for inflammation or ox-LDL (80 µg/ml) for atherosclerosis. In the inflammatory process, DTP and DPF reduced p-NF-κB, iNOS, p-JNK, and AP-1. Digested glutelin reduced 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 reduced the NF-κB translocation to nuclei. DTP and digested glutelin reduced 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 was 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 were characterized.

Conclusion: DTP and digested glutelin from chia seed reduced 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 multi-system 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- κ B), 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 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 (*Salvia 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. Materials and Methods

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), c-JUN (c-Jun N-terminal kinase, sc-74543), p-JNK (sc-6254), JNK (sc-7345), MMP-9 (matrix metalloproteinase 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'-dichlorofluorescein 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 described 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 / 35 mesh). The free-mucilage seeds were ground using a coffee grinder (Mr. Coffee®), sieved (500 μm / 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/1 h). The mixture was centrifuged (5000 g; 15 min; 25 °C) and the supernatant collected, freeze-dried and stored at -20 °C (**Supplementary Figure 1**).

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 resuspended with 0.05 mol/L Tris-HCl + 0.5 mol/L 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 $\text{Na}_2\text{B}_4\text{O}_7\text{-H}_2\text{O}$ (pH 10.0) (1:10 g: ml), processed as above, and the supernatant was then named as glutelin fraction (**Supplementary Figure 1**). 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: A: 95% of acetonitrile, 5% of water, and 0.1% of formic acid. The volume of injection was 200 μ L/min 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% CO₂/95% air using a CO₂ 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 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) 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 to 24 h at 37 °C in 5% CO₂/95%. After incubation, the cells were treated with the lipopolysaccharide (LPS) (1.0 μM) and either DTP or each DPF to 24 h.

For the analysis of anti- atherosclerosis potential, the RAW 264.7 macrophages were seeded at 2 x 10⁵ 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% CO₂/95%, followed by another 24 h starve. The cells were treated with oxidized low-density lipoprotein (ox-LDL, 80 μg/mL) 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 (NO₂), 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 NO₂⁻ 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 / 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-alpha, 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 ($y = 0.7681x - 2.4798$, $R^2 = 0.99$).

=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.

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 $-80\text{ }^{\circ}\text{C}$. 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- κ B, p65-NF- κ B, p-JNK, JNK, c-JUN, LOX-1, MMP-9 or ICAM-1) at $4\text{ }^{\circ}\text{C}$ 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). Band intensity was normalized using GAPDH antibody. All analysis were performed at least in duplicate and the results expressed in % of expression compared to a positive control.

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

Immunofluorescence and confocal laser-scanning microscopy were used to evaluate the nuclear translocation of NF- κ B 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 8-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- κ B 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 \times /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. Results were expressed in % of expression compared to a positive control.

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 3 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 *Salvia 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 the mean \pm standard deviation (SD) and analysed 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.

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 of DTP and DPF, promoted cell viability similarly as in cells without treatment (negative control-NC) (**Supplementary Figure 2A**), averaging more than 100% for both. The nitric oxide (NO) excretion was analyzed and for DTP, digested albumin, globulin and glutelin, the 1.0 mg/ml of digested protein was the best dose that reduced this inflammation marker (**Supplementary Figure 2B**). 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, showed cell viability similar or higher than NC and was used in the following experiments (**Supplementary Figure 3**). **Supplementary Table 1** 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- κ B Pathway, Reduced its Translocation to Nuclei and Reduced the Secretion of Inflammation-Mediators

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

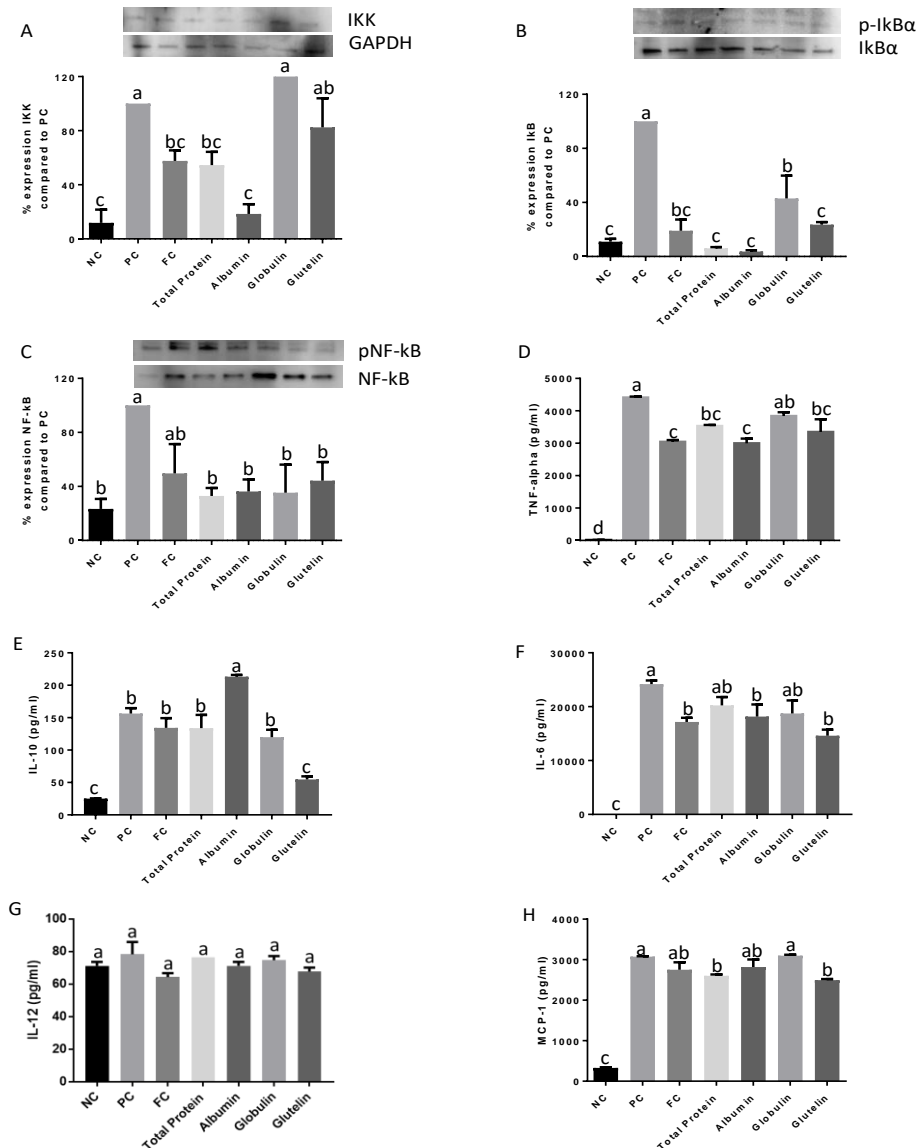


Figure 1. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of NF- κ B (A) by Western blotting, and secretion of TNF- α (B), IL-10 (C), IL-6 (D), IL-12 (E) and MCP-1 (F) by ELISA analysis, of LPS-stimulated RAW 264.7 macrophages. The net intensity 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

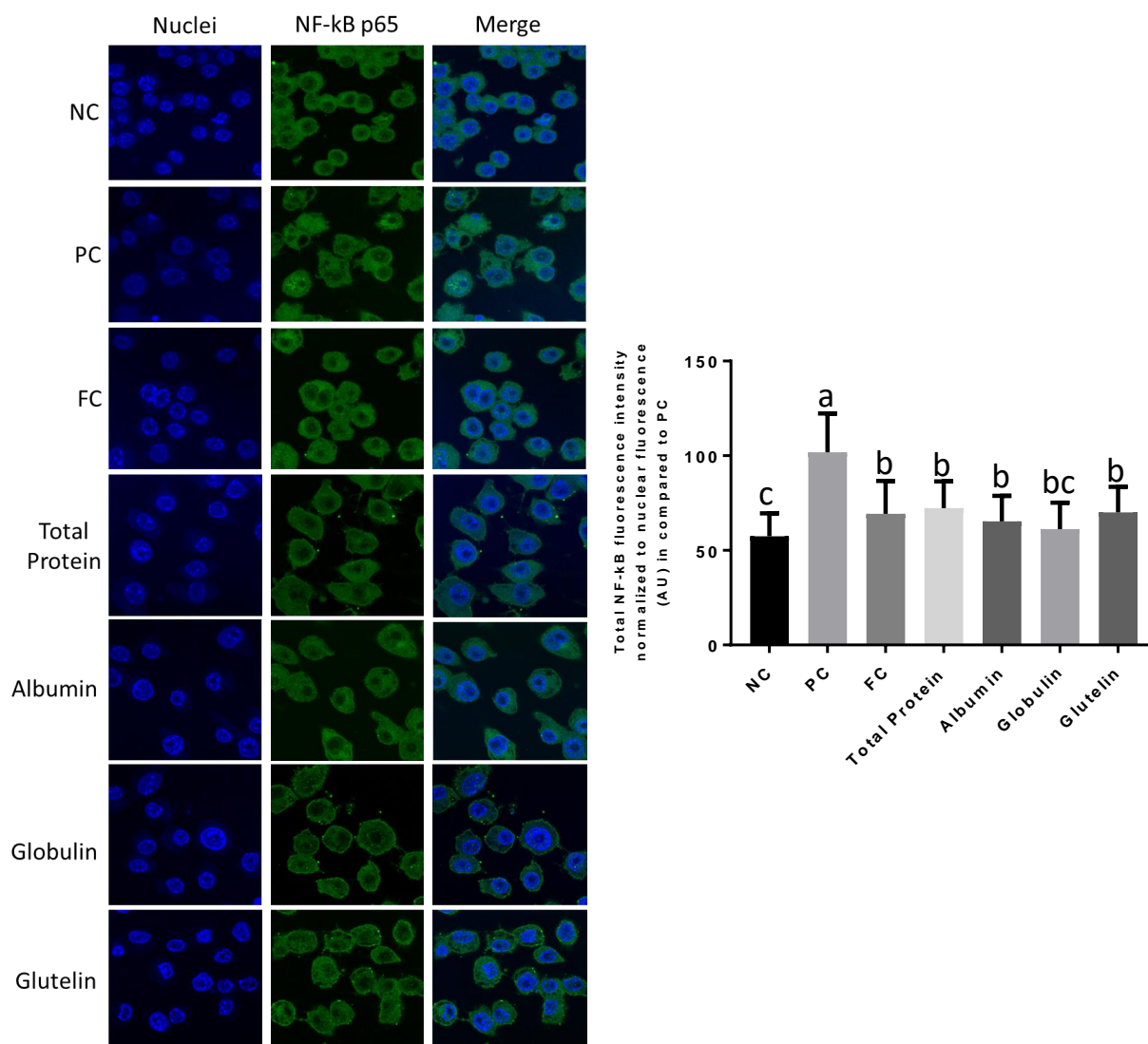


Figure 2. Confocal laser scanning microscopy depicting two-dimensional immunocytochemical localization of NF- κ B (green) normalized by nuclei (blue) in RAW264.7 macrophages after 24 h of treatment with digested total protein and digested albumin, globulin, and glutelin from chia seeds. Ten independent 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

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 3A**). All digested proteins inhibited NO secretion, especially digested glutelin, and ROS production (**Figure 3B and 3C**).

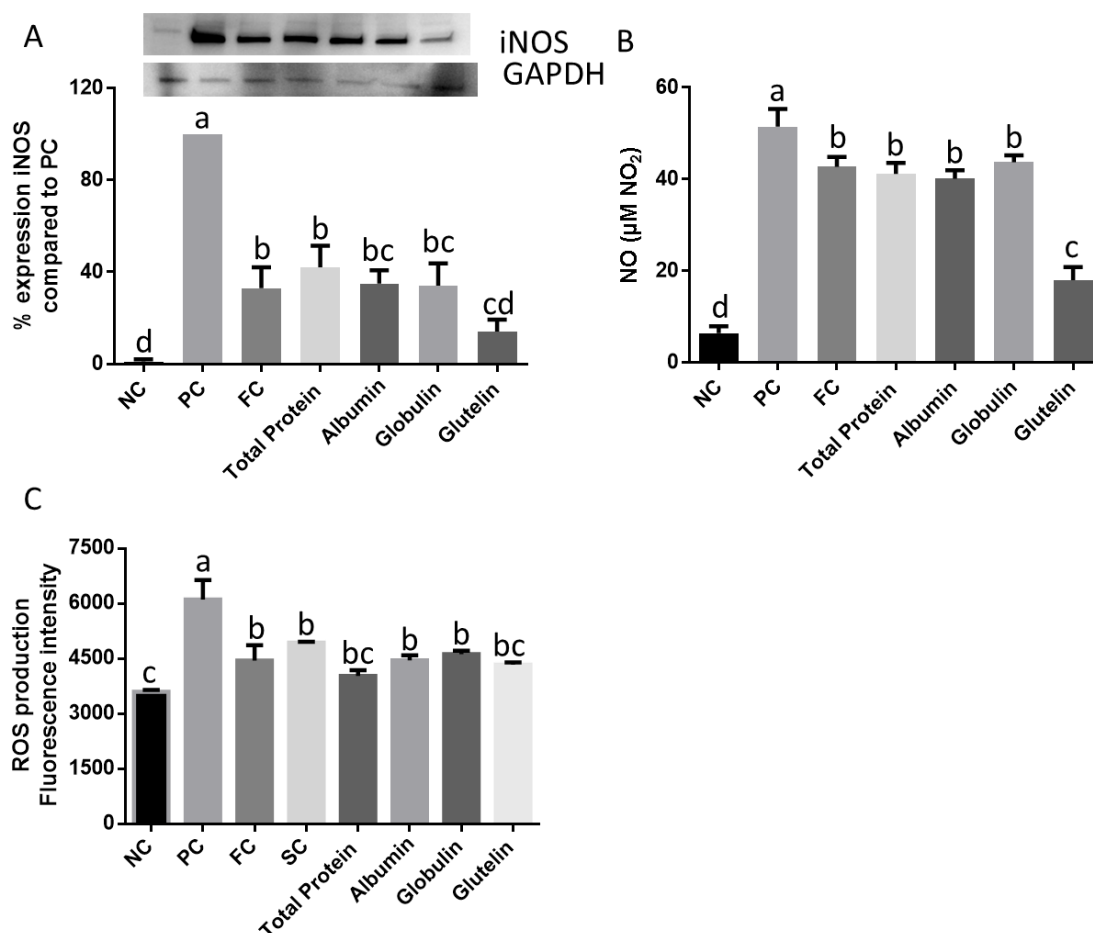


Figure 3. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of iNOS (A) by western blotting, secretion of NO (B), and ROS production (C) of LPS-stimulated RAW 264.7 macrophages. The net intensity of markers was normalized by net intensity of GAPDH (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 PGE2

DTP and DPF reduced the expression of phosphorylated JNK (**Figure 4A**) 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**).

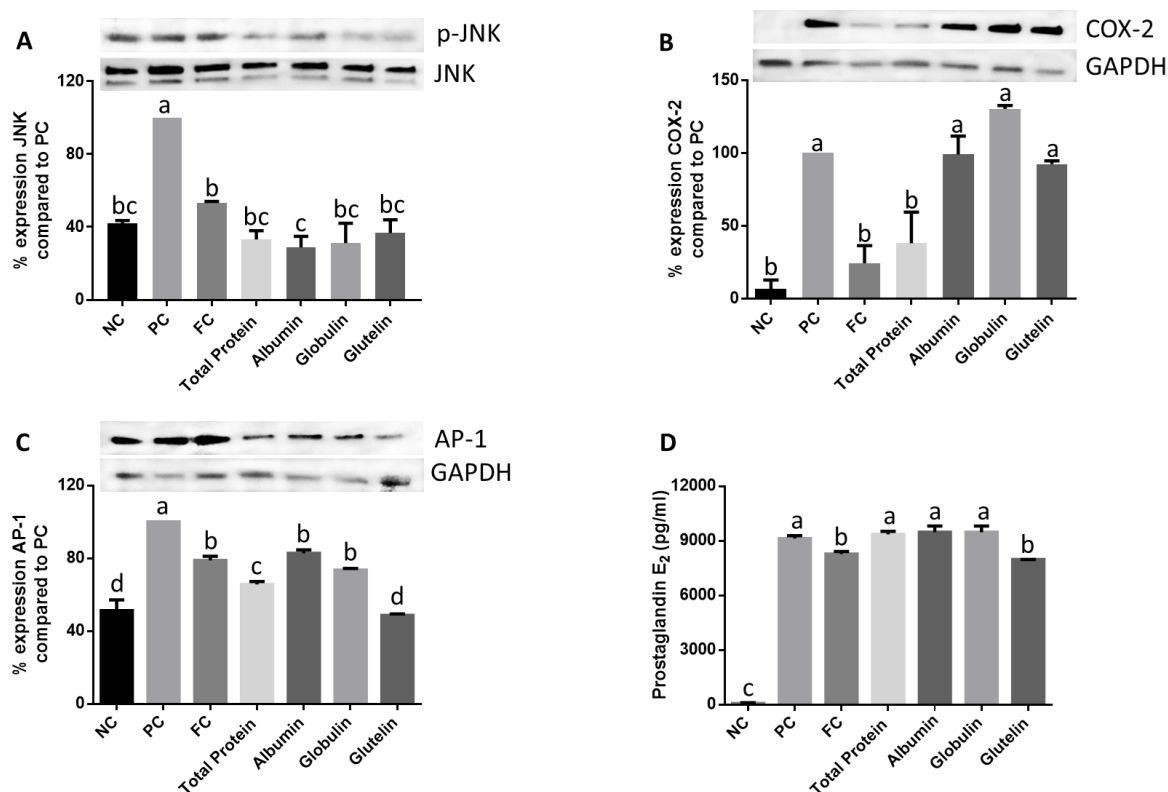


Figure 4. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of p-JNK (**A**), COX-2 (**B**), and AP-1 (**C**) by western blotting, and secretion of PGE₂ (**D**) by ELISA analysis of LPS-stimulated RAW 264.7 macrophages. The net intensity of markers 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

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 5A**) 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**).

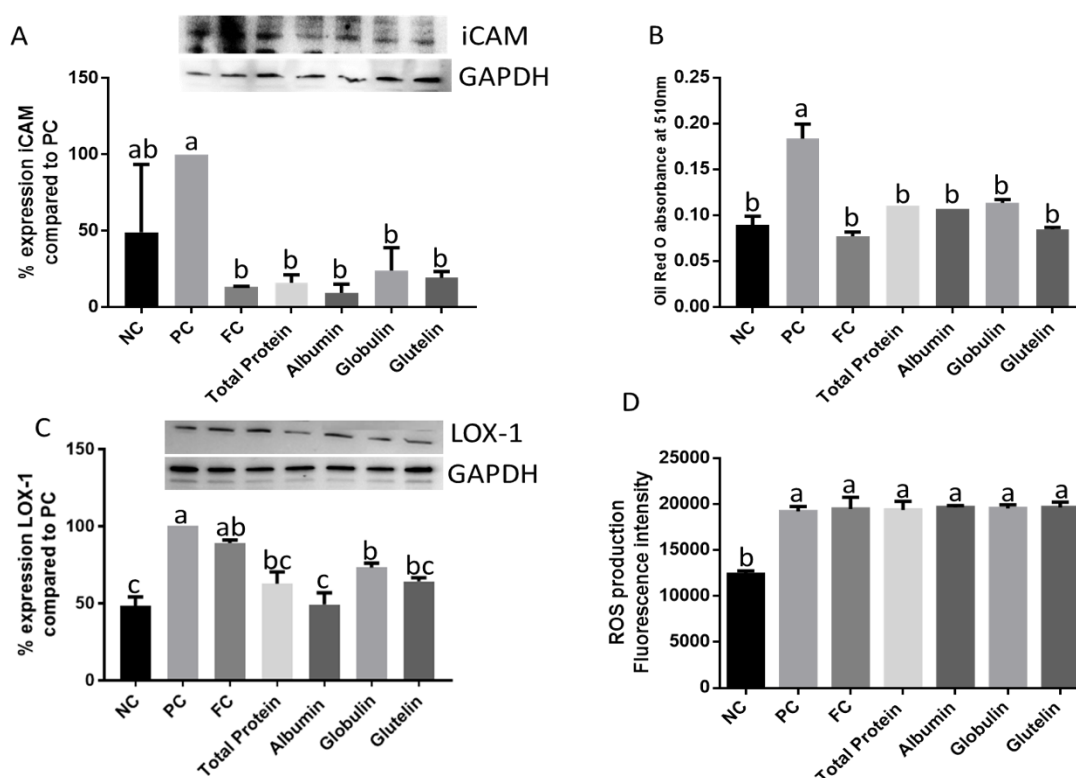


Figure 5. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of iCAM (A) by western blotting, lipids accumulation by Oil-Red O (B), LOX-1 (C) by western blotting, and ROS production (D) in ox-LDL stimulated RAW 264.7 macrophages. The net intensity 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

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- κ B (**Figure 6A**) 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**).

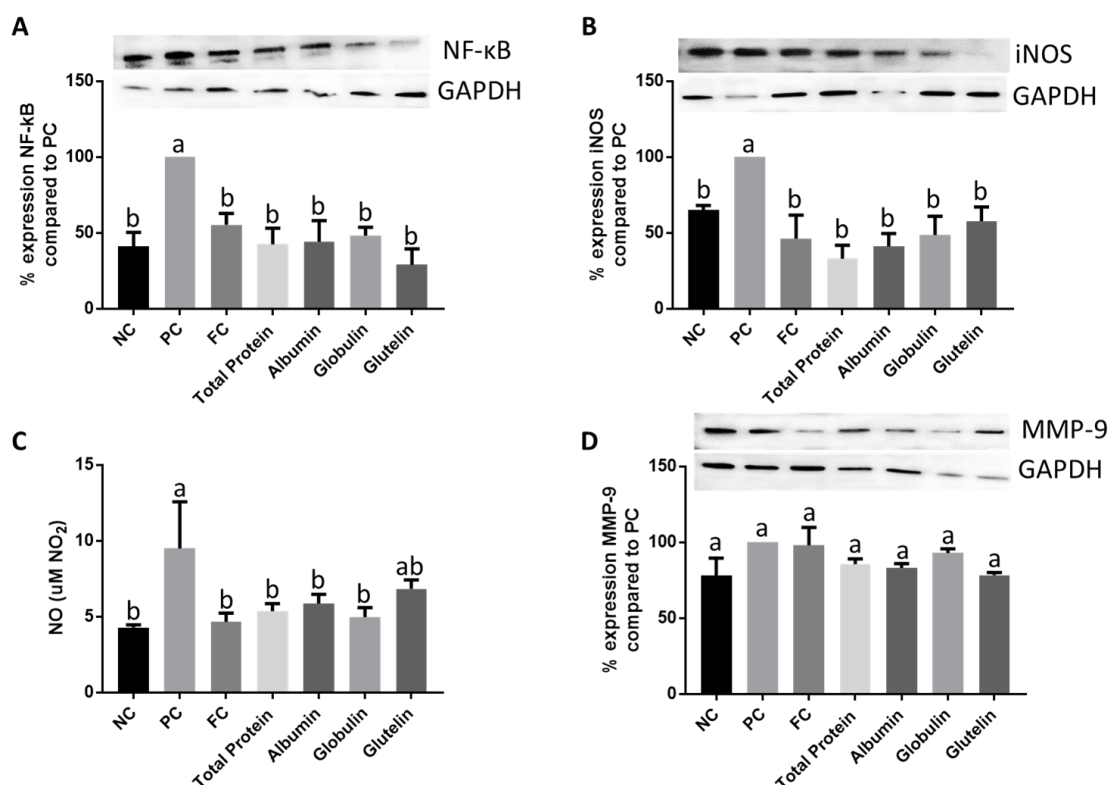


Figure 6. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on expression of NF- κ B (**A**), iNOS (**B**), NO secretion (**C**) by ox-LDL stimulated RAW 264.7 macrophages and MMP-9 (**D**) by western blotting. The net intensity of markers (A, B and D) was 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

Associated with this, all digested proteins reduced TNF- α secretion (**Figure 7A**), glutelin having the lowest value. DTP and digested glutelin had the lowest values compared to PC in PGE2 and MCP-1 secretion (**Figures 7B and 7C**, 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 (**Figures 7D and 7E**, respectively). However, the secretion of IL-6 was reduced in all samples, showing results similar to NC ($p > 0.05$, **Figure 7F**).

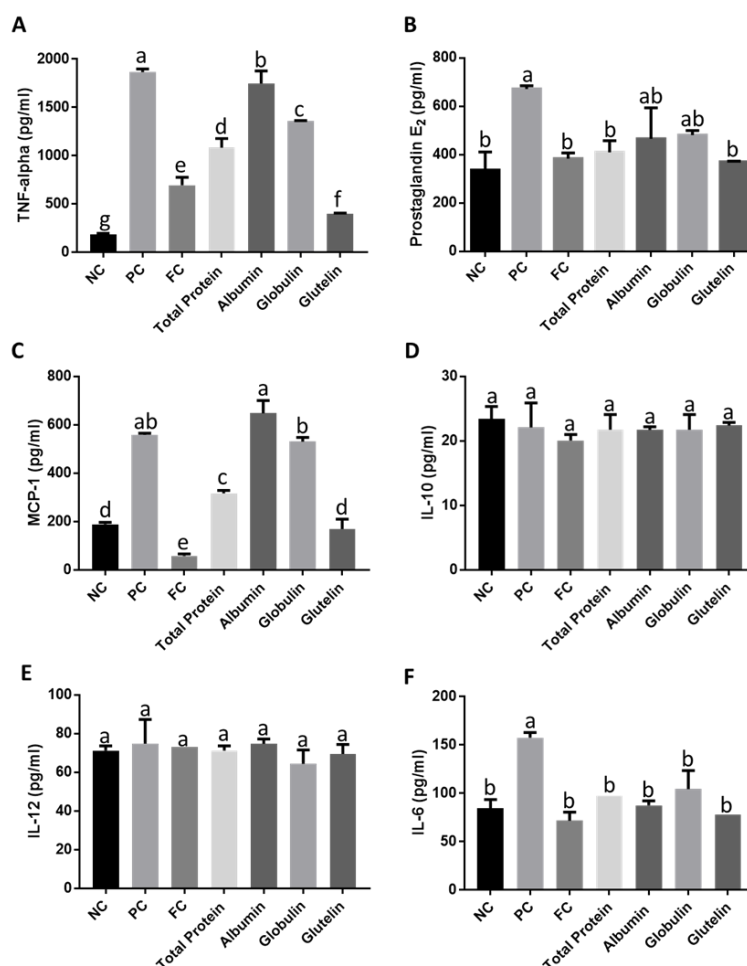


Figure 7. Effect of digested total protein and digested albumin, globulin, and glutelin from chia seeds on secretion TNF- α (**A**), PGE₂ (**B**), MCP-1 (**C**), IL-10 (**D**), IL-12 (**E**), and IL-6 (**F**) by ELISA analysis by ox-LDL stimulated RAW 264.7 macrophages. All experiments were performed 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 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

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 **Supplementary Table 1**. 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) (**Figure 8A**). This interaction was strongest in comparison with simvastatin (EFE, -4.8 kcal/mol). Peptide LPGPPATF from digested albumin and glutelin had the highest interaction with CCR2 (EFE: -8.1 kcal/mol) (**Figure 8B**); however, the pharmacological control, simvastatin, showed slightly better interaction value than this peptide (EFE: -8.4 kcal/mol).

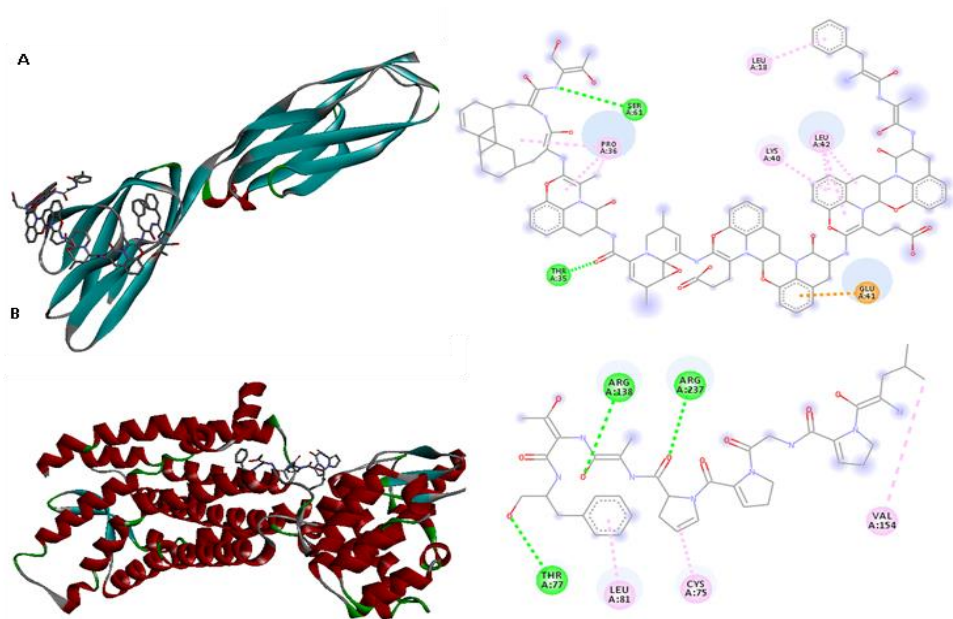


Figure 8. The *in silico* interaction of the peptide FAFFEFFELLFAFFT, found in digested glutelin, with ICAM (A) and peptide LPGPPATF, found in the digested albumin with CCR2 (B). These peptides showed the most potent interaction, by lower estimated free energies (EFE), analyzed by AutoDock Vina® and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

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 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 CD14 and 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- κ B). The activation of IKK promotes the phosphorylation and consequent degradation of NF- κ B transcription factor inhibitor (IKB- α), allowing translocation of NF- κ B from the cytosol to the nucleus. ^[34,35] In this study, we observed a block of this pathway, confirmed by reduction of NF- κ B activation, as well of its translocation to nuclei. Digested globulin and glutelin showed effect as confirmed by the reduction of NF- κ B activation, and its translocation to nuclei was reduced. These results are similar to results with amaranth hydrolysates which reduced the expression of p65 NF- κ B 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- κ B 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- κ B 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 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- κ B 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- κ B, 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 PGE₂, prostacyclin, and thromboxane A₂. ^[48] Despite NF- κ B and AP-1 reduced their expression by digested samples, only DTP was effective in reducing COX-2 expression, while digested glutelin decreased PGE₂ secretion. Other proteins related with these 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 residues per molecule, because these small peptides have a better ability to crossing the intestinal barrier and exert their biological effects. ^[53] Finally, peptides with hydrophobicity ≤ 20 kcal/mol 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 I κ B α -degradation, which allows the activation and translocation of NF- κ B. ^[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 others 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-1 β , and IL-12, and active

the enzymes iNOS and COX-2 to produce NO and PGE2, 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- κ B and iNOS, and the secretion of TNF- α , PGE2, 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- κ B 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 pathogenesises, 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.

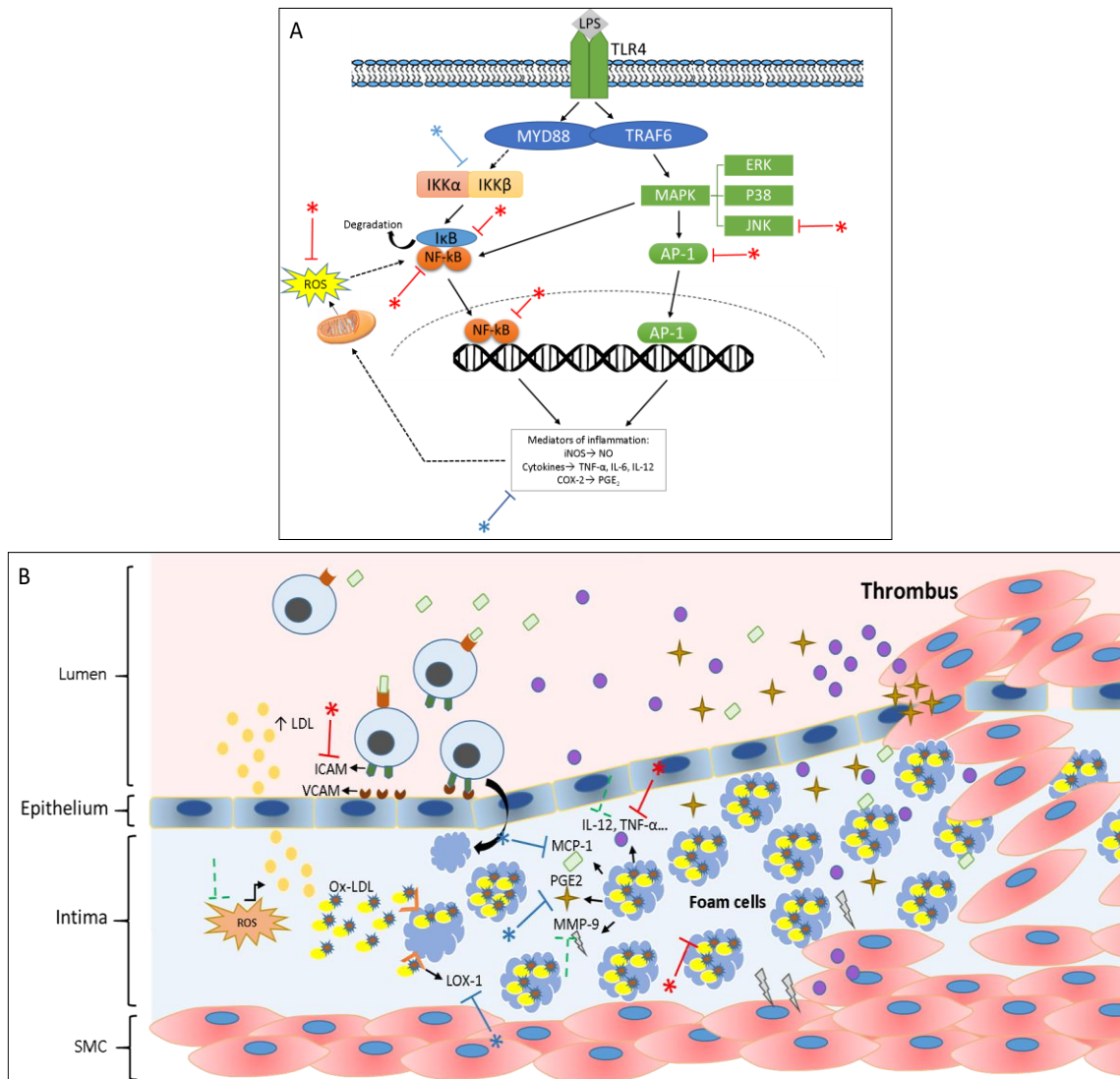


Figure 9. The proposal effects of digested total protein and digested albumin, globulin, and glutelin from chia seeds on **(A)** inflammation pathway and **(B)** atherosclerosis processes. The **red** symbols mean that every sample had effect on marker. The **blue** symbols mean that some samples had 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.

Supporting Information

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

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Conflict of interest

The authors have no conflict of interest

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Supplementary material

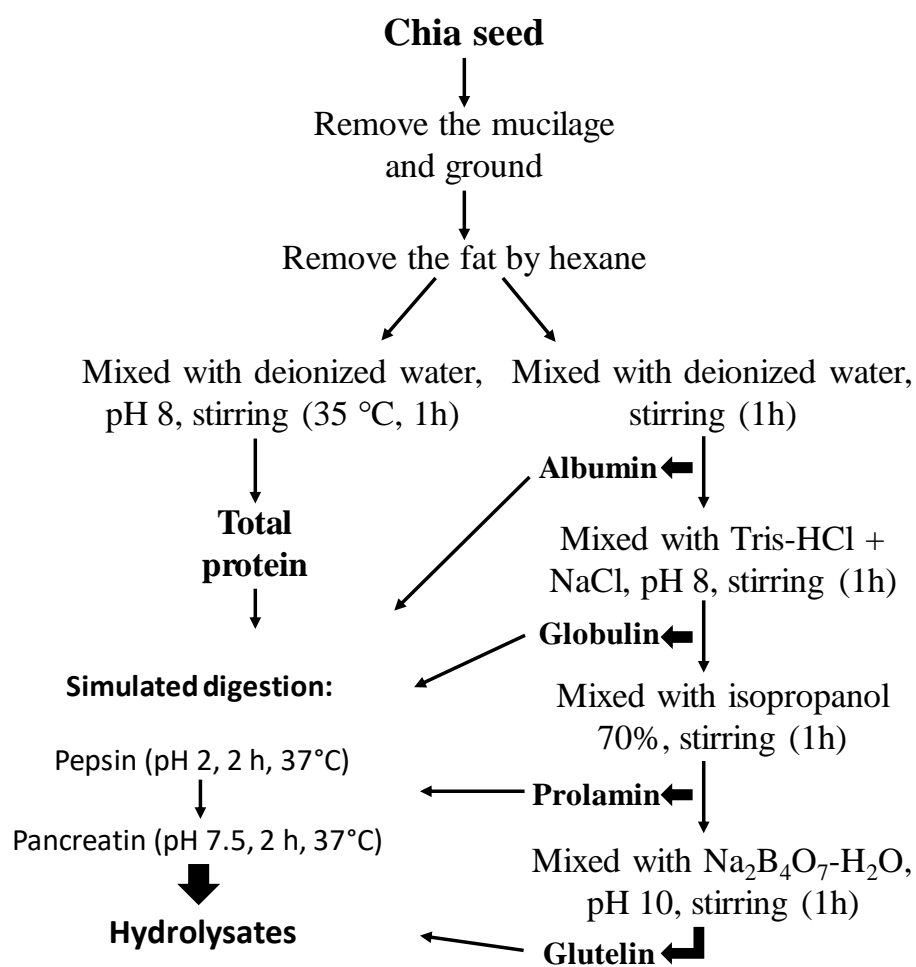


Figure 1S. The experimental process to prepare total protein, protein fractions and digests.

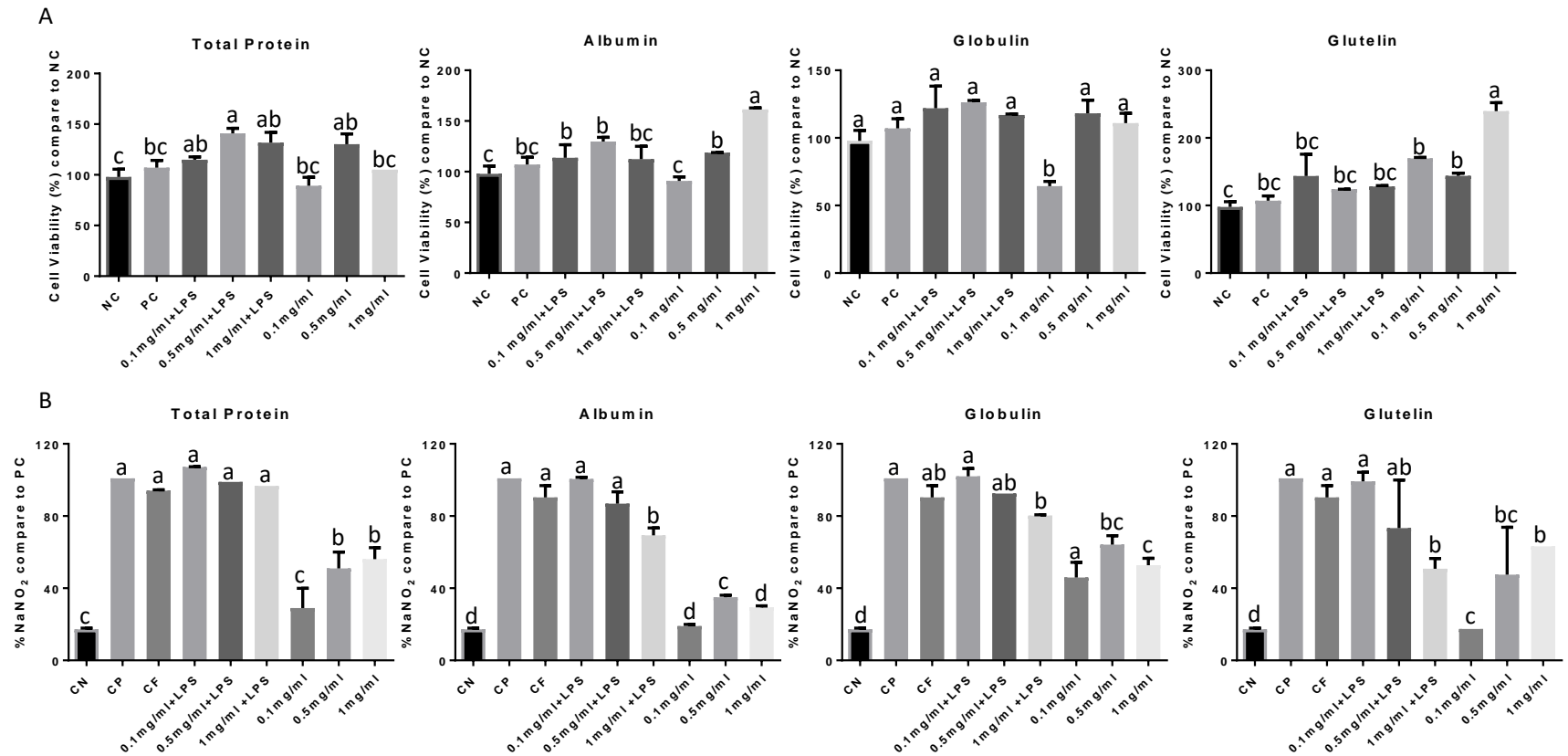


Figure 2S. The viability (A) and nitric oxide production (B) by RAW264.7 macrophages LPS-stimulated treated with digested total protein, albumin, globulin, and glutelin (0.1, 0.5, 1 mg/ml). Different letter per column means statistically different between the samples (by ANOVA and *post-hoc* Tuckey-test). All treatments contain LPS (1 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

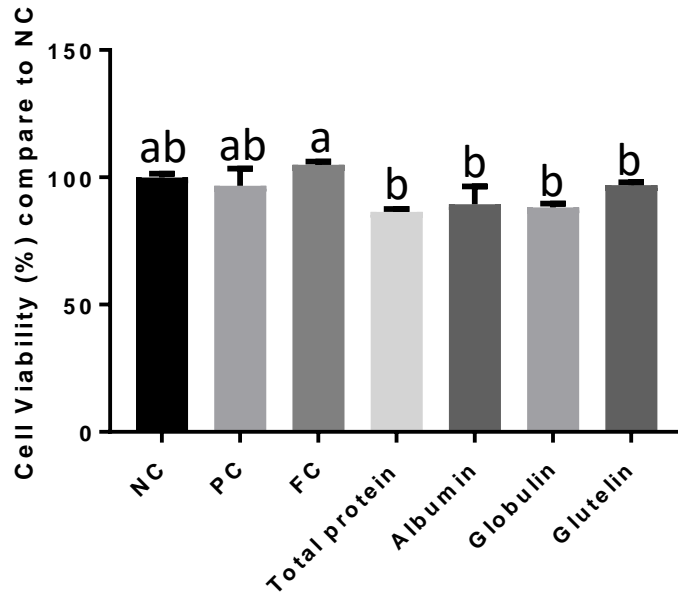


Figure 3S. The viability by RAW264.7 macrophages ox-LDL-stimulated treated with hydrolyzed total protein, albumin, globulin, and glutelin (1 mg/ml). Different letter per column means statistically different between the samples (by ANOVA and post-hoc Tuckey-test). All treatments contain ox-LDL (80 μ M) except the negative control (NC) treated only with media. NC: negative control; PC: positive control; FC: pharmacological control.

Table 1S. Estimated free energy binding and chemical interactions among the peptides present in chia seed and the pharmacological control (Simvastatin) and the catalytic site of the CCR2 and ICAM1.

Peptides	CCR2			ICAM1		
	EFC (kcal/mol)	K _I (μM)	Interacting amino acid residues	EFC (kcal/mol)	K _I (μM)	Interacting amino acid residues
			Total Protein			
Simvastatin	-8.4	0.70	MG A:401; ARG A:138; LYS A:311; LEU A:81; LEU A:134; ile A:245; ile A: 248; GLU A: 310; THR A:77; GLY A:309; TYR A:305; VAL A:244	-4.9	256.0	SER A:61; MG A: 401; GLU A:59; GLN A:58; PRO A:36
HYGGPPGGCR	-6.5	17	GLU A: 1064; ASP A: 1061; ARG A: 138; CYS A: 75; LYS A: 74; LYS A: 311; LYS A: 71; THR A:77; ASP A:78; PHE A:312; GLU A:310	-6.0	40.0	ILE A: 33; TYR A: 66; LYS A: 39; PRO A: 36; LYS A: 77; GLN A: 62; SER A: 61; GLN A: 58; GLU A: 59
SPPHGAGLGGGMT	-4.3	705	GLY A: 309; GLU A: 310; ARG A: 237; CYS A: 75; ASP A: 78; LYS A: 71; TYR A: 315; LEU A: 81; LEU A: 134; ILE A: 248; ARG A: 138; ASP A: 1061; LYS A: 74	-5.7	66.4	THR A: 35; SER A: 61; GLN A:62; GLN A: 58; GLU A: 34; TYR A: 66; LYS A:39
LPVFGLAEGNVVTYLH	95.9	1.98E+76		-5.3	130.3	GLU A: 59; ASP A: 60; SER A: 61; GLN A: 62; MET A: 64; LYS A: 77; PRO A: 36; GLU A: 34; LYS A: 39; TYR A: 52; GLU A: 41; LYS A: 40
TPPGVGGFPWGGAMLGAQ	65.3	7.34E+53		-6.3	24.1	LYS A: 40; SER A: 55; GLU A: 41; LYS A: 39; THR A: 35; PRO A: 36; SER A: 61; GLU A: 59; GLN A: 62; LYS A: 77
APSPVVLGPP	-5.1	183	ARG A: 237; ARG A: 152; ASP A: 1061; CYS A: 75; ARG A: 314; LYS A: 311; ALA A: 241; TYR A: 305; VAL A: 244; ILE A: 245; ARG A: 138; LEU	-6.5	17.2	LEU A: 31; TYR A: 66; LYS A: 39; PRO A: 36; LYS A: 77; GLN A: 62; SER A: 61; GLU A: 59
NPGGGAPSGH	-6.5	17	THR A: 77; ARG A: 138; ALA A: 241; VAL A: 244; LYS A: 311; GLY A: 309	-4.5	502.9	GLU A: 41; ILE A: 33; TYR A: 52; LYS A: 50

LHNGGPGGEY	-5.4	110	ALA A: 141; ARG A: 138; ARG A: 152; ASP A: 1061; LYS A: 74; LYS A: 71; LYS A: 311; TYR A: 315; ALA A: 241; ILE A: 245; LEU A: 134; LEU A: 81; VAL A: 244	-5.8	56.0	PRO A: 38; LYS A: 39; LYS A: 50; TYR A: 52; ILE A: 33; THR A: 35; TYR A: 66; THR A: 75
DVNPGGPAPHPWLSVNDL	75.4	1.86E+61		-4.8	303.1	MET A: 64; LYS A: 77; GLN A: 62; PRO A: 36; GLU A: 34; LYS A: 39; GLU A: 41; LEU A: 30
TGPPRPALVFPHAVVP	40.3	3.47E+35		-5.6	78.5	GLU A: 59; LEU A: 37; PRO A: 36; LYS A: 39; GLY A: 32; ILE A: 33; TYR A: 66; GLU A: 41; LYS A: 50; TYR A: 52
NSSAQYSDPFLALH	28.9	1.53E+27		-5.2	154.3	GLU A: 34; LYS A: 39; GLY A: 32; LYS A: 50; LEU A: 31; TYR A: 66; ILE A: 33
Albumin						
THPTGPAGAGPPMSL	2.2	40984794		-5.9	47.3	SER A: 5; THR A: 20; LEU A: 18; VAL A: 17; PRO A: 12; LYS A: 8; SER A: 7; VAL A: 4; LYS A: 77; PHE A: 79
RGSSSPMAGAAGDLPGSVT	68.5	1.63E+56		-5.9	47.3	SER A: 7; SER A: 3; SER A: 5; VAL A: 4; PHE A: 79; GLN A: 62; GLU A: 34; LYS A: 39; TYR A: 66; THR A: 35; GLY A: 32; LEU A: 30; ILE A: 33; PRO A: 36; ASN A: 68
YLGHPGTAN	-6.3	24	TYR A: 315; LYS A: 311; GLY A: 309; LYS A: 71; ASP A: 1061; ALA A: 141; ARG A: 138; VAL A: 244; ALA A: 241; LYS A: 240; VAL A: 308; GLU A: 310	-6.7	12.3	GLU A: 41; TYR A: 52; TYR A: 66; ILE A: 33; GLU A: 34; PRO A: 36; GLN A: 62; SER A: 61; LEU A: 37; ASN A: 56; PRO A: 38; SER A: 55
SPKDLALPPGALPPVQ	41.7	3.69E+36		-6.9	8.8	TYR A: 66; ILE A: 33; TYR A: 52; LYS A: 39; GLU A: 41; LYS A: 40; GLU A: 34; GLN A: 62; LYS A: 77
TVPLLWFRQDPTGPLNL	144.2	5.01E+111		-6.7	12.3	GLU A: 41; LYS A: 40; PHE A: 79; ; ASP A: 60
TGPSPTAGPPAPGGGTH	-6.5	17	N/A	-7.8	1.9	LYS A: 39; GLY A: 32; ILE A: 33; TYR A: 66

AVLPDLTSSSLKLDK	83.8	2.67E+67		-6.2	28.5	ARG A:49; SER A:22; SER A:7; THR A:2; PHE A:79; LYS A:77; PRO A:36; THR A:35; LYS A:39; ILE A:33; TYR A:66
RMVLPTYFSAQLE	12.5	1.45E+15		-6.1	33.8	ILE A: 33; GLU A: 41; LYS A: 40; PRO A: 38; LEU A: 37; THR A: 35; PRO A: 36; MET A: 64; GLN A: 62; ASP A: 60; GLN A: 58; GLU A: 59
RHMDLPALVFT	-3.4	3219	ARG A: 237; LYS A: 240; ALA A: 241; ARG A: 138; ALA A: 141; ASP A: 1061; CYS A: 75; TYR A: 315; ASP A: 78; TYR A: 305	-6.5	17.2	LYS A: 8; ILE A: 10; LEU A: 18; LEU A: 44; VAL A: 51; ARG A: 49; SER A: 5; SER A: 22; SER A: 3; SER A: 24
GPPDGPCTEGA	0.1	1.18E+06		-5.2	154.3	PRO A: 36; LYS A: 39; LYS A: 50; GLU A: 41; TYR A: 66
SGKVEELAELPDL	13.9	1.54E+16		-6.3	24.1	GLU A: 34; TYR A: 66; TYR A: 52; ILE A: 33; LYS A: 39; LYS A: 40; SER A: 55; PRO A: 38; ASN A: 56; GLN A: 58; GLU A: 59
LPGPPATF	-8.1	1	LEU A:81; THR A:77; ARG A:138; ARG A:237; CYS A:75; VAL A:154	-4.8	303.1	SER A: 5; SER A: 16; ILE A: 10; GLN A: 168; LYS A: 8; SER A: 7; PRO A: 6
Globulin						
KPTEPGPPVFPLAAADL	59.1	2.09E+49		-4.7	358.8	GLU A: 41; PRO A: 38; PRO A: 36; TYR A: 66; TYR A: 52; LYS A: 39
NGGGLKSGSGAPPT	-2.1	2.89E+04	ALA A: 241; ARG A: 138; GLY A: 309; LYS A: 311; ARG A: 237; CYS A: 75; LEU A: 76; LYS A: 71; TYR A: 305	-5.3	130.3	SER A: 61; PRO A: 36; THR A: 35; GLU A: 34; LYS A: 39; TYR A: 66; LEU A: 31; LYS A: 50; TYR A: 52; GLU A: 41
TLPHEPGGVAGPATNFPQ	19.5	1.97E+20		-4.2	834.4	TYR A: 66; LYS A: 40; LEU A: 42
NPGAAGALMVSFPPTGGR	36	2.45E+32		-5.9	47.3	SER A: 7; LYS A: 8; LYS A: 77; GLN A: 62; PRO A: 36; GLU A: 34; TYR A: 66; LYS A: 50; GLU A: 41
SGPPDPGLPN	-6.6	15	TYR A: 305; LEU A: 81; LEU A: 134; ILE A: 245; VAL A: 244; ARG A: 138; LYS A: 311; CYS A: 75; LYS A: 74; ARG A: 152; ASP A: 1061; LEU A:	-5.1	182.7	ARG A: 49; SER A: 5; SER A: 7; LYS A: 8; LEU A: 18; VAL A: 51; ILE A: 10

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EGGYVLAGPSPVQPPGDH	64.9	3.74E+53		-5.2	154.3	GLN A: 58; GLU A: 59; SER A: 61; LYS A: 41; GLU A: 41; LYS A: 50; TYR A: 52; LYS A: 39; PRO A: 36; LYS A: 77; ASP A: 60
SPPAGTACTY	-5.7	66	ALA A: 141; ARG A: 138; ARG A: 237; LYS A: 71; ALA A: 241; LEU A: 67; LEU A: 81; TYR A: 315; TYR A: 305	-5.7	66.4	VAL A: 17; ILE A: 10; SER A: 7; PRO A: 6; SER A: 5; ARG A: 49; THR A: 20; LEU A: 44; VAL A: 51
TPHAYPLGGGY	-1.9	4.05E+04	ALA A: 241; ARG A: 138; ARG A: 237; LYS A: 311; LYS A: 74; LYS A: 71; ASP A: 78; CYS A: 75; THR A: 77; TYR A: 305	-5.9	47.3	TYR A: 52; GLU A: 41; TYR A: 66; LYS A: 39; PRO A: 36; SER A: 55
QPGWPVAGGF	-7.8	2	CYS A: 75; LYS A: 71; LYS A: 1060; ARG A: 237; LYS A: 240; GLU A: 310; LYS A: 311; VAL A: 244; ARG A: 138; ILE A: 248; LEU A: 134; ILE A: 245; LEU A: 81; TYR A: 305	-6.6	14.5	GLU A: 34; MET A: 64; SER A: 61; GLN A: 58; PRO A: 38; PRO A: 36; THR A: 35; ILE A: 33; LYS A: 39; TYR A: 52
EPAVPPVPHVRPFPGALP	83.3	1.15E+67		-5.1	182.7	LYS A: 40; GLU A: 41; LYS A: 39; SER A: 55; LEU A: 37; PRO A: 38; PRO A: 36; THR A: 35; GLU A: 34
RKTRARLMLLATELQ	52.7	4.26E+44		-4.9	256.0	LYS A: 39; LYS A: 40; PRO A: 36; GLU A: 34; GLN A: 73; PRO A: 38; TYR A: 52; TYR A: 66; GLY A: 32; LYS A: 50
Glutelin						
KKLELTEYLYEDKK	95.1	5.12E+75		-5.4	110.1	GLN A: 62; LYS A: 77; GLU A: 34; THR A: 35; LYS A: 39; PRO A: 36; ASN A: 56
NSPGPHDVALDQ	-4.8	303	ALA A: 241; GLU A: 310; LYS A: 311; ARG A: 237; ARG A: 138; TYR A: 305; ARG A: 152; ASP A: 1061; CYS A: 75; LYS A: 71; TYR	-5.3	130.3	LYS A: 77; SER A: 61; GLN A: 62; THR A: 35; LYS A: 39; ILE A: 33; LYS A: 40; PRO A: 38; PRO A: 36

A: 315; PHE A: 312

KDSGHPNGGLFAESSP	34.6	2.30x10E+31		-6.2	28.5	SER A: 61; ASP A: 60; GLN A: 62; THR A: 35; PRO A: 36; TYR A: 66; LYS A: 39; GLU A: 41; PRO A: 38; LYS A: 40
TGPCNGAAPPAAGSVDCL	26.1	1.35E+25		-5.0	216.2	GLU A: 41; LYS A: 50; TYR A: 66; LEU A: 30; GLU A: 34; ILE A: 33; LYS A: 39; PRO A: 36; LYS A: 77; GLN A: 62; SER A: 61
QAGAGGPGAAGH	-6.9	9	ASP A: 1061; ARG A: 237; ARG A: 138; ALA A: 241; THR A: 77; ARG A: 152; LYS A: 71; LYS A: 311; ARG A: 314; TYR A: 315; THR A: 153	-5.1	182.7	GLN A: 168; ILE A: 10; LYS A: 8; SER A: 5; THR A: 20; VAL A: 51; LEU A: 42; LEU A: 18;
TPPNGLGPSGSPGPHAQ	33.4	3.04E+30		-4.5	502.9	TYR A: 52; TYR A: 66; PRO A: 36; LYS A: 40; SER A: 55; SER A: 16
RMVLPEYELLYE	16.1	6.33E+17		-5.2	154.3	LYS A: 40; LYS A: 39; TYR A: 66; LEU A: 30; LYS A: 50; TYR A: 52; GLU A: 41; LEU A: 43
RLTGTGMGAASSVLP	10.2	3.00E+13		-6.5	17.2	ASN A:56; GLU A:59; SER A:61; GLN A:62; THR A:35; LYS A:39; ILE A:33; TYR A:66; LEU A:30; ASN A:68; GLU A:34
RHMDLPLSPSSAGP	5.3	7.67E+09		-5.1	182.7	GLU A: 34; MET A: 64; GLN A: 62; ASP A: 60; LEU A: 30; LYS A: 39; TYR A: 52; PRO A: 38; PRO A: 36; GLN A: 58; GLU A: 59; SER A: 61
TTNPGVDADFPPAREL	26.3	1.90E+25		-4.8	303.1	GLU A: 34; THR A: 35; ILE A: 33; TYR A: 66; LYS A: 39; GLU A: 41; SER A: 55; ASN A: 56; PRO A: 36
QPADPNQFVYAPDTH	26.3	1.90E+25		-6.2	28.5	LYS A: 29; LYS A: 50; LEU A: 30; LYS A: 39; GLU A: 41; PRO A: 38; LEU A: 37; GLU A: 59; SER A: 61; PRO A: 36; GLU A: 34; GLN A: 62
FAFFEFFELLFAFFT	118.4	6.14E+92		-8.4	0.7	SER A: 61; PRO A: 36; THR A: 35; GLU A: 41; LYS A: 40; LEU A: 42;

TDQLGWFLGAAAFPAGQ	68.3	1.16E+56		-5.0	216.2	LEU A:18 ASP A: 60; SER A: 61; GLN A: 62; MET A: 64; LYS A: 77; THR A: 75; ALA A: 76; SER A: 7; LYS A: 8
RGSSPAPSGSELVL	6.2	3.50E+10		-4.9	256.0	ASN A: 56; SER A: 55; LYS A: 39; PRO A: 38; GLU A: 41; ILE A: 33; LEU A: 30; TYR A: 66
TDWYDPGAFQ	0.3	1.66E+06		-6.4	20.4	SER A: 3; VAL A: 4; SER A: 7; LYS A: 8; PRO A: 6; LEU A: 18; THR A: 20; SER A: 5
HAPGFLSPAHR	9.2	5.54E+12		-6.4	20.4	LEU A:18; THR A: 20; ARG A: 49; LYS A:8; VAL A:4; LYS A:77; SER A:7; SER A:3; SER A:5; GLN A:1; SER A:24
LPGPPATF	-8.1	1	LEU A: 81; THR A: 77; ARG A: 138; ARG A: 237; CYS A: 75; VAL A: 154	-4.8	303.1	SER A: 5; SER A: 16; ILE A: 10; GLN A: 168; LYS A: 8; SER A: 7; PRO A: 6

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction.

The most potent interaction between peptides and receptor is in bold.

6.4 Manuscript 4

Journal target: Scientific Reports

Protein digests and pure peptides from chia seed prevented adipogenesis and its inflammation by inhibition of PPAR γ and NF- κ B pathways

Mariana Grancieri^{1,2}, Hercia Stampini Duarte Martino¹ and Elvira Gonzalez de Mejia^{2*}

¹ Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, Viçosa, MG, Brazil

² Department of Food Science & Human Nutrition, University of Illinois at Urbana-Champaign, IL, United States.

*Correspondence and requests for materials should be addressed to E.G (postal address: 1201 W Gregory #228, Urbana, IL, 61801; email: edemejia@illinois.edu; phone: 217-244-3196; fax: +1-217-265-0925)

KEYWORDS: adipocytes, bioactive peptides, digested proteins, inflammation, obesity, *Salvia hispanica* L.

ABSTRACT

The objective was to evaluate the mechanisms of digested total proteins (DTP), albumin, glutelin, and pure peptides from chia seed (*Salvia hispanica* L.) to prevent adipogenesis and its associated inflammation in 3T3-L1 adipocytes. Preadipocytes (3T3-L1) were treated during differentiation with either DTP or digested albumin, or glutelin (1 mg/ml), or pure peptides NSPGPHDVALDQ and RMVLPEYELLYE (100 μ M). Differentiated adipocytes also received DTP, digested albumin, or glutelin (1 mg/ml), before (prevention) or after (inhibition) induced inflammation by addition of conditioned medium (CM) from inflamed macrophages. All treatments prevented adipogenesis, reducing more than 50% the expression of PPAR γ , and to a lesser extent LPL, FAS, SREBP1, lipase activity, and triglycerides. Inflammation induced by CM was reduced mainly during prevention, while DTP decreased expression of NF- κ B (-48.4%), iNOS (-46.2%), and COX-2 (-64.5%), $p < 0.05$. Secretions of nitric oxide, PGE2, and TNF α were reduced by all treatments, $p < 0.05$. DTP reduced expressions of iNOS (-52.1%) and COX-2 (-66.4%). Furthermore, digested samples and pure peptides prevented adipogenesis by modulating PPAR γ pathway, and additionally, preventing and even inhibiting inflammation in adipocytes by inhibition of PPAR γ and NF- κ B pathways. These results highlight the effectiveness of digested total proteins and peptides from chia seed against adipogenesis complications.

1. INTRODUCTION

A major cause of death and premature incapacity worldwide is cardiovascular diseases (CVD), representing around 17.3 million deaths per year ^[1]. An important modifiable risk factor for coronary heart disease is obesity ^[2]. It is estimated that over half of the global adult population is overweight or obese ^[3]. Obesity is characterized by expansion of adipose tissue starting by adipocyte hyperplasia, mediated by the recruitment and proliferation of adipogenic precursors, mainly the peroxisome proliferator-activated receptor gamma (PPAR γ), followed by adipocyte hypertrophy ^[4].

Adipose tissue is not only a fat deposit in the body. It is also an endocrine organ that synthesizes and release into systemic circulation adipokines, such as leptin, resistin, and adiponectin hormones, chemokines and cytokines ^[5]. This condition induces infiltration of activated macrophages into the adipose tissue that releases pro-inflammatory cytokines, as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-1 β , which increase local inflammation and stimulate pro-inflammatory secretion creating a vicious cycle of the inflammatory response ^[6,7]. This scenario, known as low-grade inflammation is associated with an overall impact on obesity-related complications, like insulin resistance, progression to type 2 diabetes, cardiovascular disease, metabolic syndrome, and cancer ^[8].

Obesity is caused by changes in lifestyle, including overconsumption of food and decreased physical activity ^[9]. Therefore, treatment to reduce obesity consists of lifestyle changes and reduction of energy intake ^[10]. The consumption of proteins from vegetables has been inversely related to development and progression of obesity by affecting satiety, thermogenesis, energy efficiency, and body composition ^[11]. A vegetable protein source is chia seed (*Salvia hispanica* L.) ^[12,13]. Chia is an herbaceous plant native to northern Guatemala and

southern Mexico, which produces small seeds that stand out due to their high nutritional and functional value ^[14]. They are also related with beneficial health effects as indicated by lower markers of obesity, diabetes, cancer, dyslipidemia and cardiovascular diseases ^[15–20].

Chia seed has about 20% protein ^[13]. Main storage protein fractions in chia are prolamin, glutelin, albumin and globulin, being the last two in higher concentrations ^[21,22]. After gastrointestinal digestion they generate peptides that, according to their composition and amino acid sequence, may exert antimicrobial, antihypertensive, hypocholesterolemic, antithrombotic, antioxidant, increased absorption/bioavailability of minerals, and immunomodulatory effects, among others ^[23,24].

Research has shown that digested protein of chia inhibits ACE-enzyme (angiotensin-converting enzyme) activity ^[22,24], has high antioxidant, and antibacterial effects ^[13, 24–26], shows inhibition of cholesterol synthesis ^[26]. A recent research from our group showed that digested total protein (DTP), digested albumin, globulin and glutelin from chia seed prevented against inflammation and atherosclerosis process in macrophages RAW 264.7 ^[27]. However, there is no evidence about the effect of digested proteins and pure peptides from chia seed on adipogenesis and inflammation induction to adipose tissue and the mechanisms of action. The objectives of this research were to evaluate *in vitro* the mechanisms of digested total proteins (DTP), albumin, glutelin, and pure peptides from chia seed (*Salvia hispanica* L.) to prevent adipogenesis and its associated inflammation in 3T3-L1 adipocytes. The hypothesis was that digested total proteins, protein fractions, and pure peptides from chia seed blocked PPAR γ expression, and then its pathway was inhibited and reduced the development of adipogenesis in 3T3-L1 adipocytes. Additionally, the digested chia proteins prevented and inhibited inflammation, induced by

conditioned medium from inflamed macrophages, by blocking PPAR γ and, consequently, NF- κ B pathways.

2 RESULTS

2.1 Effect of digested total protein (DTP), digested albumin and glutelin and pure peptides on adipogenesis

2.1.1 Effect on reduction of differentiation of adipocytes, lipid accumulation, and reactive oxygen species (ROS) production.

DTP, digested albumin and glutelin (**Supplementary Figure 1**) and pure peptides showed a high cell viability (**Supplementary Figure 2**) and reduced ROS production ($p<0.05$) (**Figure 1A and 1B**). DTP, digested albumin and glutelin, and Pep2 reduced expression of sterol regulatory element-binding protein 1 (SREBP1) by -63.4, -58.8, -53.3, and -46.7%, respectively ($p<0.05$) (**Figure 1C and 1D and Supplementary Figures 3 and 4**). The expression of proliferator-activated receptor gamma (PPAR γ) was reduced by DTP, digested albumin and glutelin and pure peptides, primarily by Pep2 (-77.1%; $p<0.05$) (**Figure 1E and 1F and Supplementary Figures 3 and 4**). Every treatment with DTP, digested albumin and glutelin, and pure peptides reduced lipid accumulation (**Figure 1G and 1H and Supplementary Figure 5**), especially by DTP (-37.2%; $p<0.05$). Moreover, the activity of lipase was also reduced by -58.3%, -56.9%, and -62.6% by DTP, digested albumin, and glutelin, respectively ($p<0.05$) (**Figure 2A**). However, reduction by pure peptides was -37.8%, in comparison to the positive control (**Figure 2B**). Every sample reduced ($p<0.05$) lipase lipoprotein (LPL) expression (**Figure 2C and 2D and Supplementary Figures 3 and 7**), triglyceride content (**Figure 2E and 2F**) and fatty acid

synthase (FAS) expression (**Figure 2G and 2H and Supplementary Figures 4 and 6**). DTP reduced the content of triglycerides and FAS expression by -85.7% and -71.6%, respectively ($p<0.05$).

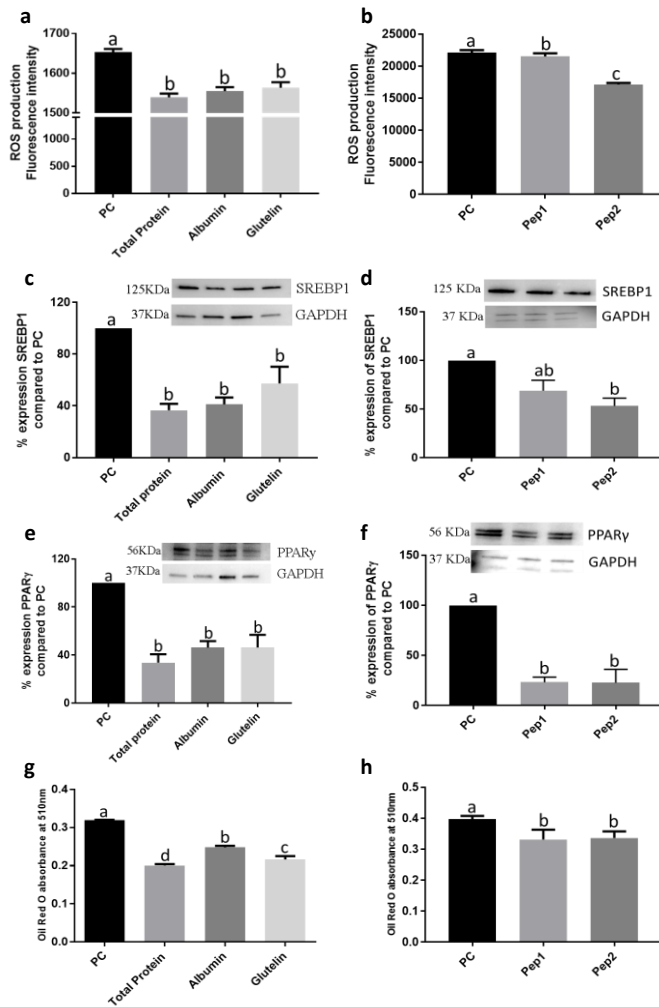


Figure 1. Effect of digested total protein, digested albumin or glutelin from chia seeds, and pure peptides NSPGPHDVALDQ (Pep1) and RMLVPEYELLYE (Pep2) from glutelin to prevent the adipogenesis process. ROS production (A, B); SREBP-1 (C, D) expression; PPAR γ expression (E, F); Oil Red-O absorbance (G, H). The proteins were added during the differentiation period (days 3, 5, 7, 8, and 10) and PC (positive control) received no treatment. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p<0.05$). ROS: Reactive oxygen species; SREBP-1: sterol regulatory element-binding protein 1; PPAR γ : peroxisome proliferator-activated receptor gamma; GAPDH: glyceraldehyde 3-phosphate; PC: Positive control.

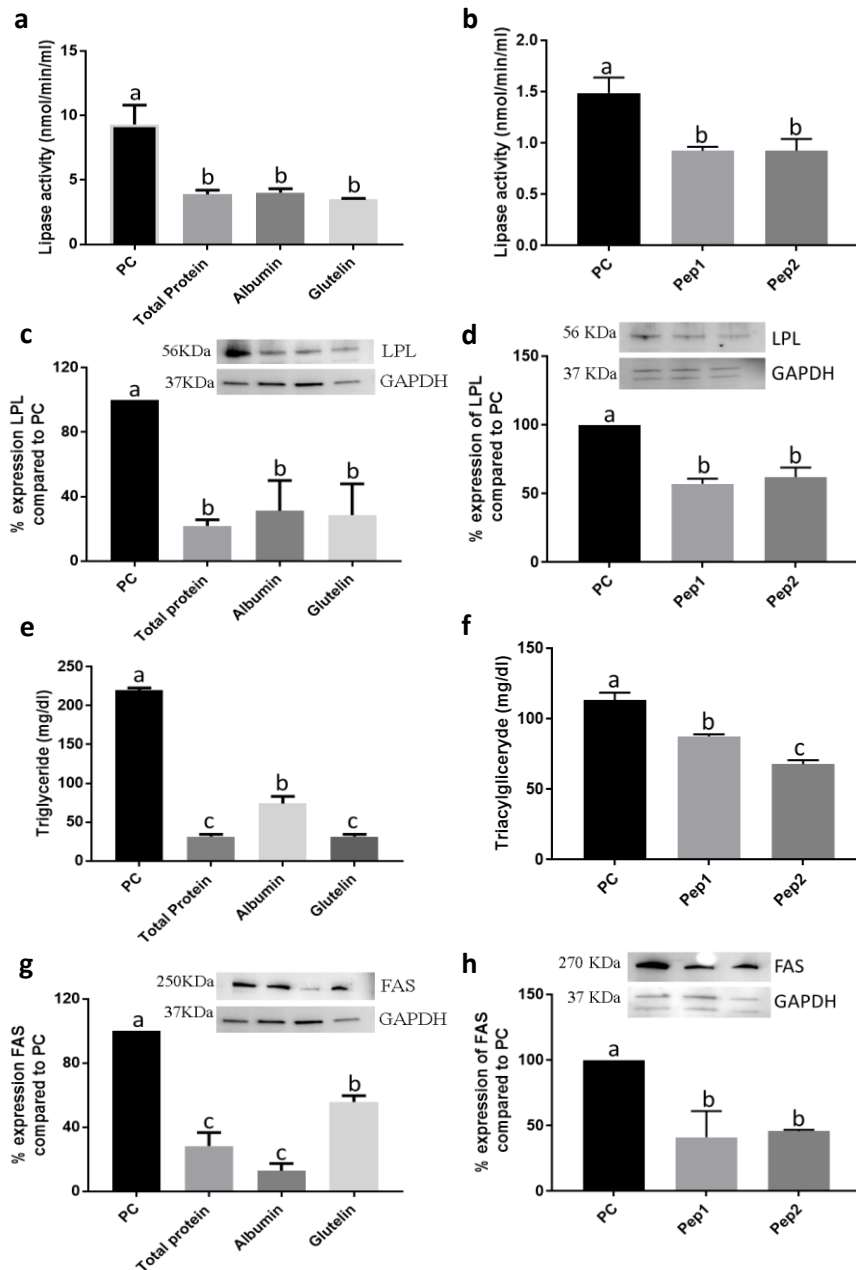


Figure 2. Effect of digested total protein, digested albumin or glutelin from chia seeds, and pure peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) from glutelin of chia seeds to prevent the adipogenesis process. Lipase activity (A, B); LPL expression (C, D); triglyceride content (E, F); FAS expression (G, H). Treatments were added during the differentiation period (days 3, 5, 7, 8, and 10) and PC received no treatment. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). LPL: lipase lipoprotein; FAS: fatty acid synthase; GAPDH: glyceraldehyde 3-phosphate; PC: Positive control.

2.1.1 Effect of digested albumin and glutelin and pure peptide 2 on reducing inflammatory markers related with the adipogenesis process.

Digested albumin and glutelin, but not DTP, were effective on reducing the secretion of PGE2 ($p < 0.05$) (**Figure 3A**). Pure peptides Pep1 and Pep2 reduced PGE2 secretion by -49.3 and -54.1%, respectively ($p < 0.05$) (**Figure 3B**). Digested albumin and glutelin also reduced the expression of cyclooxygenase-2 (COX-2) in -68.1 and -48.1%, respectively ($p < 0.05$). However, DTP and pure peptides had no effect ($p < 0.05$) (**Figure 3C and 3D and Supplementary Figures 6 and 7**). The secretion of tumor necrosis factor alpha (TNF- α) was reduced by digested albumin, glutelin and Pep2 ($p < 0.05$) (**Figure 3E and 3F**); all proteins reduced NF- κ B expression, particularly glutelin (-69.3%; $p < 0.05$) (**Figure 3G and 3H and Supplementary Figures 6 and 7**). Glutelin, Pep1 and Pep2 reduced the secretion of interleukin-6 (IL-6) in -28.4%, -18.9%, and -33.8%, respectively, as compared with PC ($p < 0.05$) (**Supplementary Table 1**). In addition, pure peptides decrease the IL-10 secretion (**Supplementary Table 1**) ($p < 0.05$). IL-12 secretion (**Supplementary Table 1**) and nitric oxide (NO) production (data not shown) were not statistically different to PC ($p > 0.05$).

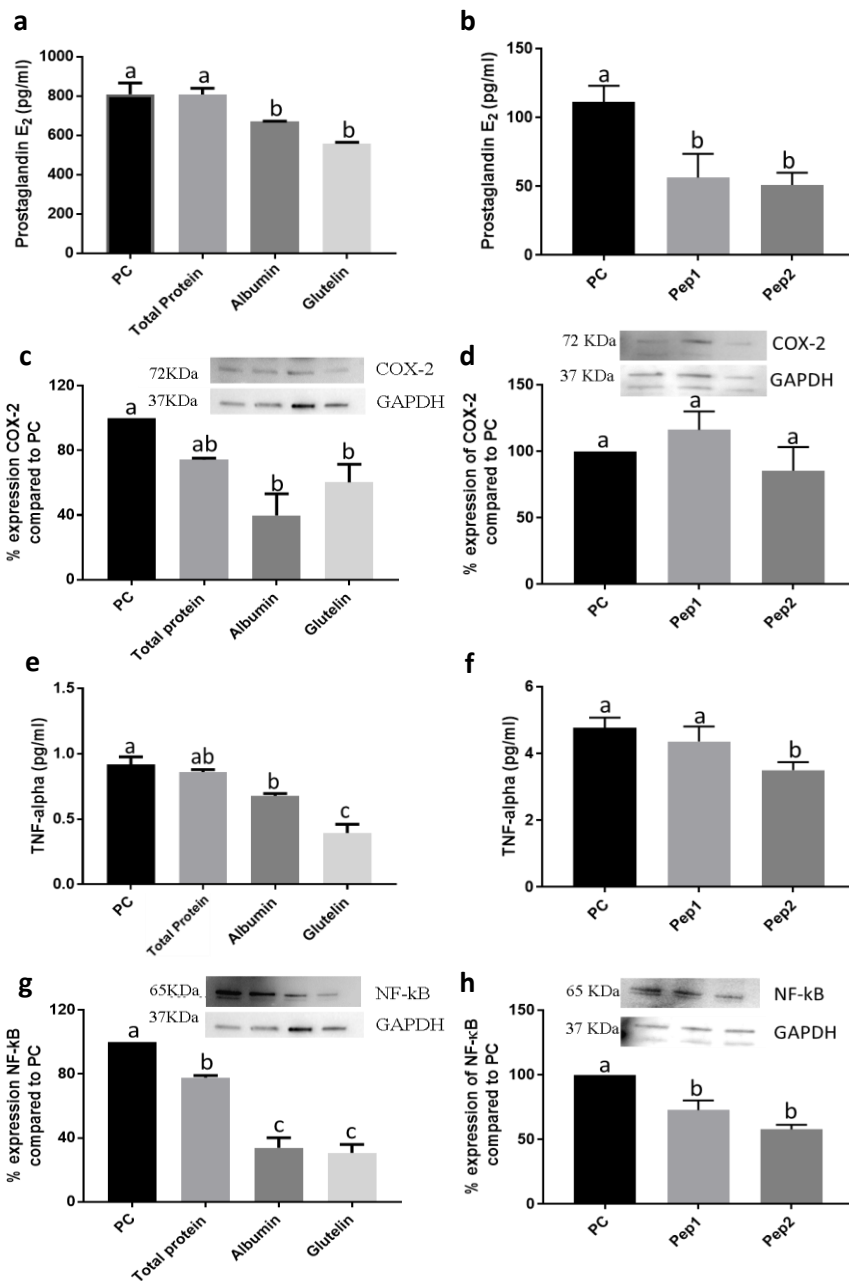


Figure 3. Effect of digested total protein, digested albumin, digested glutelin from chia seeds, and pure peptides NSPGPHDVALDQ (Pep1) and RMLVPEYELLYE (Pep2) from glutelin of chia seeds, in inflammatory markers to prevent the adipogenesis process. PGE₂ secretion (A, B); COX-2 expression (C, D); TNF- α secretion (E, F); NF- κ B expression (G, H). Treatments were added during the differentiation period (days 3, 5, 7, 8, and 10) and the PC received no treatment. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). PGE₂: prostaglandin 2; COX-2: cyclooxygenase2; TNF- α : Tumor necrosis factor alpha; NF- κ B: Factor nuclear kappa B; PC: Positive control.

2.2 Effect of DTP and digested albumin and glutelin on inflammation in mature adipocytes

2.2.1 DTP and digested albumin and glutelin fractions reduced lipid accumulation and ROS production in the prevention and inhibition of induced inflammation.

ROS production was reduced when DTP or digested albumin and glutelin were added to prevent the development of inflammation ($p < 0.05$). Glutelin, specially, reduced ROS production -53.3% ($p < 0.05$) (**Figure 4A**). However, when proteins were used to inhibit already existing inflammation, there was not effect ($p > 0.05$) (**Figure 4B**). The expression of SREBP-1 was not reduced by preventing inflammation ($p > 0.05$) (**Figure 4C and Supplementary Figure 8**), but on inhibition, DTP and digested albumin and glutelin reduced the expression of this protein in -33.0%, -40.8%, and -38.7%, respectively ($p < 0.05$) (**Figure 4D and Supplementary Figure 9**). The use of DTP and digested albumin and glutelin in both, prevention and inhibition of induced inflammation, decreased the expression of PPAR γ ($p < 0.05$). Glutelin reduced PPAR γ expression in -83.2% and -94.2% in prevention and inhibition of induced inflammation, respectively ($p < 0.05$) (**Figure 4E and 4F and Supplementary Figure 8 and 9**). The Oil red-O accumulation was reduced on prevention experimental approach by all proteins ($p < 0.05$) (**Figure 4G**), but not for the inhibition approach ($p > 0.05$) (**Figure 4H**).

Digested albumin increased the expression of LPL similar to untreated cells (**Figure 5A**) in prevention of inflammation (**Figure 5A and Supplementary Figure 8**); on inhibition, DTP and glutelin reduced the expression of LPL (-32.2% and -39.3%, respectively; $p < 0.05$) (**Figure 5B and Supplementary Figure 9**). On prevention, DTP and albumin reduced lipase activity (-40.1% and -24.6%, respectively; $p < 0.05$) (**Figure 5C**); however, the activity of lipase was not changed by any treatment in inhibition approach ($p > 0.05$) (**Figure 5D**). Digested albumin increased FAS expression in both treatment approaches, prevention (**Figure 5E and**

Supplementary Figure 8) and inhibition (**Figure 5F and Supplementary Figure 9**) of induced inflammation ($p<0.05$). Furthermore, glutelin reduced triacylceride content by -21.8% (**Figure 5G**) when preventing inflammation and DTP reduced triglyceride content also in both, prevention (**Figure 5G**) and inhibition of induced inflammation ($p<0.05$) (**Figure 5H**).

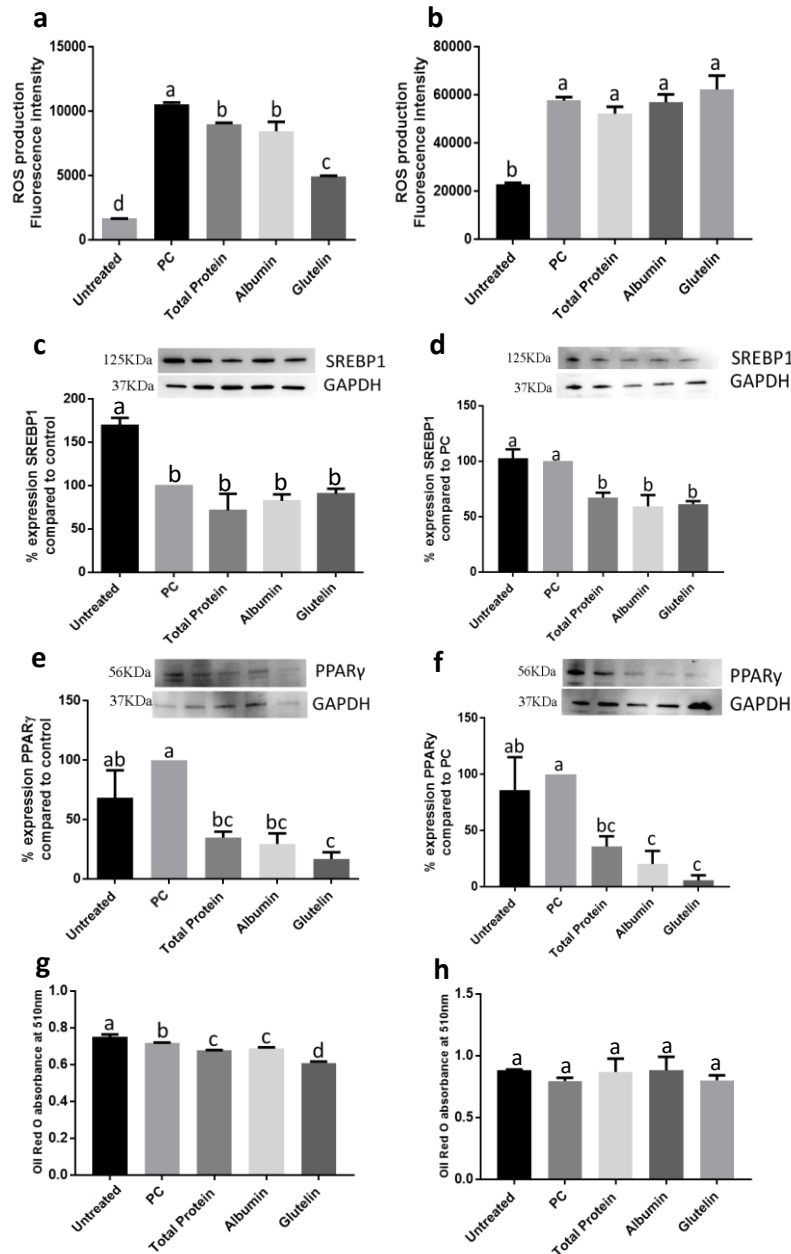


Figure 4. Effect of digested total protein and digested albumin and glutelin from chia seeds to prevent and inhibit the oxidative stress and markers of adipogenesis process in mature adipocytes stimulated by inflamed macrophages. ROS production on prevention (A) and inhibition (B); SREBP-1 expression on prevention (C) and inhibition (D); PPAR γ expression on prevention (E) and inhibition (F); Oil Red O absorbance on prevention (G) and inhibition (H). Untreated group receive any treatment, PC receive only the CM. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). CM: conditioned media from inflamed macrophages; ROS: Reactive oxygen species; SREBP-1: sterol regulatory element-binding protein 1; PPAR γ : peroxisome proliferator-activated receptor gamma; GAPDH: glyceraldehyde 3-phosphate; PC: Positive control.

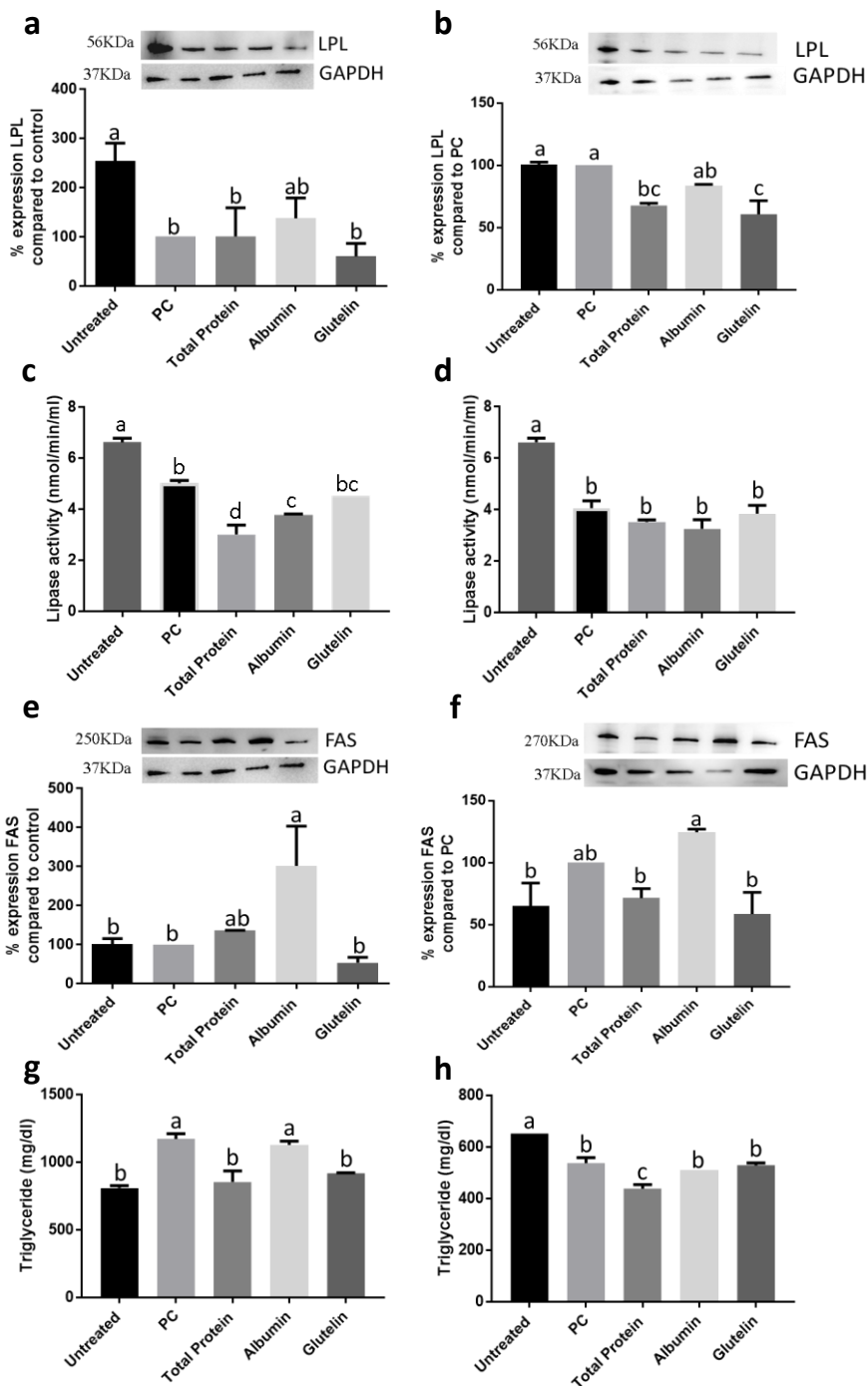


Figure 5. Effect of digested total protein and digested albumin and glutelin from chia seeds to prevent and inhibit the adipogenesis process markers in mature adipocytes stimulated by inflamed macrophages. LPL expression on prevention (A) and inhibition (B); LPL activity on prevention (C) and inhibition (D); FAS expression on prevention (E) and inhibition (F); triglyceride content on prevention (G) and inhibition (H). Untreated receive any treatment, PC receive only the CM. All experiments were performed in at least two independent trials. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). CM: conditioned media; PC: Positive control.

2.2.2 DTP and digested albumin and glutelin reduced inflammatory markers in prevention and inhibition of induced inflammation. NF- κ B expression was reduced only by DTP in the prevention treatment approach (-48.4%; $p < 0.05$) (**Figure 6A and Supplementary Figure 10**). During inhibition, any sample showed effect for this marker ($p > 0.05$) (**Figure 6B and Supplementary Figure 11**). MCP-1 secretion was reduced -35.8% and -42.8%, respectively by digested albumin and glutelin during the prevention process ($p < 0.05$) (**Figure 6C**). However, any protein reduced monocytes chemoattractant protein-1 (MCP-1) during inhibition of induced inflammation ($p > 0.05$) (**Figure 6D**). Every protein reduced TNF- α secretion in prevention (-51.6% to DTP, -66.6% to digested albumin, and -70.9% to digested glutelin; $p < 0.05$) (**Figure 6E**), and only digested albumin and glutelin reduced the secretion of this cytokine on inhibition of already produced inflammation (-53.8 to digested albumin and -77.9% to digested glutelin; $p < 0.05$) (**Figure 6F**).

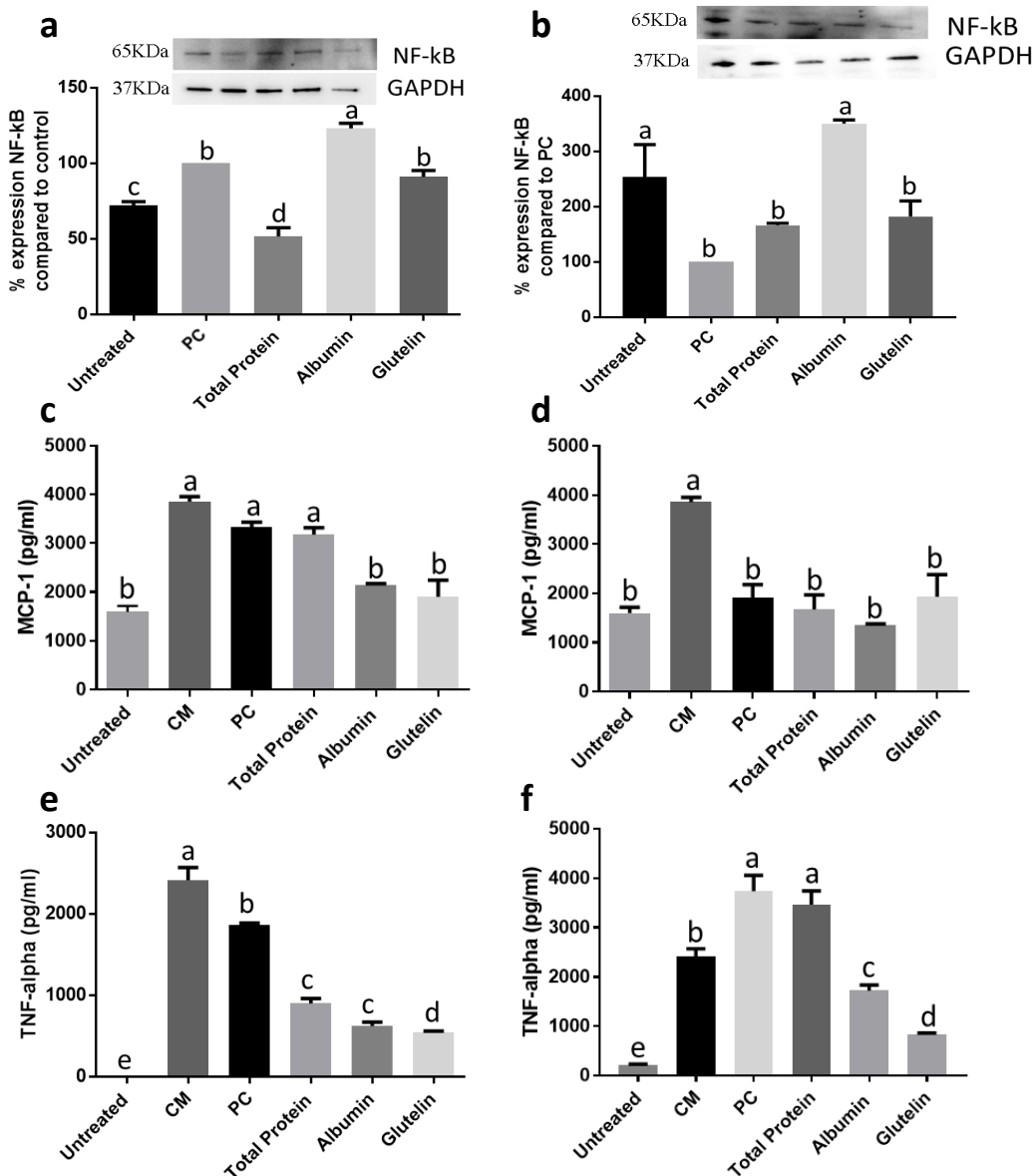


Figure 6. Effect of digested total protein and digested albumin and glutelin from chia seeds to prevent and inhibit the inflammation in mature adipocytes stimulated by inflamed macrophages. NF- κ B expression on prevention (A) and inhibition (B); MCP-1 secretion on prevention (C) and inhibition (D); TNF- α secretion on prevention (E) and inhibition (F). Untreated receive any treatment, PC receive only the CM. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). CM: conditioned media; NF- κ B: Factor nuclear kappa B; GAPDH: glyceraldehyde 3-phosphate; MCP-1: monocyte chemoattractant protein 1; TNF- α : Tumor necrosis factor alpha; PC: Positive control.

In preventing the inflammation process, DTP and digested albumin and glutelin reduced iNOS expression, mainly digested glutelin which reduced (-75%) the expression of iNOS ($p<0.05$) (**Figure 7A and Supplementary Figure 10**). Only DTP reduced iNOS expression (-52.1%) in the inhibition of induced inflammation ($p<0.05$) (**Figure 7B and Supplementary Figure 11**). NO secretion was reduced by every protein in prevention of inflammation ($p<0.05$) (**Figure 7C**). However, on inhibition just DTP and digested albumin showed effect ($p<0.05$) (**Figure 7D**). The COX-2 expression was reduced by every protein in prevention (-64.5%, DTP; -64.8%, digested albumin; -85.3%, digested glutelin; $p<0.05$) (**Figure 7E and Supplementary Figure 10**) and just by DTP (-66.4%) on inhibition of inflammation ($p<0.05$) (**Figure 7F and Supplementary Figure 11**). Secretion of PGE2 was also reduced by every sample in the prevention process ($p<0.05$) (**Figure 7G**) and digested albumin reduced PGE2 secretion (-65.5%) on inhibition ($p<0.05$) (**Figure 7H**). The secretion of cytokines had no changes in comparison with the PC, in both, prevention and inhibition of induced inflammation ($p>0.05$) (**Supplementary Table 1**).

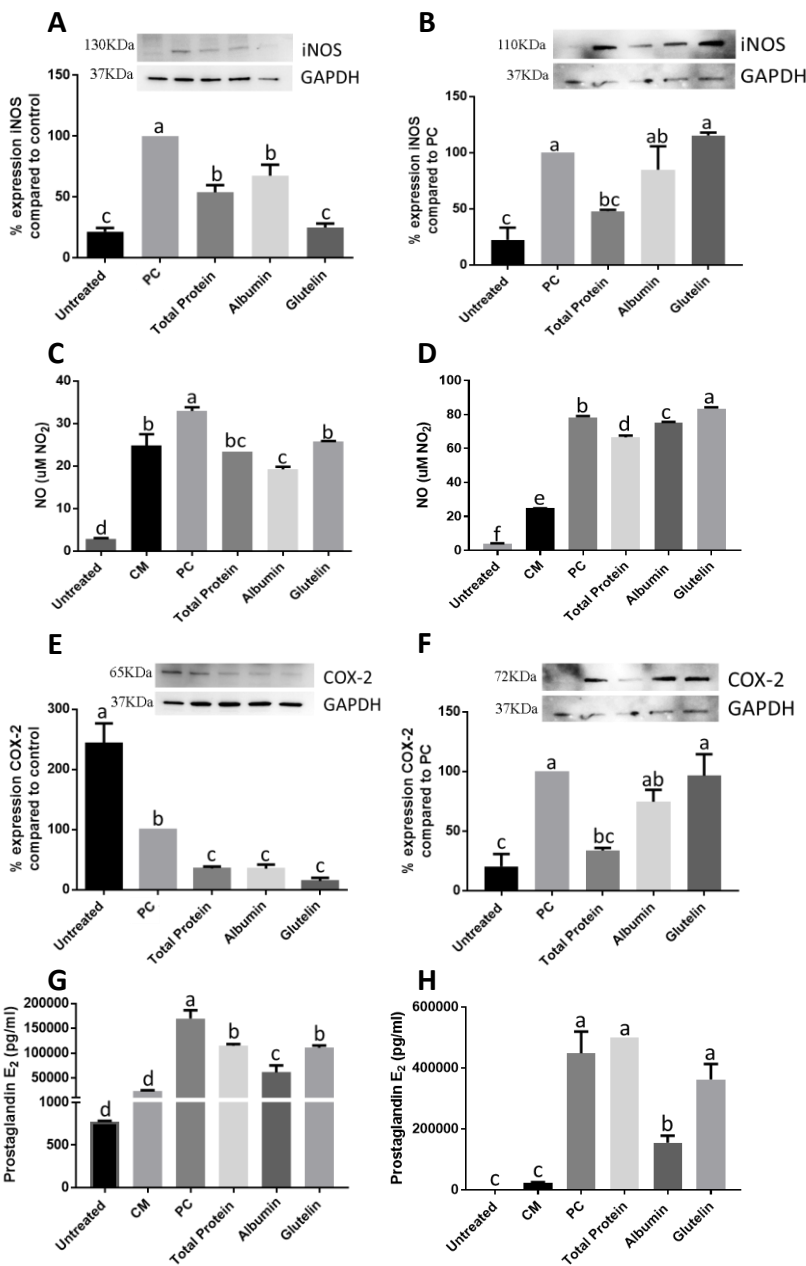
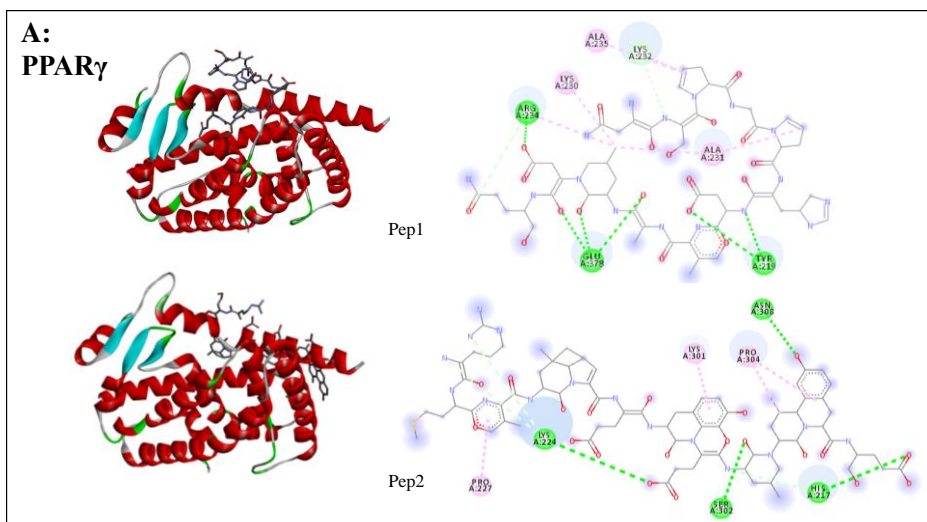


Figure 7. Effect of digested total protein and digested albumin and glutelin from chia seeds to prevent and inhibit the inflammation in mature adipocytes stimulated by inflamed macrophages. iNOS expression on prevention (A) and inhibition (B); NO secretion on prevention (C) and inhibition (D); COX-2 expression on prevention (E) and inhibition (F); PGE₂ secretion on prevention (G) and inhibition (H). Untreated receive any treatment, PC receive only the CM. All experiments were performed in at least two independent trials run with triplicate data points. Different letter per column means statistically different among the proteins by ANOVA and *post-hoc* Tukey-test ($p < 0.05$). CM: conditioned media; iNOS: inducible nitric oxide synthase; GAPDH: glyceraldehyde 3-phosphate; NO: nitric oxide; COX-2: cyclooxygenase2; PGE₂: prostaglandin 2; PC: Positive control.

2.3 *In silico* interaction of pure peptides with PPAR γ , FAS, and MAGL.

Peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) showed interactions with enzymes FAS and monoacylglycerol lipase (MAGL) and PPAR γ receptor, related to the adipogenesis process. Pep2 showed the highest interaction with PPAR γ by lowering the estimated free energy (EFE) (-6.9 kcal/mol) (**Figure 8A**) and with MAGL by also lowering EFE (-7.3 kcal/mol) (**Figure 8B**). However, Pep1 had the highest interaction with FAS (-7.3 kcal/mol) (**Figure 8C**). Other identified peptides in chia seed, peptides TGPSPTAGPPAPGGGTH and YLGAHPGTAN, both from digested albumin, showed the highest interaction with PPAR γ (-9.1 kcal/mol) and MAGL (-7.8 kcal/mol), respectively. The peptide APSPPVLGPP from DTP, showed the highest interaction with FAS (-9.8 kcal/mol) (**Supplementary Table 2**).



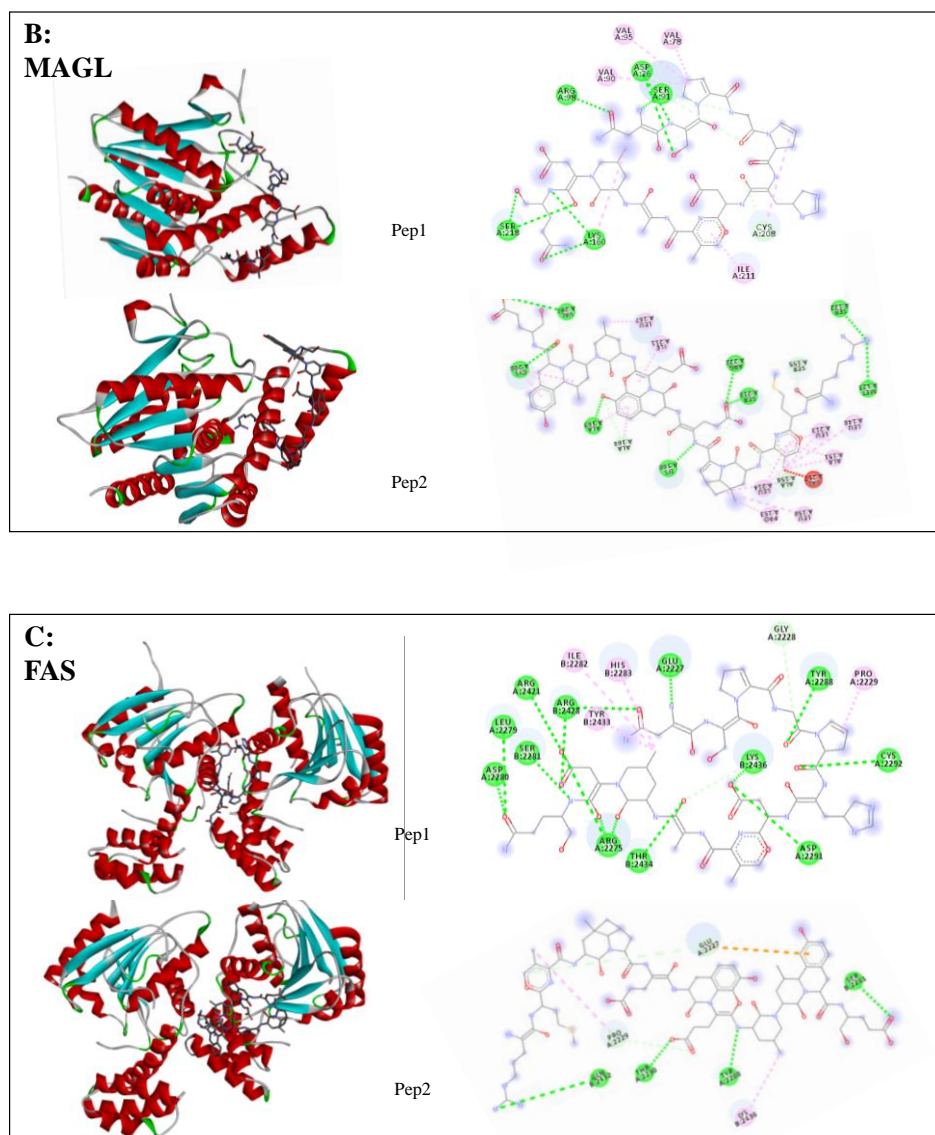


Figure 8. The *in silico* interaction of the peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) found in digested glutelin, with PPAR γ (A) MAGL (B) and FAS (C). These analyzes were performed by AutoDock Vina[®] and visualized by Discovery Studio 2016 Client[®].

3 DISCUSSION

This is the first study that shows the effects of digested proteins from chia seed, including total protein and its fractions, albumin and glutelin, on adipogenesis and inflammation of adipose tissue; markers of obesity and its complications. This investigation shows that protein fractions,

generated by digestion of chia proteins and pure peptides can prevent adipogenesis by reducing markers related with this process, and on the development of *in vitro* induced inflammation of adipose tissue. Although no study has evaluated the digested proteins and their peptides from chia seeds on markers related to obesity, some studies evaluated the whole chia seed in animal and clinical trials and found promising results against obesity [28].

In obesity, adipose tissue undergoes expansion which may ultimately compromise its function [5]. Mainly PPAR γ , considered as the ‘master regulator’ of adipogenesis, controls this expansion and its expression is sufficient to induce adipocyte differentiation. Furthermore, PPAR γ is required for maintenance of this differentiated state [4,29]. In the present research, DTP, digested albumin and glutelin, and pure peptides, reduced the expression of PPAR γ during the adipogenesis process, preventing the formation of adipocytes. In addition, DTP reduced the expression of PPAR γ in mature adipocytes exposed to media from inflamed macrophages, showing their efficacy to reverse the adipogenesis process. These results can be related to a study with rats fed a high-fat diet simultaneously fed with chia seed during 3 months which showed a reduction of PPAR γ expression on epididymal adipose tissue [30].

The PPAR γ expression can be stimulated by other factors such as the SREBP1 [31]. The expression of this protein was reduced, in the present research, when DTP, digested albumin and glutelin, or Pep2 were added to the cells during adipogenesis development. SREBP1 also mediates the induction of lipogenesis by insulin in adipocytes, together with PPAR γ , the expression of proteins, such as aP2, FAS, LPL [4,32]. LPL is a key enzyme in lipid transport and metabolism and plays a crucial role in human lipid homeostasis and energy balance. LPL is responsible for catalyzing lipolysis of triglycerides in lipoproteins, as very low-density lipoproteins (VLDL), providing free fatty acids (FFAs) which are used for lipid storage,

suggesting a role for LPL in initiation and development of obesity, becoming a marker for adipocyte differentiation ^[33] .

In this study, LPL activity and expression were reduced when DTP, digested albumin and glutelin, and pure peptides were added during adipogenesis and with DTP treatment in mature inflamed adipocytes. These results show the effectivity of the treatments, especially DTP, to reduce markers of adipogenesis. The content of triacylglycerides and Oil Red O, a differentiation parameter ^[29], was reduced drastically by every treatment when DTP, digested albumin and glutelin or pure peptides were added during the adipogenesis process, and by DTP and digested glutelin in prevention of inflammation. However, digests from albumin increased the expression of LPL and the levels of triacylglycerides. This is probably due to inflammation, mainly the presence of TNF- α , which blocks transcription of LPL ^[34]. Reduction of obesity was observed in overweight and diabetics adults that consumed chia seeds during 24 weeks ^[35] and for overweight and obesity adults during 12 weeks ^[36] . Furthermore, rats fed with chia seeds reduced the visceral adiposity index and decreased the retroperitoneal and omental fat depositions ^[37] . These effects can be associated with the antiadipogenic and antilipogenic effects of digested chia proteins observed in the present study.

Another marker stimulated by SREBP1 and PPAR γ was FAS. This enzyme catalyzes *de novo* synthesis of fatty acids by production of palmitate from malonyl-CoA and acetyl-CoA. Palmitate is subsequently used as the precursor for the synthesis of complex lipid molecules that can be used for energy storage, membrane assembly and repair, and secretion in the form of lipoprotein triglycerides ^[38,39] . Moreover, inhibition of FAS activity may block adipocyte differentiation and reduce adipogenesis ^[40] . In this research, every sample was able to reduce FAS expression on preventing adipogenesis. *Wistar* rats fed a high sucrose diet and treated with chia seeds, during 3

weeks or 5 months, reduced SREBP-1 expression, plasmatic triacylglycerol, liver triacylglycerol and FAS activity ^[41] .

Furthermore, adipocytes are not only directed to lipid storage but also to secretory cells that produce proinflammatory cytokines and adipokines, such as TNF- α , IL-6, MCP-1, adiponectin, resistin, among others. These molecules increase the local inflammation and stimulate monocytes into adipose tissue. This condition activates their differentiation to macrophages that begin to secrete pro-inflammatory cytokines and chemokines that drive to a chronic low-grade inflammatory state with systemic effects ^[29,32,42] . This process may lead to activation of NF- κ B and downstream pro-inflammatory genes involving TNF- α , IL-6 and MCP-1 and others ^[43] . In this study, the expression of NF- κ B was reduced by DTP, digested albumin and glutelin and pure peptides when added during the adipogenesis process. In mature adipocytes, NF- κ B had its expression reduced only by DTP. Despite results observed regarding NF- κ B expression, digested albumin, glutelin and Pep2 decreased TNF- α secretion when preventing adipogenesis. However, secretion of TNF- α was reduced by every treatment, while MCP-1 only by digested albumin and glutelin when preventing inflammation. When adipocytes received the digests from proteins after inflammation was established, only TNF- α had its expression reduced by digested albumin and glutelin. In addition, digested glutelin and pure peptides reduced IL-6 secretion when preventing adipogenesis and this was the only effect observed regarding cytokines in all experiments. The cytokines are regulated not only by NF- κ B pathway but also the janus kinase (JAK), signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3'-kinases (PI3K) pathways. These have been associated with the production of cytokines ⁴⁴ and may have regulated the secretion of these small

molecules in our study. It has been observed that rats fed with chia seeds during 35 days experienced an increase in levels of IL-10, an anti-inflammatory cytokine ^[45].

Another enzyme related with inflammation is COX-2 which catalyzes the conversion of arachidonic acid to prostaglandin H₂, a precursor of other inflammation mediators, as prostaglandin E₂ (PGE₂) ^[46]. This event was observed when the adipocytes received digested albumin and glutelin to prevent adipogenesis, and with every sample when preventing inflammation. In this case, both digested proteins reduced COX-2 expression and PGE₂ secretion. However, pure peptides did not change the COX-2 expression but reduced PGE₂ secretion on preventing adipogenesis. These events were perhaps due to modulation by other proteins related with the COX-2 pathway, as p38 mitogen-activated protein kinase (p38MAPK) ^[47] and cAMP-PKA-AKAP- pathway ^[48].

iNOS is another enzyme related to inflammation ^[49] that was evaluated in this research. Obesity increases expression of iNOS, which catalyzes NO synthesis and contributes to metabolic deregulation in adipocytes as well as stimulation of PPAR γ expression ^[50]. In this investigation, iNOS expression and NO secretion were reduced by every treatment when preventing inflammation. In the inhibition of inflammation, just DTP reduced iNOS expression and NO secretion; also, albumin reduced NO levels. The effectiveness of chia seed to reduce plasma nitrite was observed in hypertensive and overweight adults that ingested 35g/day of chia seeds ^[51].

Associated with the effectiveness of digested proteins on inflammation, every treatment reduced production of ROS on prevention of adipogenesis and inflammation. ROS can be generated into adipocytes from certain saturated free fatty acids (laurate, myristate, and palmitate) ^[52] and by chronic stress, glucocorticoids, mineralocorticoids, and angiotensin-II ^[53]. ROS can induce

insulin resistance and recruitment of immune cells into adipose tissue, increasing adipose tissue inflammation ^[52]. In addition, ROS increases secretion of leptin, MCP-1, IL-6, and TNF- α by adipocytes and decreases adiponectin production ^[53]. These facts may explain the poor results of inhibition with digested proteins in inflamed adipocytes. Moreover, the effectiveness of complete chia seed has been observed by the increase of IL-10 ^[45], an anti-inflammatory cytokine, as well as the antioxidant capacity in rats ^[54,55].

Based on the results, **Figure 9** presents a proposed mechanism of the effects of DTP, digested albumin, glutelin, and pure peptides in the three experimental approaches analyzed in this paper: 1) prevention of adipogenesis (experiments I and II), 2) prevention of inflammation (experiment III), and 3) inhibition of established inflammation (experiment IV). In summary, the different treatments had effects in every marker related to fibroblast differentiation in adipocytes, thus preventing the adipogenesis process. In addition, DTP and digested glutelin were most effective on reducing markers related with adipogenesis, in both, prevention and inhibition of inflammation. Moreover, DTP, digested albumin and glutelin were most effective on prevention of inflammation, DTP showing the best results.

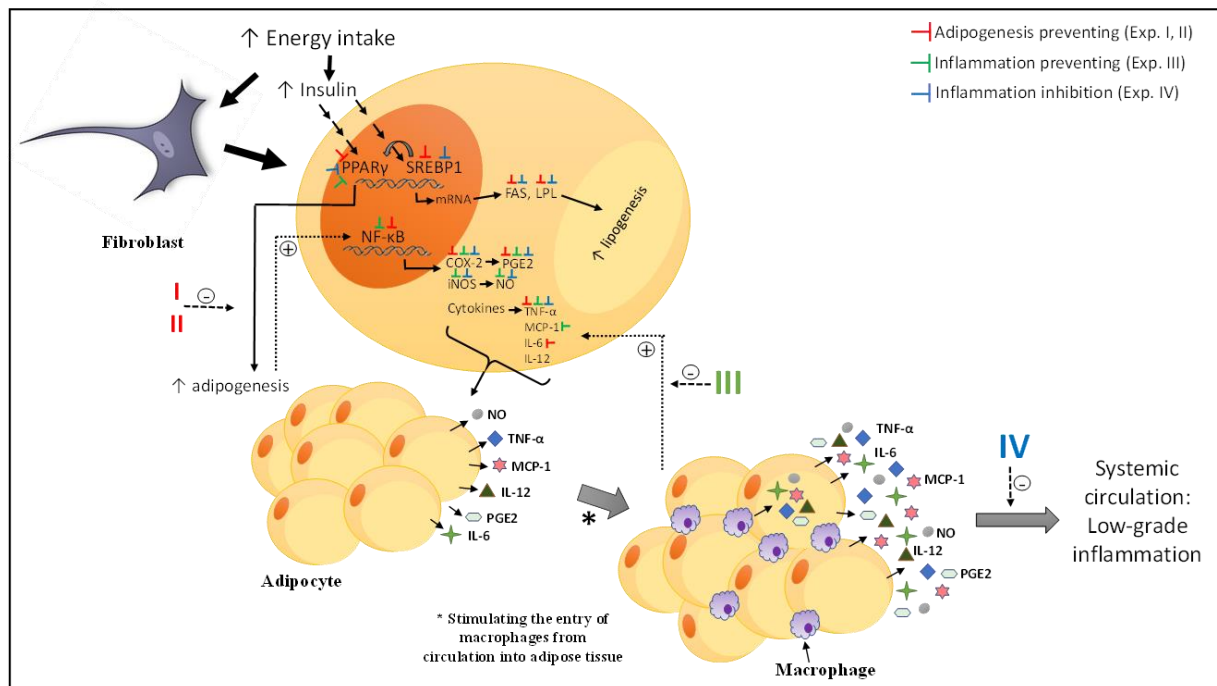


Figure 9. Proposed mechanism of the effect of DTP, digested albumin, digested glutelin, or pure peptides in the prevention of adipogenesis (experiments I and II, the red symbols), in the prevention of inflammation (experiments III, the green symbols), and inhibition of establishing inflammation (experiments IV, the blue symbols). **Red symbols:** On prevention of adipogenesis, DTP, digested albumin, glutelin, and pure peptides reduced the expression of PPAR γ , SREBP1, FAS, LPL, NF- κ B, COX-2, and iNOS and the secretion of PGE2, TNF α and albumin and pure peptides decrease the levels of IL-6. **Green symbols:** On prevention of inflammation, DTP, digested albumin and glutelin decreased the expression of PPAR γ , COX-2, and iNOS, and the NO, PGE2, TNF α secretion. DTP reduce the NF- κ B expression. Digested albumin and glutelin reduced the MCP-1 secretion. **Blue symbols:** On inhibition of inflammation, DTP, digested albumin and glutelin decreased the expression of PPAR γ and SREBP1. DTP reduce the expression of LPL, FAS, iNOS, COX-2, the triacylglycerol content, lipase activity, NO secretion. Digested albumin reduced the lipase activity and the secretion of NO, PGE2, and TNF α . Digested glutelin decreased the expression of LPL and FAS, and TNF α secretion. DTP: digested total protein; PPAR γ : peroxisome-proliferator-activated receptors gamma, SREBP1: sterol regulatory element-binding protein-1, FAS: fatty acid synthase, LPL: lipoprotein lipase, NF- κ B: factor nuclear kappa B, COX-2: ciclooxigenase-2, iNOS: inducible nitric oxide synthase, MCP-1: Monocyte chemoattractant protein-1, NO: nitric oxide, PGE2: prostaglandin E2, IL-6: Interleukin. 6.

We are reporting, for the first time, an evaluation of the effects of digested proteins from chia seeds on preventing adipogenesis and also the prevention and inhibition of inflammation on adipocytes, and additionally, suggested a mechanism of action. This study showed that digested total protein, digested albumin, digested glutelin, or pure peptides, NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2), had a significant effect on preventing adipogenesis by reducing the expression of PPAR γ . Consequently, all proteins related to this pathway were less expressed, reducing the expression of markers related to the differentiation of fibroblasts into adipocytes and the accumulation of lipids inside the cells. Furthermore, in mature adipocytes, the use of digested proteins was more effective to reduce adipogenesis and lipogenesis during prevention. Digested total protein and digested albumin and glutelin showed the best results against markers related to prevention of inflammation. The inhibition of PPAR γ blocked NF- κ B pathway, then inflammation was reduced. When the digested proteins were used to inhibit inflammation already establish, DTP stood out with the best results.

In summary, these findings suggest the ability of proteins and peptides from chia seed to block PPAR γ , causing a regulation of both, adipogenesis and inflammation. These results are promising and allow understanding the mechanisms related to the beneficial effects of chia seed consumption against obesity demonstrated in other studies with rats and humans. In addition, there is a need for clinical studies with digested proteins from chia seed to confirm the results found in this investigation. These results also suggest the value of digested chia protein fractions, including pure peptides, against adipogenesis and its associated complications by regulation of PPAR γ .

4. MATERIAL AND METHODS

4.1 Materials. 3T3-L1 and RAW 264.7 cells were purchased from American Type Culture Collection (ATCC®, Manassas, VA). The Dulbecco's modified Eagle medium (DMEM) was purchased from Corning cellgro® (Manassas, VA), fetal bovine serum (FBS), newborn calf serum (NCS), and penicillin-streptomycin (100×) were obtained from Gibco Life Technologies (Grand Island, NY). Primary antibodies NF-κB p65 (sc-8008), FAS (sc-48357), LPL (sc-373759), PPAR γ (sc-7273), SREBP-1 (sc-365513) and RIPA Lysis Buffer System were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies COX-2 (MA5-14568), GAPDH (MA5-15738), 2',7'-dichlorofluorescein diacetate (H2DCFDA) (D339), and the pure peptides were obtained from Thermo Fisher Scientific (Rockford, IL). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

4.2 Preparation of digested total protein and protein fractions. The chia seeds obtained from Rio Grande do Sul/Brazil were prepared as described by Orona-Tamayo et al. ^[22] with modifications. Total protein was extracted by alkaline precipitation, as detailed in Grancieri, Martino, de Mejia ^[13]. The storage protein fractions albumin and glutelin were obtained by solubility as according to Osbore ^[56] classification by Orona-Tamayo et al. ^[22] method and detailed in Grancieri, Martino, de Mejia ^[13]. All proteins were freeze-dried and submitted to simulated gastrointestinal digestion using the adapted procedure outlined by Megías et al. ^[57]. Briefly, each sample was suspended in deionized water (1:20 g: ml) and digested with pepsin (pH 2.0) followed by pancreatin (pH 7.5), during 1h at 37°C, each one. The process was stopped by heating in water bath (75 °C, 20 min) and the proteins were centrifuged twice at 20,000 g, 15 min, 4 °C. The supernatant was dialyzed using a 100-500 Da molecular weight cut-off membrane

(Spectra/Por®, Biotech CE Membrane), freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$ until analysis (Supplementary Figure 1).

4.3 Identification, characterization of potentially bioactive peptides. The resulting peptides from DTP (digested total protein) and digested albumin and glutelin were analyzed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) using a Q-ToF Ultima mass spectrometer (Waters, Milford, MA), according to Mojica, Chen, & de Mejia^[58]. The sequence of amino acids was identified using MassLynx V4.1 software (Waters Corp., Milford, MA). The peptides with more than 90% of probability had their biological activity predicted by BIOPEP database (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>, accessed on February 27, 2018).

Peptides NSPGPHDVALDQ and RMVLPEYELLYE from glutelin fraction were selected by their high relative abundance on chromatogram (34.6% and 92.2%, respectively), antioxidant activity (predicted by BIOPEP database), low hydrophobicity and the presence of antioxidant and certain hydrophobic amino acids. The peptide NSPGPHDVALDQ was called as Pep1 and RMVLPEYELLYE as Pep2. The pure peptides were purchase from Thermo Scientific® with > 98% of purity.

4.4 Cell culture. 3T3-L1 preadipocytes were grown in DMEM supplemented with 10% NCS (v/v) and 1% penicillin/streptomycin (v/v). The cells were seeded at a density of 6,000 viable cells/cm² and differentiated in adipocytes according to Zebisch, Voigt, Wabitsch, & Brandsch^[59]. Briefly, 3T3-L1 cells were seeded (day 1) and differentiation stimulated by incubation after 48 h with DMEM containing 10% FBS (v/v), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25

μM dexamethasone, 2 μM rosiglitazone and 1 $\mu\text{g}/\text{mL}$ insulin (day 3). After 48 h, the medium was replaced with DMEM containing 10% (v/v) FBS and 1 $\mu\text{g}/\text{mL}$ insulin (day 5). At days 7, 8, and 10, the media was replaced with 10% FBS-DMEM when 80-90% of the cells exhibited mature adipocyte phenotype. The cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

RAW 267.4 monocytes cells were cultured in DMEM supplemented with 1% penicillin/streptomycin (v/v) and 10% FBS (v/v). The cells were seed at 2.5×10^5 in a six-well plate, and the total volume was adjusted to 2 ml with growth medium and incubated to 24 h at 37 °C. After incubation, the cells were treated with the lipopolysaccharide (LPS) (1 μM) to 24 h and the media collected and designated as conditioned media (CM).

The efficiency of the cells growing in the presence of 1 mg/mL of DTP, digested albumin, digested glutelin, and 100 μM of each pure peptide was assessed by aqueous solution CellTiter 96 one proliferation assay kit- [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; MTS (Promega Corporation, Madison, WI, USA).

4.5 Experimental design and treatment of cells with digested total protein, digested albumin, glutelin or pure peptides. Four experiments were conducted in order to analyze the effects of either DTP, or digested albumin or digested glutelin, or pure peptides on adipogenesis and inflammation on adipose tissue. A preventive (before) and inhibition (after) treatment approaches regarding exposure to induced inflammation by the addition of conditioned medium (CM) from inflamed macrophages:

I- *Effects of DTP or digested albumin or glutelin in 3T3-L1 adipocytes during the differentiation process:* 3T3-L1 cells were seeded and differentiated as described above, and the DTP or digested albumin or glutelin at 1 mg/ml were added to the culture media during the differentiation process (days 3, 5, 7, 8, and 10).

II- *Effects of pure peptides in 3T3-L1 adipocytes during the differentiation process:* 3T3-L1 cells were seeded and differentiated as described above. The pure peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) at 100 μ M were added to the culture media during the differentiation process (days 3, 5, 7, 8, and 10).

III- *Effects of DTP or digested albumin or glutelin before (prevention) induced inflammation in mature adipocytes by addition of conditioned media from inflamed macrophages.* At 10th and 11th days of adipocyte differentiation, CM replaced 50% of grown media from inflamed macrophages together with DTP or digested albumin or glutelin at 1 mg/ml for 24 h.

IV- *Effects of DTP or digested albumin or glutelin after (inhibition) induced inflammation in mature adipocytes by addition of conditioned media from inflamed macrophages (CM).* At 10th and 11th days of adipocyte differentiation, CM from inflamed macrophages replaced 50% of grown media, and at days 12th and 13th DTP or digested albumin or glutelin at 1 mg/ml were added in addition to the grow media CM.

After each treatment, the growth medium and cell lysates were collected and frozen at -80 °C until analysis. All experiments were performed at least in duplicate.

4.6 Effect of either digested total protein, digested albumin or digested glutelin or pure peptides in nitric oxide and reactive oxygen species production. The accumulation of nitric oxide in the culture supernatant was measured by mixing 100 μ L of the culture supernatant with the same volume of the Griess reagent and incubated at room temperature for 10 min. The absorbance was determined at 540 nm in a microplate reader (BioTek®, Winnoski, USA) [60]. The concentration of nitrite was established from a standard curve of sodium nitrite (NaNO_2) ($y = 0.0087x + 0.0027$, $R^2 = 0.99$).

To analyze ROS production, the cells were seeded in dark 96 well plate in triplicate (as 3.4 item). At the last day of each experiment (3.5 section), the media was replaced by DCFDA in grown media (50 μ M/ total volume) and kept for 1 h in an incubator at 37 °C in 5% CO₂. 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.

4.7 Effect of either digested total protein, or digested albumin, glutelin or pure peptides on cellular lipid accumulation. The 3T3-L1 cells were seeded in 12-well plates and induced to differentiation. At the last day of each experiment (4.5 section), the cells were fixed with 10% formalin, washed with 60% isopropanol and a working solution of Oil Red O was added to each well (0.35% w/v Oil Red O in isopropanol overnight). Then, pictures were taken, and Oil Red O staining was eluted with 100% isopropanol for detection at 510 nm in a plate reader ^[29].

4.8 Impact of either digested total protein, or digested albumin, glutelin or pure peptides in PGE2, TNF- α , MCP-1, and cytokines secretion. Commercial kits were used to analyze prostaglandin 2 (PGE2) (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 and according Grancieri, Martino, and de Mejia ^[27]. The cell culture supernatants were diluted 1:5 (v/v, sample: buffer) for experiments I and II and 1:25 (v/v, sample: buffer) for experiments III and IV. The amount of PGE2 ($y = -0.2766x + 0.3636$, $R^2 = 0.99$), 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 were run at the same time as treatments. Absorbance was determined at 450 nm and results were expressed in pg/mL.

4.9 Influence of either digested total protein, or digested albumin, glutelin or pure peptides in the expression of proteins related to adipogenesis and inflammation processes.

The expression of proteins related with the adipogenesis and inflammation was measured by western blotting and according Grancieri, Martino, and de Mejia ^[27]. Cell culture were collected after each treatment and lysed with RIPA lysis buffer, sonicated, and added with Laemmli buffer (Bio-Rad) containing 5% β -mercaptoethanol, then the cell lysed were frozen at -80 °C. Protein concentration was quantified using RC-DC Assay (Bio-Rad) and 20 μ g loaded in 4–20% Tris–HCl gels (Bio-Rad) for protein separation. Proteins were transferred to a PVDF membrane (polyvinylidene difluoride membrane, Hybond-P, Millipore, Billerica, MA, USA) and incubated overnight with respective primary antibodies (1:500) (COX-2, p65-NF, PPAR γ , FAS, LPL or SREBP1) at 4 °C. The membranes were incubated with secondary antibody for 2 h (if required) and the protein bands visualized with a GL 4000 Pro Imaging system (Carestream Health Inc.). Band intensity was normalized using GAPDH antibody. All analyses were performed in duplicate and results expressed in % of expression compared to positive control.

4.10 Effects of either digested total protein, or digested albumin, glutelin or pure peptides on inhibition of lipase activity and triglyceride content.

3T3-L1 cells were cultured and treated as in sections 4.5. On the last day of each experiment, the cells were collected with RIPA buffer and stored at -80 °C. Inhibition of lipase activity was determined using a lipase activity assay kit (Cayman Chemical Item No. 700640), following manufacturer's instructions. Results

were expressed as nmol/min/ml. Triglyceride content was analyzed using a triglyceride colorimetric assay kit (Cayman Chemical Item No. 10010303) according to manufacturer's instructions. Results were expressed as mg/dl.

4.11 Potential inhibitory interactions of PPAR γ , FAS, and MAGL by peptides from digested total protein, digested albumin or glutelin: *In silico* analyses. Interactions of generated peptides from DTP and digested albumin and glutelin, including Pep1 and Pep2, with PPAR γ , FAS, and monoacylglycerol lipase (MAGL) were evaluated by *in silico* analysis. The peptides were designed using Instant MarvinSketch (ChemAxon Ltd). The crystal structure files of PPAR γ , FAS, and MAGL were obtained from the Protein Data Bank (PDB: 5DSH, 2PX6, and 3PE6 respectively). Flexible torsions, charges, and grid size were assigned by AutoDock Tools and docking calculations were performed using AutoDock Vina ^[61]. The binding pose with the lowest binding energy (highest binding affinity) was selected as a representative image to be visualized in the Discovery Studio 2016 Client (Dassault Systemes Biovia Corp®).

4.12 Statistical analysis. Results were expressed as the mean \pm standard deviation (SD), and analyzed by *one-way* analysis of variance (ANOVA) and *post hoc* Tukey test. Differences were considered significant at $p < 0.05$. Data analyses were performed in triplicate, from at least two independent experiments, using GraphPad Prism 7.

ABBREVIATIONS

ACE-enzyme, angiotensin-converting enzyme; aP2, adipocyte fatty acid-binding protein; COX-2, cyclooxygenase-2; CVD, cardiovascular diseases; DMEM, Dulbecco's modified Eagle medium;

DTP, digested total protein; FAS, fatty acid synthase; FBS, fetal bovine serum; FFAs, providing free fatty acids; H2DCFDA, 2',7'-dichlorofluorescein diacetate; IL, interleukin; LPL, lipoprotein lipase; MAGL, monoacylglycerol lipase; MCP-1, monocytes chemoattractant protein-1; NCS; newborn calf serum; NO, nitric oxide; PPAR γ , proliferator-activated receptor gamma; ROS, reactive oxygen species; SREBP-1, sterol regulatory element-binding protein 1; TNF- α , tumor necrosis factor alpha; VLDL, low-density lipoproteins.

Data Availability

Availability of data and material: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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AUTHOR CONTRIBUTIONS

M.G.: major contributor in writing the manuscript, performed the experiments and analyzes, and created the figures and tables.

H.S.D.: critical analysis and helped with the editing process of the manuscript.

E.G.M: overall concept, editing of the manuscript, critical analysis, and provided scientific guidance throughout the study.

All authors critically revised the manuscript and gave their attention, conclusions, and final approval for submission.

ADDITIONAL INFORMATION

Conflict of interest: The authors declare that they have no conflict of interest.

Protein digests and pure peptides from chia seed prevented adipogenesis and its inflammation by inhibition of PPAR γ and NF- κ B pathways

Mariana Grancieri, Hercia Stampini Duarte Martino and Elvira Gonzalez de Mejia

Supplementary material

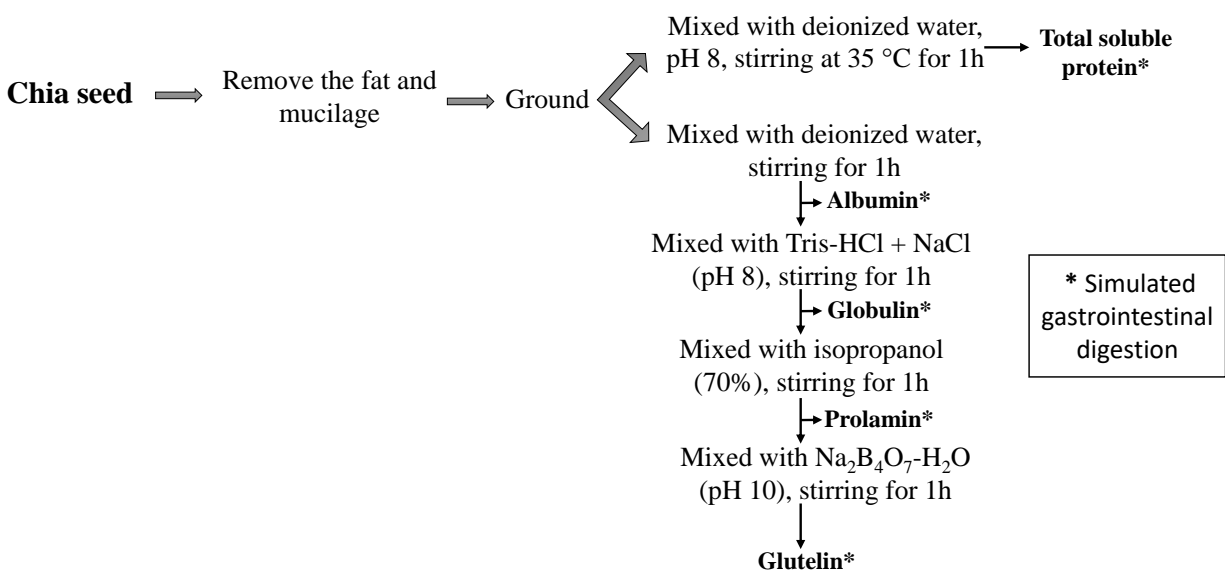


Figure 1. Samples preparation

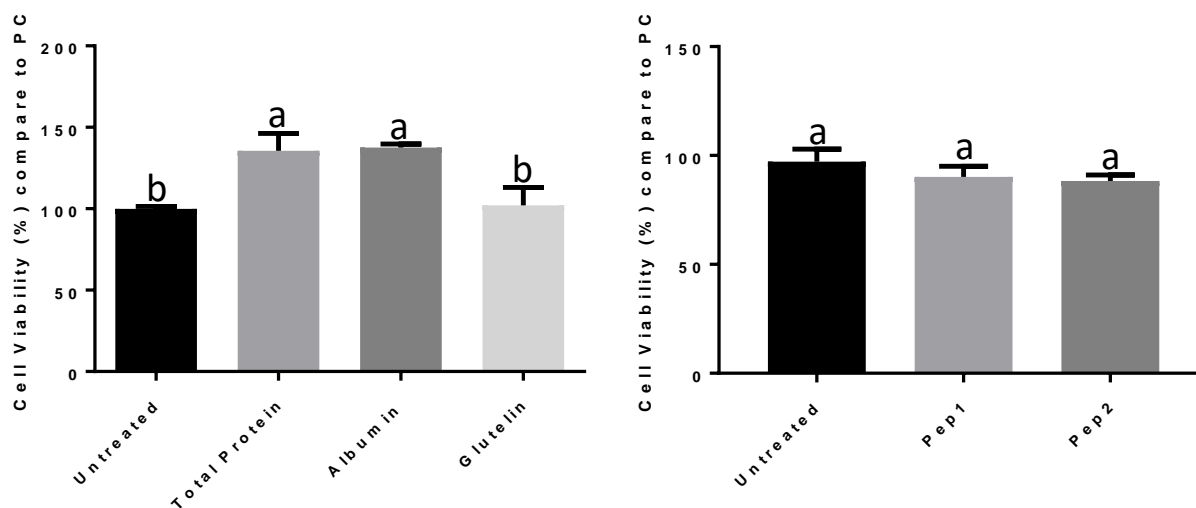


Figure 2. Effect of digested total protein (1 mg/ml), digested albumin and glutelin (1mg/ml) and the pure peptides Pep1 (NSPGPHDVALDQ) and Pep2 (RMVLPEYELLYE) (100 μ M) on 3T3-L1 cell viability. Analyzed by CellTiter 96 one proliferation assay kit- MTS (Promega Corporation, Madison, WI, USA).

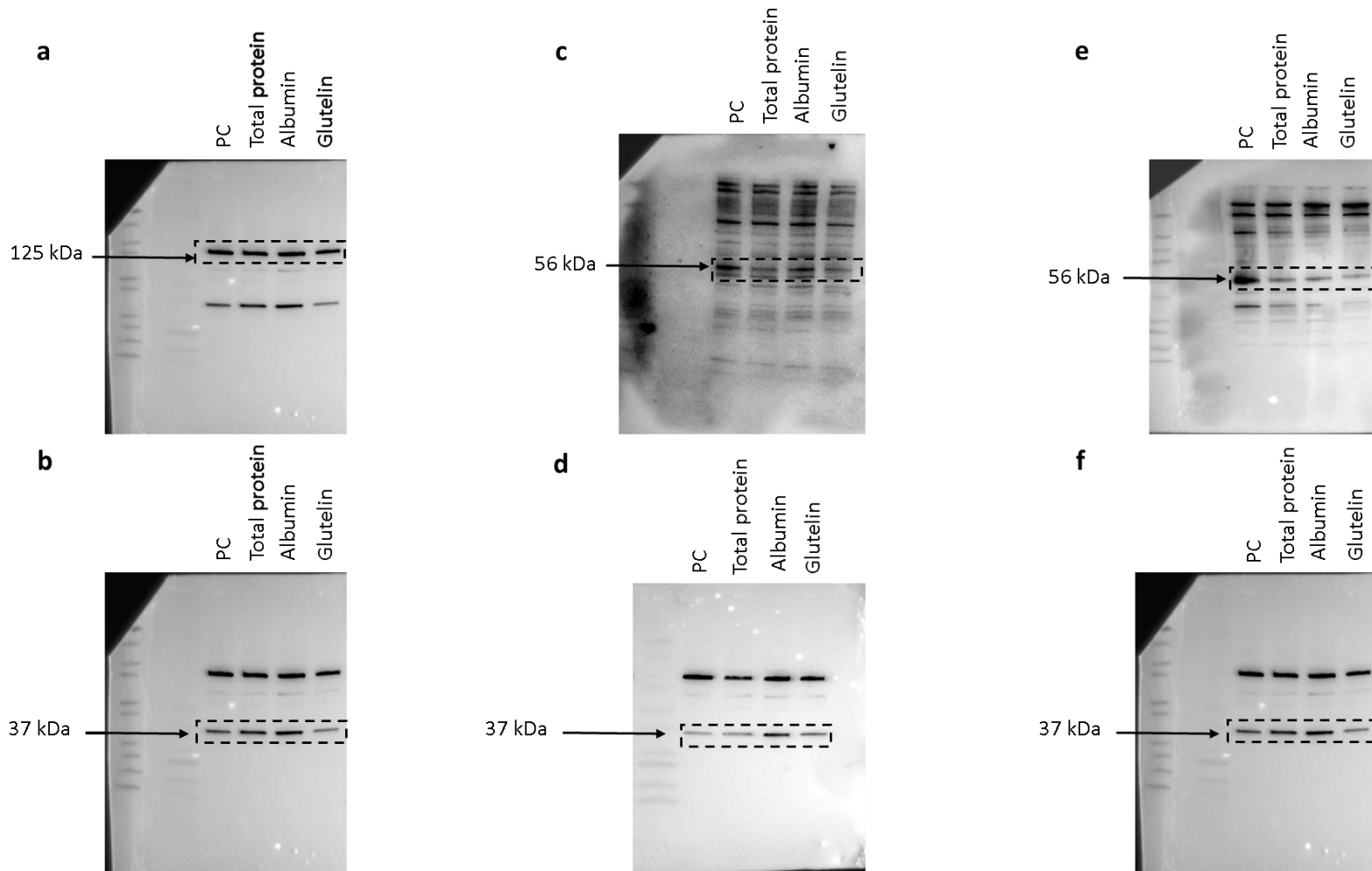


Figure 3. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 1 and 2. a) SREBP1; b) GAPDH of SREBP1; c) PPAR γ ; d) GAPDH of PPAR γ ; e) LPL; f) GAPDH of LPL. PC (positive control) received no treatment. All experiments were performed in at least two independent trials. SREBP: sterol regulatory element-binding protein 1; GAPDH: glyceraldehyde 3-phosphate; PPAR γ : peroxisome proliferator-activated receptor gamma ; LPL: lipase lipoprotein; PC: Positive control.

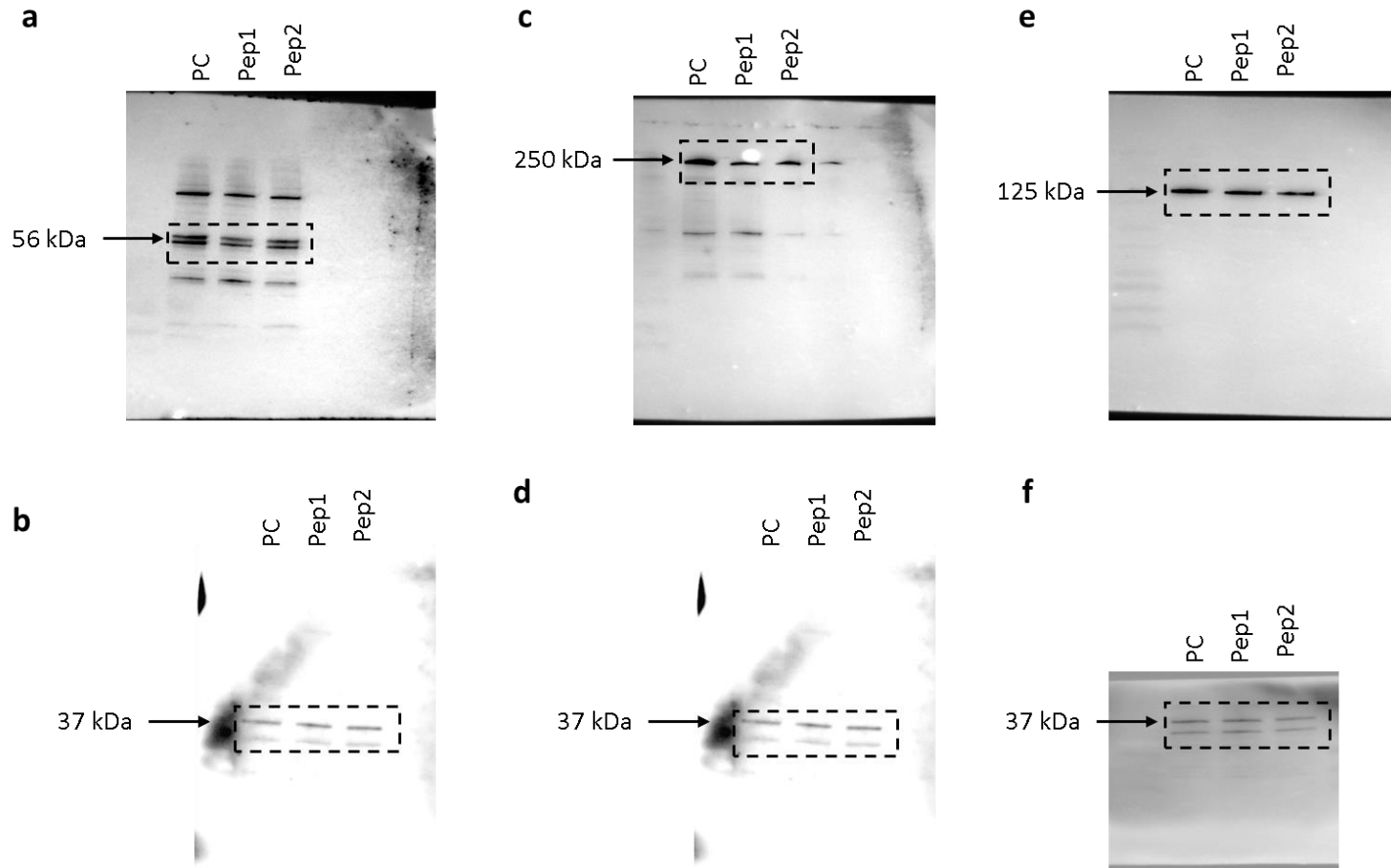


Figure 4. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 1 and 2. a) PPAR γ ; b) GAPDH of PPAR γ ; c) FAS; d) GAPDH of FAS; e) SREBP1; f) GAPDH of SREBP1. PC (positive control) received no treatment. All experiments were performed in at least two independent trials. PPAR γ : peroxisome proliferator-activated receptor gamma; GAPDH: glyceraldehyde 3-phosphate; FAS: fatty acid synthase; SREBP: sterol regulatory element-binding protein 1; PC: Positive control; Pep1: NSPGPHDVALDQ; Pep2: RMVLPEYELLYE.

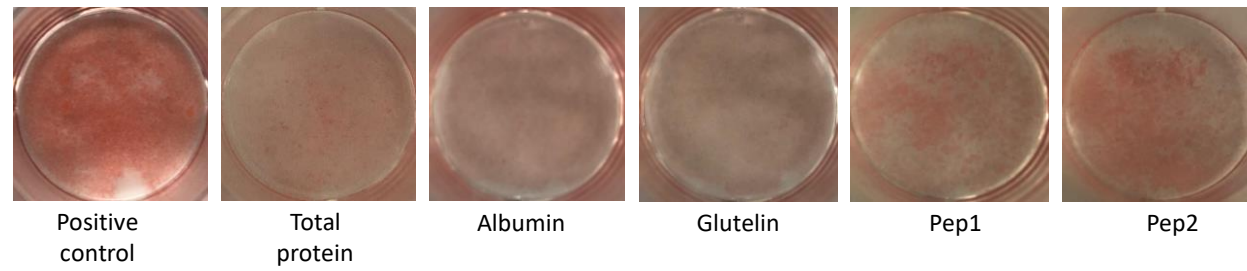


Figure 5. Effect of digested total protein (1 mg/ml), digested albumin and glutelin (1mg/ml) and the pure peptides (NSPGPHDVALDQ and RMVLPEYELLYE) (100 μ M) added during the differentiation of adipocytes on lipids accumulation.

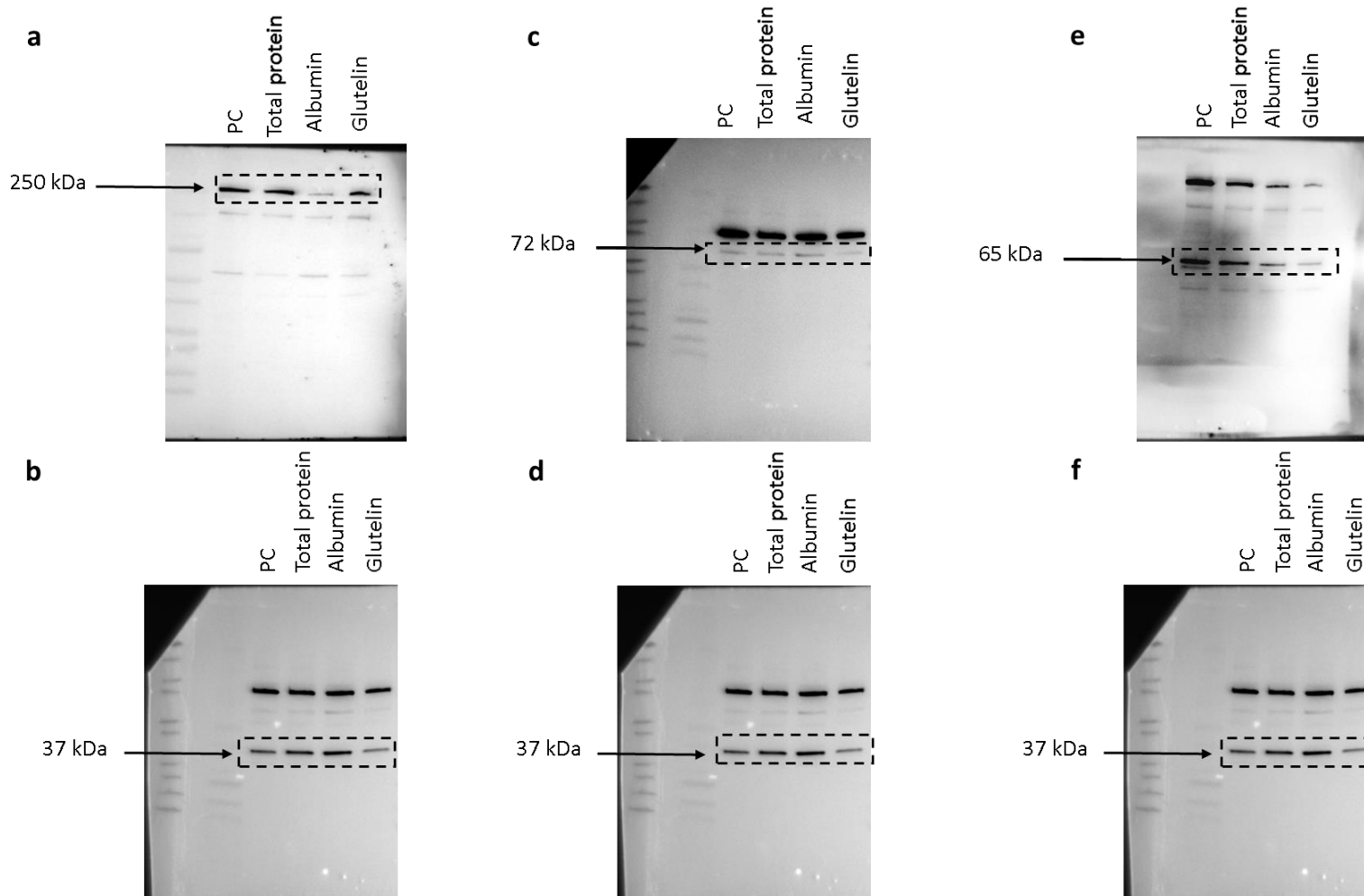


Figure 6. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 2 and 3. a) FAS; b) GAPDH of FAS; c) COX-2; d) GAPDH of COX-2; e) NF-κB; f) GAPDH of NF-κB. PC (positive control) received no treatment. All experiments were performed in at least two independent trials. FAS: fatty acid synthase; GAPDH: glyceraldehyde 3-phosphate; COX-2: cyclooxygenase-2; NF-κB: Factor nuclear kappa B; PC: Positive control.

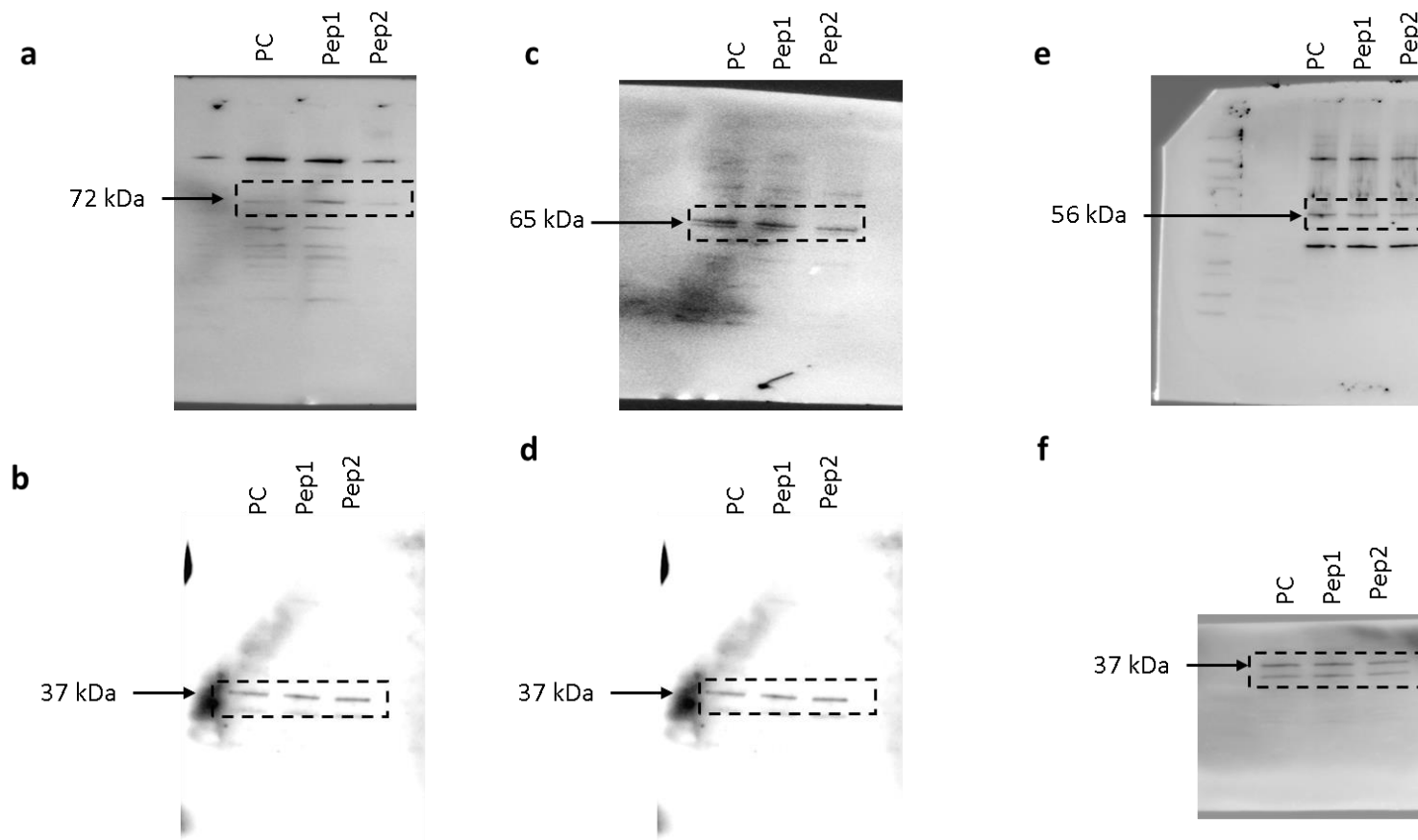


Figure 7. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 2 and 3. a) COX-2; b) GAPDH of COX-2; c) NF- κ B; d) GAPDH of NF- κ B; e) LPL; f) GAPDH of LPL. PC (positive control) received no treatment. All experiments were performed in at least two independent trials. COX-2: cyclooxygenase-2; GAPDH: glyceraldehyde 3-phosphate; NF- κ B: Factor nuclear kappa B; LPL: lipase lipoprotein; PC: Positive control; Pep1: NSPGPHDVALDQ; Pep2: RMVLPEYELLYE.

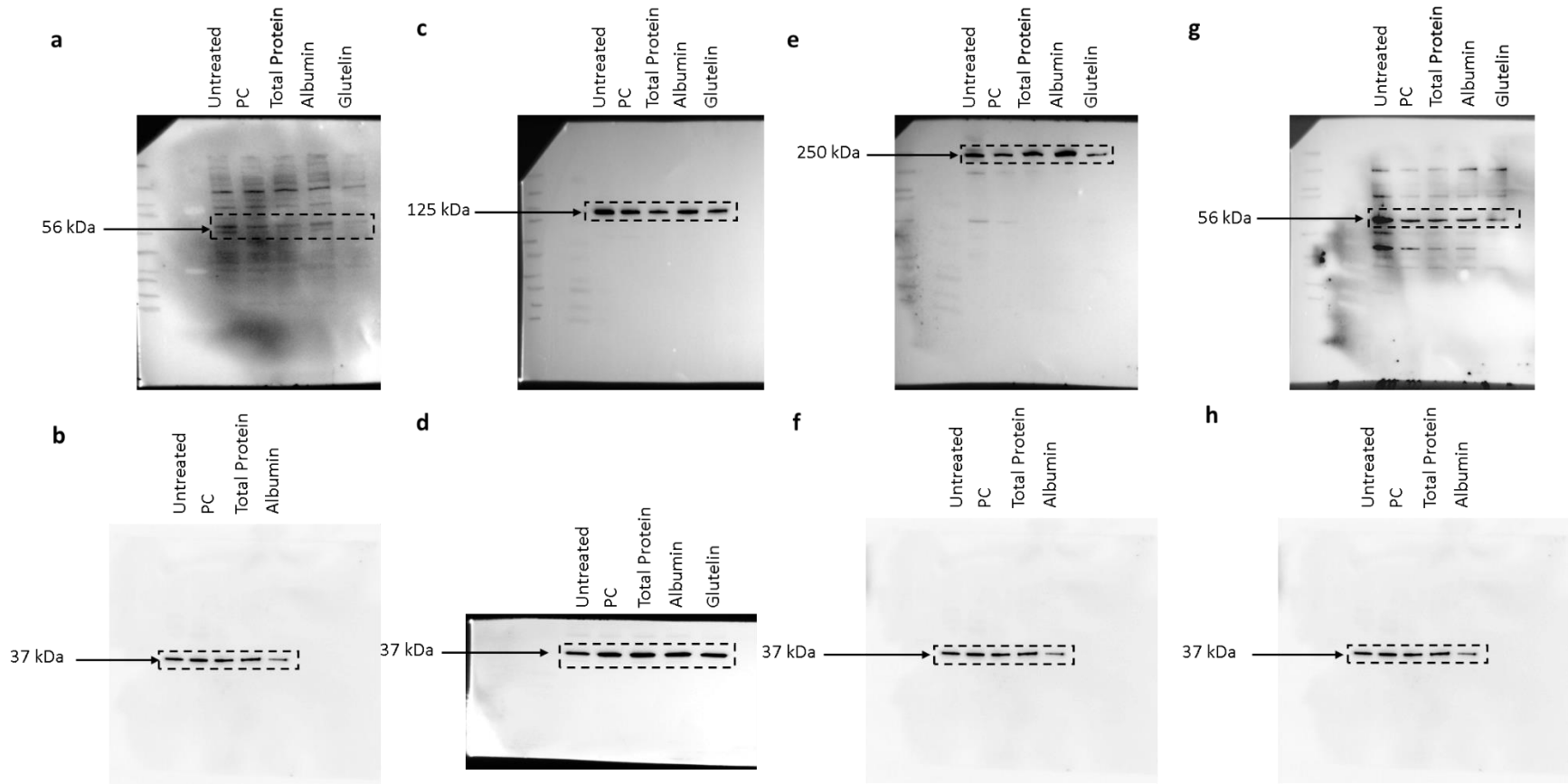


Figure 8. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 4 and 5. a) PPAR γ ; b) GAPDH of PPAR γ ; c) SREBP1; d) GAPDH of SREBP1; e) FAS; f) GAPDH of FAS; g) LPL; h) GAPDH of LPL on prevention of inflammation in adipocytes. Untreated receive any treatment, PC receive only the CM (conditioned media). All experiments were performed in at least two independent trials. PPAR γ : peroxisome proliferator-activated receptor gamma; GAPDH: glyceraldehyde 3-phosphate; SREBP: sterol regulatory element-binding protein 1; FAS: Fatty acid synthase; LPL: lipase liprotein; PC: Positive control.

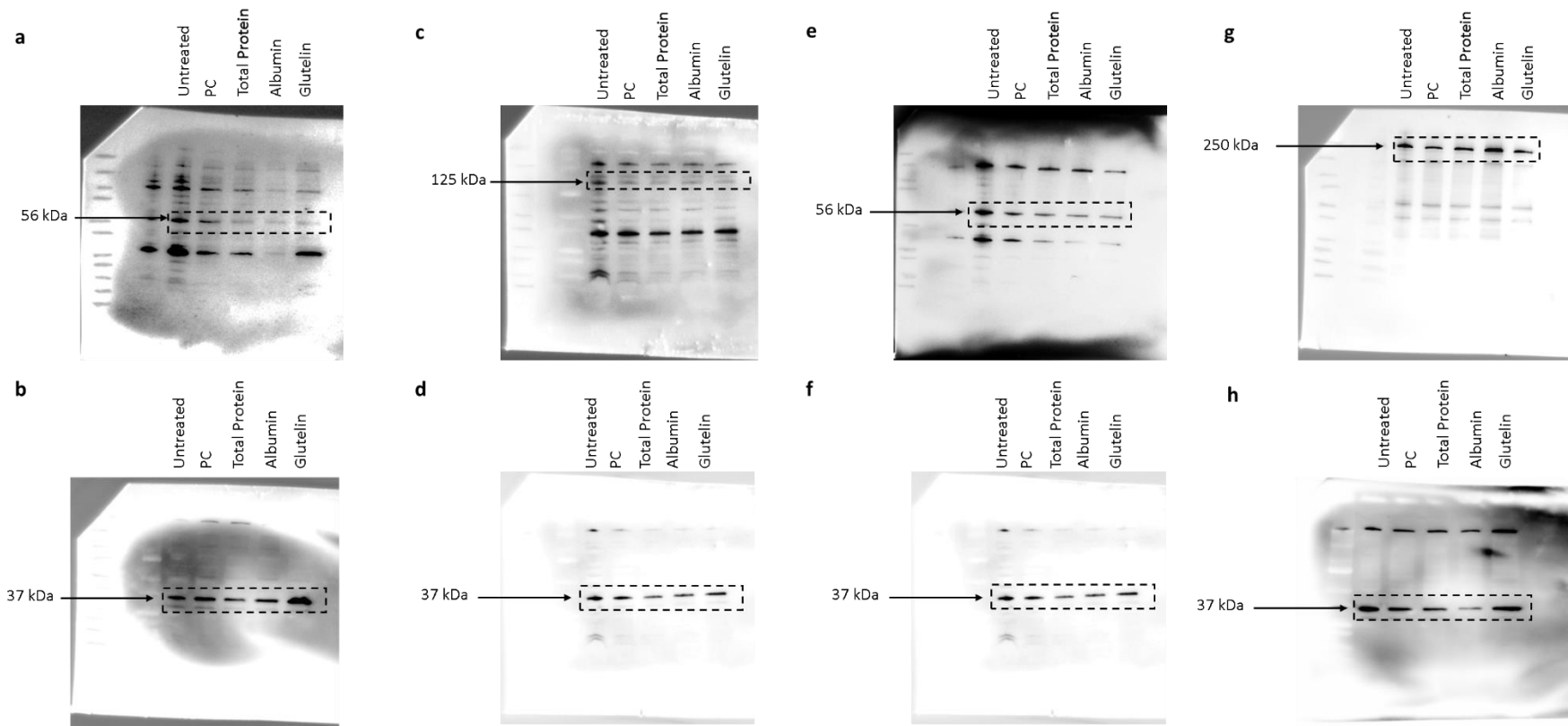


Figure 9. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 4 and 5. a) PPAR γ ; b) GAPDH of PPAR γ ; c) SREBP1; d) GAPDH of SREBP1; e) LPL; f) GAPDH of LPL; g) FAS; h) GAPDH of FAS on inhibition of inflammation in adipocytes. Untreated receive any treatment, PC receive only the CM (conditioned media). All experiments were performed in at least two independent trials. PPAR γ : peroxisome proliferator-activated receptor gamma; GAPDH: glyceraldehyde 3-phosphate; SREBP: sterol regulatory element-binding protein 1; LPL: lipase liprotein; FAS: Fatty acid synthase; PC: Positive control.

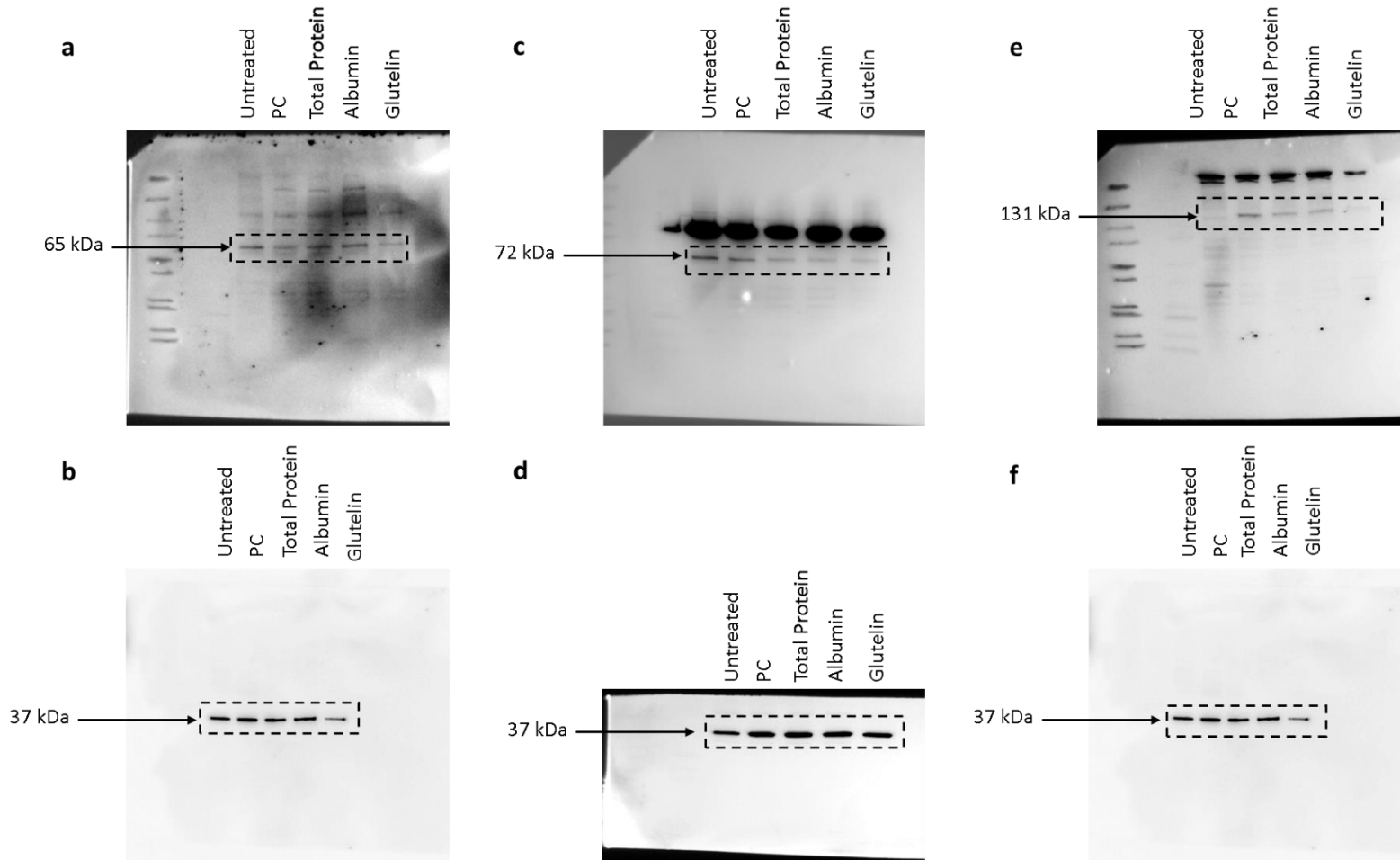


Figure 10. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 6 and 7. a) NF- κ B; b) GAPDH of NF- κ B; c) iNOS; d) GAPDH of iNOS; e) COX-2; f) GAPDH of COX-2 on prevention of inflammation in adipocytes. Untreated receive any treatment, PC receive only the CM (conditioned media). All experiments were performed in at least two independent trials. NF- κ B: Factor nuclear kappa B; GAPDH: glyceraldehyde 3-phosphate; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; PC: Positive control.

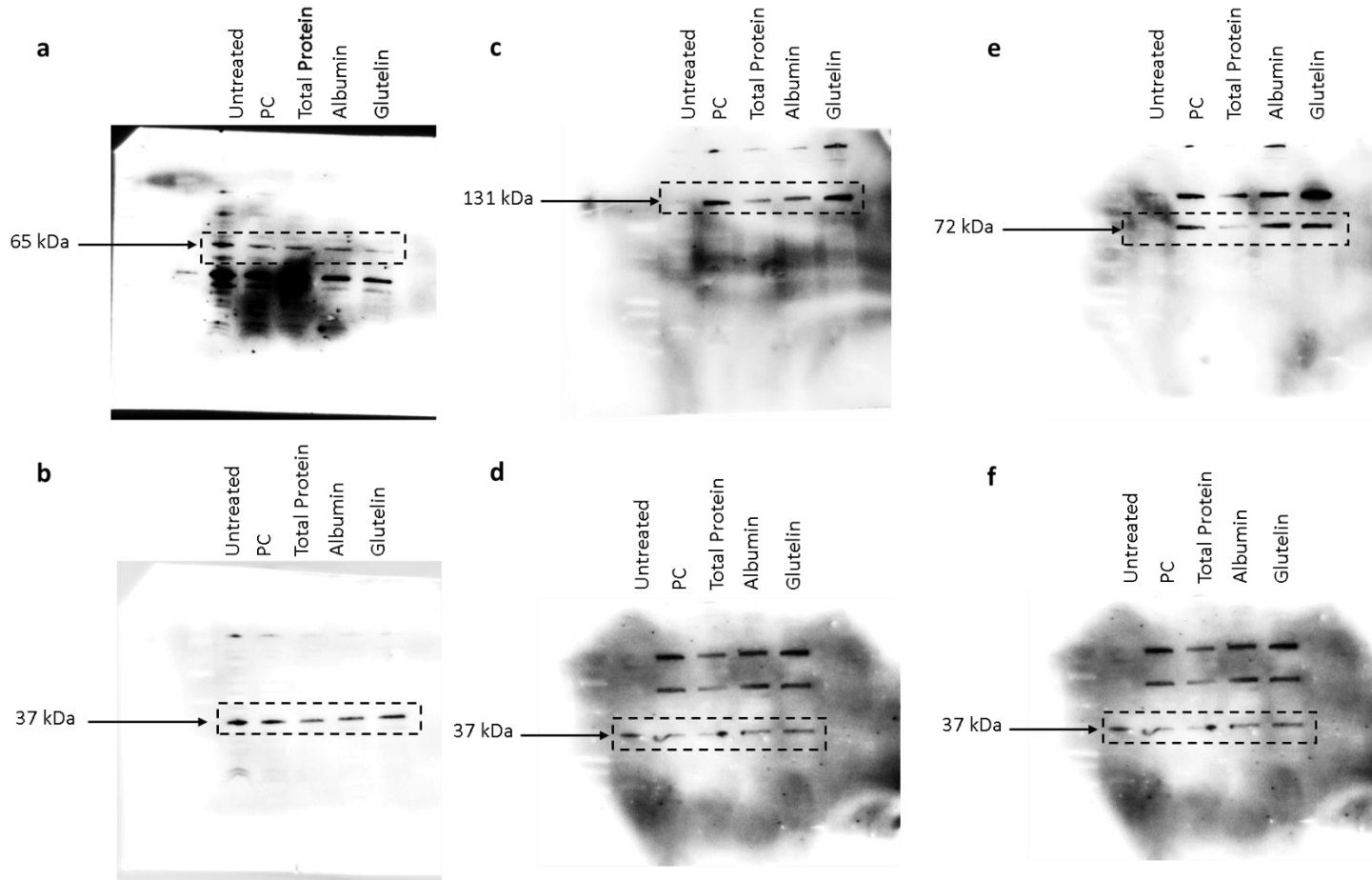


Figure 11. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 6 and 7. a) NF- κ B; b) GAPDH of NF- κ B; c) iNOS; d) GAPDH of iNOS; e) COX-2; f) GAPDH of COX-2 on inhibition of inflammation in adipocytes. Untreated receive any treatment, PC receive only the CM (conditioned media). All experiments were performed in at least two independent trials. NF- κ B: Factor nuclear kappa B; GAPDH: glyceraldehyde 3-phosphate; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; PC: Positive control.

Table 1. The effects of either digested total protein, or digested albumin, or glutelin, or pure peptides on cytokines secretion.

Experiments	Cytokines	Treatment groups							
		Untreated	CM	PC	Total protein	Albumin	Glutelin	Pep1	Pep2
Adipocytes during differentiation receiving DTP and digested albumin and glutelin (Experiment 1)	IL-6			150±15.6 ^a	149.9±5.2 ^a	121.1±4.9 ^{ab}	107.3±4.8 ^b		
	IL-10			108.1±11.1 ^a	112.9±15.1 ^a	110.9±15.3 ^a	104.9±5.2 ^a		
	IL-12			72.9±4.9 ^a	81.8±2.5 ^a	72.9±4.9 ^a	72.9.9±0.1 ^a		
Adipocytes during differentiation receiving pure peptides (Experiment 2)	IL-6			10.8±0.7 ^a				8.7±0.1 ^b	7.1±0.7 ^b
	IL-10			35.1±2.4 ^a				31.6±1.3 ^b	29.5±1.5 ^b
	IL-12			13.5±0.8 ^a				13.5±0.1 ^a	12.6±0.8 ^a
Inflammation prevention in mature adipocytes stimulated with CM (Experiment 3)	IL-6	154.6±52.3 ^c	24197±677 ^a	8721±1161.7 ^b	8462±1392 ^b	7330.6±2774.8 ^b	7871.3±1551 ^b		
	IL-10	30.1±0.5 ^b	156.4±8.3 ^a	24.4±0.5 ^b	26.2±1 ^b	27.2±0.5 ^b	28.7±2.5 ^b		
	IL-12	85.4±2.6 ^a	71.2±2.5 ^a	76.5±5 ^a	87.3±5.2 ^a	81.6±7.6 ^a	91±0.1 ^a		
Inflammation inhibition in mature adipocytes stimulated with CM (Experiment 4)	IL-6	139±10.2 ^a	23148.4±167.5 ^a	10608.2±405.4 ^a	8475±1048 ^a	8479.8±1339.7 ^a	8579.3±1117.5 ^a		
	IL-10	30.1±0.5 ^b	156.4±8.4 ^a	33.1±1.6 ^b	36.5±3.3 ^b	29.4±0.5 ^b	32.3±0.5 ^b		
	IL-12	85.4±2.6 ^b	71.2±2.5 ^b	121.9±11.3 ^a	138.1±0.1 ^a	119.8±8.4 ^a	127.8±2.9 ^a		

Experiment 1: Effects of digested total protein (DTP) and digested protein fraction (DPF) in 3T3-L1 adipocytes during the differentiation process. **Experiment 2:** Effects of pure peptides NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2) in 3T3-L1 adipocytes during the differentiation process. **Experiment 3:** Effects of DTP and DPF for prevent of inflammation in mature adipocytes stimulated with conditioned media from inflamed macrophages. **Experiment 4:** Effects of DTP and DPF for inhibition of inflammation in mature adipocytes stimulated with conditioned media from inflamed macrophages. Different letter per row means statistically different between the proteins (by ANOVA and post-hoc Tukey-test). CM: conditioned media; PC: Positive control

Table 2. Estimated free energy binding (EFE) and chemical interactions among the peptides present in digested total protein and digested albumin and glutelin from chia seeds.

Peptides	PPAR γ		MAGL		FAS	
	EFE	Interacting amino acid residues	EFE	Interacting amino acid residues	EFE	Interacting amino acid residues
	Total Protein					
HYGGPPGGCR	-7.0	ARG A: 280; ARG A: 288; GLU A: 343; GLU A: 295; GLU A: 298; SER A: 225	-7.5	ASN A: 215; GLN A: 212; ALA A: 163; ILE A: 211; ALA A: 151; LYS A: 160; VAL A: 217; ALA A: 156; MET A: 123; SER A: 122; HIS A: 269	-8.3	LEU A: 2231; THR A: 2230; TYR A: 2288; LYS B: 2436; LEU A: 2279; SER B: 2281; SER B: 2284; ASP B: 2280; ARG A: 2275
SPPHGAGLGGGMT	-6.3	ASN A: 375; ARG A: 234; THR A: 238; HIS A: 425; LEU A: 377; SER A: 428; LYS A: 434; LYS A: 373; ALA A: 376	-6.7	LYS A: 160; PRO A: 153; ALA A: 151; ALA A: 156; SER A: 176; SER A: 155; LEU A: 184; SER A: 241; ILE A: 179; ALA A: 51	-6.8	PHE A: 2418; ARG A: 2421; SER A: 2422; ALA A: 2277; ARG A: 2275; LYS B: 2436; SER B: 2284; TYR A: 2288; CYS A: 2292; GLN B: 2432; GLU A: 2227; TYR A: 2425; ALA B: 2287
LPVFGLAAEGNVVTYLH	-7.7	GLU A: 427; ASP A: 380; ALA A: 231; TYR A: 219; ILE A: 223; PRO A: 227; SER A: 225	-6.0	LYS A: 206; PRO A: 172; VAL A: 170; CYS A: 208; LEU A: 167; ALA A: 164; LYS A: 160; SER A: 218; ARG A: 222; ARG A: 219; ASN A: 215; GLN A: 212; SER A: 91	-5.9	LYS B: 2436; HIS B: 2283; THR B: 2434; ARG A: 2421; THR A: 2230; PRO A: 2229; ARG A: 2275; GLU A: 2227; TYR A: 2288; CYS A: 2292; ALA A: 2276
TPPGVGGFPWGGAMLGAQ	-7.0	ASN A: 424; GLU A: 427; SER A: 382; GLN A: 420; LYS A: 216; ARG A: 212; ALA A: 231; ALA A: 235; TYR A: 219	-5.8	SER A: 91; ASP A: 92; ASP A: 26; LEU A: 167; ASN A: 168; ALA A: 164; ALA A: 163; ILE A: 211; LEU A: 214; LYS A: 160; SER A: 218; GLN A: 212	-5.3	PRO A: 2229; LEU A: 2231; CYS A: 2292; GLN A: 2295; ASP A: 2291; ALA A: 2276; ARG A: 2275; PRO A: 2278; ASP B: 2280; SER B: 2281; LYS B: 2436; ARG A: 2421; LEU A: 2279; TYR A: 2425; HIS B: 2283; GLU B: 2316; SER A: 2284; HIS A: 2283

APSPVVLGPP	-7.1	GLU A: 295; SER A: 225; PHE A: 287; HIS A: 466; GLN A: 294; THR A: 297	-6.2	ALA A: 164; LYS A: 160; LEU A: 214; ASN A: 215; ILE A: 211; GLN A: 212; SER A: 91	-9.8	TYR A: 2269; THR A: 2230; LEU A: 2231; TYR A: 2288; ARG A: 2275; GLN B: 2432; HIS B: 2283; ARG A: 2421; PHE A: 2418; SER A: 2281; SER B: 2284; TYR A: 2425
NPGGGAPSGH	-5.9	ARG A: 288; GLU A: 295; ALA A: 292; ILE A: 326; MET A: 329; LEU A: 228	-6.8	ALA A: 51; MET A: 123; SER A: 122; CYS A: 242; LEU A: 241; LEU A: 205; ILE A: 179; SER A: 176; PHE A: 159	-5.2	ALA A: 2361; GLU A: 2364; TYR A: 2424
LHNGGPGGEY	-7.1	LEU A: 228; MET A: 329; ALA A: 292; GLU A: 295; ARG A: 288; ILE A: 326; MET A: 348; ILE A: 341; CYS A: 285; SER A: 342; GLN A: 294; HIS A: 466; PHE A: 287; VAL A: 290	-7.2	HIS A: 121; HIS A: 269; MET A: 123; SER A: 122; SER A: 155; ALA A: 151; SER A: 218; LYS A: 160	-6.7	PRO A: 2229; CYS A: 2292; ARG A: 2275; TYR B: 2433; GLN B: 2432; SER B: 2281; ARG A: 2421; HIS B: 2283; ASP A: 2280; GLN B: 2320; TYR A: 2425; LEU A: 2279
DVNPGGPAPHPWLSVNDL	-7.4	ALA A: 213; HIS A: 217; GLY A: 305; PRO A: 304; SER A: 302; GLU A: 295; ILE A: 223; PRO A: 227; LYS A: 232; LYS A: 224; TYR A: 219; LYS A: 216; ALA A: 215	-6.2	VAL A: 166; VAL A: 207; LEU A: 167; ASN A: 168; CYS A: 208; LYS A: 160; LEU A: 150; ALA A: 156; VAL A: 217; ILE A: 211; ALA A: 164; GLN A: 212; SER A: 91	5.1	
TGPPRPALVFPHAVVP	-5.7	ILE A: 223; ASP A: 220; LYS A: 216; ARG A: 212; TYR A: 219; LYS A: 232; ILE A: 236; ALA A: 235	-4.9	LEU A: 214; ASN A: 215; LYS A: 160; ILE A: 211; ASP A: 92; CYS A: 208; LEU A: 167; ARG A: 219	-8.2	SER B: 2284; SER B: 2281; LYS B: 2436; ALA A: 2277; HIS B: 2283; ARG A: 2275; GLN B: 2432; ALA A: 2276; TYR A: 2288; CYS A: 2292; LEU A: 2231; THR A: 2230; GLN A: 2272; PRO A: 2229
NSSAQYSDPFLALH	-6.6	LYS A: 232; ILE A: 223; THR A: 229; TYR A: 219; ALA A: 231; ARG A: 212; PRO A: 426; GLU A: 427; HIS A: 425; GLU A: 378; ARG A: 234; ASN A: 375; LYS A: 230; ASP A: 380	-5.2	LYS A: 160; SER A: 218; LEU A: 214; ARG A: 219; ARG A: 222; ASN A: 215; ASP A: 92; SER A: 91; ILE A: 211; LEU A: 167	-3.1	TYR B: 2288; ASP A: 2280; LEU A: 2279; SER B: 2281; ASP B: 2280; THR B: 2434; SER B: 2284; TYR A: 2425; ALA B: 2287; HIS B: 2283; LYS B: 2436; ARG A: 2275; ALA A: 2276; TYR A: 2288; CYS A: 2292; PRO A: 2229; VAL A: 2296; LEU A: 2231

Albumin						
THPTGPAGAGPPMSL	-5.7	TYR A: 219; ILE A: 223; LYS A: 232; ALA A: 235; GLU A: 378; LYS A: 230; ARG A: 234	-6.0	VAL A: 207; CYS A: 208; VAL A: 89; SER A: 91; GLN A: 212; ASN A: 215; ASP A: 92; ARG A: 219; ILE A: 211; LYS A: 160; LEU A: 214; SER A: 218; ARG A: 222; GLU A: 221; VAL A: 217; LEU A: 150	-5.6	ASP A: 2291; TYR A: 2288; CYS A: 2292; LEU A: 2231; HIS B: 2283; SER B: 2281; ARG A: 2275; LEU A: 2279; TYR A: 2425
RGSSSPMAGAAGDLPGSVT	-5.8	ASN A: 424; HIS A: 425; GLU A: 378; ALA A: 231; THR A: 241; TYR A: 219; ASP A: 380; LEU A: 379; ARG A: 234; ASN A: 375; ASN A: 335; LYS A: 336; VAL A: 372	-5.3	ARG A: 222; ASN A: 215; SER A: 218; ILE A: 211; ALA A: 163; GLN A: 212; SER A: 91; LEU A: 167; CYS A: 208; LYS A: 206	-2.3	ASN A: 2225; GLN B: 2432; PRO A: 2229; ARG A: 2233
YLGHPGTAN	-7.5	TYR A: 219; LYS A: 216; GLN A: 420; LEU A: 423; GLU A: 378; GLU A: 427; ASN A: 335; ASN A: 375; ARG A: 234	-7.8	GLN A: 212; ARG A: 219; ASN A: 215; ARG A: 222; SER A: 218; LYS A: 160; ALA A: 151; ALA A: 156; VAL A: 217; LEU A: 205; ILE A: 179; LEU A: 241; MET A: 123; SER A: 122; ALA A: 51; GLY A: 50; TYR A: 194; VAL A: 270; LEU A: 184; SER A: 181	-7.5	ASP A: 2280; LEU A: 2279; HIS B: 2283; ASP B: 2280; SER B: 2281; GLN B: 2432; ARG A: 2275; THR B: 2434; THR A: 2230
SPKDLALPPGALPPVQ	-7.7	THR A: 238; ARG A: 234; ALA A: 231; HIS A: 425; ASP A: 380; TYR A: 219; ILE A: 223; SER A: 225; VAL A: 307; LYS A: 301	-7.3	SER A: 91; GLN A: 212; ASN A: 215; ARG A: 219; ILE A: 211; LEU A: 167; LEU A: 171; VAL A: 166; VAL A: 170; CYS A: 208; LYS A: 206; SER A: 175; ASN A: 173	-7.0	ARG A: 2275; HIS B: 2283; GLU A: 2227; ALA A: 2276; TYR A: 2288; THR A: 2230; PRO A: 2229; ASN A: 2225; LEU A: 2223; THR A: 2255; ARG A: 2233
TVPLLWFRQDPTGPLNL	-6.9	N/A	-6.8	NA	12.7	
TGPSPTAGPPAPGGGTH	-9.1	ARG A: 234; LYS A: 230; ALA A: 231; ASP A: 380; ASP A: 381; TYR A: 219; HIS A: 425; GLU A: 378	-7.7	NA	9.7	

AVLPDLTSSSLKLDK	-6.2	ARG A: 234; TYR A: 219; THR A: 241; ALA A: 231; LYS A: 232; ILE A: 223; PRO A: 227; LYS A: 224; SER A: 225	-5.7	ARG A: 219; ASN A: 215; SER A: 218; LYS A: 160; ILE A: 211; ALA A: 163; LEU A: 167; VAL A: 207; CYS A: 208; LEU A: 174	-3.7	PRO A: 2229; LEU A: 2231; TYR A: 2288; GLN B: 2432; THR B: 2434; SER B: 2281; TYR A: 2425; LEU A: 2279; ASP A: 2280; ARG A: 2275; HIS B: 2283; LYS B: 2436; PRO A: 2278; ARG A: 2421; GLU B: 2316; SER B: 2284
RMVLPTYFSAQLE	-6.8	TYR A: 219; THR A: 229; ASP A: 220; ARG A: 212; SER A: 382; GLN A: 420; ASN A: 424; PRO A: 426; ALA A: 235; LYS A: 230; ARG A: 234	-7.1	SER A: 218; ARG A: 219; ARG A: 222; GLN A: 212; LEU A: 214; LYS A: 160; ILE A: 211; VAL A: 170; VAL A: 207; LEU A: 167; CYS A: 208; PRO A: 172; LYS A: 206; LEU A: 174; ASN A: 173; SER A: 175	-7.1	ARG A: 2233; MET A: 2232; PRO A: 2229; ASN A: 2225; GLN A: 2272; VAL A: 2296; LEU A: 2231; PRO A: 2278; HIS B: 2283; ARG A: 2275
RHMDLPALVFT	-8.9	GLU A: 298; SER A: 225; PRO A: 227; SER A: 342; ARG A: 288; LEU A: 228; SER A: 289; HIS A: 323	-6.9	LYS A: 160; ALA A: 164; ASN A: 215; GLN A: 212; CYS A: 208; VAL A: 89; LYS A: 206; ASN A: 173; PRO A: 172; LEU A: 167	-7.0	LYS B: 2436; THR B: 2434; HIS B: 2283; GLU A: 2227; GLY A: 2228; ARG A: 2233
GPPDGPCTEGA	-6.8	ARG A: 212; LYS A: 216; TYR A: 219; ALA A: 235; ALA A: 231; ALA A: 376	-5.8	LYS A: 160; ALA A: 163; ILE A: 211; ASN A: 215; GLN A: 212; SER A: 91; CYS A: 208; VAL A: 89; GLU A: 86; LYS A: 206; VAL A: 207; LEU A: 167; VAL A: 170	-8.5	ARG A: 2421; ASP B: 2280; PHE A: 2418; ARG A: 2275; LEU A: 2279; TYR A: 2425; SER B: 2281; SER B: 2284; GLN B: 2432; THR B: 2434; ALA A: 2276; TYR A: 2288; THR A: 2230; LEU A: 2231
SGKVEELAEPLDL	-7.0	THR A: 238; LYS A: 240; ALA A: 235; THR A: 241; TYR A: 219; SER A: 382; ASP A: 380; ALA A: 231; LYS A: 230; ARG A: 234; GLU A: 378; ASN A: 375	-6.7	GLU A: 86; CYS A: 208; VAL A: 170; LEU A: 167; ILE A: 211; ALA A: 163; GLN A: 212; ASN A: 215; ARG A: 219; LYS A: 160; ALA A: 156; VAL A: 217; PRO A: 153	-7.2	THR B: 2434; SER B: 2281; LYS B: 2436; ASP B: 2280; TYR A: 2425; ARG A: 2275; CYS A: 2292; THR A: 2230; LEU A: 2231; GLU A: 2227
LPGPPATF	-6.1	TYR A: 219; ARG A: 212; ASP A: 380; ASN A: 424; HIS A: 425; LEU A: 379; ARG A: 234; ALA A: 235; ALA A: 231; LYS A: 240; THR A: 238; LYS A: 230; ASP A: 381; SER A: 382; GLN A: 420; LYS A: 216; GLU A: 378	-5.8	ILE A: 211; LEU A: 214; SER A: 218; ARG A: 222; ARG A: 219; GLN A: 212; ASN A: 215	-9.0	CYS A: 2292; TYR A: 2288; THR A: 2230; ALA A: 2276; LYS B: 2436; HIS B: 2283; ARG A: 2275; LEU A: 2279; PHE A: 2418; ASP B: 2280; SER B: 2281; SER B: 2284

Glutelin						
KKLELTEYLYEDKK	-6.3	LYS A: 301; PRO A: 227; LYS A: 232; SER A: 225; THR A: 229; LYS A: 224; ILE A: 223; TYR A: 219; ASP A: 220; ASP A: 380; ALA A: 231	-6.0	ALA A: 151; GLU A: 221; SER : 218; ARG A: 222; LEU A: 214; ARG A: 98; SER A: 91; LEU A: 167; VAL A: 166; VAL A: 207; CYS A: 208	16.4	
NSPGPHDVALDQ	-5.6	LYS A: 230; ALA A: 235; LYS A: 232; ALA A: 231; TYR A: 219; GLU A: 378; ARG A: 234	-6.4	ARG A: 98; ASP A: 26; SER A: 91; VAL A: 90; VAL A: 95; VAL A: 78; CYS A: 208; ILE A: 211; SER A: 218; LYS A: 160	-7.3	GLU A: 2227; GLY A: 2228; TYR A: 2288; PRO A: 2229; CYS A: 2292; LYS B: 2436; ASP A: 2291; THR B: 2434; ARG A: 2275; ARG A: 2421; ARG B: 2428; TYR B: 2433; ILE B: 2282; HIS B: 2283; SER B: 2281; LEU A: 2279; ASP A: 2280
KDSGHPNGGLFAESSP	-6.0	PRO A: 227; TYR A: 219; LYS A: 232; THR A: 241; ASP A: 380; LYS A: 230; ALA A: 231; ALA A: 235; LYS A: 240	-6.8	ALA A: 164; VAL A: 166; VAL A: 170; ASN A: 215; GLN A: 212; ASP A: 92; ARG A: 219; ARG A: 222; SER A: 218; GLU A: 221; ALA A: 156; ALA A: 151; VAL A: 217	-5.2	ARG A: 2275; ARG A: 2421; SER B: 2281; GLN B: 2432; GLU A: 2227; THR B: 2434; LYS B: 2436; PRO A: 2229; CYS A: 2292; TYR A: 2288; ALA B: 2437; PRO B: 2435
TGPCNGAAPPAAGSDCL	-6.6	LYS A: 438; LYS A: 434; SER A: 428; GLU A: 427; ASN A: 424; TYR A: 219; THR A: 229; ALA A: 235; LYS A: 240; GLU A: 378; ASN A: 375; LYS A: 230	-5.2	LYS A: 206; ASN A: 173; LEU A: 174; VAL A: 207; CYS A: 208; VAL A: 166; VAL A: 170; LEU A: 167; ILE A: 211; ALA : 163; ASN A: 215; LYS A: 160; SER A: 218; ARG A: 222	-5.8	SER A: 2253; ASN A: 2225; PRO A: 2229; CYS A: 2292; GLU A: 2227; GLN B: 2432; SER B: 2281; ARG A: 2275; PRO A: 2278; TYR A: 2425
QAGAGGPGAAGH	-7.8	SER A: 332; ARG A: 288; SER A: 342; SER A: 225; GLU A: 298; LYS A: 224; ASP A: 220	-6.7	SER A: 218; ASN A: 215; LYS A: 160; THR A: 157; PRO A: 153; ALA A: 151; MET A: 123	-8.3	THR A: 2230; THR A: 2274; ALA A: 2276; TYR A: 2288; CYS A: 2292; THR B: 2434; SER B: 2281; ARG A: 2275; GLN B: 2432; ARG A: 2421; SER B: 2284; TYR A: 2425; LYS B: 2436
TPPNGLGPSGSPGPHAQ	-6.0	SER A: 225; PRO A: 227; ILE A: 223; LYS A: 232; THR A: 229; TYR A: 219; ALA A: 231; ARG A: 234; LYS A: 230; ASP A: 380; LYS A: 240; THR A: 241; ASP A: 243	-5.3	SER A: 91; VAL A: 89; GLN A: 212; ASN A: 215; ARG A: 219; LEU A: 171; VAL A: 207; LYS A: 206; CYS A: 208; LEU A: 174; ASN A: 173; LYS A: 160; ALA A: 163; LEU A: 214; ILE A: 211; PHE A: 159	-6.0	LEU A: 2231; VAL A: 2296; ARG A: 2233; CYS A: 2292; THR A: 2230; TYR A: 2288; ALA A: 2276; ARG A: 2275; ASP B: 2280; TYR A: 2425; LEU A: 2279

RMVLPEYELLYE	-6.9	GLN A: 420; TYR A: 219; LYS A: 224; ILE A: 223; LYS A: 232; THR A: 241; ALA A: 231; ARG A: 234; GLU A: 378; ASP A: 380; HIS A: 425	-7.3	MET A: 123; SER A: 122; SER A: 155; LEU A: 148; LEU A: 213; ALA A: 151; ALA A: 156; LEU A: 214; LEU A: 150; PRO A: 153; SER A: 218; ARG A: 222; LYS A: 160; ALA A: 164; ALA A: 163; ILE A: 211; LEU A: 167; CYS A: 208; VAL A: 207	-5.9	SER B: 2281; GLU A: 2227; LYS B: 2436; TYR A: 2288; THR A: 2230; PRO A: 2229; GLN B: 2432
RLTGTGMGAASSVLP	-7.7	ILE A: 326; CYS A: 285; SER A: 342; PHE A: 287; GLN A: 294; HIS A: 466; LEU A: 468; LEU A: 318; THR A: 297	-6.2	LYS A: 160; SER A: 218; THR A: 157; VAL A: 217; ALA A: 164; CYS A: 208; GLN A: 212; ASN A: 215; ARG A: 219	-4.9	GLN A: 2272; MET A: 2232; PRO A: 2229; THR B: 2434; GLU A: 2227; TYR A: 2288; CYS A: 2292
RHMDLPLSPSSAGP	-5.8	GLN A: 420; ARG A: 212; LYS A: 216; TYR A: 219; ASN A: 424; HIS A: 425; ARG A: 234; LYS A: 230; ALA A: 231; LYS A: 240; THR A: 241; THR A: 229	-5.2	ALA A: 163; ALA A: 164; SER A: 218; LYS A: 160; LEU A: 214; ILE A: 211; LEU A: 167; CYS A: 208; ASN A: 215; GLN A: 212; ARG A: 219; ARG A: 222	56.9	
TTNPGVDADFPAREL	-5.9	GLU A: 298; SER A: 225; GLU A: 343; PRO A: 227; LYS A: 232; ILE A: 223; THR A: 229; ASP A: 381; ALA A: 231; LYS A: 230; ARG A: 234; THR A: 238	73.2		-4.7	PRO A: 2229; GLU A: 2227; ALA A: 2276; THR B: 2434; LYS B: 2436; CYS A: 2292; THR A: 2274; THR A: 2230; GLY A: 2228; GLN A: 2295
QPADPNQFVYAPDTH	-5.5	SER A: 429; SRG A: 443; ASP A: 396; ASP A: 475	-5.6	ARG A: 222; SER A: 218; LYS A: 160; ALA A: 163; ILE A: 211; CYS A: 208; GLN A: 212; ASN A: 215; SER A: 91	-6.2	GLU A: 2227; PRO A: 2229; LEU A: 2231; LYS B: 2436; ALA A: 2276; GLN B: 2432; SER B: 2281
FAFFEFFELLFAFFT	-8.5	ARG A: 234; ALA A: 231; ASP A: 380; HIS A: 425; TYR A: 219; ASP A: 220; LYS A: 224; PRO A: 227	-6.5	LYS A: 160; ALA A: 164; ALA A: 163; SER A: 218; ARG A: 222; CYS A: 208; LYS A: 206; ALA A: 203; ARG A: 87	9.2	
TDQLGWFLGAAAFPAQ	-6.4	GLU A: 298; LYS A: 224; GLN A: 294; GLY A: 284; SER A: 342	-5.5	CYS A: 208; VAL A: 207; VAL A: 170; VAL A: 166; ALA A: 163; PHE A: 159; LEU A: 214; LYS A: 160; ILE A: 211; ASP A: 92; GLN A: 212; ARG A: 219	-4.9	GLU A: 2415; THR A: 2255; ARG A: 2233; GLU A: 2227; ARG A: 2275; HIS B: 2283; ARG A: 2421

RGSSPAPSGSELVL	-4.9	GLN A: 420; ARG A: 212; LEU A: 423; PRO A: 426; ASN A: 424; HIS A: 425; GLU A: 427; LYS A: 240; GLU A: 378; LYS A: 230; ASP A: 380; ALA A: 231; TYR A: 219	-5.9	PRO A: 172; ASN A: 173; ILE A: 174; CYS A: 208; SER A: 91; ASP A: 92; GLN A: 212; ARG A: 219; ARG A: 222; SER A: 218; LEU A: 167; VAL A: 170	-7.2	LEU A: 2231; PRO A: 2229; GLU A: 2227; ALA A: 2276; THR A: 2274; PRO A: 2226; CYS A: 2292; LYS B: 2436; SER B: 2281; LEU A: 2279; SER B: 2284; TYR A: 2425; ARG A: 2275; ARG A: 2421
TDWYDPGAFQ	-6.7	ILE A: 232; TYR A: 219; LYS A: 216; ASP A: 220; ILE A: 223; LYS A: 224; SER A: 225; SER A: 221; TYR A: 299; SER A: 302; GLU A: 298	-7.5	LYS A: 160; SER A: 218; ARG A: 222; ARG A: 219; ASN A: 215; LEU A: 214; ILE A: 211; GLN A: 212; ASN A: 173; VAL A: 207; CYS A: 208	-7.3	CYS A: 2292; LEU A: 2231; THR B: 2434; LYS B: 2436; HIS B: 2283; TYR B: 2433; SER B: 2281; GLN B: 2432; ARG B: 2428
HAPGFLSPAHR	-6.2	TYR A: 219; LYS A: 216; ASP A: 380; ALA A: 231; ASN A: 424	-6.5	SER A: 218; ALA A: 163; ILE A: 211; GLN A: 212; CYS A: 208; LYS A: 206; LEU A: 174	-7.5	TYR A: 2288; CYS A: 2292; LEU A: 2231; ALA A: 2276; GLY A: 2228; GLU A: 2227; ARG A: 2275; GLN B: 2432; ASP B: 2280; SER B: 2281; ARG A: 2421; LEU A: 2279; SER B: 2284; TYR A: 2425
LPGPPATF	-6.1	TYR A: 219; ARG A: 212; ASP A: 380; ASN A: 424; HIS A: 425; LEU A: 379; ARG A: 234; ALA A: 235; ALA A: 231; LYS A: 240; THR A: 238; LYS A: 230; ASP A: 381; SER A: 382; GLN A: 420; LYS A: 216; GLU A: 378	-5.8	ILE A: 211; LEU A: 214; SER A: 218; ARG A: 222; ARG A: 219; GLN A: 212; ASN A: 215	-9.0	CYS A: 2292; TYR A: 2288; THR A: 2230; ALA A: 2276; LYS B: 2436; HIS B: 2283; ARG A: 2275; LEU A: 2279; PHE A: 2418; ASP B: 2280; SER B: 2281; SER B: 2284

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction.

7. GENERAL CONCLUSIONS

The chia seed is a good protein and essential amino acids source. From the total of 20 protein in chia seed, 8 were related to production and storage of plant lipids. Then, we correlated, for the first time, that the lipids content of chia seeds, especially linolenic acid, can be associated with its protein composition. Furthermore, those proteins are a source of peptides with biological effects, mainly dipeptidyl peptidase-IV inhibitors, angiotensin-converting enzyme inhibitors, and antioxidant capacity. These results can be correlated with benefits of chia seeds consumption in animals and human studies, such as antioxidant capacity, and hypotensive, hypoglycemic, and anticholesterolemic effects.

Moreover, we are reporting, also for the first time, which are the peptides formed after simulated gastrointestinal digestion of chia seeds, as well as the chemical characteristics and the effects of those peptides against the markers of inflammation, atherosclerosis, and adipogenesis biochemically, *in silico* and *in vitro*. Digested total protein (DTP), and digested albumin, globulin, and glutelin showed antioxidant capacity by scavenging capacity for superoxide, hydrogen peroxide, nitric oxide and DPPH. Moreover, these samples inhibited the 5-LOX, COX-1-2, and iNOS enzymes by biochemical analyses and high interaction with COX-2, TLR-4, p65-NF- κ B, and LOX-1 by *in silico* analysis, showing the anti-inflammatory and anti-atherosclerotic potentials of the peptides from chia seeds. However, the prolamin had no promissory effects and, because of that, its fraction was not utilized on cells study.

The samples DTP, digested albumin, globulin, and glutelin showed anti-inflammatory effects by modulation of NF- κ B and AP-1 pathway, confirmed by reduction of NF- κ B translocation to nuclei. The ROS was also reduced, showing the antioxidant effect of the digested proteins. In addition, the DTP and digested protein fractions had anti-atherosclerotic effect by reduction of expression of proteins related to atherosclerosis development, as iCAM and LOX-1, and the lipid accumulation into macrophages. Then, once inflammation is a key for most of diseases, including CVD, and atherosclerosis aggravates it, these samples can be a promising protection to the organism.

Besides, the digested total protein, digested albumin and globulin, and two pure peptides had a strong effect against adipogenesis by reduction of lipids into adipocytes. Furthermore, these samples reduced the expression of PPAR γ , the mainly marker related with adipogenesis. Other markers were also reduced, as LPL, FAS, SREBP1, which are confirm the antiadipogenic effects of digested proteins from chia seed and their possible effects against obesity development.

Finally, to observe the effects of samples on inflammation associated with obesity, it was observed that the samples had most powerful effects when they were added together of conditioned media, in other words, in prevention of inflammation. When the cells were first inflamed with conditioned media and then treated with the samples (inhibition of inflammation), the effects were milder. These results demonstrated that the digested samples are more effective on prevention of adipogenesis and its associated inflammation than to inhibit an inflammation already established.

Then, we observed that the digested total protein, albumin, globulin, and glutelin, had effects against the markers of inflammation, atherosclerosis and adipogenesis. The total protein, in general, had the best effects in comparison with isolated fractions. These results prove that total protein is more powerful than separated proteins. Additionally, the pure peptides selected, NSPGPHDVALDQ (Pep1) and RMVLPEYELLYE (Pep2), had a significant effect on preventing adipogenesis, which indicates the necessity of news studies to investigate their effects on other markers. The **Figure 3** shows a synthesis of this study and a proposal effects of digested proteins on organism.

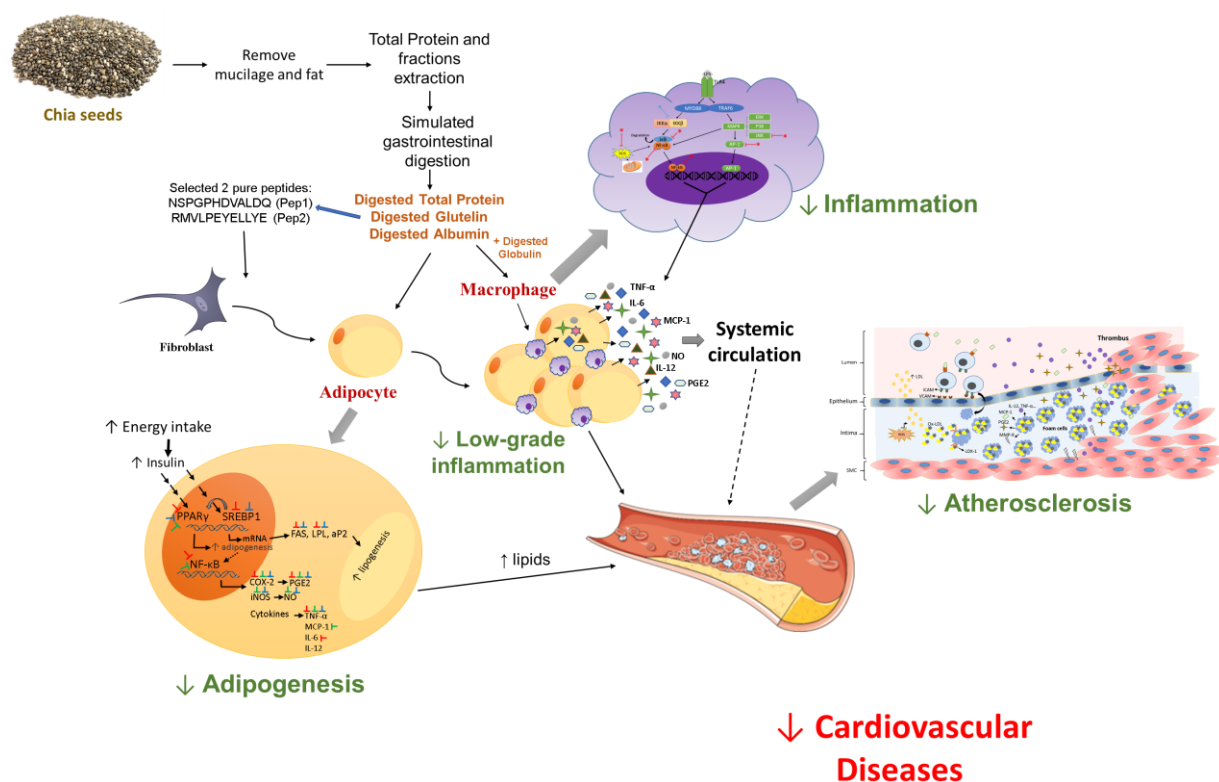


Figure 3. Proposal effect about how the digested proteins from chia seeds and its pure peptides can act on organism. The digested proteins and their peptides are able to reduce the

inflammation on macrophages and the adipogenesis process in adipose tissue; consequently, less macrophages migrate into adipose tissue, reducing the low-grade inflammation. Furthermore, less free lipids and macrophages are available to start the atherosclerosis process. All these conditions reduce the chances to cardiovascular diseases development.

8. FINAL CONSIDERATIONS

These results are promising and allow understanding the mechanisms related to the beneficial effects of chia seed consumption against chronic diseases and its complications, especially cardiovascular diseases, demonstrated in other studies with rats and humans. Moreover, this study highlighted, for the first time, the expressive effects of proteins from chia against the markers of CVD, which can act together other compounds present in the chia and more studied, as dietary fiber and lipids. In addition, there are not other studies testing the digested proteins from chia seeds *in vitro* or *in vivo* to compare with our results.

Then, the results obtained in this study using cells and molecular docking can be allow future studies with animal and human trials that may show the effects of the digested protein from chia seed in inflammation, atherosclerosis, oxidative stress, and adipogenesis in a complex organism. Since better results were found when the samples were used to prevent inflammation, both in macrophages and adipocytes, future studies are suggested to investigate the effect of these samples, but especially DTP in preventing obesity, as it is shown as the main triggering of CVD.

Moreover, the peptide profile of chia showed in this study suggested other possible benefits of the peptides from chia seeds, as hypotensive and hypoglycemic. Then, future studies in cells and animals are necessary to understanding all mechanisms of digested proteins on the physiological process in the organism. All these results will contribute to new knowledge for the Nutrition Science, being able to explain the benefits obtained with the consumption of chia seed.