BÁRBARA NERY ENES

EFFECT OF CHIA (*Salvia hispanica* L.) ON GLUCOSE METABOLISM, AND THEIR MOLECULAR MECHANISMS ON INSULIN RESISTANCE CONDITIONS, *IN VIVO* AND *IN VITRO*

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

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Assent:

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ABSTRACT

ENES, Bárbara Nery, D.Sc., Universidade Federal de Viçosa, February, 2020. **Effect of chia (*Salvia hispanica* L.) on glucose metabolism, and their molecular mechanisms on insulin resistance conditions, *in vivo* and *in vitro*.** Adviser: Hércia Stampini Duarte Martino. Co-Advisers: Érica Aguiar Moraes, Helen Hermana Miranda Hermsdorff and Maria Eliza de Castro Moreira.

Chia (*Salvia hispanica* L.) is known for its high concentration of alpha linolenic acid omega 3, dietary fiber, proteins, vitamins, minerals and phytochemicals. These components are considered responsible for the improvement of biological markers related to non-communicable diseases. However, it is not clear whether there is an interaction between the compounds, or if a specific compound is responsible for the health benefits generated by the consumption of chia. In addition, the biochemical mechanisms and pathways involved in the chemoprevention of diseases caused by chia remain unknown. Therefore, the first investigations was, to compile evidence regarding the effects of chia seed (flour or oil) in studies with animals fed with unbalanced diets, and to understand the effects and molecular mechanisms in the modulation of metabolic biomarkers. Additional aims were to evaluate the influence of chia flour and oil on glucose metabolism in insulin-resistant *Wistar* rats, as well as to determine the effect of a phenolic extract from chia on glucose metabolism in insulin-resistant HepG2 cells. A systematic review was carried out on electronic databases, following the recommendations of PRISMA. The risk of bias and quality was assessed using the SYRILE toll and ARRIVE guidelines. The *in vivo* study was carried out with 40 male adults *Wistar* rats, initially divided into two groups: AIN-93M (n=10) and high fat and fructose content (HFHF) (n=30), during 8 weeks. Then, the HFHF group was divided into three groups (n=10): HFHF, HFHF with chia flour (14.7%) and HFHF with chia oil (4%). The parameters evaluated were: food consumption; adiposity; intraperitoneal tolerance to glucose and insulin; mRNA levels of enzymes and proteins involved on glucose metabolism (glycokinase, phosphofructokinase, pyruvate kinase, monophosphate-adenosine protein kinase, insulin receptor, protein transcription factor Forkhead box O1 and protein kinase B), as well as phosphorylated AKT (AKT1 [pS473]) protein level. The *in vitro* study was carried out with HepG2 cells, treated with glucose (25 mM) and palmitate (1mM) for 24 h, to induce insulin resistance. Insulin-resistant HepG2 cells were next, treated with hydrolyzed phenolics extract obtained from chia (20, 40 and 80 ppm). The hydrolyzed phenolics extract from chia were

obtained by ultrasound liquid-liquid extraction, followed by hydrolysis with HCl (1 M). The characterization and quantification of phenolics from the hydrolyzed extract were assessed by reverse phase HPLC. To check effects from chia's phenoliscs on insulin resistance, were analyzed the gene expression of AKT protein and enzymes involved in glycogenogenesis (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) and glycolysis (phosphofructokinase and pyruvate kinase). Throughout the 17 studies included in the systematic review, details on randomization and allocation concealment in studies were insufficient, as well as information on protocols. Among the studies, there was a lack of information about the sample size, the dose of chia used, and the number of animals evaluated for each parameter evaluated. However, summarizing the results, chia were associated with AMPK modulation, improment on glucose and insulin tolerance, lipogenesis, antioxidant activity and inflammation. The original *in vivo* study demonstrated that chia (seed and oil) increased AKT1 [pS473]. Chia oil improved glucose and insulin tolerance, increased AMPK, insulin receptor, FOX01, glycolysis enzymes and decreased gluconeogenesis enzymes. The *in vitro* experiment demonstrated that the phenolics of chia seeds decreased the expression of gluconeogenic enzymes without affecting the phosphorylation of AKT. Based on data from experimental studies, systematic review, chia presents bioactive potential and its daily consumption can reduce the risk of developing chronic diseases, mainly due to the antioxidant, antinflammatory, hypoglycemic and hypolipidemic effects of the seed. The original *in vivo* study indicated the potential of chia to improve glucose tolerance and positively affect insulin, neverthelesses, *in vitro* study showed that hydrolyzed phenolics from chia did not show effects. From the three fractions investigated in this research (flour, oil and hydrolyzed phenolics), the oil from chia demonstrated superior results regarding glucose metabolism in insulin resistance condition.

Keywords: Chia flour. Chia oil. Alpha-linolenic acid. Phenolic compounds. AMPK. AKT.

RESUMO

ENES, Bárbara Nery, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Efeito da chia (*Salvia hispanica* L.) no metabolism de glicose, e mecanismos moleculares em condições de resistência à insulina, *in vivo* e *in vitro*.** Orientadora: Hércia Stampini Duarte Martino. Coorientadoras: Érica Aguiar Moraes, Helen Hermana Miranda Hermsdorff e Maria Eliza de Castro Moreira.

Chia (*Salvia hispanica* L.) é conhecida por sua alta concentração de ácido alfa linolênico ômega 3, fibra alimentar, proteínas, vitaminas, minerais e fitoquímicos. Esses componentes são considerados os responsáveis pela melhora de marcadores biológicos relacionados à doenças crônicas não transmissíveis. Embora ainda não esteja claro se há uma interação entre os compostos, ou se um composto específico é responsável pelos benefícios à saúde gerados pelo consumo da chia. Além disso, os mecanismos e vias bioquímicas envolvidos na quimioprevenção de doenças pela semente de chia permanecem desconhecidos. O objetivo deste trabalho foi, em primeiro lugar, compilar evidências referente aos efeitos da chia (farinha ou óleo) em estudos com animais alimentados com dietas desequilibradas, e entender os efeitos e mecanismos moleculares na modulação de biomarcadores metabólicos. Segundo, avaliar a influência da farinha e óleo de chia no metabolismo da glicose em ratos *Wistar* resistentes à insulina, e ainda o efeito de um extrato hidrolisado de fenólicos extraídos da chia, no metabolismo da glicose em células HepG2 resistentes à insulina. Uma revisão sistemática foi realizada em bancos de dados eletrônicos, seguindo as recomendações do PRISMA. O risco de viés e qualidade foi avaliado usando as diretrizes SYRILE toll e ARRIVE. O estudo *in vivo* foi conduzido por 8 semanas utilizando 40 ratos *Wistar* adultos, machos, divididos inicialmente em dois grupos: AIN-93M (n=10) e alto conteúdo de gordura e de frutose (HFHF) (n=30). Em seguida, o grupo HFHF foi dividido em três grupos (n=10): HFHF, HFHF com farinha de chia (14,7%) e HFHF com óleo de chia (4%). Foram analisados o consumo alimentar; adiposidade; tolerância intraperitoneal à glicose e insulina. No fígado, foram avaliados a expressão gênica de enzimas e proteínas envolvidas no metabolismo de glicose: glicoquinase, fosfofrutoquinase, piruvato quinase, monofosfato-adenosina proteína quinase (AMPK), receptor de insulina (INSR), fator de transcrição proteína Forkhead box 01 (FOX01) e proteína quinase B (AKT), e expressão proteica de AKT fosforilada (AKT1[pS473]). O estudo *in vitro* foi realizado com células HepG2, tratadas com glicose (25 mM) e palmitato (1 mM) durante 24 horas, para induzir resistentência à insulina. As células,

foram em seguida, tratadas com fenólicos hidrolisados obtidos da semente de chia (20, 40 e 80 ppm). O extrato de fenólicos hidrolisados da chia foi obtido por meio de extração líquido-líquido com ultrassom, seguida de hidrólise com HCl (1 M). A caracterização e quantificação dos fenólicos contidos no extrato foi realizada utilizando HPLC de fase reversa. Para avaliar o efeito dos fenólicos da chia na resistência à insulin, foram analisados a expressão gênica de AKT, e das enzimas envolvidas na glicogenogênese (fosfoenolpiruvato carboxiquinase e glicose-6-fosfatase) e glicólise (fosfofrutoquinase e piruvato quinase). Dentre os 17 artigos incluídos na revisão sistemática, detalhes sobre a randomização e ocultação de alocação nos estudos foram considerados insuficientes, assim como informações sobre os protocolos utilizados. Entre os estudos, foram encontrados ausência de informações sobre o tamanho amostral, doses de chia e o número de animais utilizados para cada parâmetro avaliado. Entretanto, os principais efeitos da chia foram associados à modulação da AMPK, melhora da tolerância à glicose e insulina, lipogênese, atividade antioxidante e inflamação. O estudo original *in vivo* demonstrou que a chia (farinha e óleo) aumentou os níveis de AKT1[pS473]. O óleo de chia melhorou a tolerância à glicose e insulina, aumentou a AMPK, o receptor de insulina, FOX01, as enzimas glicólise e diminuiu as enzimas gliconeogênese. O experimento *in vitro* demonstrou que os fenólicos das sementes de chia diminuíram a expressão das enzimas gliconeogênicas sem afetar a fosforilação do AKT. Com base em dados de estudos experimentais e da revisão sistemática, nota-se que a chia tem potencial bioativo e seu consumo diário pode reduzir o risco de desenvolvimento de doenças crônicas, principalmente devido aos efeitos antioxidantes, anti-inflamatórios, hipoglicêmicos e hipolipidêmicos da semente. O estudo original *in vivo* indicou potencial da chia em melhorar a tolerância à glicose, embora o estudo *in vitro* tenha demonstrado que os fenólicos da chia não apresentaram tais efeitos. Dentre as três frações investigadas no presente estudo (farinha, óleo e fenólicos hidrolisados), o óleo demonstrou resultados superiores, em relação ao metabolismo de glicose em condições de resistência á insulina.

Palavras-chave: Farinha de Chia. Óleo de chia. Ácido alfa-linolênico. Compostos fenólicos. AMPK. AKT.

LIST OF ABBREVIATIONS

ACC: acetyl-coA carboxylase

AKT: protein kinase B

ALA: alpha linolenic acid

ALP: alkaline phosphatase

ALT: alanine aminotransferase

AMPK: adenosine monophosphate-activated protein kinase

ARRIVE: animal research reporting of in vivo experiment

AST: aspartate aminotransferase

AT: adipose tissue

AUC: area under the curve

BSA: bovine serum albumin

CAT: catalase

CK: creatinine

CPT-1: carnitine-palmitoyl transferase-1

CRP: C-reactive protein

DBP: diastolic blood pressure

DG: diacylglyceride

DMEM: Dulbecco´s modified Eagle´s medium

FA: fatty acid

FAS: fatty acid synthase

FAT/DC36: Fatty acid translocase

FBS: fetal bovine serum

FFA: free fatty acid

Fox01: forkhead box protein O1

FRAP: ferric reducing ability of plasma

G6Pase: glucose-6-phosphate

G6PD: glucose-6-phosphate dehydrogenase

GAE: gallic acid equivalents

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GIR: glucose infusion rate

GK: glucokinase

GLUT-4: glucose transporter type 4

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSK-3β: glycogen synthase kinase 3β

HDLc: high density lipoprotein

HFD: high fat diet

HFHF: high-fat and high-fructose

HOMA-IR: homeostasis model assessment: insulin resistance

HSP25: heat shock protein 25

HSP60: heat shock protein 60

HSP70: heat shock protein 70

iGTT: intraperitoneal glucose tolerance test

IL-10: interleukin 10

IL-6: interleukin 6

INSR: insulin receptor

IR: insulin resistance

IRS-1: insulin receptor substrate 1

IRS-2: insulin receptor substrate 2

ITT: insulin tolerance test

LCA-CoA: long-chain scyl-coA

LCMS: liquid chromatography–mass spectrometry

LDH: lactate dehydrogenase

LDLc: low density lipoprotein

LILACS: Latin American and Caribbean Center on Health Sciences Information

n3: omega 3

n6: omega 6

NEFA: nonesterified fatty acids

NF-κB: factor nuclear kappa B

Nrf2: nuclear factor (erythroid-derived 2)-like 2

PA: palmitic acid

p-AKT: phospho-protein kinase B

pAMPK: phosphorylation of adenosine monophosphate-activated protein kinase

PBS: phosphate-buffered saline

PC: positive control

PEPCK: phosphoenolpyruvate carboxykinase

PDHa: pyruvate dehydrogenase E1 component subunit alpha

PFK: phosphofructokinase

PGC-1α: peroxisome proliferator-activated receptor-γ coactivator

pIRS-1(Tyr)/IRS-1: phosphorylation on Tyr989 of IRS-1

PK: phosphokinase

PPAR: peroxisome proliferator-activated receptor

PRISMA: preferred reporting items for systematic reviews and meta-analysis

PPAR-α: peroxisome proliferator-activated receptor alpha

PPAR-γ: peroxisome proliferator-activated receptor gamma

PVDF: polyvinylidene difluoride

RA: rosmarinic acid

RD: reference diet

RD-RD: offspring from dams fed a reference diet (RD) and fed the RD after weaning

ROS: reactive oxygen species

RT-PCR: real-time polymerase chain reaction

SAMP8 HFD: senescence accelerated mouse - fed a high fat diet

SAMP8 LFD: Senescence Accelerated Mouse - fed a low fat diet

SAMR1 LFD: senescence-accelerated mouse - resistant 1 - fed a low fat diet

SBP: systolic blood pressure

SD: standard diet

SFA: saturated fatty acids

SOD: superoxide dismutase

SRD: sucrose rich diet

SRD-SRD: offspring from SRD-fed dams fed an SRD after weaning

SRD-SRDC: offspring from SRD-fed dams fed an SRD+chia after weaning

SREBP1: sterol regulator element-binding protein 1

SYRCLE: systematic review centre for laboratory animal experimentation

TAC: total antioxidant capacity

TBARS: thiobarbituric acid reactive substances

TBP: TATA box binding protein

TBS-T: tris-buffered saline twin

TC: total cholesterol

TE: total energy

TG: triacylglycerol

TNF-α: tumor necrosis factor alpha

VLDLc: very low density lipoprotein

w: weeks

XO: xanthine oxidase.

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SUMMARY

1. GENERAL INTRODUCTION

Noncommunicable diseases, such as obesity, type 2 diabetes and cardiovascular diseases are responsible for three in five deaths in the world (GBD 2016 RISK FACTORS COLLABORATORS, 2017). These diseases are derived from high blood pressure, high cholesterol, and overweight (WORLD HEALTH ORGANIZATION, 2017). Epidemiological studies indicated strong correlation between obesity and high caloric diets (characterized by high amounts of sugar, fructose and saturated lipids). This dietary pattern is often termed as "western diet", characterized by high intakes of fats, animal-source foods, refined carbohydrates and added sugar (POPKIN; ADAIR; NG, 2011; POPKIN; NIELSEN; BRAY, 2004).

The "western diet" is considered an unbalanced diet, since the macronutrients distribution is not adequate to human requirements (INSTITUTE OF MEDICINE, 2005). Sedentary lifestyle and unbalanced diets lead to overweight (WORLD HEALTH ORGANIZATION, 2017), which is associated with increased oxidative stress and chronic inflammation, and major biochemical and metabolic changes, which trigger a series of events associated with the development of comorbidities such as insulin resistance (IR) and type 2 diabetes mellitus (POZZA; ISIDORI, 2018). The disrupted glucose metabolism is considered key for other events such as atherosclerosis, cardiovascular diseases, high blood pressure, kidney injuries and nonalcoholic fatty liver disease (ISMAIEL; DUMITRAŞCU, 2019).

IR is known as the failed response to insulin by cells. It is a pathological condition, in which cells are unable to use insulin or the hormone production is impaired by pancreas, leading to high blood glucose (SCHENK; SABERI; OLEFSKY, 2008). Eventually, IR may cause hyperinsulinemia in attempt to maintain blood glucose at normal levels. These conditions also lead to increased gluconeogenesis activity and glucose output in liver. Additionally, a high caloric diet is related to the upregulation of Fox01 transcriptional factor, which controls the transcription of gluconeogenesis enzymes. Under these conditions, the downregulation of PI3K/AKT pathway may occur leading to the insulin/AKT pathway disruption and impaired glucose receptor (GLUT) translocation to the plasmatic membrane. These changes in the homeostatic scenario are accompanied by decrease in AKT phosphorylation (CZECH, 2018).

In current context of increased obesity and comorbidities, plant food has been investigated as source of bioactive compounds that might act as a coadjutant in decreasing risk of noncommunicable diseases (BOROWSKA; BRZÓSKA, 2016; GOMES *et al*.,

2019; MOREIRA *et al*., 2019; RIBEIRO *et al*., 2019). The search for mechanisms through which bioactive compounds would improve obese health and decrease comorbidities risk has increased the number of animal studies fed unbalanced diets, since it is a way to mitigate the human food intake pattern that contributes to overweight. Animal studies can be considered an important screening for testing new plant food and their different doses and fractions, attempting a deep investigation into molecular pathways using tissues, which is not possible to accomplish in research involving humans (CHALVON-DEMERSAY *et al*., 2017).

Research studies have demonstrated that food or bioactive compounds could attenuate or prevent the damage generated by obesity and IR (SKROVANKOVA *et al*., 2015). Among these plant foods and their bioactive, an alternative is chia seed (*Salvia hispanica* L.), which belongs to Salvia genus (ARCTOS SPECIMEN DATABASE, 2018), native to northern Guatemala and southern Mexico (BUENO *et al*., 2010), used by Pre-Colombian populations as nutritional food (AYERZA, 2009). Although chia is native from Central America, nowadays the seeds are cultivated all over the world. The largest exportations are from China (14.9%), followed by United Sates (8.9%) and Kazakhstan (7.6%) (TRIGDE SOURCING SOLUTIONS, 2019). The consumption of chia seed has been increasing over the years, due to its nutritional, physicochemical and sensory properties. The adition of chia seed in preparations, such as breads and cakes, improve their concentrations of proteins, unsaturated fatty acids, antioxidants, and dietary fiber. The gum content of chia flour increases its ability to hold water and oil, acting as an emulsifier and stabilizer. The absence of gluten also increases the demand for chia seed use (GRANCIERI; MARTINO; DE MEJIA, 2019).

This seed is also considered the highest botanical source of alpha-linolenic acid (ALA) omega 3 (n-3) and is a good source of dietary fiber, proteins, vitamins, minerals, and phytochemicals, such as phenolic compounds (MARINELI *et al*., 2014; SILVA *et al*., 2017). These components present in chia seed have been considered responsible for the improvement of biological markers related to diseases, presenting properties including: anti-inflammatory (HAMEDI *et al*., 2016), antioxidant (SCAPIN *et al*., 2016), hypotensive (TOSCANO *et al*., 2014), hypocholesterolemic (TAVARES TOSCANO *et al*., 2015) and hypoglycemic (FONTE-FARIA *et al*., 2019; MARINELI *et al*., 2015; VUKSAN *et al*., 2017). The doses used in animal studies vary from 3 to 41.3% for chia seed, and 0.15 to 10% for chia oil (ENES *et al*., 2020a). In clinical trials, the doses varied from 25g/day to 30g/1000 kcal (GRANCIERI; MARTINO; DE MEJIA, 2019).

The potential benefits of chia seed and oil on glucose metabolism control have been investigated in the last few years (FONTE-FARIA *et al*., 2019; MARINELI *et al*., 2015; SILVA *et al*., 2016) and some components of chia seed can be related. For example, rosmarinic acid (RA) is the major phenolic compound in chia seed (OLIVEIRA-ALVES *et al*., 2017) and it has been related to hypoglycemic effect (INUI *et al*., 2016). The high content of fiber and fat of chia seed could contribute to its potential in decreasing blood glucose. Rats fed high-fat and high-fructose diet (HFHF) added of chia seed (13.3%) or chia oil (4%) improved glucose tolerance and insulin resistance (MARINELI *et al*., 2015). Mice fed high-fat diet (HFD) had insulin cascade activated in skeletal muscle tissue after adding chia oil (0.15%) to the diet (FONTE-FARIA *et al*., 2019).

However, it remains unclear, if there is an interaction between chia's bioactive compounds, or if a specific compound is responsible for its health benefits. Regarding the hypoglycemic effect, remains unknown whether it is related only to skeletal muscle tissue or if other insulin dependent tissues may be affected. In addition remains unknown which fraction of chia seed (whole seed, oil or phenolic compounds) is responsible for the improvement on glucose metabolism. Since liver is the major site related to glucose metabolism control, our study aims to elucidate if chia and its fractions can modulate liver glucose control.

2. OBJECTIVES

2.1 General objective

Investigate the effect of different fractions (flour, oil and phenolic compounds) of chia (*Salvia hispanica* L.) on glucose metabolism, and their molecular mechanisms on insulin resistance conditions, *in vivo* and *in vitro*.

2.2 Specifics objectives

- Review the molecular mechanisms involved on chia effect on metabolic biomarker modulation, in animal studies;
- Identify and quantify the phenolic compounds in chia seed hydrolyzed extract;
- Evaluate the effect of chia flour and chia oil on food intake, weigh control and glucose metabolism in liver of rats fed HFHF diet;
- Evaluate the effect of chia seed hydrolyzed phenolics extract on glucose metabolism in insulin resistant hepatoma cells.

3. HYPOTESIS

Hypothesis 1. Chia flour and chia oil, improve glucose metabolism by modulating proteins involved on insulin cellular signanling, and glycolysis and gluconeogenesis enzymes, on liver of *Wistar* rats fed high-fat high-fructose diet.

Hypothesis 2. Hydrolyzed phenolic compounds from chia seed can attenuate insulin resistance, by regulating glycolysis and gluconeogenesis enzymes, on insulin resistant HepG2 cells.

4. MATERIAL AND METHODS

This study can be considered an umbrella trial, since the same experimental design will be investigated in differents approachs, in attempt to find different effects of chia seed in *Wistar* rats fed HFHF diet. The first investigation was based on the potential of chia on health, focoused on molecular mechanisms in animal studies, in a systematic review (ENES *et al*., 2020a). Based on these results, we were able to evaluate the lack of knowlodge involving chia seed. Therefore, our research team conducted different investigations involving the same animal experiment. The first work was developed by Moreira (2019), focousing on chia's (flour and oil) effects on histomorphometry, inflammation and oxidative stress. The present study, therefore is the second work derived from the animal experiment, in which, we focoused on chia flour and chia oil effects on liver glucose metabolism.

The content of this thesis include the review study (ENES *et al.*, 2020a), and the original investigation about chia and its fraction (flour, oil and phenolics) effects on glucose metabolism, and their molecular mechanisms on insulin resistance conditions, *in vivo* and *in vitro* (ENES *et al*., 2020b).

4.1 Preparation of Chia Fractions and Characterization

4.1.1 Preparation of Chia Flour and Extration of Oil from Chia Seeds

The seeds used in the present study were cultivated in Rio Grande do Sul, Brazil, crop of 2017. Seeds were storage at -20 ºC until use.

Chia seed was added to the diet as a flour that was obtained each 15 days in order to prepare experimental diets and to extract the oil from chia, also used to prepare one of the diets used in this protocol.

To obtain the flour, seeds were ground using a blender (PHILIPS WALITA®, model RI2035 500W), at level 4, room temperature, for 2 min. The flour was stored in black polypropylene bags, at -20 ºC until diet preparation or oil extraction.

The oil was weekly obtained, by cold pressing chia flour throughout a hydraulic press (Carver Laboratory Press, ModelC 22400-36 - USA). During the oil extraction, precaution was taken to avoid light exposure. The oil extraction efficiency was 8%. The oil was collected, filtered using filter paper, centrifuged at 1050 x g, for 15 minutes at 7 °C, and stored in amber bottle glass at -20 °C until the moment of chemical characterization analyses, or its use for experimental diets preparation.

4.1.2. Chemical Characterization of Chia Flour and Chia Oil

The chemical composition of chia seed was determined according to analytical procedures recommended by the Association of Official Analytical Chemistry - AOAC (2012). The caloric density value was determined by the conversion factors 4 kcal. g^{-1} for carbohydrates and proteins and 9 kcal. g^{-1} for lipids.

The fatty acid profile of chia oil was determined by gas chromatography as proposed by Murrieta; Hess; Rule (2003), adapted for vegetable oil.

4.1.3 Extraction and Hydrolyses of Phenolics From Chia Flour

Phenolic extraction was performed using an ultrasound liquid–liquid extraction method proposed by Oliveira-Alves *et al*., (2017). Briefly, 2 g of chia flour and 10 mL of methanol: water (80:20, v/v) were vortexed for 10 s and placed in an ultrasound water bath (Model 3510R-MT, Branson Ultrasonic Corporation, Danbury, USA) operated at 42 kHz of ultrasound frequency, 100 W of power, for 60 min at 25 °C ± 3. This was followed by centrifugation at 1792g for 30 min. Collected supernatant was dried using a rotary evaporator, at 50 °C. The dried extracted phenolics were subjected to acid hydrolysis by dissolving in 2 mL of acidified water (formic acid 0.01%), then mixed with 1 M HCl (2 mL) and kept in water bath (100 °C) for 60 min. Hydrolyzed phenolics were purified using solid phase extraction (SPE). Solvent was evaporated to dryness at ± 50 °C under reduced pressure. Dried CHPE were stored at -20 °C with nitrogen gas for future analysis and cell culture experiments.

4.1.4 Chia Hydrolyzed Phenolics Extract Characterization

The total phenolic content of chia hydrolyzed phenolics extract (CHPE) was assessed according to Folin-Ciocalteu's (SWAIN; HILLIS, 2006), method with modifications (ABDERRAHIM *et al*., 2011). The results were quantified using a standard curve ranging 0 to 300 ppm of gallic acid and expressed in milligrams of gallic acid equivalents per milliliter of extract (mg GAE.mL^{-1}) ($y = 1.6229x - 0.0227$; $R2 = 0.9805$). The content of total phenolics was used to establish the treatment doses in cell culture experiment.

The polyphenolic profile and quantification was assessed by reversed phase HPLC with a Waters 2695 Alliance system (Waters Corp.,Milford, MA). Identification and quantitation of polyphenolics were based on their spectral characteristics and retention time, as compared to authentic standards (Sigma Chemical Co., St. Louis, MO).

Compound identities were further confirmed by mass spectrometric analyses, performed on a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Separations were conducted using the Phenomenex (Torrance, CA) Synergi 4 μHydro-RP 80A (2 × 150 mm; 4 μm; S/N) 106273–106275) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in 50:50 methanol and acetonitrile (phase B) run at 0.25 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 30% in 5 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min and was held isocratic for 20 min. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N2), 60 units/min; auxiliary gas (N2), 5 units/ min; spray voltage, 3.3 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V.

4.2 Systematic Review Study

4.2.1 Protocol and Registration

The systematic review was carried out and reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (LIBERATI *et al*., 2009). The review has been registered at the PROSPERO (www.crd.york.ac.uk/prospero/): CRD42019119667.

4.2.2 Literature Search

Studies were identified by searching electronic databases: Latin American and Caribbean Center on Health Sciences Information (LILACS), PubMed/MEDLINE, Embase and Science Direct. The following terms were used to search in each database: (“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L.”) AND (Obesity OR Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” OR Diabetes OR “Cardiovascular Diseases” OR Hypertension OR “Metabolic syndrome” OR “Nonalcoholic fatty liver disease” OR “Nonalcoholic steatohepatitis”). Full search strategy employed in the electronic databases is presented in Table 1. Filters were used, if available, for animal studies. The reason of that choice was based on our purpose of investigating the mechanisms and pathways through which chia seed improves metabolic parameters. The results included original studies conducted with chia seeds and their fractions (oil, fiber, or both), regardless of study duration, dose or concentration used, or experimental design. To

be included in this review the study protocol should have an unbalanced diet group and a control group (standard diet). We excluded studies that used mixtures containing chia seeds, those involving other foods in the same treatment as chia, and those that used chia seed in a healthy diet context.

Table 1. Search strategy for different databases

A. PubMed
((“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L” OR “Chia seed” OR “Chia seeds OR “*Salvia hispanica* L”) AND (Obesity OR Hyperglycemia OR Inflammation O Dyslipidemias OR “Insulin Resistance” OR Diabetes OR “Cardiovascular Diseases” O Hypertension OR “Metabolic syndrome” OR “Nonalcoholic fatty liver disease” O “Nonalcoholic steatohepatitis”))
B. Embase
((“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L”) AND (Obesity O Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” O Diabetes OR “Cardiovascular Diseases” OR Hypertension OR “Metabolic syndrome” ((“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L”) AND (“Nonalcoholic fatty liv disease” OR “Nonalcoholic steatohepatitis”))
C. COCHRANE
((“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L”) AND (Obesity O Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” O Diabetes OR “Cardiovascular Diseases” OR Hypertension OR “Metabolic syndrome” O “Nonalcoholic fatty liver disease” OR “Nonalcoholic steatohepatitis”))
D. LILACS
(tw:(“Chia seed” OR “Chia seeds” OR “*Salvia hispanica* L”)) AND (tw:(Obesity)) O (tw:(Hyperglycemia)) OR (tw:(Inflammation)) OR (tw:(Dyslipidemias)) OR (tw:(“Insul Resistance”)) OR (tw:(Diabetes)) OR (tw:(“Cardiovascular Diseases”)) O (tw:(Hypertension)) OR (tw:(“Metabolic syndrome”)) OR (tw:(“Nonalcoholic fatty liv disease”)) OR (tw:(“Nonalcoholic steatohepatitis”))

4.2.3 Study Selection and Data Collection Process

Following the recommendations of PRISMA (Liberati *et al*., 2009), two researchers independently assessed the eligibility of studies that evaluated the effect of chia consumption. Any discrepancies between reviewers were resolved through consensus. For

studies that fulfilled the inclusion criteria (as described in section 4.2.2), data extraction was performed by two independently working reviewers who were not blinded to author identity and study origin. Titles and abstracts were examined first, followed by full text screening. The reviewers extracted relevant information from each study: authors, publication date, animal model, chia fraction and dose, duration of the study, study protocol, pathway investigated, and main results. This information was summarized in a standardized model of data extraction.

4.2.4 Quality Assessment

The risk of bias was assessed using the Systematic Review Centre for Laboratory animal Experimentation Risk of Bias (SYRCLE RoB) tool (HOOIJMANS, CARLIJN R ROVERS *et al*., 2014), which is based on the Cochrane Collaboration. The SYRCLE RoB tool was developed to evaluate method quality and to measure the bias involving animal studies (HIGGINS *et al*., 2011). The SYRCLE RoB toll consider ten entries that are related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and others. For each included study the 6 bias types were classified as "high" (+, if one or more criteria were not attended), "low" (-, if all criteria were attended) or "unclear" (?, one or more criteria were partly attended).

To increase the strength of the present systematic review, the quality of the included studies was assessed using the Animal Research Reporting of in Vivo Experiment (ARRIVE) guidelines (KILKENNY *et al*., 2010). The ARRIVE guidelines are intended to standardize and improve the quality of reporting animal studies. It consists of 20 criteria which evaluate everything from the title to the conclusion of the report with special attention to the methods involved in the study and the discussion of results, as well as the relevance to human biology and the limitations of the study. Each of the included studies was evaluated and for each criteria, "0" or "1" was graded for "not reported" and "reported" information, respectively, and the frequency was calculated.

4.3 *In Vivo* Study

The *in vivo* study was conducted from June to October of 2018, in Experimental Nutrition Laboratory, of Universidade Federal de Viçosa, using chia flour and chia oil.

4.3.1 Study Design and Diets

This study was approved by the Ethics Commission on Animal Use (CEUA/UFV, protocol no. 31/2018, date of approval: April 26th, 2018) (Appendix 1) and followed university guidelines for animal use for experimental studies. Forty adults male *Wistar* rats (*Rattus norvegicus*), obtained from the Central Animal Facility of the Center for Biological Sciences and Health at Universidade Federal de Viçosa, Minas Gerais, Brazil. Rats remained in individual stainless-steel cages, with free access to water and diet, under controlled conditions (22 ºC ± 2, 12h light/dark cycle).The animals were randomized by body weight and divided into 2 experimental groups, that received standard diet (AIN-93M) (n= 10; 156 g ± 17) and HFHF diet (n=30; 156.5 g ± 17.9), respectively, for 8 weeks, to induce insulin resistance (Phase I). In order to assess the effect of chia flour and chia oil on metabolic disorders (Phase II), animals fed HFHF (b.w. = 358.04 ± 33.00 g, n = 30) were randomized into 3 experimental groups and fed HFHF (b.w. = 366.8 ± 35 g, n = 10); chia flour HFHF (b.w. = 362.4 ± 35.4 g, n = 10), or chia oil HFHF (b.w. = 362.4 ± 34.8 g, n = 10) for 10 weeks. The lean control group (b.w. = 350 ± 29.1 g, n = 10) fed AIN93M diet continued on same diet for 10 weeks.The experimental design is presented in Figure 1.

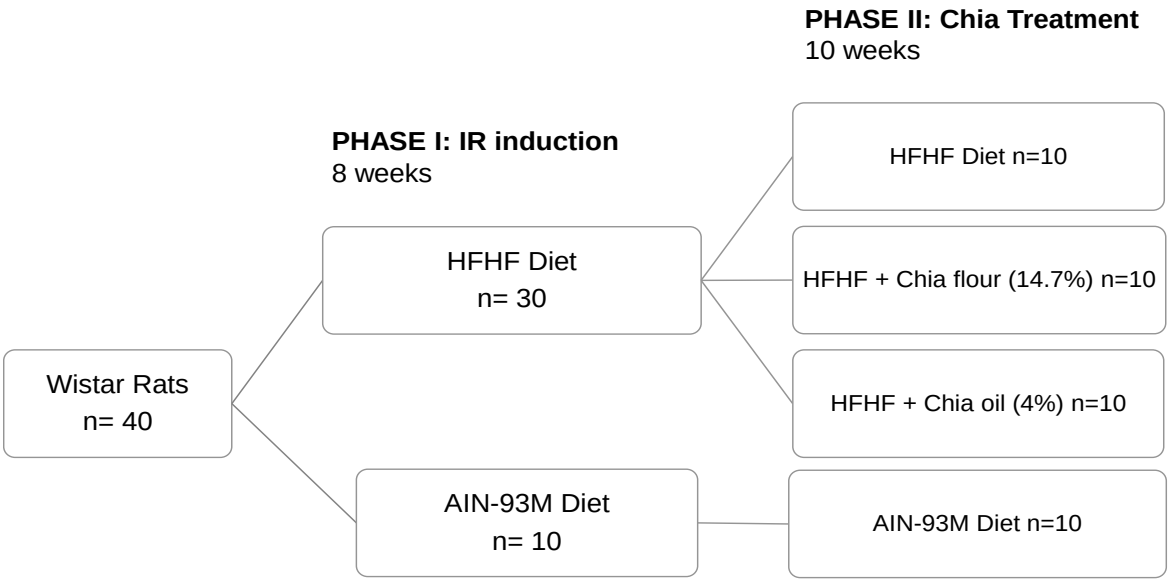

Figure 1 Figure 1. In vivo study experimental design.The experiment was divided into two phases. Phase I: Animals were divided in 2 groups: Control received a standard diet (AIN-93M); high-fat and highfructose group (HFHF) containing 4% (w/w) soybean oil, 31% (w/w) lard and 20% fructose (w/w) (Table 1) for 8 wk. Phase II: HFHF group was divided into 3 groups: HFHF; chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil). Animals from control received AIN-93M diet on phase II. IR: insulin resistance.

Diets were based on the roedent standard AIN-93M diet (REEVES; NIELSEN; FAHEY GEORGE C., 1993), or high-fat and high-fructose diet (MARINELI *et al*., 2015). The control group (AIN-93M) received the standard diet from AIN-93M; the HFHF group received a diet containing 4% (w/w) of soybean oil, 31% (w/w) of lard, and 20% (w/w) of fructose; the chia flour group received the HFHF diet with 14.7% (w/w) of chia flour; the chia oil group received HFHF with 4% (w/w) of chia oil. For chia flour and chia oil groups the soybean oil was replaced by the fat content of chia (flour or oil). The content of protein and carbohydrate and dietary fiber of chia seed was taken into account to balance the chia seed diet. For dietary fiber adequacy, it was considered that the amount of chia flour to prepare 1000 g of chia flour diet (147 g/1000 g) offered 5.6% of dietary fiber. In order to standardize dietary fiber amount for all diets, based on chia flour diet amount, it was added cellulose for the other experimental diets (AIN-93M, HFHF and HFHF with chia oil) (Table 2). Diets were prepared every 15 days, packed in dark polyethylene bags and stored at -20 ºC to minimize fatty acids oxidation. Diets and water were replaced every week. The food intake and weight gain of the animals were monitored weekly. Diets were prepared every 15 days, packed in dark polyethylene bags and stored at -20 ºC to minimize fatty acid oxidation. Water and diet consumption were daily checked, and replaced every week,

with exception of chia oil diet, that was replaced every two days in attempt to avoid lipid oxidation. The food intake and animal weight gain were monitored weekly.

Table 2. Nutritional composition of experimental diets.

Ingredients (g/kg of diet)	Experimental Diets			
	AIN-93M	**HFHF**	**Chia flour**	**Chia oil**
Albumin*	136.4	136.4	101.8	136.4
Dextrinized starch	155	45.4	45.4	45.4
Corn starch	463.5	135	116.8	135
Sucrose	100	28.6	29.3	28.6
Fructose	-	200	200	200
Soybean oil	40	40	-	-
Chia flour	-	-	147.3	-
Chia oil	-	-	-	40
Lard	-	310	310	310
Microcrystalline Cellulose	55.8	55.8	0.00	55.8
Vitamin mix	10	10	10	10
Mineral mix	35	35	35	35
L-cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5
Nutritional Composition				
Macronutrients				
Carbohydrates (%)	77.4	31.0	30.1	31.0
Protein (%)	12.9	9.1	9.2	9.1
Lipids (%)	9.7	59.8	60.4	59.8
Energetic density (kcal.g^{-1})	3.71	5.26	5.21	5.26
Fatty acids (g.kg^{-1})**				
Linoleic (C18:2 n-6)	20.2	58.8	46.5	46.5
α-Linolenic (C18:3 n-3)	3.3	10.2	31.8	31.8
n-6/n-3 ratio	6.17:1	5.77:1	1.46:1	1.46:1

AIN-93M (Reeves, Nielsen e Fahey George C., 1993): standard diet group; HFHF (Marineli, *et al.*, 2015): high fat and high-fructose group; Chia flour group: HFHF with 14.7% (w/w) chia flour; Chia oil group: HFHF with 4% (w/w) chia oil.

* Amount was calculated based on protein content equal to 88% to provide 12 g protein.100 g-1 of diet (MOREIRA, 2019).

** Fatty acids expressed in g/kg diet and determined by gas chromatography (MOREIRA, 2019).

4.3.2 Intraperitoneal Glucose Tolerance Test and Insulin Tolerance Test

Intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT) were performed after 9th and 10th weeks of chia flour or oil administration. Briefly, blood glucose levels were measured with a handheld glucometer (Accu-Chek®, Roche) using appropriate test strips. For the iGTT, D-glucose solution 50% (2 g.kg^{-1} body weight) was injected into the peritoneal cavity. Blood glucose levels were measured after 30, 60, 90, and 120 min from the tail vein. The area under the curve (AUC) of glucose was calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA). ITT was accessed after intraperitoneal human insulin injection (0.75 U.kg^{-1} body weight, Novolin®, Novo Nordisk) through glucose blood levels after 5, 10, 15, 20, 25, and 30 min of insulin injection. The formula [0.693/(t1/2)] was used to calculate the constant rate of glucose disappearance. The glucose t1/2 was calculated from the slope of the least-squares analysis of the glucose concentrations during the linear phase (BONORA *et al*., 1987).

4.3.3 Euthanasia and Tissue Collection

The experiment was finished by euthanasia of animals at the end of 10 weeks of diets administration (no fast was performed). The animals were euthanized by cardiac puncture, after anesthetized with isoflurane (Isoforine, Cristália®). The liver was collected, weighted and immediately frozen with liquid nitrogen, prior storage at -80 °C for subsequent analysis. Adipose tissue was collected and weighted to calculate percentage of adiposity using the following formula: (visceral + retroperitoneal + epididymal adipose tissues)/total body weight × 100 (SILVA *et al*., 2018).

4.3.4 Gene Expression in Liver Tissue

Expression levels of genes involved on insulin and glucose metabolism in the liver were analyzed by RT-qPCR using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacture's protocol. Briefly, liver tissue (~100 mg) grinded in liquid nitrogen were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) (1 mL) following manufacturer’s protocol. Extracted mRNA was reverse transcribed into cDNA using the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY). The analyses were performed on the StepOneTM Real-TimePCR System (Thermo Fisher Scientific, Waltham, MA) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). Primers were purchase from Integrated DNA Technologies

(IDT DNA, Coralville, IA) to amplify AMPK, INSR, FOXO1, AKT, GK, PFK, PK and β-actin. β-actin was used as endogenous control to normalize the relative expression of mRNA. The primer sequences are presented in Table 4

Table 3. Sequencing primers used in the RT-qPCR analysis for in vivo study.

Genes	Oligonucleotide (5´-3´)	
	Forward	**Reverse**
AMPK	TGAAGCCAGAGAACGTGTTG	ATAATTTGGCGATCCACAGC
INSR	TCATGGATGGAGGCTATCTGGA	TCCTTGAGCAGGTTGACGATTTC
FOX01	GATAAGGGCGACAGCAACAG	TGAGCATCCACCAAGAACTT
AKT	GGGCCACGGATACCATGAAC	AGCTGACATTGTGCCACTGA
GK	AGTATGAACCGGATGGTGGATGAA	CCAGCTTAAGCAGCACAAGTCGTA
PFK	CCACCTGGAGGCCATTGTAGA	GGGATGACGCACATGACGA
PK	ATCTGGGCAGATGATGTGGA	ATAGGGTGTAACTGGGTCAGAATGG
β-actin	GTCGTACCACTGGCATTGTG	CTCTCAGCTGTGGTGGTAA

AMPK: AMP-activated protein kinase; INSR: Insulin receptor; FOX01: Forkhead box protein 01; AKT: Protein kinase B; GK: Glucokinase; PFK: Phosphofructokinase, PK: Pyruvate kinase.

4.3.5 Phospho-AKT Assessment

Phospho-AKT protein levels were quantified using the AKT1[pS473] Ultrasensitive ELISA sandwich Kit (Invitrogen, ThermoFisher Scientific, MA, USA), according to the manufacture's protocol, on liver homogenate. Liver tissue was homogenized with phosphate buffer (1:10) 50 mM, at pH 7.4 containing 1 mM EDTA, and protease inhibitor (Protease Inhibitor Cocktail powder, Sigma Aldrich). The absorbance of samples was read at 450 nm (Multiskan Microplate Photometer, ThermoFisher Scientific, MA, USA).

4.4 *In Vitro* Study

The *in vitro* study was carried out in Laboratory of Nutritional Biochemistry, located in Texas A&M University, Texas, United States. The *in vitro* study was conducted in attempt to test the phenolics from chia, using an extract. The in vitro model was chosen to test phenolics from chia, since this was the first time that phenolics fraction of chia seed was used in order to check its effects in biological systems. Since no data were available

regarding its safety, we conserided to use an in vitro model, once based on chia phenolics characterization studies, the seed presents high content of phenolics, and it could be harmful to animals. Otherwise, botanical extracts are usually chemically unstable. Using them in an *in vivo* model would be a risk since animal studies protocol's take at least weeks and extract changes would impair the studie results and conclusions.

4.4.1 Cell line

The human hepatoma (HepG2) cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dullbecco's modified Eagle's medium (DMEM) containing 5.5 mM Dglucose (GIBCO, Thermo Fisher, Waltham, MA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C, 5% CO2.

HepG2 cells were used since for the *in vivo* study we inbestigated the effects of chia flour and chia oil on liver glucose metabolism. The aim was to focus this research on the effects of differents fractions of chia on liver glucose metabolism. Therefore, we choose hepatoma human cells to conduct the *in vitro* protocol.

4.4.2 Cell Viability

Cells were seeded at 80% confluence in a 96 well plate for 24 h to allow cell attachment. Followed by chia phenolics treatments (20 to 320 ppm) quantified as total phenolics (ABDERRAHIM *et al.*, 2011) against a standard curve of gallic acid (0–300 ppm) and expressed in milligrams of gallic acid equivalents per milliliter of extract (mg GAE.mL^{-1}). After 48 h incubation, cell viability was determined using the in vitro toxicology assay kit resazurin following the manufacture's protocol (Sigma-Aldrich, San Luis, MO).

4.4.3 Insulin resistance protocol

HepG2 cells were maintained in DMEM medium at 37 °C, 5% CO2 for 24 h. One day after plating, the medium was replaced for FBS free DMEM for 24 h. The cells were then treated with high glucose (25 mM) FBS free medium containing palmitic acid (PA) (1 mM) for 24 h (LEE; CHO; KWON, 2010; NAKAJIMA *et al.*, 2000). After 24 h, insulin (100 µM) was added for 15 min. Cells cultured in 5.5 mM D-glucose DMEM, FBS free medium were used as negative controls (with and without insulin).

4.4.4 Gene Expression

A preliminary test was conducted with chia phenolics extract (20, 40, 80 ppm). Insulin resistance induced cells as described in 2.6.3 were treated with CHPE extract (80 ppm) for 24 h followed by insulin (100 µM) was added for 15 min. Total RNA from HepG2 cells was extracted using Quick-RNA Mini Prep (Zymo Research, Irvine, CA), according to the manufactureŕs protocol. cDNA was synthesized using iScript Reverse Transcription Supermix (BioRad, Hercules, CA). cDNA was subjected to quantitative Real-time polymerase chain reaction (RTPCR) using the iTaq Universal SYBR Green Supermix Kit (BioRad, Hercules, CA) according to the manufactureŕs protocol. RT-PCR data was analyzed with the delta CT method (Schmittgen and Livak, 2008) using TBP as the housekeeping gene. Primers were purchased from Sigma-Aldrich (San Luis, MO, USA) to amplify PFK, PK, PEPCK and G6Pase. Primers sequence are presented in Table 5.

Table 4. Sequencing primers used in the RT-qPCR analysis for in vitro study.

Genes	Oligonucleotide (5´-3´)	
	Forward	Reverse
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
PFK	GGTAAGATCTCAGAGACTACAG	AGAAGTCGTTATCGATGGAG
PK	AAAATTGAGAACCACGAAGG	CAATCATCATCTTCTGAGCC
PEPCK	TGGAAGAATAAGGAGTGGAG	CTTCATAGACTAGAGGGACAC
G6Pase	ACTGTGCATACATGTTCATC	TGAATGTTTTGACCTAGTGC

TBP: TATA box binding protein; PFK: Phosphofructokinase; PK: Phosphokinase; PEPCK: Phosphoenolpyruvate carboxykinase; G6Pase: Glucose-6 phosphatase.

4.4.5 Phospho-AKT Assessment

Insulin resistance induced cells as described in 2.6.3 were treated with CHPE extract (20, 40 and 80 ppm) for 24 h followed by insulin (100 µM) for 15 min. Negative controls were prepared as detailed in 2.7.3. Cells lysates were obtained adding X Tractor buffer (Takara Bio Company, CA, USA). Solid cellular debris was removed by centrifugation at 25,200g for 10 min at 4 °C. The supernatant was collected and stored at -80 °C. Protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Cell lysates (60 mg protein diluted with Laemmli's loading buffer and boiled for 5 min) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for 2 h. The proteins were transferred by wet

blotting onto a 0.2 mm PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked using 5% non-fat milk in a 0.1% tween-phosphate buffer solution (T-PBS) for 30 min and incubated with the primary antibody (1:1000) in a 3% bovine serum albumin in T-PBS overnight at 4 °C with gentle shaking to assess the protein level of p-AKT(Ser473) (Cell Signalling, Danvers, MA) and βactin (Sigma-Aldrich, San Loius, MO). This was followed by incubation with the anti-rabbit IgG secondary antibody (1:2000) (Cell Signalling, Danvers, MA) in 5% milk T-PBS for 2 h. The reactive bands were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) after 1 min of incubation, using an Omega Ultra Lum Molecular Imaging System (model Omega10gD) and band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, UDA; https://imagej.nih.gov/ij/download.html).

4.5 Statistical Analysis

Quantitative data were expressed as mean ± standard error or standard deviation of the mean. Effect of treatments were analyzed using one-way analysis of variance (ANOVA) followed by NewmanKeuls post hoc test for parametric data and Kruskal-Wallis followed by Dun's for non-parametric. Tukey test was used to compare glucose blood levels means during different times on iGTT and ITT tests. The significance level was set at $p < 0.05$. GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used to perform the analysis.

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5. RESULTS

5.1 Paper 1

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Concise Reviews & Hypotheses in Food Science

Chia seed (*Salvia hispanica L.*) effects and their molecular mechanisms on unbalanced diet experimental studies: A systematic review

Bárbara N. Enes, Luiza P. D. Moreira, Bárbara P. Silva, Mariana Grancieri, Haira G. Lúcio, Vinícius P. Venâncio, Susanne U. Mertens-Talcott, Carla O. B. Rosa, and Hércia S. D. Martino

Abstract: The aim of this review was to compile evidence and understand chia seed effects on unbalanced diet animal studies and the molecular mechanisms on metabolic biomarker modulation. A systematic review was conducted in electronic databases, following PRISMA recommendations. Risk of bias and quality was assessed using SYRCLE toll and ARRIVE guidelines. Seventeen articles were included. Throughout the studies, chia's main effects are associated with AMPK modulation: improvement of glucose and insulin tolerance, lipogenesis, antioxidant activity, and inflammation. Details about randomization and allocation concealment were insufficient, as well as information about blind protocols. Sample size, chia dose, and number of animals evaluated for each parameter were found to be lacking information among the studies. Based on experimental study data, chia has bioactive potential, and its daily consumption may reduce the risk of chronic disease development, mainly due to the antioxidant, anti-inflammatory, hypoglycemic, and hypolipidemic effects of the seed.

Keywords: alpha linolenic acid, chia seed, dyslipidemia, glucose tolerance, *Salvia hispanica* L.

Practical Application: The consumption of chia seeds may improve lipid profile, insulin and glucose tolerance, and reduce risk of cardiovascular disease. Whole seed or its oil presents positive effect, but the effects of chia oil can act faster than the seed.

1. INTRODUCTION

Noncommunicable diseases, such as cardiovascular diseases, cancer, and diabetes, are responsible for three in five deaths in the world (Wang et al., 2016). These diseases are derived from high blood pressure, high cholesterol, and ultimately overweight and obesity (World Health Organization, 2017). Among various factors that may contribute to weight excess, epidemiological studies indicated strong correlation between the consumption of sugar, fructose, and saturated lipids—high content products with obesity. This pattern of food consumption is often termed as the "western diet," characterized by high intakes of fats, animal-source foods, refined carbohydrates, and added sugar. (Popkin, Adair, & Ng, 2011; Popkin, Nielsen, & Bray, 2004).

The "western diet" is considered an unbalanced diet, since the macronutrients distribution is not adequate to human requirements (Institute of Medicine, 2005). Sedentary lifestyle and unbalanced diets lead to overweight (World Health Organization, 2017), which is associated with increased oxidative stress and chronic inflammation, and major biochemical and metabolic changes, which trigger a series of events associated with the development of comorbidities, such as insulin resistance (IR), type 2 diabetes mellitus, and cardiovascular diseases (Pozza & Isidori, 2018).

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In the current context of increased obesity and comorbidities, plant food has been investigated as a source of bioactive compounds that might act as a coadjutant in the prevention and treatment of diseases (Borowska & Brzóska, 2016). The search for mechanisms through which bioactive compounds would improve obese health and decrease comorbidities risk has increased animal studies fed unbalanced diets, since it is a way to mitigate the human food intake pattern that contributes to overweight. Experimental studies involving Rodents are widely used in the nutrition research field since their physiology is similar to human's, including gastrointestinal (Hatton, Yadav, Basit, & Merchant, 2015). Animal studies can be considered an important screening for testing new plant food and their different doses and fractions, attempting a deep investigation into molecular pathways using tissues, which is not possible to accomplish in research involving humans (Chalvon-demersay, Blachier, Tomé, & Blais, 2017). Well-planned animal studies are considered a step before treatment application in clinical trials, which can be more successful when previous data are available to design efficient outcomes (Everitt, 2015).

A plant food alternative is chia seed (*Salvia hispanica* L.), which belongs to Salvia genus (Arctos Specimen Database, 2018), native to northern Guatemala and southern Mexico (Busilacchi et al., 2013), used by Pre-Colombian populations as nutritional food (Ayerza, 2009). This seed is known for its high concentration of alpha linolenic acid (ALA) omega 3 (n-3), dietary fiber, proteins, vitamins, minerals, and phytochemicals, such as phenolic compounds (da Silva et al., 2017; Marineli et al., 2014).

These components present in chia seed have been considered responsible for the improvement of biological markers related to diseases, presenting properties, including anti-inflammatory

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Chia seed (*Salvia hispanica L.*) effects and their molecular mechanisms on unbalanced diet experimental studies: a systematic review

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Running Head: Chia seed effects on unbalanced diet

Abstract

The aim of this review was to compile evidence and understand chia seed effects on

unbalanced diet animal studies and the molecular mechanisms on metabolic biomarker modulation. A systematic review was conducted in electronic databases, following PRISMA recommendations. Risk of bias and quality was assessed using SYRCLE toll and ARRIVE guidelines. 17 articles were included. Throughout the studies, chia´s main effects are associated to AMPK modulation: improvement of glucose and insulin tolerance, lipogenesis, antioxidant activity, and inflammation. Details about randomization and allocation concealment were insufficient, as well as information about blind protocols. Sample size, chia dose and number of animals evaluated for each parameter were found to be lacking information among the studies. Based on experimental study data, chia has bioactive potential, and its daily consumption may reduce the risk of chronic disease development, mainly due to the antioxidant, anti-inflammatory, hypoglycemic, and hypolipidemic effects of the seed.

Keywords: *Salvia hispanica* L., Chia seed, Glucose tolerance, Dyslipidemia, Alpha linolenic acid.

Practical Application: The consumption of chia seeds may improve lipid profile, insulin and glucose tolerance, and reduce risk of cardiovascular disease. Whole seed or its oil present positive effect, but the effects of chia oil can act faster than the seed.

1. INTRODUCTION

Noncommunicable diseases, such as cardiovascular diseases, cancer and diabetes are responsible for three in five deaths in the world (Wang *et al*., 2016). These diseases are derived from high blood pressure, high cholesterol, and ultimately overweight and obesity (World Health Organization, 2017). Among various factors that may contribute to weight excess, epidemiological studies indicated strong correlation between the consumption of sugar, fructose and saturated lipids-high content products with obesity. This pattern of food consumption is often termed as the "western diet", characterized by high intakes of fats, animal-source foods, refined carbohydrates and added sugar (Popkin, Adair, & Ng, 2011; Popkin, Nielsen, & Bray, 2004).

The "western diet" is considered an unbalanced diet, since the macronutrients distribution is not adequate to human requirements (Institute of Medicine, 2005). Sedentary lifestyle and unbalanced diets lead to overweight (World Health Organization, 2017), which is associated with increased oxidative stress and chronic inflammation, and major biochemical and metabolic changes, which trigger a series of events associated with the development of comorbidities such as insulin resistance, type 2 diabetes mellitus, and cardiovascular diseases (Pozza & Isidori, 2018).

In current context of increased obesity and comorbidities, plant food has been investigated as source of bioactive compounds that might act as a coadjutant in prevention and treatment of diseases (Borowska & Brzóska, 2016). The search for mechanisms through which bioactive compounds would improve obese health and decrease comorbidities risk has increased animal studies fed unbalanced diets, since it is a way to mitigate the human food intake pattern that contributes to overweight. Experimental studies involving roedents are widely used in the nutrition research field since their physiology is similar to human´s, including gastrointestinal (Hatton, Yadav, Basit, & Merchant, 2015). Animal studies can be considered an important screening for testing new plant food and their different doses and fractions, attempting a deep investigation into molecular pathways using tissues, which is not possible to accomplish in research involving humans (Chalvon-demersay, Blachier, Tomé, & Blais, 2017). Well-planned animal studies are considered a step before treatment application in clinical trials, which can be more successful when previous data are available to design efficient outcomes (Carolina, 2015).

A plant food alternative is chia seed (*Salvia hispanica* L.) which belongs to Salvia genus (Arctos Specimen Database, 2018), native to northern Guatemala and southern

Mexico (Busilacchi *et al*., 2013), used by Pre-Colombian populations as nutritional food (Ayerza, 2009). This seed is known for its high concentration of alpha linolenic acid (ALA) omega 3 (n-3), dietary fiber, proteins, vitamins and minerals, and phytochemicals, such as phenolic compounds (da Silva *et al*., 2017; Marineli *et al*., 2014).

These components present in chia seed have been considered responsible for the improvement of biological markers related to diseases, presenting properties including: anti-inflammatory (Hamedi, Jamshidzadeh, Ahmadi, Sohrabpour, & Zarshenas, 2016), antioxidant (Scapin, Schmidt, Prestes, & Rosa, 2016), hypotensive (Toscano *et al*., 2014), hypoglycemic (Vuksan *et al*., 2017; Vuksan *et al*., 2010), and hypocholesterolemic (Toscano, Toscano, Tavares, & Oliveira Silva, 2015). Though it remains unclear if there is an interaction between them, acting in a synergic way or, if a specific compound is responsible for its health benefits. Besides that, the mechanisms and pathways involved in the chemoprevention of diseases by chia seeds remains unknown.

Thus, the aim of this review was to compile evidence of chia seed effects on unbalanced diet animal studies and to understand the effects and the molecular mechanisms on metabolic biomarker modulation.

2. METHODS

2.1. Protocol and registration

This systematic review was carried out and reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (Liberati *et al*., 2009). The review has been registered at the PROSPERO (www.crd.york.ac.uk/prospero/): CRD42019119667.

2.2. Literature Search

Studies were identified by searching electronic databases: Latin American and Caribbean Center on Health Sciences Information (LILACS), PubMed/MEDLINE, Embase and Science Direct. The following terms were used to search in each database: (“chia seed” OR “chia seeds” OR “salvia hispanica L.”) AND (obesity OR Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” OR diabetes OR “Cardiovascular Diseases” OR Hypertension OR “metabolic syndrome” OR “nonalcoholic fatty liver disease” OR “nonalcoholic steatohepatitis”). Full search strategy employed in the electronic databases is presented in **Table S1** in Supplementary Material. Filters were

used, if available, for animal studies. The reason of that choice was based on our purpose of investigating the mechanisms and pathways through which chia seed improves metabolic parameters. The results included original studies conducted with chia seeds and their fractions (oil, fiber, or both), regardless of study duration, dose or concentration used, or experimental design. To be included in this review the study protocol should have an unbalanced diet group and a control group (standard diet). We excluded studies that used mixtures containing chia seeds, those involving other foods in the same treatment as chia, and those that used chia seed in a healthy diet context.

2.3. Study Selection and Data Collection Process

Following the recommendations of PRISMA (Liberati *et al*., 2009), two researchers independently assessed the eligibility of studies that evaluated the effect of chia consumption. Any discrepancies between reviewers were resolved through consensus. For studies that fulfilled the inclusion criteria (as described in section 2.1), data extraction was performed by two independently working reviewers who were not blinded to author identity and study origin. Titles and abstracts were examined first, followed by full text screening. The reviewers extracted relevant information from each study: authors, publication date, animal model, chia fraction and dose, duration of the study, study protocol, pathway investigated, and main results. This information was summarized in a standardized model of data extraction.

2.4. Quality assessment

The risk of bias was assessed using the Systematic Review Centre for Laboratory animal Experimentation Risk of Bias (SYRCLE RoB) tool (Hooijmans *et al*., 2014) which is based on the Cochrane Collaboration. The SYRCLE RoB tool was developed to evaluate method quality and to measure the bias involving animal studies. (Higgins et al., 2011). The SYRCLE RoB toll consider ten entries that are related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and others. For each included study the 6 bias types were classified as “high” (+, if one or more criteria were not attended), “low” (-, if all criteria were attended) or “unclear” (?, one or more criteria were partly attended).

To increase the strength of the present systematic review, the quality of the included studies was assessed using the Animal Research Reporting of in Vivo Experiment (ARRIVE) guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). The

ARRIVE guidelines are intended to standardize and improve the quality of reporting animal studies. It consists of 20 criteria which evaluate everything from the title to the conclusion of the report with special attention to the methods involved in the study and the discussion of results, as well as the relevance to human biology and the limitations of the study. Each of the included studies was evaluated and for each criteria, "0" or "1" was graded for "not reported" and "reported" information, respectively, and the frequency was calculated.

3. RESULTS AND DISCUSSION

3.1 Included studies and characteristics

The search yielded a total of 165 records from which 17 duplicates were removed. The selection of the remaining studies was made by reading their titles and abstracts and after this step, 19 studies that met the inclusion criteria were retrieved for full-text reading. From the full-text reading, 16 studies attended all the inclusion criteria and were included in this review. During the full-text reading, one study was identified by snowballing adding up to 17 studies included. Most records excluded were review publications, chemical characterization research, studies related to technological properties of chia, and absence of use of chia as study treatment **(Figure 1)**. From 17 included records, only one was conducted with Flanders hybrid rabbits (Sierra, Roco, Alarcon, & Medina, 2015). The others were conducted with rodents: *Wistar* rats (da Silva *et al*., 2018; Fortino, Oliva, Rodriguez, Lombardo, & Chicco, 2017; Creus, Benmelej, Villafañe, & Lombardo, 2017; Creus *et al*., 2016; Marineli *et al*., 2015a; Marineli, Lenquiste, Moraes, & Maróstica, 2015b; Oliva, Ferreira, Chicco, & Lombardo, 2013; Poudyal, Panchal, Ward, & Brown, 2013; Poudyal, Panchal, Waanders, Ward, & Brown, 2012a; Poudyal, Panchal, Ward, Waanders, & Brown, 2012b; Chicco, D'Alessandro, Hein, Oliva, & Lombardo, 2009; Ayerza & Coates, 2007), C57BL/6 mice (Fonte-Faria *et al*., 2019), Swiss mice (de Miranda *et al*., 2019), and SAMR1/SAMP8 mice (Rui, Yang, Chen, Qin & Wan, 2018).

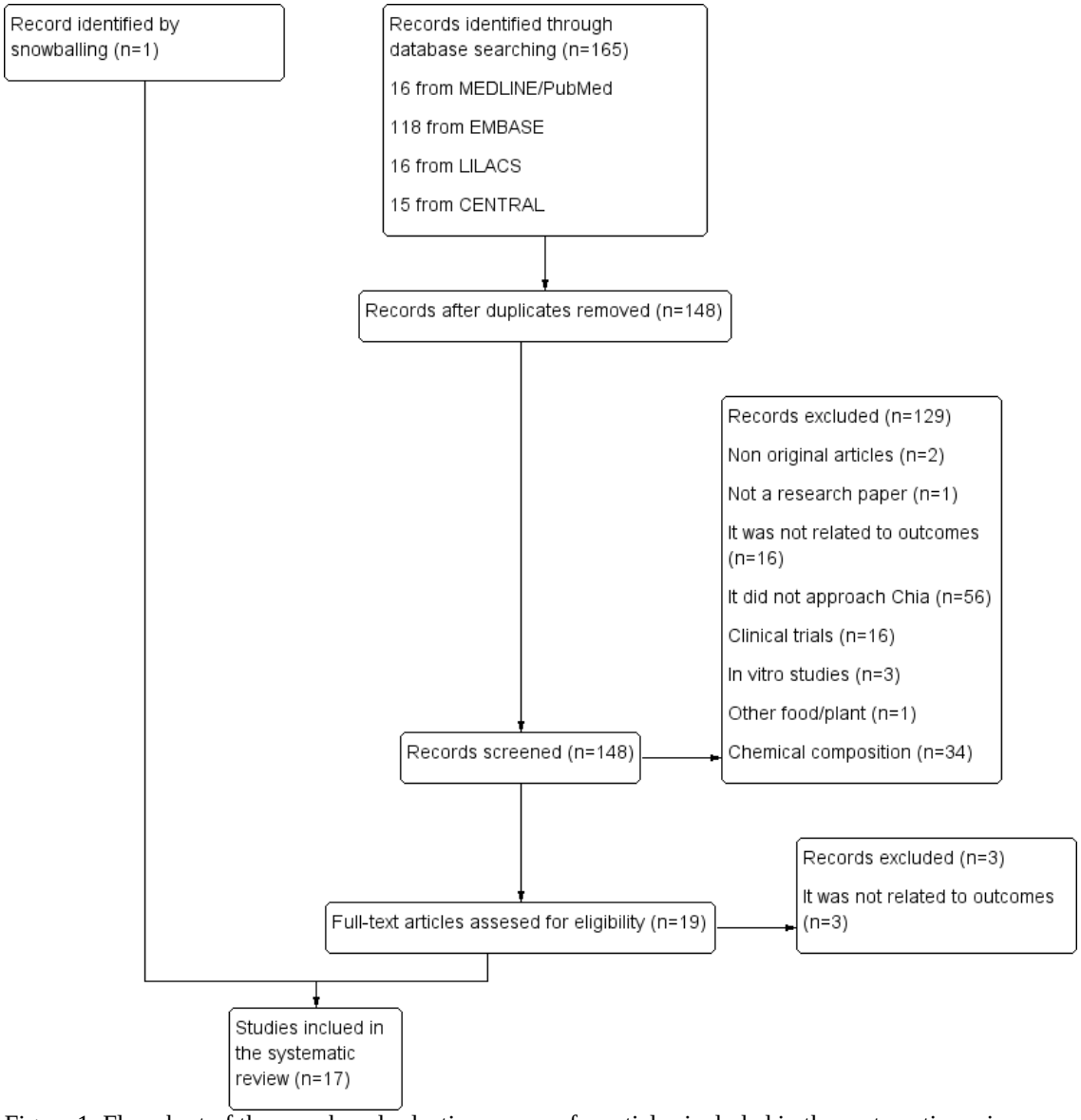

Figure 1: Flowchart of the search and selection process for articles included in the systematic review, according to PRISMA recommendation.

A total of 9 studies evaluated glucose metabolism, 10 studies evaluated the lipogenesis pathway, 5 studies analyzed the antioxidant effects, 3 studies analyzed the inflammation and 1 analyzed the atherosclerosis pathway. Regarding the used fractions, 11 studies used chia seed and the dose varied between 3 and 41.7%. The lowest dose, 3%, was able to increase the HDL-c of the animals (de Miranda *et al*., 2019), and the highest dose, 41.7%, was able to increase the antioxidant capacity, decrease the inflammatory markers (cytokines and NF-κB expression), and decrease cholesterol (da Silva *et al*., 2018). The whole seed was offered to animals as flour since particle size acts directly on digestion and metabolic processes (Slavin, 2003). Furthermore, the whole seed flour has every component present in the grain, like endosperm, bran, germ and coat (Arvola *et al*., 2007) and the grinding allows bioaccessibility of bioactive antioxidant, such as phenolic acids to

cells which increase health benefits (Rosa, Dufour, Lullien-pellerin, & Micard, 2013).

Six studies used chia oil and the doses varied between 0.15% and 10%. The lowest concentration (0.15%) had effects that improved insulin resistance (IR), as increased glucose and insulin tolerance induced insulin receptor substrate (IRS) phosphorylation and GLUT-4 translocation to plasma membrane, and also reduced the serum fasting insulin levels (Fonte-Faria *et al*., 2019). Treatment duration varied between 3 and 24 weeks and all studies observed positive effects of chia seeds or oil. Chia seed has the highest known amount of n-3, ranged between 64.8% to 56.9% (Ayerza & Coates, 2011) which may explain the promising results found in animal studies. A summary of each publication is presented in **Table 1**.

Although there are published clinical trials with chia seeds, this review included only animal studies. Our choice was based on our purpose which was to investigate the mechanisms and pathways through which chia seed improves metabolic parameters. Most of the clinical trial results are limited to improvement on lipid profile (Vuksan *et al*., 2017; Toscano *et al*., 2015; Toscano *et al*, 2014; Nieman *et al*., 2012; Nieman *et al*., 2009), oxidative stress (Vuksan *et al*., 2017; Toscano *et al*., 2014; Vuksan *et al*., 2007), blood pressure (Toscano *et al*., 2014; Vuksan *et al*., 2007), and weight loss (Vuksan *et al*., 2017; Toscano *et al*., 2015), but all these parameters were not reproducible. This lack of major results may be due to feasible doses of chia used in these studies, which can be considered low when compared to doses offered to animals (20g/kg body weight) (Creus *et al*., 2017). The amount of chia seed (as food) expected to improve metabolic parameters may be too high for human consumption.

Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Fonte-Faria *et al*. (2019)	C57 BL/6 mice, male	- Control (n=8) - HFD (n=8) - HFD (n=8) followed by HFD + Chia oil	Chia oil 0.15%	19 weeks	Glucose metabolism Insulin signaling	↑ Glucose and insulin tolerance ↓ Serum fasting insulin levels ↓ Serum leptin ↓ Serum TG ↑ HDLc ↓ Body fat mass and ↑ Body lean mass ↑ pIRS-1(Tyr)/IRS-1 ↑ GLUT-4 translocation to plasma membrane
da Silva *et al*. (2018)	*Wistar* rats, male	- Control + calcium carbonate (n=8) - Control + Chia seed (n=8) - HFD + calcium carbonate (n=8) - HFD + Chia seed (n=8)	Chia seed 41.3%	5 weeks	Antioxidant status Inflammation	↓ Total, LDLc and VLDLc cholesterol ↑ SOD activity (liver) ↑ CAT (plasma) ↑ PPAR-α ↓ NFκB mRNA expression ↓ NFκB (liver) ↓ TNF and IL-10
de Miranda *et al*. (2018)	Swiss mice	- Control (n=6) - Control + Chia seed (n=6) - HFD (n=6) - HFD + Chia seed (n=6)	Chia seed 3%	16 weeks	Glucose metabolism Antioxidant status Lipogenesis Inflammation	- Did not modify glucose metabolism ↑ HDLc
Yehua Rui *et al*. (2018)	SAMR1 mice SAMP8 mice	- SAMR1 LFD (n=8) - SAMP8 LFD (n=8) - SAMP8 HFD (n=8) - SAMP8 HFD + Chia seed (n=8)	Chia seed 10%	18 weeks	Glucose metabolism	↓ Insulin (plasma) ↓ HOMA-IR

Continuation Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Creus *et al*. (2017)	*Wistar* rats, male	- Control (n=24) - SRD (n=24) - SRD (n=24) followed by SRD + Chia seed	Chia seed 36.2%	24 weeks	AMPK Glucose metabolism	↓ pAMPK/AMPK ↓ TG, FFA, glucose (plasma) ↑ G-6-P, glycogen (heart muscle) ↑ Hexokinase and PDHa activities (heart muscle) ↑ GLUT-4, IRS-1 (Heart muscle) ↓ Collagen and hydroxyproline contents in left ventricle ↓ SBP
Fortino *et al*. (2017)	*Wistar* rats, male	Male offspring of RD-fed dams: - RD-RD (control) Male offspring of SRD - fed dams: - SRD-SRD (n=30) - SRD-SRD Chia seed (n=30)	Chia seed 20%	20 weeks	Glucose metabolism Lipogenesis	↓ TC, TG (plasma) ↓ n6:n3 ratio ↑ CPT-1 ↓ ACC ↓ Glucose (plasma) ↑ Glucose tolerance ↓ SBP and DBP
Ferreira *et al*. (2016)	*Wistar* rats, male	- Control (n=24, 24 w) - SRD (n= 24, 24 w) - SRD (n=24) followed by SRD + Chia seed	Chia seed 36.2%	12 weeks	Antioxidant status Lipogenesis Inflammation	↓ Epididymal fat and ↑ PPAR-γ (epididymal fat) ↓ TG, FFA, Uric acid, glucose, n3:n6 ratio (plasma) and ↑ GIR ↓ TNF, IL-6 (plasma) ↓ TBARS, protein carbonyl groups, XO activity and ROS ↑ GPx and SOD activity ↑ Nrf2 mRNA

Continuation Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Creus *et al*. (2016)	*Wistar* rats, male	- Control (n=20) - SRD (n=20) - SRD (n=20) followed by SRD + Chia seed	Chia seed 36.2%	24 weeks	Lipogenesis	↓Visceral Adiposity Index (%) ↑ GIR ↓TG, FFA, cholesterol, glucose (plasma) ↓ TG, LCA-CoA, DAG and ↑ PDHa (Intramyocardial lipid) ↑ CPT-1 ↑ FAT/CD36 ↓ Cardiac lipotoxicity ↑ Glucose oxidation ↓ SBP
Marineli *et al*. (2015)b	*Wistar* rats, male	- Control (n=6) - HFHF (n=6) - HFHF followed by HFHF + Chia seed (n=6) - HFHF followed by HFHF + Chia oil (n=6) - HFHF + Chia seed (n=6) - HFHF + Chia oil (n=6)	Chia seed 13.3% Chia Oil 4%	Short treatment: 6 weeks Long treatment: 12 weeks	Antioxidant status	↑TAC (plasma) ↑ GSH ↑ GR, GPx (liver and plasma) ↑ FRAP (liver and plasma) ↓ TBARS (plasma) ↓ 8-isoprostane
Marineli *et al*. (2015)a	*Wistar* rats, male	- Control (n=6) - HFHF (n=6) - HFHF followed by HFHF + Chia seed (n=6) - HFHF followed by HFHF + Chia oil (n=6) - HFHF + Chia seed (n=6) - HFHF + Chia oil (n=6)	Chia seed 13.3% Chia Oil 4%	Short treatment: 6 weeks Long treatment: 12 weeks	Antioxidant status Glucose metabolism Lipogenesis	↑ Glucose and insulin tolerance ↑ Expression of HSP70, HSP25 ↓ HSP60 (skeletal muscle) ↑ SOD and GPx ↑ TAC ↑ Expression of PGC-1α ↓ NEFA ↓ ALT e AST

Continuation Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Sierra *et al*. (2015)	*Flanders* hybrids rabbits	- Control (n=8) - Chia oil (n=8) - Control + cholesterol 1% (n=8) - Chia oil + cholesterol 1% (n=8)	Chia oil 10%	6 weeks	Atherosclerosis	↓ TG (plasma) ↑ ALA (plasma) ↑ Endothelium relaxation ↓ Angiotensin II and noradrenaline
Oliva *et al*. (2013)	*Wistar* rats, male	- Control (n= 6) - SRD (n= 6) - SRD (n= 6) followed by SRD + Chia seed	Chia seed	24 weeks	Glucose metabolism	↓ TG, FFA, Glucose (plasma) ↑ GIR ↓ Epididymal AT ↓ TG (Adipocyte) ↓ FAS, G6PD, Malic enzyme ↑ Hexokinase, PDHa (gastrocnemius muscle) ↓ TG, Long-chain acyl CoA and DG (gastrocnemius muscle)
Poudyal *et al*. (2013)	*Wistar* rats, male	- Control (n=12) - Control followed by Control + Chia oil (n=12) - HFHF (n=12) - HFHF followed by HFHF + Chia oil (n=12)	Chia oil 3%	16 weeks	Lipogenesis	↓ Visceral adiposity (%), Abdominal circumference and Retroperitoneal adipose tissue ↓ Liver weight ↓ Lipid content in liver, heart and skeletal muscle ↓ TC, TG and NEFA (plasma) ↓ Insulin (plasma) ↑ Glucose and insulin tolerance ↑ n3:n6 (heart, liver, skeletal muscle, retroperitoneal adipose tissue) ↓ AST, LDH, ALP, CK ↑ SBP

Continuation Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Poudyal *et al*. (2012)a	*Wistar* rats, male	- Control (n=12) - Control + Chia (n=12) - HFHF (n=12) - HFHF + Chia seed (n=12)	Chia seed 5%	24 weeks	Lipogenesis Glucose metabolism	↓Visceral adiposity (%), retroperitoneal and omental adipose tissue ↓ Lipid content in liver and ↑ in heart ↑ TG and ↓ NEFA (plasma) ↑ n3:n6 (plasma and retroperitoneal AT) ↑ Glucose and insulin tolerance ↓ ALT and ↑ ALP ↓ Inflammatory cells in the left ventricle, collagen deposition, diastolic rigidity, fibrosis ↑ SBP
Poudyal *et al*. (2012)b	*Wistar* rats, male	- Control (n=12) - Control + Chia seed (n=12) - HFHF (n=12) - HFHF + Chia seed (n=12) 8w	Chia seed 5%	8 weeks	Lipogenesis Glucose metabolism	↓ Visceral adiposity (%), Body fat (%), abdominal circumference, retroperitoneal and omental adipose tissue and ↑ Total body lean mass ↓ Lipid content in skeletal muscle ↑ Lipid content in heart ↑ Glucose and insulin tolerance ↓ Uric acid, LDH, CRP, AST ↑ TG, ALP and n3:n6 ratio (plasma) ↓ Liver fibrosis ↑ SBP
Chicco *et al*. (2008)	*Wistar* rats, male	Experimental design 1: - Control (n=24) - SRD (n=24) - SRD + Chia seed (n=24) Experimental design 2: - Control (n=24) - SRD (n=72) divided into three subgroups: 1. immediately killed (n=24) 2. SRD (n=24) 3. SRD + Chia seed (n=24)	Chia seed 36.2%	Experimental design 1: 3 weeks Experimental design 2: 20 weeks	Lipogenesis	↓Epididymal and retroperitoneal AT ↓ TG, NEFA, TC (plasma) ↓ TG (liver) ↓ Glucose ↑ Glucose tolerance ↑ n3 total ↓ n6:n3 ratio (plasma)

Continuation Table 1: Characteristics of the animal studies

References	Animal model	Study protocol	Fraction and Dose	Study duration	Pathway	Main Results
Ayerza; Coates (2007)	*Wistar* rats, male	- Control (n=8, 4 w) - Whole chia seed (n=8) - Ground chia seed (n=8) - Chia oil (n=8)	Chia seed: 16% Chia oil: 5,34%	4 weeks	Lipogenesis	↓ TG, total SFA (plasma) ↑ HDLc and n3 (plasma)

↑: increase; ↓: decrease; ACC: acetyl-CoA Carboxylase; ALA: alpha linolenic acid; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMPK: adenosine monophosphate-activated protein kinase; AST: aspartate aminotransferase; AT: adipose tissue; CAT: catalase; CK: creatinine; CPT-1: carnitine-palmitoyl transferase-1; CRP: C-reactive protein; DBP: diastolic blood pressure; DG: diacylglyceride; FAS: fatty acid synthase; FAT/DC36: Fatty acid translocase; FFA: free fatty acid; FRAP: ferric reducing ability of plasma; G-6-P: glucose-6-phosphate; G6PD: glucose-6-phosphate dehydrogenase; GIR: glucose infusion rate; GLUT-4: glucose transporter type 4; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; HDLc: high density lipoprotein; HFD: high fat diet; HOMA-IR: homeostasis model assessment: insulin resistance; HSP25: heat shock protein 25; HSP60: heat shock protein 60; HSP70: heat shock protein 70; IL-10: interleukin 10; IL-6: interleukin 6: IRS-1: insulin receptor substrate 1; LCA-CoA: Long-chain Acyl-CoA; LDH: lactate dehydrogenase; LDLc: low density lipoprotein; n3: omega 3; n6: omega 6; NEFA: nonesterified fatty acids; NF-κB: factor nuclear kappa B; Nrf2: nuclear factor (erythroid-derived 2)-like 2; pAMPK: phosphorylation of adenosine monophosphate-activated protein kinase; PDHa: pyruvate dehydrogenase E1 component subunit alpha; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator; pIRS-1(Tyr)/IRS-1: phosphorylation on Tyr989 of IRS-1; PPAR-α: peroxisome proliferator-activated receptor alpha; PPAR-γ: peroxisome proliferator-activated receptor gamma; RD: Reference diet; RD-RD: offspring from dams fed a reference diet (RD) and fed the RD after weaning; ROS: reactive oxygen species; SAMP8 HFD: Senescence Accelerated Mouse - fed a high fat diet; SAMP8 LFD: Senescence Accelerated Mouse - fed a low fat diet; SAMR1 LFD: senescence-accelerated mouse - resistant 1 - fed a low fat diet; SBP: systolic blood pressure; SD: standard diet; SFA: saturated Fatty *acids*; SOD: superoxide dismutase; SRD: sucrose rich diet; SRD-SRD: offspring from SRD-fed dams fed an SRD after weaning; SRD-SRDC: offspring from SRD-fed dams fed an SRD+Chia after weaning; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive substances; TC: total cholesterol; TG: triacylglycerol; TNF-α: tumor necrosis factor alpha; VLDLc: very low density lipoprotein; w: weeks; XO: xanthine oxidase.

3.3 Impact of chia on metabolic and associated disorders

3.3.1 Glucose metabolism

The introduction of chia to rats fed with an unbalanced diet improved glucose tolerance and insulin sensitivity (Fonte-Faria *et al*., 2019; Rui *et al*., 2018; Fortino *et al*., 2017; Creus *et al*., 2017; Creus *et al*., 2016; Ferreira *et al*., 2016; Marineli *et al*., 2015a; Marineli *et al*., 2015b; Oliva *et al*., 2013; Poudyal *et al*., 2012a; Poudyal *et al*., 2012b; Chicco *et al*., 2009). These effects were confirmed by additional data of chia seed intake such as increased expression of heat shock proteins 25 (HSP25) and HSP70 in soleus muscle (Marineli *et al*., 2015a), increased insulin-stimulated phosphorylation on IRS-1 and GLUT-4 translocation on gastrocnemius (Fonte-Faria *et al*., 2019) and heart (Creus *et al*., 2017), increased AMP-actived protein kinase (AMPK) phosphorylation on gastrocnemius (Fonte-Faria *et al*., 2019), cardiac muscles (Creus *et al*., 2017), epididymal and subcutaneous adipose tissue (Rui *et al*., 2018).

AMPK is one of the most important proteins involved to the cellular energy balance and it works as sensor of energy-deprivation. Its decrease leads to noticeable effects on animal energy metabolism, among which are the oxidative pathways of glucose and lipids (Nelson & Cox, 2014). It is already well established that diets high in fat and sucrose (or both) reduce AMPK (Lindholm *et al*., 2013; Yang, Miyahara, Takeo, & Katayama, 2012), and HSP expression (Chung *et al*., 2008). Therefore, chia seed and its compounds present a promising role in energetic metabolism since animals fed unbalanced diets, when fed chia, had AMPK phosphorylation restored as control animals. Besides that, chia modulates other markers, including carnitine palmitoyltransferase 1 (CTP-1) (Creus *et al*., 2017; Fortino *et al*., 2017), peroxisome proliferator-activated receptor-γ coactivator 1-alpha (PGC-1α) and HSP expression (Marineli *et al*., 2015a), signaling improvement in mitochondrial activity pattern, fatty acid oxidation, adipogenesis control and prevention of the overexpression of inflammatory mediators (da Silva *et al*., 2018; Creus *et al*., 2016; Musch, Kapil, & Chang, 2004).

From the included studies, we hypothesize that chia and its fractions mitigate obesity-induced insulin sensitivity by regulating AMPK and IRS-1 phosphorylation, which improve GLUT-4 translocation and increase hexokinase and glucose 6-phosphate enzymatic activity (Creus *et al*., 2017; Fortino *et al*., 2017; Ferreira *et al*., 2016; Marineli *et al*., 2015a; Oliva *et al*., 2013; Poudyal *et al*., 2012b; Chicco *et al*., 2009), which would restore glucose utilization as an energetic fuel. This hypothesis is supported by the increase

in CPT-1 activity (Creus *et al*., 2016), PGC-1α expression and peritoneal glucose tolerance as a response to consumption of chia seed and oil (Marineli *et al*., 2015a) (**Figure 2)**.

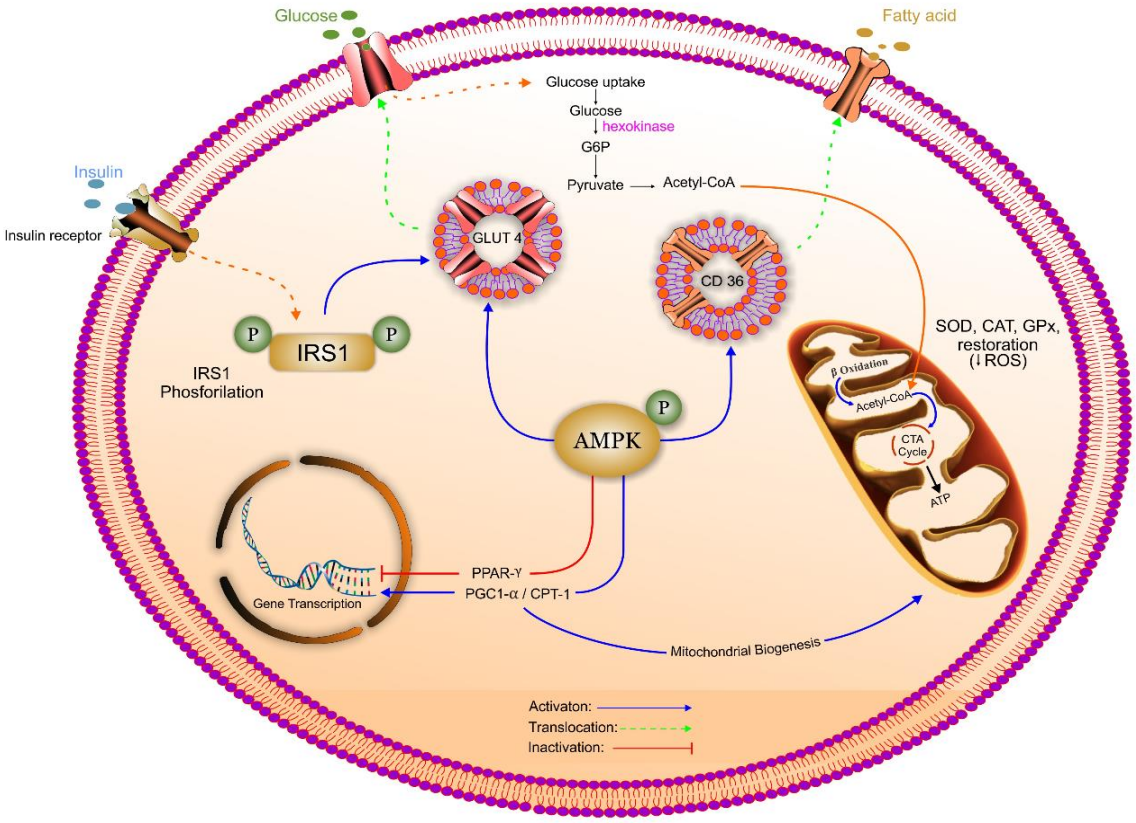

Figure 2. Hypothesis of chia´s seeds mechanism of action based on unbalanced diet experimental studies.IRS1: Insulin receptor substrate 1; G6PD: Glucose 6 phosphate; AMPK: AMP-activated protein kinase; PPAR-γ: Peroxisome Proliferator-Activated Receptor Gamma; PGC1-α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CTP-1: Carnitine palmitoyl transferase I; SOD: Superoxide dismutase; GPx: Gluthatione peroxidase; ROS: Reative reative oxygen species; CTA: Tricarboxylic acid.

3.3.2 Lipid profile and lipolysis

The consumption of chia improved the lipid profile in animals fed nutritionally inadequate diets (high in simple carbohydrates like sucrose and fructose, high in saturate fat, or both). These results are related to improving plasma cholesterol profile (Fonte-Faria *et al*., 2019; da Silva *et al*., 2018; de Miranda *et al*., 2019; Creus *et al*., 2016; Sierra *et al*., 2015; Poudyal *et al*., 2013) and decreasing plasma triglycerides (Fonte-Faria *et al*., 2019; Creus *et al*., 2017; Fortino *et al*., 2017; Creus *et al*., 2016; Sierra *et al*., 2015; Oliva *et al*., 2013; Poudyal *et al*., 2013; Poudyal *et al*., 2012a; Chicco *et al*., 2009; Ayerza & Coates, 2007). Additionally, chia seed increased the plasmatic levels of n-3 and α-linoleic (n-6) fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), n-3/saturated fatty acids and n-3/n-6 ratio (Creus *et al*., 2017; Fortino *et al*., 2017; Sierra *et al*., 2015;

Oliva *et al*., 2013; Poudyal *et al*., 2012a; Chicco *et al*., 2009; Ayerza & Coates, 2007).

Among plant foods chia seed is the major source of n-3 (da Silva *et al*., 2017; Ayerza, 2009; Ayerza & Coates, 2005) and despite being the primary fatty acid of the n-3 pathway, its conversion to EPA and DHA has been widely held as inefficient (Ratnayake & Galli, 2009). Studies with chia seed as a source of ALA have been demonstrating that its physiological effects are different than that produced by EPA and DHA in rats with metabolic syndrome. They also showed that although ALA was not efficient in reducing total body fat (Poudyal *et al*., 2013; Poudyal *et al*., 2012a), it has induced fat redistribution away from the abdominal area (Fonte-Faria *et al*., 2019; Oliva *et al*., 2013; Poudyal *et al*., 2013; Poudyal *et al*., 2012a; Poudyal *et al*., 2012b), decreasing risk of cardiovascular diseases.

Moreover, the modulation of glucose metabolism markers by chia seed had impact on lipogenesis, evidenced by the increase of peroxisome proliferator-activated receptor (PPAR) (da Silva *et al*., 2018; Creus *et al*., 2016), improvement in serum lipid profile, reduction of visceral adiposity, and decrease of epididymal adipose tissue weight (Fortino *et al*., 2017; Creus *et al*., 2017; Creus *et al*., 2016; Ferreira *et al*., 2016; Oliva *et al*., 2013; Sierra *et al*., 2015; Chicco *et al*., 2009; Ayerza & Coates, 2007) (**Figure 2**).

Regarding lipogenesis, rats fed a high-fat-diet (HFD) had impaired recruitment of FAT/CD36 by insulin as well as CTP-1 activity (Creus *et al*., 2016) and PPAR-α protein mass (da Silva *et al*., 2018; Creus *et al*., 2016). These findings are related to chronic high exposure to fatty acids provided by HFD, disrupting the balance between lipid oxidation-storage. The recruitment of FAT/CD36 by insulin is a key mechanism for beta-oxidation and for restoring mitochondrial activity. Chia seed was able to revert this condition by recruiting FAT/CD36 to the sarcolemma through insulin signaling (Creus *et al*., 2016), increasing PPAR-α (da Silva *et al*., 2018), increasing PGC1- α expression (Marineli *et al*., 2015a) and decreasing visceral adiposity (Creus *et al*., 2016; Ferreira *et al*., 2016; Poudyal *et al*., 2013; Poudyal *et al*., 2012b)

Our review allowed us to develop a hypothesis about how chia seed can improve lipid biomarkers. Unbalanced diets induce insulin intolerance and this is a key event for lipogenesis. Chia seed reversed abnormal glucose homeostasis and peripheral insulin insensitivity (Fonte-Faria *et al*., 2019; Rui *et al*., 2018; Fortino *et al*., 2017; Creus *et al*., 2017; Creus *et a*l., 2016; Ferreira *et al*., 2016; Marineli *et al*., 2015a; Marineli *et al*., 2015b; Oliva *et al*., 2013; Poudyal *et al*., 2012a; Poudyal *et al*., 2012b; Chicco *et al*., 2009) and insulin induced the translocation of FAT/CD36 (Creus *et al*., 2016) to the plasmatic

membrane. Our hypothesis is that the normalization of insulin response is one of the most important events involved in chia seed improvement on lipogenesis, recovering beta-oxidation and balancing fuel utilization. Thus, the normalization of insulin response by chia seed may reverse triggers for lipogenesis.

3.3.3 Impact of chia on oxidative stress and inflammation

Several authors described chia`s effects on decreasing oxidative stress by increasing total antioxidant capacity, restoring antioxidant enzymes (da Silva *et al*., 2018; Marineli *et al*., 2015a; Marineli *et al*., 2015b), and decreasing reactive oxygen species (ROS) and lipid peroxidation (Ferreira *et al*., 2016; Marineli *et al*., 2015b). The decrease of the plasma lipid peroxidation may be connected to hypoglycemic effect of chia that is associated to decrease of LDL oxidation, probably due to a reduction on its plasmatic levels (Marineli *et al*., 2015b). With exception of de Miranda *et al*. (2019), all the studies included in this review that evaluated glucose metabolism, plasma lipids, or its peroxidation and ROS production found simultaneously glycaemia decrease and plasmatic lipid level improvement (Fonte-Faria *et al*., 2019; Fortino *et al*., 2017; Creus *et al*., 2017; Creus *et al*., 2016; Ferreira *et al*., 2016; Oliva *et al*., 2013; Poudyal *et al*., 2013; Poudyal *et al*., 2012a). Three other papers showed decrease in peroxidation markers (Ferreira et al., 2016; Marineli *et al*., 2015a; Marineli *et al*., 2015b).

Compounds in chia, such as phenolic acids (rosmarinic acid, caffeic acid, danshensu, chlorogenic acid, quercetin, myricetin and kaempferol) and lipophilic compounds (carotenoids, tocopherols, phospholipids and ALA) were associated with antioxidant effects (da Silva *et al*., 2017; Oliveira-Alves *et al*., 2017; Ixtaina *et al*., 2010; Reyes-Caudillo, Tecante & Valdivia-López, 2008). Although antioxidant activity and phenolic compounds quantities between chia seeds and oil are different (Oliveira-Alves *et al*., 2017), studies have highlighted similar effects of both seed and oil in the modulation of oxidative stress (Marineli *et al*., 2015b). The beneficial effect demonstrated for both fractions may be due to interactions between the chemical components in the seed, and synergistic activity between the lipophilic compounds in chia oil. The variability observed between the studies may be due to the distinct composition of chia from different locations and cultivation conditions (da Silva *et al*., 2017).

Regarding the effect of chia seed on inflammation, some studies showed reduction on inflammation biomarkers (da Silva *et al*., 2018; Creus *et al*., 2017; Creus *et al*., 2016; Poudyal *et al*., 2012a; Poudyal *et al*., 2012b) or on associated ones, such as glutamic-

oxalacetic transaminase, alanine aminotransferase (Marineli *et al*., 2015a; Poudyal *et al*., 2013; Poudyal *et al*., 2012a; Poudyal *et al*., 2012b), liver fibrolisis (Poudyal *et al*., 2012a) and HSP72 upregulation, which is associated to preventing the overexpression of inflammatory mediators (Moura, Lollo, Morato & Amaya-Farfan, 2018). All of them interestingly, were associated to other improvements such as glucose and insulin tolerance and lipogenesis control. This finding strengthens our previous hypothesis about insulin activity normalization and positive effects of chia seed on health.

3.4. Impact of chia's bioactive compounds on metabolic and associated disorders

All studies included in this review mentioned the bioactive compounds of chia seed as potential in improving metabolic disorders developed by unbalanced diets. However, none of them are able to point out a compound as responsible for a specific action. Previous studies using other food sources or isolated bioactive compounds evidenced that phenolic compounds (e.g. quercetin, chlorogenic acid, caffeic acid, rosmarinic acid), dietary fiber, fatty acid (e.g. ALA), and others, play a role in repairing health conditions triggered by unbalanced diets (Sadeghi, Seyyed Ebrahimi, Golestani & Meshkani, 2017; Gonzalez-Manan *et al*., 2012; Pal & Ghosh, 2012;). Nevertheless, regarding chia seed, it is hard to link the effect on metabolism to one specific compound. Even the studies that used fractions like oil or fiber cannot discern which "compound" is associated to that action.

The fractions of the seed have different components that result in distinct actions. Apparently hydrolyzed extracts of chia as well as extracts from the seed and its fiber present higher antioxidants (Oliveira-Alves *et al*., 2017), and flavonoid bioaccessibility may be impaired by the seed´s fat (Pellegrini *et al*., 2018). Nevertheless, studies conducted with chia oil presented improvement in inflammation (Poudyal *et al*., 2012a) and restored the antioxidant system (Marineli *et al*., 2015b). Studies that compared chia´s fraction during different times showed that chia oil presents a faster action than chia seed (Marineli *et al*., 2015a; Marineli *et al*., 2015b), but more studies must be conducted to confirm it. Although chia oil seems to be superior to the seed, the extraction methods should be observed in order to avoid losses regarding antioxidant compounds. Özcan *et al* (2019a) showed that chia oil obtained from roasted seeds presents lower content of α-Tocopherol, β+γ-Tocopherol, δ-Tocopherol, β-Tocotrienol and γ-Tocotrienol compared with non-roasted seeds, regardless of the method used to obtain the oil: cold press or Soxhlet extraction. Recent data showed that heat acts negatively on physical-chemical and

bioactive properties of chia oil, where the content of fatty acids and phenolic compounds was decreased by microwave roasting (Özcan *et al*., 2019b).

Therefore, it still remains unclear if there is a specific bioactive compound or the synergism of them are implicated in the improvement of biomarkers evidenced by the studies. None of them make a direct association to a certain compound with the results obtained. However, most of the studies mentioned that the major action of chia on health conditions is due to the improvement of glucose uptake, its oxidation and restored tissue sensibility to insulin and its regulation of gene expression (PPARα, CTP-1, PGC1-α) related to lipogenesis and mitochondrial activity.

Based on the few studies that evaluated the effects of chia on metabolic pathways, we hypothesized that chia compounds increase AMPK expression which increases the PGC1-α and CTP-1, increasing expression of PPARα, reducing the lipogenesis, increasing mitochondrial activity and consequently fatty acid oxidation. Therefore, the AMPK together with IRS, stimulates the translocation of GLUT-4 to plasmatic membrane and allows the entrance of glucose inside the cells, reducing the IR. Moreover, AMPK allows the translocation of FAT/CD36 to the sarcolemma in muscle cells, facilitating the FA oxidation by mitochondria. Chia´s effects on restoring antioxidant defense may come from the improvement on glucose tolerance, from the reduction of oxidative stress or the improvement on mitochondrial dysfunction, but it is unknown how chia seed is able to increase these anti-oxidative enzymes (**Figure 2**).

3.5. Reporting Quality and Risk of Bias

Among the 17 studies evaluated, 1 study did not show the dose of chia administered to the animals (Chicco *et al*., 2009). None of them discussed potential adverse effects of the doses and/or compounds used in the intervention, as well as insufficient information on the limitations of the study, especially concerning extrapolation of data to humans (Creus *et al*., 2017; Fortino *et al*., 2017; Sierra *et al*., 2015; Oliva *et al*., 2013; Poudyal *et al*., 2013; Poudyal, *et al*., 2012a; Poudyal, *et al*., 2012b) (**Table 2**). Our findings are consistent to Kilkeny *et al*. (2009) who claims that there is a lack of important information regarding experimental and statistics methods applied in research regarding animals and in publications.

Table 2. Risk of bias from experimental studies

References	Marineli et. al., 2015a	Marineli et. al., 2015 b	Oliva et. al., 2013	Poudyal et.al., 2012 a	Creus et. al., 2017	Creus et. al., 2016	Chicco et. al., 2008	Sierra et. al., 2015	Ayerza; Coates, 2007	Fortino et. al., 2017	Poudyal et. al., 2012b	Ferreira et. al., 2016	Poudyal et. al., 2013	Yehua et al. 2018	de Miranda et al. 2018	da Silva et al. 2018	Fonte-Faria et al. 2019	Percentage (%)
(1) Title	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	94.1
(2) Abstract	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	88.2
Introduction																		
(3) Background information	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(4) Primary and secondary objectives	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	94.1
Methods																		
(5) Ethical statement	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(6) Study design, allocation concealment, blinding and randomization	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(7) Experimental procedure	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	94.1
(8) Experiment animals details including species, gender, age, weight and source	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(9) Housing and husbandry conditions	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(10) Sample size	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	88.2
(11) Allocation of animals to experimental groups	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(12) Experiment outcomes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(13) Statistical analysis	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	94.1
Results																		
(14) Baseline data	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(15) Number of animals analyzed	1	1	0	0	0	0	0	1	1	1	0	1	1	0	1	0	1	52.9

(16) Outcomes and estimation, results for each animal	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(17) Adverse events (details of all important adverse)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Discussion																		
(18) Interpretation, scientific implications, study limitations	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
(19) Generalizability and translation, relevance to human biology	1	1	0	1	0	1	0	0	1	0	0	1	1	1	1	1	1	64.7
(20) Funding	1	1	0	1	0	1	0	1	1	0	1	0	0	1	1	1	1	64.7

Moreover, about the risk of bias (**Figure 3**), none of the studies reported about blinding the investigators involved in the research. Only one study (da Silva *et al*., 2018) reported details about the animal's randomization to groups. 16 studies failed to report information about allocation concealment strategies. The lack of information reported in studies may reveal significant short-comings on designing animal studies, which in turn may impair the scientific community to obtain reliable data from previous studies, generating a negative effect on laboratory research. Our data about lack of blinding is consent with findings demonstrating little blinding in experimental research (Holman, Head, Lanfear & Jennions, 2015). Selection and measurement bias can be solved with randomization and blinded assessment of outcome (Macleod *et al*., 2015), since they are related to the randomness of outcomes. We suggest researchers involved in animal studies to follow the ARRIVE guidelines to avoid misinformation in their reports.

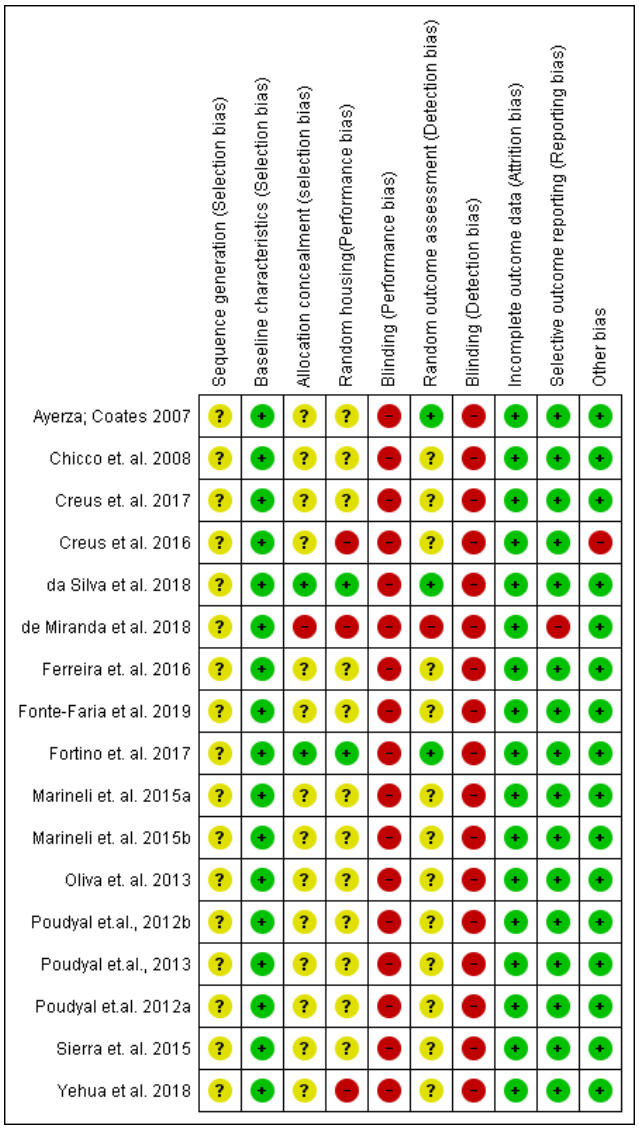

Figure 3. Risk of bias summary: review authors' judgments about each risk of bias item for each included study.

4. CONCLUSIONS

This review supports the prospective use of chia in the prevention and treatment of comorbidities associated to unbalanced diets. Despite the limitations in extrapolating the results to humans, we consider chia seed a potential bioactive food, as the realistic consumption of chia seeds and oil could be able to prevent and attenuate metabolic changes. We highlight the lack of data about which compound would be responsible to stimulate or down regulate the pathways discussed in this review as well as the dose that would present an efficient and secure effect on human health. We reinforce the need of future clinical studies that consider the dose of chia seed consumed daily and the seed fraction that best impacts health. Thus, the biological effects of the seed and its components can be clinically confirmed and may represent a dietary strategy to prevent

and treat chronic health problems. The use of tools for quality assessment identified methodological gaps that suggest operational improvements on running experimental research for future high quality controlled trials.

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Conflict of interest

The authors state they have no conflict of interest.

Author Contribution

Bárbara N. Enes researched prior studies, evaluated each study, compiled data, interpreted the results and drafted the manuscript.

Luiza P. D. Moreira researched prior studies, evaluated each study, interpreted the results and drafted the manuscript.

Bárbara P. Silva compiled data and drafted the manuscript.

Mariana Grancieri drafted the manuscript and reviewed the manuscript.

Haira G. Lúcio researched prior studies and compiled data.

Vinícius P. Venâncio drafted and reviewed critically the manuscript.

Susanne U. Mertens-Talcott reviewed critically the manuscript.

Carla O.B. Rosa reviewed critically the manuscript.

Hércia S.D. Martino reviewed critically the manuscript and approved the final and revised version.

All authors have read and approved the final manuscript.

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Supplemental material

Table S1. Search strategy for different databases

A. PubMed
((“chia seed” OR “chia seeds” OR “salvia hispanica L” OR chia seed OR chia seeds OR salvia hispanica L) AND (obesity OR Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” OR diabetes OR “Cardiovascular Diseases” OR Hypertension OR “metabolic syndrome” OR “nonalcoholic fatty liver disease” OR “nonalcoholic steatohepatitis”))
B. Embase
((“chia seed” OR “chia seeds” OR “salvia hispanica L”) AND (obesity OR Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” OR diabetes OR “Cardiovascular Diseases” OR Hypertension OR “metabolic syndrome”)) ((“chia seed” OR “chia seeds” OR “salvia hispanica L”) AND (“nonalcoholic fatty liver disease” OR “nonalcoholic steatohepatitis”))
C. COCHRANE
((“chia seed” OR “chia seeds” OR “salvia hispanica L”) AND (obesity OR Hyperglycemia OR Inflammation OR Dyslipidemias OR “Insulin Resistance” OR diabetes OR “Cardiovascular Diseases” OR Hypertension OR “metabolic syndrome” OR “nonalcoholic fatty liver disease” OR “nonalcoholic steatohepatitis”))
D. LILACS
(tw:(“chia seed” OR “chia seeds” OR “salvia hispanica L”)) AND (tw:(obesity)) OR (tw:(Hyperglycemia)) OR (tw:(Inflammation)) OR (tw:(Dyslipidemias)) OR (tw:(“Insulin Resistance”)) OR (tw:(diabetes)) OR (tw:(“Cardiovascular Diseases”)) OR (tw:(Hypertension)) OR (tw:(“metabolic syndrome”)) OR (tw:(“nonalcoholic fatty liver disease”)) OR (tw:(“nonalcoholic steatohepatitis”))

5.2 Paper 2

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Effect of different fractions of chia (*Salvia hispanica* L.) on glucose metabolism, *in vivo* and *in vitro*

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ABSTRACT

The influence of chia (*Salvia hispanica* L.) flour and oil on glucose metabolism (GM) in insulin resistant (IR) Wistar rats and the effect of chia hydrolyzed phenolics extract (CHPE) on GM in IR HepG2 cells were evaluated. *In vivo* study: animals were divided into four groups: AIN-93M, high-fat and high-fructose (HFHF), HFHF with chia flour (14.7%) or chia oil (4%). *In vitro* study: IR HepG2 cells were treated with CHPE (80 ppm). *In vivo*, chia flour and oil reduced adiposity and increased AMPK mRNA. Chia oil improved glucose tolerance, increased AKT1[pS473] protein level, mRNA of insulin receptor, FOXO1 and glycolysis enzymes. *In vitro*, CHPE decreased gluconeogenesis enzymes mRNA. Chia flour and oil decreased adiposity, but only chia oil was able to improve glucose tolerance and restore energy fuel system in liver of rats fed HFHF diet. CHPE decreased mRNA levels of gluconeogenesis enzymes.

1. Introduction

Three in five deaths in the world are related to chronic diseases like obesity, type 2 diabetes and cardiovascular diseases (GBD 2016 Risk Factors Collaborators, 2017). These outcomes are derived from hypertension, high cholesterol and overweight (World Health Organization, 2017). Moreover, obesity leads to an impaired glucose metabolism caused by insulin resistance (IR) and type 2 diabetes (Samuel, 2011). The disrupted glucose metabolism is considered key for other events such as atherosclerosis, cardiovascular diseases, high blood pressure, kidney injuries and nonalcoholic fatty liver disease (Ismaiel & Dumitraşcu, 2019).

In this context, IR is known as the failed response to insulin by cells. It is a physiological condition, in which cells are unable to use insulin or the hormone production is impaired by pancreas, leading to high blood glucose (Schenk, Saberi, & Olefsky, 2008). Eventually, IR may cause hyperinsulinemia in attempt to maintain blood glucose at normal levels. These conditions also lead to increased gluconeogenesis activity and glucose output in liver. Epidemiological studies indicate a strong correlation between obesity and high caloric diets (high in sugar, fructose, and saturated fat) (Popkin, Adair, & Ng, 2011; Popkin, Nielsen, & Bray, 2004). Additionally, a high caloric diet is related to the upregulation of FOXO1 transcriptional factor, which controls the transcription of gluconeogenesis enzymes. Under these conditions, the

Abbreviations: AKT, protein kinase B; p-AKT, phospho-protein kinase B; ALA, alpha linolenic acid; AMPK, AMP-activated protein kinase; AUC, area under the curve; BSA, bovine serum albumin; BW, body weight; CHPE, chia hydrolyzed phenolics extract; DMEM, Dulbeccós modified Eaglés medium; FAT/CD36, Fatty acid translocase; FBS, fetal bovine serum; FoxO1, forkhead box protein O1; G6Pase, glucose-6 phosphatase; GAE, gallic acid equivalents; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GK, glucokinase; GSK-3β, glycogen synthase kinase 3β; HFHF, high-fat and high-fructose; HFD, high-fat diet; iGTT, intraperitoneal glucose tolerance test; INSR, insulin receptor; IR, insulin resistance; IRS2, insulin receptor substrate 2; ITT, insulin tolerance test; LCMS, liquid chromatography–mass spectrometry; PA, palmitic acid; PBS, phosphate-buffered saline; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PK, phosphokinase; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; RA, rosmarinic acid; RT-PCR, real-time polymerase chain reaction; SREBP1, sterol regulator element-binding protein 1; TBP, TATA box binding protein; TBS-T, tris-buffered saline twin

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Effect of different fractions of chia (*Salvia hispanica* L.) on glucose metabolism, *in vivo* and *in vitro*

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ABSTRACT

The influence of chia (*Salvia hispanica* L.) flour and oil on glucose metabolism (GM) in insulin resistant (IR) Wistar rats and the effect of chia hydrolyzed phenolics extract (CHPE) on GM in IR HepG2 cells were evaluated. *In vivo* study: animals were divided into four groups: AIN-93M, high-fat and high-fructose (HFHF), HFHF with chia flour (14.7%) or chia oil (4%). *In vitro* study: IR HepG2 cells were treated with CHPE (80 ppm). *In vivo*, chia flour and oil reduced adiposity and increased AMPK mRNA. Chia oil improved glucose tolerance, increased AKT1[pS473] protein level, mRNA of insulin receptor, FOXO1 and glycolysis enzymes. *In vitro*, CHPE decreased gluconeogenesis enzymes mRNA. Chia flour and oil decreased adiposity, but only chia oil was able to improve glucose tolerance and restore energy fuel system in liver of rats fed HFHF diet. CHPE decreased mRNA levels of gluconeogenesis enzymes.

Keywords: Glucose tolerance, AKT, AMPK, Chia oil, Chia phenolics.

ABREVIATION

AKT: protein kinase B; p-AKT: phospho-protein kinase B; ALA: alpha linolenic acid; AMPK: AMP-activated protein kinase; AUC: area under the curve; BSA: bovine serum albumin; BW: body weight; CHPE: chia hydrolyzed phenolics extract; DMEM: Dulbecco´s modified Eagle´s medium; FAT/CD36: Fatty acid translocase; FBS: fetal bovine serum; FoxO1: forkhead box protein O1; G6Pase: glucose-6 phosphatase; GAE: gallic acid equivalents; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; GK: glucokinase; GSK-3β: glycogen synthase kinase 3β; HFHF: high-fat and high-fructose; HFD: high-fat diet; iGTT: intraperitoneal glucose tolerance test; INSR: insulin receptor; IR: insulin resistance; IRS2: insulin receptor substrate 2; ITT: insulin tolerance test; LCMS: Liquid chromatography–mass spectrometry; PA: palmitic acid; PBS: phosphate-buffered saline; PEPCK: phosphoenolpyruvate carboxykinase; PFK: phosphofructokinase; PK: phosphokinase; PPAR: peroxisome proliferator-activated receptor; PVDF: polyvinylidene difluoride; RA: rosmarinic acid; RT-PCR: real-time polymerase chain reaction; SREBP1: sterol regulator element-binding protein 1; TBP: TATA box binding protein; TBS-T: tris-buffered saline twin.

1. INTRODUCTION

Three in five deaths in the world are related to chronic diseases like obesity, type 2 diabetes and cardiovascular diseases (GBD 2016 Risk Factors Collaborators, 2017). These outcomes are derived from hypertension, high cholesterol and overweight (World Health Organization, 2017). Moreover, obesity leads to an impaired glucose metabolism caused by insulin resistance (IR) and type 2 diabetes (Samuel, 2011). The disrupted glucose metabolism is considered key for other events such as atherosclerosis, cardiovascular diseases, high blood pressure, kidney injuries and nonalcoholic fatty liver disease (Ismaiel & Dumitraşcu, 2019).

In this context, IR is known as the failed response to insulin by cells. It is a physiological condition, in which cells are unable to use insulin or the hormone production is impaired by pancreas, leading to high blood glucose (Schenk, Saberi, & Olefsky, 2008). Eventually, IR may cause hyperinsulinemia in attempt to maintain blood glucose at normal levels. These conditions also lead to increased gluconeogenesis activity and glucose output in liver. Epidemiological studies indicate a strong correlation between obesity and high caloric diets (high in sugar, fructose, and saturated fat) (Popkin, Adair, & Ng, 2011; Popkin, Nielsen, & Bray, 2004). Additionally, a high caloric diet is related to the upregulation of FOXO1 transcriptional factor, which controls the transcription of gluconeogenesis enzymes. Under these conditions, the downregulation of PI3K/AKT pathway may occur leading to the insulin/AKT pathway disruption and impaired glucose receptor (GLUT) translocation to the plasmatic membrane. These changes in the homeostatic system are accompanied by the decrease in AKT phosphorylation (Czech, 2018).

Research studies have demonstrated that food or bioactive compounds could attenuate or prevent the damage generated by obesity and IR (Skrovankova *et al*., 2015). Chia seed (*Salvia hispanica* L.) is considered the highest botanical source of alpha linolenic acid (ALA) omega 3 (n-3). In addition, chia seeds are good source of dietary fiber, proteins, vitamins, minerals, and phytochemicals, such as phenolic compounds (Silva *et al*., 2017; Marineli *et al*., 2014). Chia seed and oil have been investigated in the last few years for their potential benefits on glucose metabolism control (Marineli *et al*., 2015; Fonte-Faria *et al*., 2019). For example, rosmarinic acid (RA), is the major phenolic compound in chia seed (Oliveira-Alves *et al*., 2017), has been related to hypoglycemic effect (Runtuwene *et al*., 2016). Rats fed high-fat and high-fructose diet (HFHF) improved glucose tolerance and insulin resistance after chia seed (13.3%) and chia oil (4%) intake

(Marineli *et al*., 2015). Mice fed high-fat diet (HFD) supplemented with chia oil (0.15%) for 90 to 135 days, showed activation of the insulin cascade in skeletal muscle (Fonte-Faria *et al*., 2019).

However, it remains unknown if the hypoglycemic effect of chia oil targets only skeletal muscle, and which fraction of chia (flour, oil or phenolic compounds) are responsible for glucose metabolism improvement on liver. This study aims to investigate the effects of chia flour and chia oil on liver glucose metabolism of rats fed a high-fat and high-fructose diet and the *in vitro* influence of chia hydrolyzed phenolics extract (CHPE) on glucose metabolism of HepG2 cells.

2. MATERIALS AND METHODS

The chia seeds were cultivated in Rio Grande do Sul, Brazil, crop of 2017. Seeds were storage at -20 ºC until use.

2.1 Chia flour prepare

Chia seeds were used for diet preparation in the form of flour (seeds were ground in a blender for approximately 2 min). Chia flour diet was prepared every 15 days with freshly ground chia seeds and stored at -20 ºC to prevent lipid oxidation.

2.2 Extraction of phenolics from chia flour

Phenolic extraction was performed using an ultrasound liquid-liquid extraction method Oliveira-Alves *et al*. (2017). Briefly, 2 g of chia flour and 10 mL of methanol: water (80:20, v/v) were vortexed for 10 s and placed in an ultrasound water bath (Model 3510R-MT, Branson Ultrasonic Corporation, Danbury, USA) operated at 42 kHz of ultrasound frequency, 100 W of power, for 60 min at 25 ºC ± 3. This was followed by centrifugation at 1792 g for 30 min. Collected supernatant was dried using a rotary evaporator, at 50 ºC. The dried extracted phenolics were subjected to acid hydrolysis by dissolving in 2 mL of acidified water (formic acid 0.01%), then mixed with 1 M HCl (2 mL) and kept in water bath (100 ºC) for 60 min. Hydrolyzed phenolics were purified using solid phase extraction (SPE). Solvent was evaporated to dryness at ± 50 ºC under reduced pressure. Dried CHPE were stored at -20 ºC with nitrogen gas for future analysis and cell culture experiments.

2.3 Oil extraction

Chia seeds were ground and cold pressed throughout a hydraulic press (Carver Laboratory Press, ModelC 22400-36 - USA) to obtain the oil. During the oil extraction, precaution was taken to avoid light exposure. Extracted oil was stored at -20 ºC until use in diet preparation to prevent lipid oxidation.

2.4 Extract chemical analysis

The total phenolic content of CHPE was assessed according to Folin-Ciocalteu's (Swain & Hillis, 2006), method with modifications (Abderrahim *et al*., 2011). The results were quantified using a standard curve ranging 0 to 300 ppm of gallic acid and expressed in milligrams of gallic acid equivalents per milliliter of extract (mg GAE.mL^{-1}) (y = 1,6229x - 0,0227; R2 = 0.9805). The content of total phenolics was used to establish the treatment doses in the HepG2 cells experiment.

2.5 Chia phenolics characterization and quantification

The polyphenolic profile and quantification was assessed by reversed phase HPLC with a Waters 2695 Alliance system (Waters Corp.,Milford, MA). Identification and quantitation of polyphenolics were based on their spectral characteristics and retention time, as compared to authentic standards (Sigma Chemical Co., St. Louis, MO). Compound identities were further confirmed by mass spectrometric analyses, performed on a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher,San Jose, CA). Separations were conducted using the Phenomenex (Torrance, CA) Synergi 4 μHydro-RP 80A (2 × 150 mm; 4 μm; S/N) 106273-106275) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in 50:50 methanol and acetonitrile (phase B) run at 0.25 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 30% in 5 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min and was held isocratic for 20 min. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N2), 60 units/min; auxiliary gas (N2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V.

2.5 Animal Study

2.5.1 Study design and diets

This study was approved by the Ethics Commission on Animal Use (CEUA/UFV, protocol no. 31/2018, date of approval: April 26th, 2018) and followed university guidelines for animal use for experimental studies. Forty male Wistar rats (*Rattus norvegicus*), 45-50 days old, were obtained from the Central Animal Facility of the Center for Biological Sciences and Health at Federal University of Viçosa, Minas Gerais, Brazil. The animals remained in individual stainless-steel cages, with free access to water and diet, under controlled conditions (22 ºC ± 2, 12h light/dark cycle). Animals were randomized according to body weight and fed with standard diet (AIN-93M) (b.w.=156 ± 17.0 g, n=10) or HFHF diet (b.w.=156.5 ± 17.9 g, n=30) for 8 wk to induce insulin resistance (Phase I). In order to assess the effect of chia flour and chia oil on metabolic disorders (Phase II), animals fed HFHF (b.w.=358.04 ± 33.00 g, n=30) were randomized into 3 experimental groups and fed HFHF (b.w.=366.8 ± 35 g, n=10); chia flour HFHF (b.w.=362.4 ± 35.4 g, n=10), or chia oil HFHF (b.w.=362.4 ± 34.8 g, n=10) for 10 weeks. The lean control group (b.w.=350 ± 29.1 g, n=10) fed AIN93M diet continued on same diet for 10 wk. The experimental design is presented in Figure 1. Diet composition and ingredients are presented in Table 1. Chia flour and oil composition were taken into account in order to prepare diets. Diets were prepared every 15 days, packed in dark polyethylene bags and stored at -20 ºC to minimize fatty acid oxidation. Water and diet consumption were daily checked, and replaced every week, with exception of chia oil diet, that was replaced every two days in attempt to avoid lipid oxidation. The food intake and animal weight gain were monitored weekly.

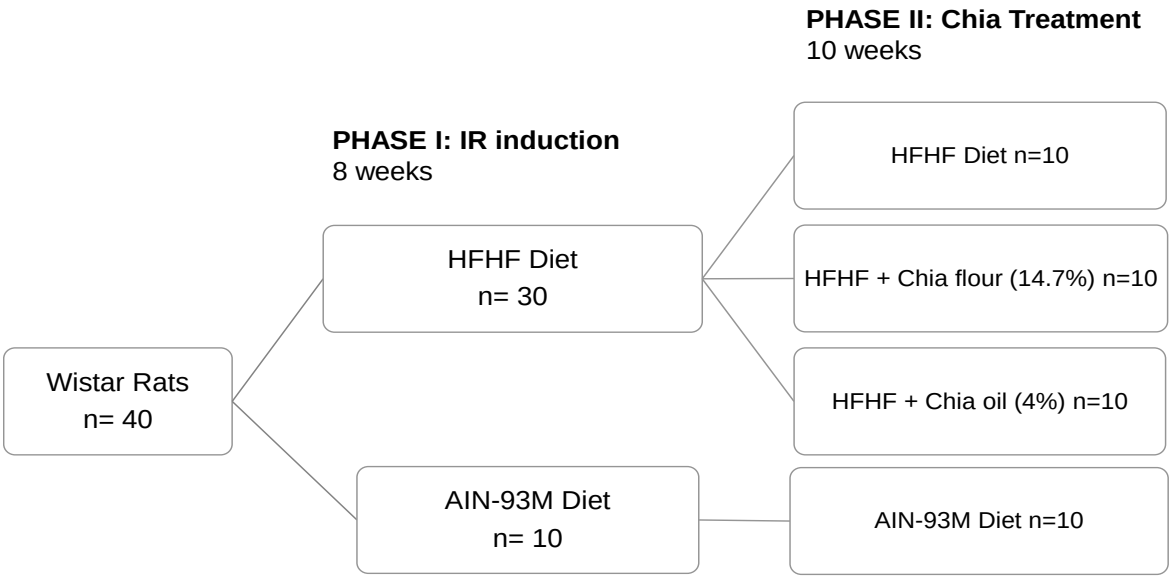


Figure 1. Experimental design. The experiment was divided into two phases. Phase I: Animals were divided in 2 groups: Control received a standard diet (AIN-93M) ; high-fat and high-fructose group (HFHF) containing 4% (w/w) soybean oil, 31% (w/w) lard and 20% fructose (w/w) (Table 1) for 8 wk. Phase II: HFHF group was divided into 3 groups: HFHF; chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil). Animals from control received AIN-93M diet on phase II. IR: insulin resistance.

Table 1. Nutritional composition of experimental diets.

Ingredients (g/kg diet)	Experimental Diets			
	AIN-93M	**HFHF**	**Chia seed**	**Chia oil**
Albumin*	136.4	136.4	101.8	136.4
Dextrinized starch	155	45	45.4	4
Corn starch	463.5	135	116.8	135
Sucrose	100	28.6	29.3	28.6
Fructose	-	200	200	200
Soybean oil	40	40	-	-
Chia seed	-	-	147.3	-
Chia oil	-	-	-	40
Lard	-	310	310	310
Microcrystalline Cellulose	55.8	55.8	55.8	55.8
Vitamin mix	10	10	10	10
Mineral mix	35	35	35	35
L-cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5
Nutritional Composition				
Macronutrients				
Carbohydrates (%)	77.4	31.0	30.1	31.0
Protein (%)	12.9	9.1	9.2	9.1
Lipids (%)	9.7	59.8	60.4	59.8
Energetic density (kcal.g^{-1})	3.71	5.26	5.21	5.26
Fatty acids (g.kg^{-1})**				
Linoleic (C18:2 n-6)	20.2	58.8	46.5	46.5
α-Linolenic (C18:3 n-3)	3.3	10.2	31.8	31.8
n-6/n-3 ratio	6.17:1	5.77:1	1.46:1	1.46:1

AIN-93M (Reeves, Nielsen, Fahey George, 1993): standard diet group; HFHF (MARINELI *et al*., 2015): high fat and high-fructose group; Chia flour group: HFHF with 14.7% (w/w) chia flour; Chia oil group: HFHF with 4% (w/w) chia oil.

* Amount was calculated based on protein content equal to 88% to provide 12 g protein.100 g-1 of diet.

** Fatty acids expressed in g/kg diet and determined by gas chromatography (MOREIRA, L.P.D., 2019)

2.5.2 Intraperitoneal glucose tolerance test and insulin tolerance test

Intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT) were performed on 9th and 10th week of chia flour (n=10) or chia oil (n=10) administration, respectively. Briefly, blood glucose levels were measured with a handheld glucometer (Accu-Chek®, Roche) using appropriate test strips. For the iGTT, D-glucose solution 50% (2 g.kg^{-1} body weight) was injected into the peritoneal cavity after 12 h fasting. Blood glucose levels were measured at baseline (time 0) and after 30, 60, 90, and 120 min from the tail vein. The area under the curve (AUC) of glucose was calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA). ITT was accessed on animals fasted for 12 h, after intraperitoneal human insulin injection (0.75 U.kg^{-1} body weight, Novolin®, Novo Nordisk). Glucose blood levels were measured on baseline (time 0) and after 5, 10, 15, 20, 25, and 30 min of insulin injection. The formula [0.693/(t1/2)] was used to calculate the constant rate of glucose disappearance. The glucose t1/2 was calculated from the slope of the least-squares analysis of the glucose concentrations during the linear phase (Bonora, Manicardi, Zavaroni, Coscelli, & Butturini, 1987). The euthanasia was performed 7 days after the ITT test.

2.5.3 Euthanasia and Tissue collection

The experiment was finished by euthanasia of animals at the end of 10 weeks of diets administration (no fast was performed). The animals were euthanized by cardiac puncture, after anesthetized with isoflurane (Isoforine, Cristália®). The liver was collected, weighted and immediately frozen with liquid nitrogen, prior storage at -80 ºC for subsequent analysis. Adipose tissue was collected and weighted to calculate percentage of adiposity using the following formula: (visceral + retroperitoneal + epididymal adipose tissues)/total body weight × 100.

2.5.4 Gene expression in liver tissue

Expression levels of genes involved on insulin and glucose metabolism in the liver were analyzed by RT-qPCR using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacture´s protocol. Briefly, liver tissue (~100 mg) grinded in liquid nitrogen were homogenized in TRIzol reagent (Invitrogen, Carlsbad,CA, USA) (1 mL) following manufacturer's protocol. Extracted mRNA was reverse transcribed into cDNA using the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY). The analyses were performed on the StepOneTM Real-

TimePCR System (Thermo Fisher Scientific, Waltham, MA) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). Primers were purchase from Integrated DNA Technologies (IDT DNA, Coralville, IA) to amplify AMPK, INSR, FOXO1, AKT, GK, PK, PFK PEPCK, G6Pase and β-actin (Supplementary materials Table S1). β-actin was used as endogenous control to normalize the relative expression of mRNA. The primer sequences are presented in supplementary material (Table S1).

2.5.5 Phospho-AKT assessment

Phospho-AKT protein levels were quantified using the AKT1[pS473] Ultrasensitive ELISA sandwich Kit (Invitrogen, ThermoFisher Scientific, MA, USA), according to the manufacture´s protocol, on liver homogenate. Liver tissue was homogenized with phosphate buffer (1:10) 50 mM, at pH 7.4 containing 1 mM EDTA, and protease inhibitor (Protease Inhibitor Cocktail powder, Sigma Aldrich). The absorbance of samples was read at 450 nm (Multiskan Microplate Photometer, ThermoFisher Scientific, MA, USA).

2.6 *In Vitro* Study

2.6.1 Cell line

The human hepatoma (HepG2) cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco´s modified Eagle´s medium (DMEM) containing 5.5 mM D-glucose (GIBCO, Thermo Fisher, Waltham, MA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 ºC, 5% CO_2.

2.6.2 Cell Viability

Cells were seeded at 80% confluence in a 96 well plate for 24 h to allow cell attachment. Followed by chia phenolics treatments (20 to 320 ppm) quantified as total phenolics (Abderrahim et al., 2011) against a standard curve of gallic acid (0-300 ppm) and expressed in milligrams of gallic acid equivalents per milliliter of extract (mg GAE.mL^{-1}). After 48h incubation, cell viability was determined using the in vitro toxicology assay kit resazurin following the manufacture´s protocol (Sigma-Aldrich, San Luis, MO).

2.6.3 Insulin resistance protocol

HepG2 cells were maintained in DMEM medium at 37 ºC, 5% CO_2 for 24 h. One day after plating, the medium was replaced for FBS free DMEM for 24 h. The cells were then treated with high glucose (25 mM) FBS free medium containing palmitic acid (PA) (1 mM) for 24 h (Nakajima, K. *et al*., 2000; Lee, J., Cho, H., Kwon, Y., 2010). After 24 h, insulin (100 µM) was added for 15 min. Cells cultured in 5.5 mM D-glucose DMEM, FBS free medium were used as negative controls (with and without insulin).

2.6.4 Gene Expression

A preliminary test was conducted with chia phenolics extract (20, 40, 80 ppm). Insulin resistance induced cells as described in 2.6.3 were treated with CHPE extract (80 ppm) for 24 h followed by insulin (100 µM) was added for 15 min. Total RNA from HepG2 cells was extracted using Quick-RNA Mini Prep (Zymo Research, Irvine, CA), according to the manufacturer´s protocol. cDNA was synthesized using iScript Reverse Transcription Supermix (BioRad, Hercules, CA). cDNA was subjected to quantitative Real-time polymerase chain reaction (RT-PCR) using the iTaq Universal SYBR Green Supermix Kit (BioRad, Hercules, CA) according to the manufacturer´s protocol. RT-PCR data was analyzed with the delta CT method (Schmittgen and Livak, 2009) using TBP as the housekeeping gene. Primers were purchased from Sigma-Aldrich (San Luis, MO, USA) to amplify PFK, PK, PEPCK and G6Pase. Primers sequence are presented in supplementary material (Table S2).

2.6.5 Phospho-AKT assessment

Insulin resistance induced cells as described in 2.6.3 were treated with CHPE extract (20, 40 and 80 ppm) for 24 h followed by insulin (100 µM) for 15 min. Negative controls were prepared as detailed in 2.6.3. Cells lysates were obtained adding X Tractor buffer (Takara Bio Company, CA, USA). Solid cellular debris was removed by centrifugation at 25,200 g for 10 min at 4 ºC. The supernatant was collected and stored at -80 ºC. Protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Cell lysates (60 mg protein diluted with Laemmli's loading buffer and boiled for 5 min) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for 2 h. The proteins were transferred by wet blotting onto a 0.2 mm PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked using 5% non-fat milk in a 0.1% tween-phosphate buffer solution (T-PBS) for 30

min and incubated with the primary antibody (1:1000) in a 3% bovine serum albumin in T-PBS overnight at 4 ºC with gentle shaking to assess the protein level of p-AKT(Ser473) (Cell Signalling, Danvers, MA) and β-actin (Sigma-Aldrich, San Loius, MO). This was followed by incubation with the anti-rabbit IgG secondary antibody (1:2000) (Cell Signalling, Danvers, MA) in 5% milk T-PBS for 2 h. The reactive bands were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) after 1 min of incubation, using an Omega Ultra Lum Molecular Imaging System (model Omega10gD) and band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, UDA; https://imagej.nih.gov/ij/download.html).

2.7 Statistical Analysis

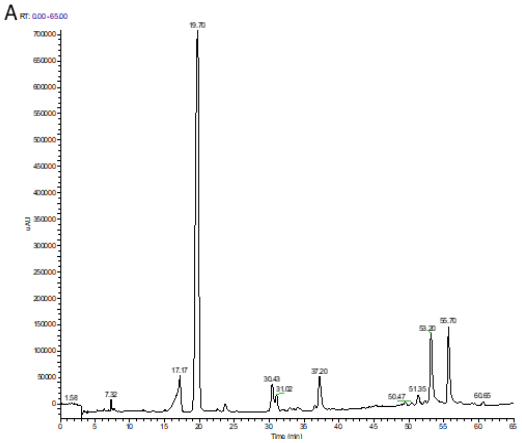
Quantitative data were expressed as mean ± standard error or standard deviation of the mean. Effect of treatments were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test for parametric data and Kruskal-Wallis followed by Dun´s for non-parametric. Tukey test was used to compare glucose blood levels means during different times on iGTT and ITT tests. The significance level was set at $p<0.05$. GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used to perform the analysis.

3. RESULTS

3.1 Chia phenolics, identification and quantification

The chromatogram of CHPE and the content of phenolic compounds are presented in Figure 2. CHPE presented 28,000 mg GAE. mL^{-1} of total phenolic. The main phenolic compounds on the extract were rosmarinic acid, danshensu glycoside, ferulic acid and caffeic acid.

A



B

Retention Time (min)	[M–H]⁻ m/z	Compound	Concentration (mg/L)
23.4	197	Danshensu	569.00
36.94	179	Caffeic acid	1,016.00
53.03	359	Rosmarinic acid	3,519.00
55.00	373.07	Danshensu glycoside	1,614.00
30.56	211	Danshensu Methyl ester	181.00
40.25	153	Protocatachuic acid	61.00
48.69	521	Rosmarinic acid glycoside	311.00
47.91	717	Salvianolic acid	110.00
51.75	717	Salvianolic acid	501.00
51.75	535	Rosmarinic acid glycoside	482.00
44.78	355	Ferulic acid glycoside	66.00
50.59	193	Ferulic acid	1,351.00
58.98	357	Caffeic acid Derivative	241.00

Figure 2. Hydrolyzed Phenolics from Chia Characterization. (A). Chromatographic profile at 280 nm of the chia hydrolyzed phenolics extract from chia flour used in the *in vitro* study. (B). Quantification of phenolic compounds by LCMS from chia hydrolyzed phenolics extract.

3.2.1 Chia oil administration decreased body weight and body fat accumulation

Results from food and energy intake showed higher food intake in control group (AIN-93M), but energy intake was similar among all experimental groups (Figure 3A and B). Body weight gain in animals fed chia oil was lower compared to HFHF, while chia flour group was similar to AIN-93M (Figure 3C). Furthermore, adiposity in chia oil and chia flour groups were 5.1% and 6.9%, respectively, lower than HFHF (7.8%) control, and similar to AIN-93M group (4.2%) ($p<0.05$) (Figure 3D).

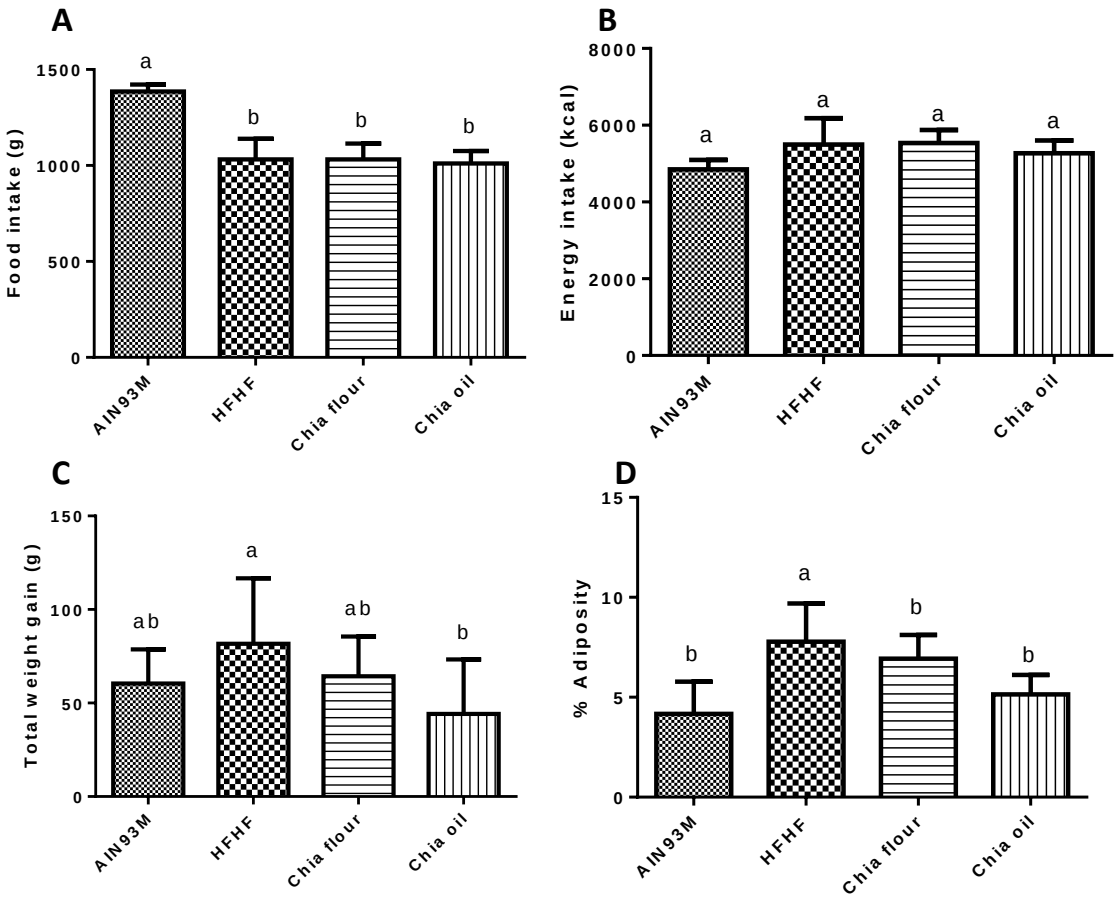


Figure 3. Effect of chia flour and oil consumption on (A) food intake, (B) energy intake, (C) total weight gain, (D) % adiposity. Data are expressed as mean ± standard deviation. Different letters represent significant differences ($p<0.05$) by Newman-Keuls test. HFHF: high-fat and high-fructose, chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil).

3.2.2 Glucose and insulin tolerance tests

Results showed that chia oil decreased significantly the blood glucose levels and the AUC to levels that were similar to those on AIN-93M group. Blood glucose of chia oil group was lower than HFHF and chia flour groups, and similar to control group at times 30, 60 and 90 min (Figure 4A and B). Consistently, kITT showed a similar pattern, with chia oil administration presenting faster glucose disappearing as well as AIN-93M group (Figure 4C). Blood glucose levels were similar to AIN-93M group at 20 min (Figure 4D).

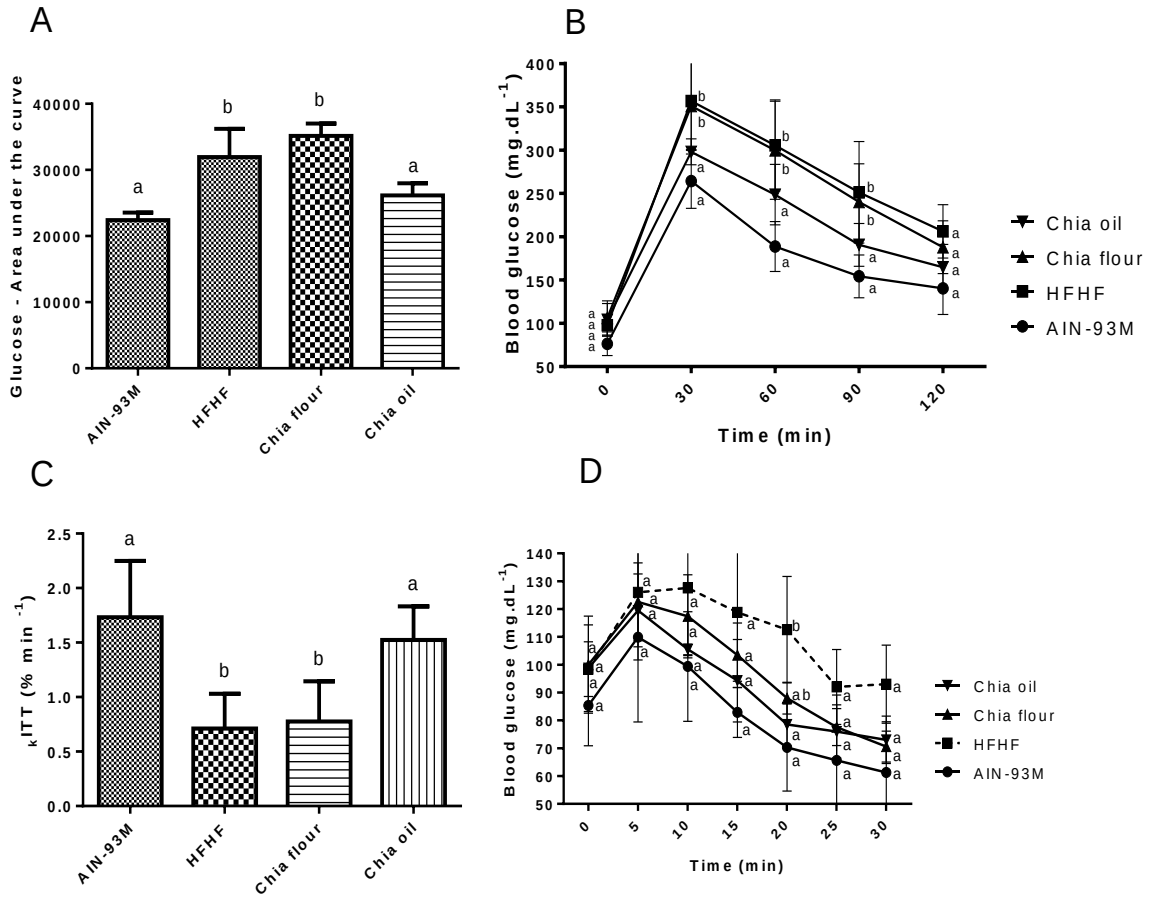


Figure 4. Chia oil decreased hyperglycemia and insulin resistance. Glucose and insulin tolerance were accessed through intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT). (A) glucose area under the curve during iGTT, (B) mean blood glucose levels after intraperitoneal infusion of glucose solution, (C) kITT during ITT, (D) mean blood glucose levels after insulin intraperitoneal infusion. Data are expressed as mean and standard deviation. Different letters represent significant differences ($p<0.05$) by Tukey´s test. HFHF: high-fat and high-fructose, chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil). kITT: constant rate of glucose disappearance in ITT test.

3.2.3 Gene expression and protein in liver tissue

Results showed that chia oil and chia flour at lesser extend modulated the expression of biomarkers that play an important role in liver IR. Among the assessed biomarkers (AMPK, INSR, AKT, FOXO1, GK, PFK and PK), chia oil upregulated the mRNA levels of all of them ($p<0.05$) (7.3, 5.3, 1.9, 4.7, 10.5, 5.18 and 1.9-fold of HFHF control, respectively); while chia flour upregulated only AMPK and AKT (3.8 and 2.0-fold of HFHF control, respectively) mRNA levels (Figure 5). Moreover, chia oil prevented HFHF-induced downregulation of AMPK, INSR, and FOXO1 mRNA maintaining those levels similar to AIN-93M group (Figure 5A, 5B, 5C) upregulated the mRNA levels. These results suggest that chia seed may help to ameliorate or delay the onset of IR, while chia oil is the active compound in chia seeds with potential to reverse or prevent this condition.

AKT1[pS473] protein level was increased for chia oil group. For HFHF and lean control groups, the AKT1 [pS473] protein levels were similar (Figure 5H) and consistent with mRNA levels (Figure 5D).

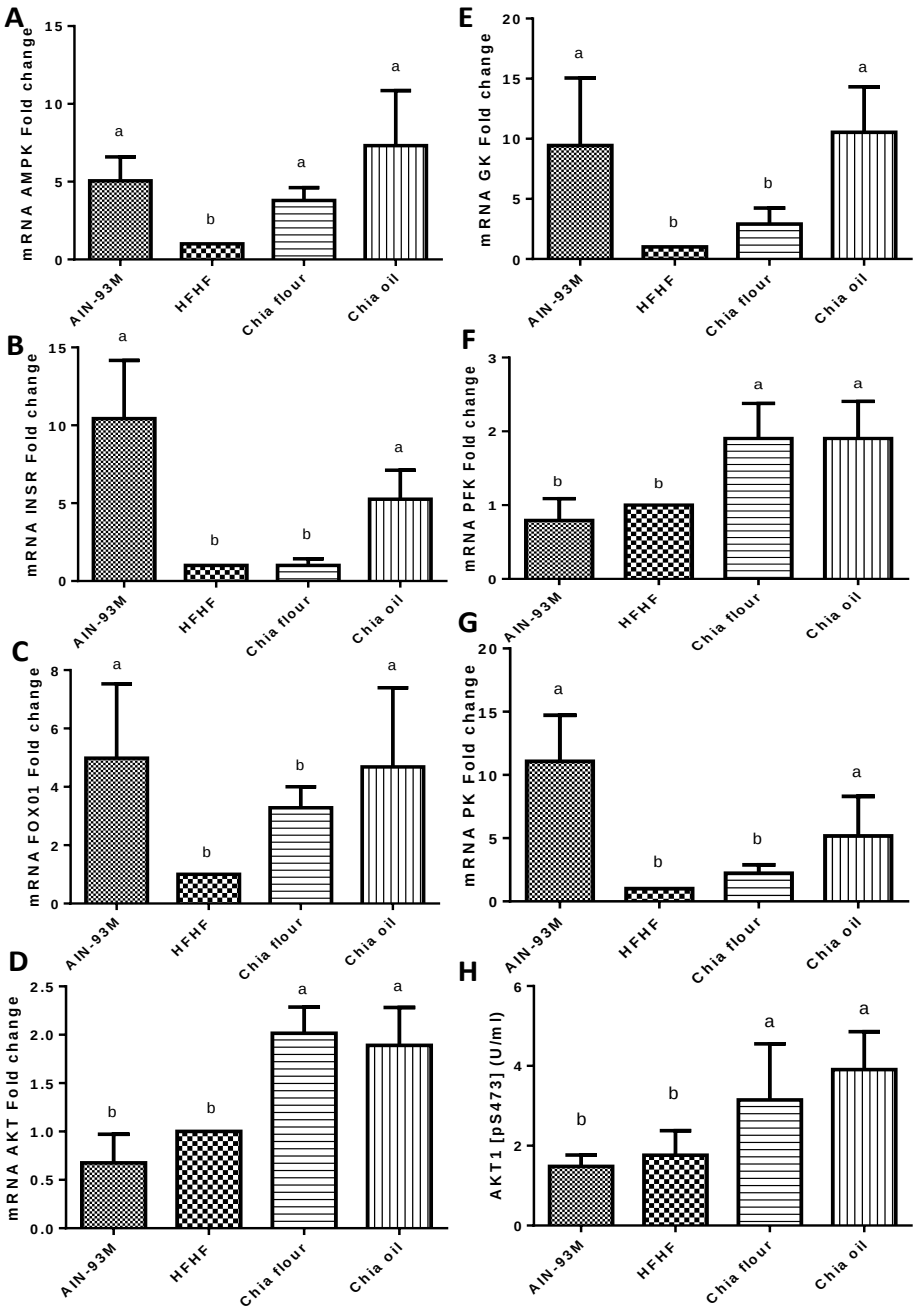


Figure 5. Chia oil modulated gene expression of biomarkers for IR. Chia (flour and oil) increased p-AKT. (A) AMPK, (B) INRS, (C) FOX01, (D) AKT, (E) GK, (F) PFK, (G) PK, and (H) AKT[pS473]. Gene expression of key proteins related to glucose metabolism and glycolysis enzymes. mRNA was extracted from Wistar rats liver and analyzed by qRT-PCR. Protein levels of AKT1[pS473] were quantified in Wistar rats liver homogenates by ELISA. Data are mean ± standard error of the mean (SEM). Different letters represent significant differences ($p<0.05$) by Newman-Keuls test. AMPK adenosine monophosphate-activated protein; INSR insulin receptor; FOX01 Forhead box protein 01; AKT: protein kinase B; GK glucokinase; PFK phosphofructo kinase; PK pyruvate kinase. HFHF: high-fat and high-fructose, chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil).

3.3. *In vitro* study

3.3.1 Cell viability

HepG2 cell viability was not inhibited by CHPE at dose range 10-80 ppm (Figure S1). Therefore, this dose range was used for future in vitro experiments.

3.3.2 Chia phenolics improved mRNA levels of gluconeogenic enzymes but did not modulate PI3K/AKT pathway in HepG2 cells

Results showed that mRNA levels of gluconeogenic and glycolysis enzymes were modulated by CHPE (Figure 6). The mRNA levels of glycolytic and gluconeogenic enzymes indicate if these pathways are involved on CHEP glucose modulation. In general, enzyme mRNA levels were upregulated in positive control (PC) upon insulin resistance induction by palmitate and high glucose indicating a cell homeostatic response, and chia phenolics decreased this cell response. This indicates that chia phenolics lessened the cell insults and the cell response needed to maintain homeostasis. Protein levels of p-AKT were decreased as expected under conditions resembling insulin resistance induced by palmitic acid (1 mM) associated to high glucose media (25 mM) as shown in PC. However, chia phenolics failed to reverse this condition (Figure 6E).

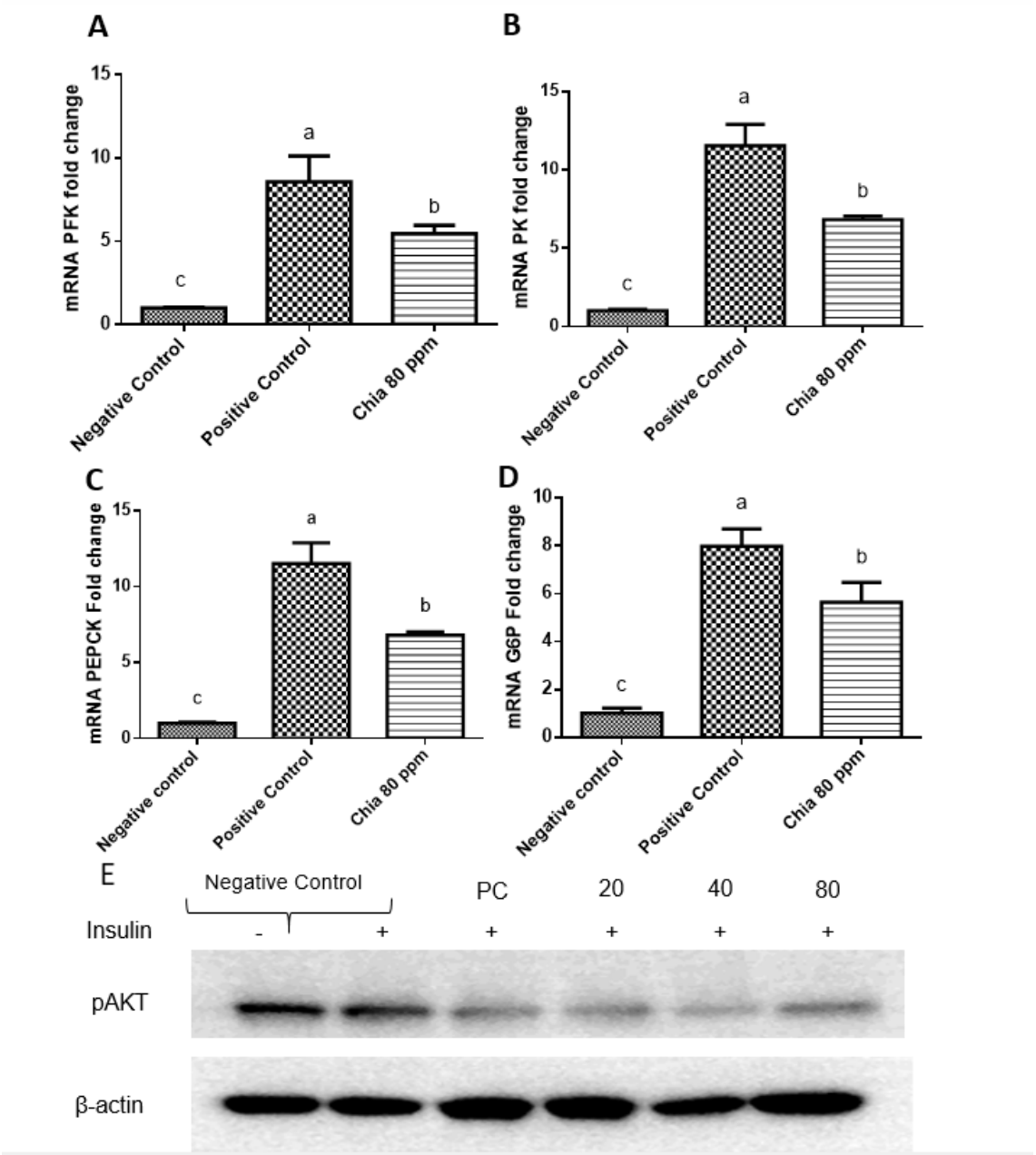


Figure 6. Chia hydrolyzed phenolics extract decreased gluconeogenesis and glycolysis enzymes activity. Chia hydrolyzed phenolics extract did not increase pAKT protein levels. (A) PFK, (B) PK, (C) PEPCK, (D) G6Pase, (E) p-AKT(Ser473). Gene expression of gluconeogenesis enzymes. mRNA was extracted from HelpG2 cells and analysed by qRT-PCR. Negative control: HepG2 cells treated with low glucose media. Positive Control: HepG2 cells treated with high glucose media (25 mM) and Palmitate (1mM) for 24 h followed by hydrolyzed chia extract for 12 h and insulin for 15 min. Chia 80 ppm: dose of chia hydrolyzed phenolics extract (based on total phenolics content). Protein expression was extracted from HepG2 cells and analyzed by western blot (β-actin as control). Negative control: HepG2 cells treated with low glucose media. Positive Control (PC): HepG2 cells treated with high glucose media (25 mM) and Palmitate (1mM) for 24 h followed by hydrolyzed chia extract for 12 h and insulin for 15 min. 20, 40, 80: doses of chia hydrolyzed phenolics extract (based on total phenolics content). Data are mean ± standard error of the mean (SEM). Different letters represent significant differences ($p<0.05$) by Newman-Keuls test. PFK Phosphofructokinase; PK: pyruvate kinase; PEPCK Phosphoenolpyruvate carboxykinase; G6Pase: Glucose-6 phosphatase.

4. DISCUSSION

This study brought new insights into the chia seeds health properties regarding different fractions (flour, oil or phenolics) on insulin pathway activation and blood glucose control in nutritional unbalanced status.

Regarding the *in vivo* study, food intake in HFHF groups was lower than in AIN-93M group, however, energy intake was similar (Figure 3A and B). This outcome could be explained due to the higher energy density of HFHF diet. These results were consistent with the study reported by Marineli *et al*. (2015).

Besides the same energy consumption among experimental groups, chia oil group showed the lowest body weight gain compared with chia flour and HFHF groups (Figure 3C). However, adiposity was lower in chia flour and oil groups compared to HFHF group and similar to AIN93M group (Figure 3D). These results are consistent with previous reports (Fonte-Faria *et al*., 2019; Creus *et al*., 2017; Poudyal *et al*., Brown, 2012; Poudyal, Panchal, Ward, & Brown, 2013; Poudyal, Panchal, Ward, *et al*., 2012). The reduced body adiposity may be associated with ALA intake, since the consumption of chia has been associated to a lipid redistribution with FAT/CD36 recruitment to plasmatic membrane, mitochondrial activity and beta-oxidation (Creus *et al*., 2016; Marineli *et al*., 2015; Poudyal, Panchal, Waander, Ward & Brown, 2012). The lipid redistribution could also contribute to glucose tolerance improvement (Adeva-andany, Noemi, & Fern, 2016).

Glucose and insulin tolerance intraperitoneal tests showed that only chia oil was able to repair glucose metabolism. This finding is supported by Fonte-Faria *et al*. (2019), which found that chia oil restored glucose and insulin tolerance in mice fed HFD diet. Nevertheless, Marineli *et al*., (2015) found that both, chia oil and flour, improved glucose and insulin tolerance in rats fed HFHF diet. In contrast, Miranda *et al*. (2018) did not find improvement on glucose tolerance by chia flour administration in mice fed HFD. Although the high content of dietary fiber in chia seeds (37.9%, data not showed) was expected to contribute to glucose and insulin tolerance reestablishment, results indicate that ALA in chia oil could be the bioactive compound that contributed to improve glucose and insulin resistance. However, its content may be influenced by chia seed variety, and growing conditions (temperature, climate and soil) (Silva *et al*., 2017). In our study, ALA content in animals' diet was 31.8 $g.kg^{-1}$. It was higher than reported by Marineli *et al*. (2015), Miranda *et al*. (2018), Fonte-Faria *et al*. (2019) (25.87 $g.kg^{-1}$, 4.93 $g.kg^{-1}$, and 1.7 $g.kg^{-1}$, respectively). It should be highlighted that the chia seeds were grown in different locations.

Besides the GTT and ITT tests outcomes, chia flour and oil increased liver AMPK gene expression. AMPK, considered an energy-deprivation sensor, is downregulated during the onset of obesity and metabolic syndrome, and under unbalanced diet conditions, as HFHF, disrupting energy balance. However, exercise, caloric restriction and anti-diabetic compounds activate this pathway (Kim *et al*., 2016). Our results showed that liver AMPK gene expression increased after chia flour and oil intake (3.8- and 7.3-fold of HFHF, respectively). Chia oil group showed increment even higher than in AIN93M group (5-fold). It suggests ALA as AMPK activator as reported by Wang *et al*. (2018). This outcome should be highlighted because AMPK activation could decrease blood glucose levels by AS160 protein phosphorylation. This protein plays a role on trafficking glucose transporter, increasing glucose uptake in an insulin-independent pathway (Bradley, *et al*., 2015). Therefore, both, chia flour and oil, seem to modulate AMPK expression as insulin-independent mechanism to improve glucose metabolism, although only the oil have impacted on GTT and ITT. To our knowledge, this is the first study which chia flour and oil intake induced upregulation of AMPK gene expression in liver.

In addition, chia flour and oil were effective on increasing AKT and PFK mRNA levels. While chia oil also upregulated gene expression of INSR, FOXO1, GK and PK. These results imply the enhanced activity of chia oil on glucose metabolism modulation in dependent and independent insulin pathways. The upregulation of INSR and AKT mRNA, followed by phospho-AKT protein level for chia oil group may indicate increasing in glucose uptake and oxidation, glycolysis (according to the increment of GK, PK and PFK mRNA expression), and decreasing gluconeogenesis (indicated by FOXO1 upregulation). These data and chia oil effect on glucose metabolism are reinforced by ITT test (Figure 4C).

Even though the oil content of chia flour and oil diets were similar, the availability of oil compounds may be impaired by other components of chia, such as dietary fiber, protein and calcium, which could bound compounds, like fatty acids, decreasing its availability to digestive enzymes and absorption (SILVA *et al*., 2017, 2018). These findings draw attention to chia oil and to its effect on glycolysis modulation, which are associated with AKT and AMPK activation, and may represent an important outcome on glucose metabolism.

The *in vitro* results showed that CHPE, simultaneously, downregulated mRNA of enzymes involved on gluconeogenesis and glycolysis, PEPCK and G6Pase, and PFK and PK, respectively. Opposite to the *in vivo* results, the *in vitro* studies showed CHPE do not

contribute to activate the AKT signaling cascade in HepG2 cells. These results suggest that chia phenolics may reduce obesity-related metabolic disorders linked to the impaired gluconeogenesis pathway. As an example, mice treated with ferulic acid (25 $mg.kg^{-1}$) had improved insulin resistance associated with decreased protein expression of PEPCK and G6Pase enzymes, which are involved on gluconeogenesis (Naowaboot, Piyabhan, Munkong, & Parklak, 2016). Moreover, Rosmarinic acid, has been associated with glucose improvement related to AMPK phosphorilation, with no involvement of PI3K – AKT signaling cascade (Vlavcheski, Naimi, Murphy, Hudlicky, & Tsiani, 2017). Also, caffeic acid from propolis, improved glucose uptake in HepG2 cells and decreased G6Pase expression in insulin resistant HepG2 cells (Nie *et al*., 2017). In this research, the evaluation of chia phenolics effects on HepG2 IR cells were limited to p-AKT protein levels and mRNA of enzymes involved on gluconeogenesis and glycolysis. We reinforce the need for further investigations regarding enzymes activity as wells as the crosstalk pathways.

Considering the *in vitro* and *in vivo* research models used in this study, the proposed underlying mechanisms modulated by chia in insulin resistance are illustrated in Figure 7. Among the different components of chia, the oil showed a major impact on glucose and insulin tolerance. This may be related to its lipid profile highlighted by ALA. Some of the underlying mechanisms modulated by chia oil were the mRNA upregulation of INSR. This is linked to improvement of insulin affinity for its receptor on hepatocyte membrane, activating AKT phosphorylation, contributing to glucose uptake and glucose oxidation as major energy fuel (indicated by GK, PFK and PK glycolysis enzymes mRNA expression). Both, chia flour and oil, upregulated AMPK gene expression, which may implicate in an insulin-independent mechanism on glucose metabolism control. Chia phenolics may contribute to glucose control by decreasing gluconeogenesis activity, linked to PEPCK and G6Pase mRNA levels downregulation (Figure 7).

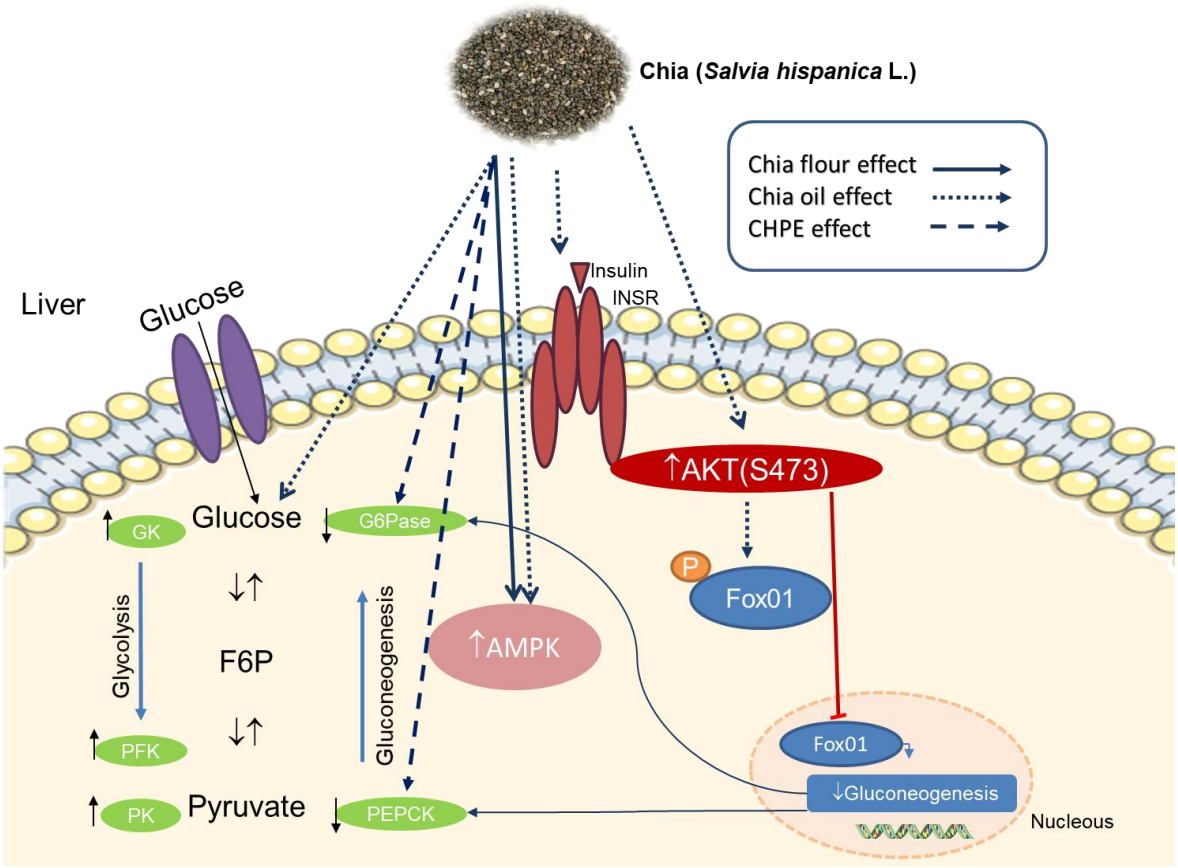


Figure 7. Proposed effect of Chia (Salvia hispanica L.) on glucose homeostasis by controlling AKT activation, gluconeogenesis and glycolysis pathways. Chia oil increased insulin receptor expression, contributing to insulin bind to its, signaling to phosphorylate AKT. P-AKT blocks FOX01 to migrate to nucleous and to initiate transcription of gluconeogenesis enzymes, controlling glucose synthetase. Chia hydrolyzed phenolics contributes to decrease gluconeogenic pathway. Chia flour and chia oil increased AMPK expression, which increases glucose uptake and oxidation, as well as glycolysis enzymes, resulting in glucose tolerance improvement.

5. CONCLUSION

Chia flour and oil decreased adiposity and may modulate glucose metabolism throughout insulin independent mechanism (AMPK) in rats fed HFHF diet. However, only chia oil improved glucose and insulin tolerance and restored energy fuel system on hepatocytes, increasing AKT phosphorylation and improving glycolysis. Chia hydrolyzed phenolics extract was associated with gene expression modulation of gluconeogenic and glycolytic enzymes on HepG2 insulin resistant cells. The present study provides information with the positive effects of chia components on glucose metabolism in the liver under insulin resistance conditions.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary material

Table S1. Sequencing primers used in the RT-qPCR analysis for in vivo study

Genes	Oligonucleotide (5´-3´)	
	Forward	**Reverse**
AMPK	TGAAGCCAGAGAACGTGTTG	ATAATTTGGCGATCCACAGC
INSR	TCATGGATGGAGGCTATCTGGA	TCCTTGAGCAGGTTGACGATTTC
FOX01	GATAAGGGCGACAGCAACAG	TGAGCATCCACCAAGAACTT
AKT	GGGCCACGGATACCATGAAC	AGCTGACATTGTGCCACTGA
GK	AGTATGAACCGGATGGTGGATGAA	CCAGCTTAAGCAGCACAAGTCGTA
PK	ATCTGGGCAGATGATGTGGA	ATAGGGTGTAACTGGGTCAGAATGG
PFK	CCACCTGGAGGCCATTGTAGA	GGGATGACGCACATGACGA
β-actin	GTCGTACCACTGGCATTGTG	CTCTCAGCTGTGGTGGTAA

AMPK: AMP-activated protein kinase; INSR: Insulin receptor; FOX01: Forkhead box protein O1; AKT: Protein kinase B; GK: Glucokinase; PK: Pyruvate kinase; PFK: Phosphofructokinase.

Table S2. Sequencing primers used in the RT-qPCR analysis for in vitro study.

Genes	Oligonucleotide (5´-3´)	
	Forward	Reverse
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
PFK	GGTAAGATCTCAGAGACTACAG	AGAAGTCGTTATCGATGGAG
PK	AAAATTGAGAACCACGAAGG	CAATCATCATCTTCTGAGCC
PEPCK	TGGAAGAATAAGGAGTGGAG	CTTCATAGACTAGAGGGACAC
G6Pase	ACTGTGCATACATGTTCATC	TGAATGTTTTGACCTAGTGC

TBP: TATA box binding protein; PFK: Phosphofructokinase; PK: Phosphokinase; PEPCK: Phosphoenolpyruvate carboxykinase; G6Pase: Glucose-6 phosphatase.

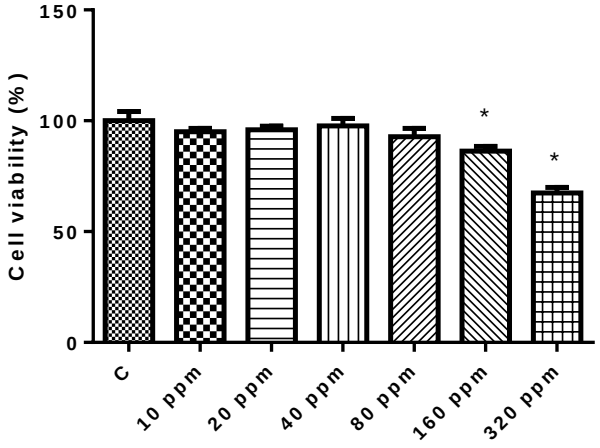

Figure S1. Chia hydrolyzed phenolics up to 80 ppm did not exert HepG2 cell toxicity. Cell viability (% of control). Cells were treated with chia phenolics (10 – 320 ppm) for 48 h and cell viability was determined with resazurin test as detailed in materials and methods. Data are mean ± standard error of the mean (SEM). * represent significant difference ($p<0.05$) by Dunnett´s test.

6. GENERAL CONCLUSIONS

This work brough some new information about chia seed effects on health, regarding molecular pathways. We first demonstrated by systematic review the hypothesis formulated after analyzing animal studies results, which received chia (seed or oil) accompanied of unbalanced diet, throughtout a systematic review. The data extracted allowed us to conclude that AMPK is the central protein affected by chia seed. The consumption of chia seed and chia oil by animals, increased the expression of AMPK, which increased the expression of biomarkers related to mithocondrial activity and fatty acid oxidation. In addition, activated AMPK stimulate insulin receptor phosphorylation, that together, translocate GLUT4 to celular membrane, increasing glucose uptake and oxidation, and lowering blood glucose. Moreover than results about chia's effect, the review demonstrated the lack of information about the methods used to carrrie the experiment. The absence of certain data, such as bliding protocolos, information about randomization, sample size, dose of chia consumed, and number of animals evaluated on statistical analysis. The quality of data extracted from experimental studies is important for confident results and to plan future experiments, including clinical trials.

Second, we demonstrated throughout original data, for the first time, that chia's fractions (whole seed, oil and phenolics) have different effects on health, especially, on glucose metabolism. Our data provided information claiming that the oil from chia presents superior activity on glucose metabolism, improving glucose tolerance and positively affecting insulin resistance conditions. Chia oil improved blood glucose and insulin tolerance, increased AKT gene expression and AKT[pS473] protein. Besides that, the gene expression of AMPK, FOX01, INSR and enzymes involved on glycolysis (glucokinase, phosphofructo kinase, and pyruvate kinase). The whole seed increased AKT gene expression, AKT[pS473] protein, and AMPK gene expression, but blood glucose and insulin tolerance was not affected, as well as, gene expression of FOX01, INSR and glycolysis enzymes. The phenolics from chia modulated the expression of gluconeogenesis enzymes, but this event was not sufficient to improve central biomarkers related to glucose control. The phenolics however, may have other properties, such as antioxidant activity, cited in published literature, but not investigated in this work.

From the three fractions investigated in this research (whole seed, oil and phenolics), the oil from chia demonstrated superior results regarding glucose metabolism in insulin resistance conditions.

7. FINAL CONSIDERATIONS

These results from these studies are promising and allow understanding about the mechanisms related to the beneficial effects of chia seed consumption related to unbalanced diets, especially on insulin resistance conditions. Until the present moment, other studies have investigated only two fractions of chia, whole seed and oil, but this is the first time that the effects of phenolics from chia were investigated.

Phenolics did not improve glucose metabolism on insulin resistance conditions, but they might demonstrate positive effects on other conditions, such as, inflammation. We strongly suggest an investigation about chia's phenolics compounds on inflammatory biomarkers, since, previous studies demonstrated improvement on inflammation status after chia intake. However, since these studies were performed using the oil or the whole seed, and phenolics can be found on both fractions, the isolation of these compounds and treatment with it is recommended.

Then, the results obtained in this work can allow future studies, including clinical trials, in attempt to establish adequate doses of chia oil or seed that could prevent and attenuate biomarkers related to diseases, such as type 2 diabetes or its previos status, glucose and insulin resistance.

We suggest for our research group, the investigation of the intestinal microbiota of these animals, since recent data showed that chia seed soluble extract positively modulated intestinal health and functionality. We also suggest that lipogenesis pathway on liver should be investigated, since, chia seed and oil increased AMPK expression, which can indicate enhance on energy fuel utilization, that usually is disrupted by unbalanced diets, such as HFHF, used in this research.

The results obtained from this work will contribute to knowledge for Nutrition Science, regarding functional foods. These data are able to explain the mechanisms related to chia seed consumption on insulin resistance conditions.

APPENDIX 1

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 31/2018, intitulado **"Propriedades funcionais da fração oleosa da semente de chia (*Salvia hispanica* L.), na inflamação, estresse oxidativo, adipogênese e modulação da microbiota intestinal em modelos de obesidade"**, coordenado pela professora Hércia Stampini Duarte Martino do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 26/04/2018, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 31/2018, named **"Functional properties of the oil fraction of chia seed (Salvia hispanica L.), inflammation, oxidative stress, adipogenesis and modulation of the intestinal microbiota in models in obesity"**, is in agreement with the an actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on April 26, 2018 valid for 12 months.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV