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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

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*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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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, Sumczynski, Mlcek, Jurikova, & Sochor, 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 (Marineli et al., 2014; Silva et al., 2017). Chia seed and oil have been investigated in the last few years for their potential benefits on glucose metabolism control (Fonte-Faria et al., 2019; Marineli, Lenquiste, Moraes, & Maróstica, 2015; Marineli, Moura, et al., 2015). 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, Lenquiste, et al., 2015; Marineli, Moura, et al., 2015). Mice fed high-fat diet (HFD) supplemented with chia oil (0.15%) for 90-135 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 °C 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 °C 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 °C  $\pm$  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  $\pm$  50 °C under reduced pressure. Dried CHPE were stored at -20 °C 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 °C 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<sup>-1</sup>) (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  $\mu$ Hydro-RP 80A (2  $\times$  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.

#### 2.6. Animal study

#### 2.6.1. 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 °C  $\pm$  2, 12 h light/dark cycle). Animals were randomized according to body weight and fed with standard diet (AIN-93M) (b.w. =  $156 \pm 17.0$  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. =  $358.04 \pm 33.00$  g, n = 30) were randomized into 3 experimental groups and fed HFHF (b.w. =  $366.8 \pm 35$  g, n = 10); chia flour HFHF (b.w. =  $362.4 \pm 35.4$  g, n = 10), or chia oil HFHF (b.w. =  $362.4 \pm 34.8$  g, n = 10) for 10 weeks. The lean control group (b.w. = 350  $\pm$  29.1 g, n = 10) fed AIN93M diet continued on same diet for 10 wk. The experimental design is presented in Fig. 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 °C 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



Fig. 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 <sup>-1</sup> )	3.71	5.26	5.21	5.26
Fatty acids $(g.kg^{-1})^{**}$				
Linoleic (C18:2n-6)	20.2	58.8	46.5	46.5
α-Linolenic (C18:3n-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: high fat and high-fructose group (Marineli, Lenquiste, et al., 2015; Marineli, Moura, et al., 2015); Chia flour group: HFHF with 14.7% (w/w) chia flour; Chia oil group: HFHF with 4% (w/w) chia oil.

 $^{\ast}\,$  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, 2019).

avoid lipid oxidation. The food intake and animal weight gain were monitored weekly.

#### 2.6.2. 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<sup>®</sup>, Roche) using appropriate test strips. For the iGTT, D-glucose solution 50% (2 g.kg<sup>-1</sup> 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<sup>®</sup>, 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.

#### 2.6.3. 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  $\times$  100.

#### 2.6.4. 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).

#### 2.6.5. 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).

#### 2.7. In vitro study

#### 2.7.1. 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 D-glucose (GIBCO, Thermo Fisher, Waltham, MA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C, 5%  $CO_2$ .

#### 2.7.2. 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<sup>-1</sup>). After 48 h incubation, cell viability was determined using the *in vitro* toxicology assay kit resazurin following the manufacturés protocol (Sigma-Aldrich, San Luis, MO).

#### 2.7.3. Insulin resistance protocol

HepG2 cells were maintained in DMEM medium at 37 °C, 5% CO<sub>2</sub> 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  $\mu$ M) 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).

#### 2.7.4. 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  $\mu$ M) 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, 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 supplementary material (Table S2).

#### 2.7.5. 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  $\mu$ M) 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–po-lyacrylamide 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).

#### 2.8. Statistical analysis

Quantitative data were expressed as mean  $\pm$  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 Fig. 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.

#### 3.2. In vivo study

### 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 (Fig. 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 (Fig. 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-93 M group (4.2%) (p < 0.05) (Fig. 3D).

#### 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 (Fig. 4A and B). Consistently, kITT showed a similar pattern, with chia oil administration presenting faster glucose disappearing as well as AIN-93M group (Fig. 4C). Blood glucose levels were similar to AIN-93M group at 20 min (Fig. 4D).

#### 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 (Fig. 5).



Fig. 2. (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.

Moreover, chia oil prevented HFHF-induced downregulation of AMPK, INSR, and FOXO1 mRNA maintaining those levels similar to AIN-93M group (Fig. 5A, B, C) 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 (Fig. 5H) and consistent with mRNA levels (Fig. 5D).

#### 3.3. In vitro study

#### 3.3.1. Cell viability

HepG2 cell viability was not inhibited by CHPE at dose range 10–80 ppm (Fig. 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 (Fig. 6). The mRNA levels of glycolytic and gluconeogenic enzymes indicate if these pathways are involved on CHEP glucose modulation. In general, enzyme mRNA levels



**Fig. 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  $\pm$  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).



**Fig. 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.

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 (Fig. 6E).

#### 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 (Fig. 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, Lenquiste, et al. (2015); Marineli, Moura, 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 (Fig. 3C). However, adiposity was lower in chia flour and oil groups compared to HFHF group and similar to AIN93M

group (Fig. 3D). These results are consistent with previous reports (Creus et al., 2017; Fonte-Faria et al., 2019; Poudyal, Panchal, Waanders, Ward, & Brown, 2012; Poudyal, Panchal, Ward, & Brown, 2013; Poudyal, Panchal, Ward, Waanders, & Brown, 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, Ferreira, Oliva, & Lombardo, 2016; Marineli, Lenquiste, et al., 2015; Marineli, Moura, et al., 2015; Poudyal, Panchal, Waanders, et al., 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, de 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<sup>-1</sup>. It was higher than reported by Marineli, Lenquiste, et al.



Fig. 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: highfat and high-fructose, chia flour (HFHF with 14.7% of chia flour) and chia oil (HFHF with 4% of chia oil).

(2015); Marineli, Moura, et al. (2015), Miranda et al. (2018), Fonte-Faria et al. (2019) (25.87 g.kg<sup>-1</sup>, 4.93 g.kg<sup>-1</sup>, and 1.7 g.kg<sup>-1</sup>, respectively). It should be highlighted that the chia seeds were grown in different locations.

Besides the GTT e 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, Yang, Kim, Kim, & Ha, 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.,



**Fig. 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 (1 mM) 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 ( $\beta$ -actin as control). Negative control: HepG2 cells treated with low glucose media (25 mM) and Palmitate (1 mM) 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 ( $\beta$ -actin as control). Negative control: HepG2 cells treated with low glucose media (25 mM) and Palmitate (1 mM) 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  $\pm$  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.



Fig. 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 phosphorvlate 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.

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 (Fig. 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 (2018; Silva et al., 2017). 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<sup>-1</sup>) 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 Fig. 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 (Fig. 7).

#### 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.

#### **Ethics statements**

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.

#### CRediT authorship contribution statement

Bárbara Nery Enes conducted the in vivo and in vitro study, obtained, compiled, analyzed data, and drafted the manuscript. Luiza de Paula Dias Moreira conducted the in vivo study, obtained, compiled and analyzed data. Renata Celi Lopes Toledo contributed to conduct the in vivo experiment, and conducted analyses of gene and protein expression. Érica Aguiar Moraes contributed to design the in vivo experiment, to analyze data and reviewed critically the manuscript. Maria Eliza de Castro Moreira contributed to design the in vivo experiment and reviewed critically the manuscript. Helen Hermana Miranda Hermsdorff contributed to design the in vivo experiment and reviewed critically the manuscript. Giuliana Noratto contributed to conduct the in vitro manuscript, analyze data and reviewed critically the manuscript. Susanne Ursula Mertens-Talcott contributed to design the in vitro experiment and analyzed data. Stephen Talcott contributed to obtain the chia hydrolyzed phenolics extract and to analyze its content. Hércia Stampini Duarte Martino contributed to design the in vivo and in vitro experiment, reviewed critically and approved the final version of the manuscript.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.jff.2020.104026.

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