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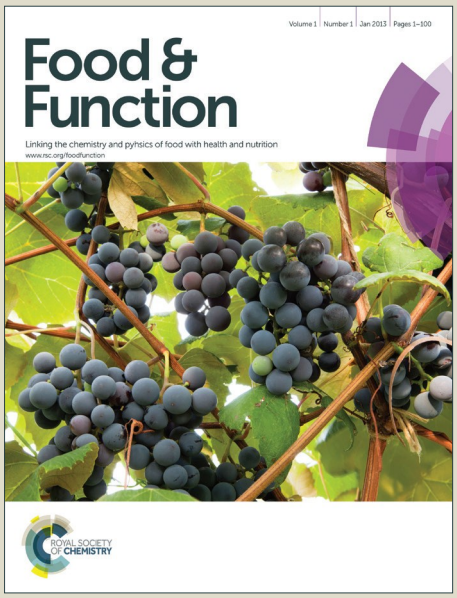

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Grape pomace and grape pomace extract improves insulin signaling in high-fat-fructose fed rats-induced metabolic syndrome.

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Abreviations: **Ctrl,** control; **ERK,** extracellular signal-regulated kinase; **DF,** dietary fiber; **GPE,** grape pomace extract; **GP,** grape pomace; **HFF**, high fat-fructose; **IRS1**, Insulin receptor substrate 1; **JNK,** c-jun N-terminal kinase; **MAPK**, mitogen-activated protein kinase; **MetS,** metabolic syndrome; **CRP,** c- reactive protein; **SBP,** systolic blood pressure; **TNFα,** tumor necrosis factor alpha; **TPC,** total phenolic content; **VAT,** visceral adipose tissue

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SUMMARY

In this study the effect of diet supplementation with grape pomace (GP) and grape pomace extract (GPE) on insulin sensitive tissues (adipose, liver and muscle) was evaluated in an experimental model of metabolic syndrome (MetS). MetS was developed by giving a high-fat-fructose (HFF) diet to Wistar rats. Six weeks of HFF diet induced weight gain, which was partially attenuated by GP (1 g $Kg^{-1}day^{-1}$) and GPE (300 mg Kg^{-1} day^{-1}) supplementation. HFF diet increased systolic blood pressure, triglycerides, insulin resistance (HOMA:IR) and inflammation (c-reactive protein (CRP)). Supplementation with GP prevented SBP, triglycerides and CRP increased and partially attenuated insulin resistance. On the other hand, GPE partially reduced SBP and triglycerides and significantly prevented insulin resistance and inflammation. Also, HFF diet induced higher triglycerides content and enhanced NADPH oxidase activity in liver. As well, HFF diet increased epididymal adipose tissue weight, enlarged adipocytes size, and c-jun N-terminal kinase (JNK) activation, probably contributing with a pro-inflammatory cytokine pattern (higher resistin) and lower adiponectin protein expression. These alterations may result in an impairment of insulin signaling cascade observed in adipose, liver and muscle tissue (IRS1, Akt, and extracellular signal-regulated kinases (ERK1/2)) from HFF rats. Supplementation with GP and in a greater extent GPE attenuated liver triglyceride content and adiposity and restored adipose, liver and muscle response to insulin. These findings show that supplementation with GP and GPE in a greater extent can counteract adiposity, inflammation, liver damage and impaired insulin signaling associated to MetS,

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supporting the utilization of winemaking residues in food industry/human health due to their high amount of bioactive compounds.

INTRODUCTION

Increased adipocytes size plays an important role in the insulin resistance-induced metabolic syndrome (MetS) [1, 2], a pathological state characterized by hyperglycemia, dyslipidemia (higher levels of triglycerides and lower levels of HDL cholesterol), abdominal obesity and hypertension. Hypertrophy of adipocytes, particularly from visceral adipose tissue, causes an increase in the production and secretion of pro-inflammatory adipocytokines such as tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein 1 (MCP-1), resistin, interleukin 6, and decrease release of the anti-inflammatory cytokine adiponectin, therefore constituting a self feeding circle of inflammation [3]. This inflammatory condition may alter the insulin signaling cascade in the liver, skeletal muscle and adipose tissue that includes insulin receptor substrate 1 (IRS1) phosphorylation at the serine307 residue and consequently decrease of AKT and extracellular signal-regulated kinases 1/2 (ERK) phosphorylation. Akt activation plays a key role in insulin actions, such as enhancement of GLUT4 translocation and glucose transport, glycogen and protein synthesis and lipolysis decrease [4, 5].

Grape pomace (GP) is a waste product, containing predominantly a left-over of skins and seeds, generated in the winemaking process which contains relatively high amounts of bioactive components like polyphenols and dietary fiber (DF,

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especially glycans, cellulose and pectins) [6-8]. Polyphenols are bioactive compounds found in many fruits and vegetables such as grape, cocoa, and tea [9]. Several studies have shown that consumption of polyphenols or food-rich in polyphenols attenuate the complications associated with MetS [10-13].

The recovery of bioactive compounds such as phenolics from GP and its application has been reviewed [8] and the characterization of Malbec grape pomace extract (GPE) was recently reported by Antoniolli et al. [14], showing the potentiality of this by-product for a wide range of applications as food ingredients [15, 16]. In human adipocytes stimulated with TNFα, grape powder extracts attenuate inflammation and insulin resistance [17]. Regarding GPE, Hogan et al. [18] observed that a daily dose of Norton GPE 250 mg Kg^{-1} of body weight reduced plasma C-reactive protein (CRP) in mice fed at high fat diet. On the other hand, pomace ethanol extract (450 mg kg^{-1} day) reduced the expression of lipogenic genes in liver and plasma triglycerides in rats with high-fat diet [19].

In this study it was hypothesized that GP and its extract (GPE), both rich in bioactive compounds, may attenuate metabolic alterations associated with a high-fat-fructose (HFF) diet-induced MetS, focused on adiposity (epididymal adipose tissue), liver damage and insulin signaling cascade from liver, skeletal muscle and adipose tissue.

RESULTS

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Chemical characterization and antioxidant activity of GPE and GP

The TPC, dietary fiber and antioxidant activity of GPE and GP are shown in **Table 1**. As it is observed, GP presented higher content of DF than GPE, mainly as insoluble DF which is because GP is the original crude material. The levels of TPC and ORAC were higher in GPE than GP, which is a logical fact taking into account that GP represent a crude material not a concentrated extract such as GPE.

The concentration of low-molecular-weight phenolics found in GPE and GP is presented in **Table 2**. The most abundant compounds quantified in the studied samples were the flavan-3-ols (+)-catechin and (–)-epicatechin as well as the flavonol quercetin. Amongst non-flavonoids, stilbenes are important because of their reported biological properties and nutritional applications. The stilbene *trans*-resveratrol was found in GPE and GP samples. The *trans*-resveratrol contents were higher to those reported by Casazza et al. for GPEs of cv. Pinot Noir (11.6 – 22.4 μg g^{-1}) [20].

Effects of GPE and GP on metabolic variables

The effect of GPE and GP supplementation on metabolic parameters was investigated in HFF rats as shown in **Table 3**. The evolution of systolic blood pressure (SBP) and body weight is shown in **Fig. 1A and B**, respectively. The daily food consumption and water intakes were similar among groups. HFF diet induced increase in body weight compared with Ctrl rats, which was partially attenuated in

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HFF rats supplemented with GPE and GP. In addition, chronic administration of fat and fructose during 6 weeks induced several metabolic alterations such as increased SBP, plasma triglycerides, insulin resistance (higher insulin and the index of insulin resistance HOMA:IR), and inflammation (higher levels of CRP) in comparison with those fed the standard chow. Supplementation with GPE to HFF rats partially attenuated SBP and triglycerides, while significantly prevented insulin resistance. On the other hand, the addition of GP to HFF rats significantly decreased SBP, and triglycerides, but partially reduced the insulin levels and HOMA:IR index. Also, GPE and GP supplementation to HFF rats increased the levels of HDL and reduced CRP compared with HFF rats. No differences were observed in plasma levels of glucose among groups **(Table 3)**.

Supplementation with GPE and GP reduced liver weight, triglycerides content and NADPH-oxidase activity.

Rats receiving HFF diet during 6 weeks increased liver triglycerides content compared with those receiving a standard diet. In contrast, HFF supplemented with GPE and GP significantly and partially reduced liver triglycerides content, respectively and reduced liver weight compared with HFF rats **(Fig. 2A and B)**.

Next, the activity of NADPH oxidase and the expression of NADPH subunit p67phox were evaluated in membrane and cytosolic fraction, respectively. HFF rats significantly increased NADPH oxidase activity in liver with respect to Ctrl rats. By other hand, NADPH oxidase activity was markedly attenuated in HFF rats

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supplemented with GPE and GP **(Fig. 2C)**. Accordingly, p67 protein expression was significantly lower in HFF, suggesting an increased translocation of p67 to membrane fraction compared with HFF rats supplemented with GPE and GP **(Fig. 2D)**.

Together, the augmented liver triglycerides and enhanced NADPH oxidase activity may contribute to an altered insulin signaling. Therefore, we evaluated the insulin signaling cascade in liver tissue. Ctrl, HFF and HFF supplemented with GPE and GP were injected with saline or insulin solutions (as explained in the experimental section), and then basal and insulin-stimulated signaling was determined. HFF diet induced phosphorylation of IRS1 (Ser307) in insulin-stimulated rats that was prevented in HFF upon GPE and GP supplementation. Basal and insulin-stimulated Akt (Ser473) phosphorylation was increased in Ctrl compared with HFF rats. Only GPE supplementation to HFF rats prevents insulin-induced Akt decrease. No differences were observed on pERK (Thr202/Tyr204) among groups **(Fig 3)**.

Effect of GPE and GP supplementation on adipocyte area in epididymal adipose tissue

Adipocyte hypertrophy is proposed as the leading cause of inflammation and insulin resistance. High-fat-fructose diet induced significant increase in epididymal adipose tissue weight in comparison to the Ctrl group, which was markedly alleviated by administration of both GPE and GP **(Fig. 4A)**. Then, the adipocyte

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size in epididymal adipose tissue among groups was evaluated **(Fig. 4B)**. It was observed that frequency distribution of adipocyte area was different between Ctrl and HFF rats. HFF rats had higher adipocyte area compared to Ctrl group **(Fig. 4C and D)**. In contrast, the area of adipocytes from HFF rats supplemented either with GPE or GP tended to have a similar distribution of Ctrl group **(Fig. 4C and D)** demonstrating that GPE and GP supplementation reduced both adipocytes weight and size.

Supplementation with GPE and GP improves the balance of adipocytokine expression and enhanced insulin sensitivity in the epididymal adipose tissue

Enlarged adipocytes are linked with adipose tissue dysfunction (altered adipocytokine balance) and insulin resistance. Then, the protein expression of adiponectin and resistin, which are respectively related with anti-inflammatory and pro-inflammatory properties, was evaluated. Adiponectin expression was lower and resistin was higher in rats fed a HFF diet compared with Ctrl group, which was prevented in HFF rats supplemented with GPE and GP **(Fig. 5A)**.

To investigate whether enlarged and pro-inflammatory adipocytes are associated with altered insulin signaling cascade, the molecular basis involved in insulin signaling in epididymal adipose tissue was explored. HFF diet induced phosphorylation of IRS1 at ser307 in insulin-stimulated rats compared to Ctrl rats, which was attenuated in HFF rats supplemented with both GPE and GP. Phosphorylation of IRS1 in serine residues is in part regulated by JNK activation.

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JNK (Thr183/Tyr185) phosphorylation was increased in insulin-stimulated HFF rats compared to Ctrl rats, which was prevented in HFF rats supplemented with GPE. Insulin-stimulated Akt (Ser472) and ERK phosphorylation (Thr202/Tyr204) was decreased in rats fed a HFF diet compared with Ctrl group and HFF rats supplemented with GPE, while GP supplementation to HFF rats elevated pAkt and attenuated pERK **(Fig 5B)**. These results indicate that supplementation with GP and GPE in a larger extent attenuated adipose inflammation and improved insulin sensitivity.

Effect of GPE and GP supplementation on insulin signaling in skeletal muscle tissue from HFF rats.

Muscle is one of the most important target tissues of insulin. Therefore, we evaluated the insulin signaling cascade in muscle tissue. No differences were observed on IRS1 (Ser307) phosphorylation in basal and insulin-stimulated rats. Basal and insulin-stimulated Akt (Ser473) and ERK (Thr202/Tyr204) phosphorylation was decreased in HFF compared with Ctrl rats. GPE supplementation to HFF rats significantly prevents insulin-induced Akt and ERK decrease, while GP partially attenuated pAKT **(Fig 6)**.

DISCUSSION

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This study showed that supplementation with GP and GPE in a greater extent improved insulin sensitivity due to (at least in part) i) decreased adiposity, adipocyte size, and restoring altered adiponectin and resistin protein expression; and ii) reduced liver triglyceride content and NADPH-oxidase activity, overall improving the metabolic alterations associated with high fat-fructose diet in rats.

High fat-fructose diet has been described as a model of diet induced-MetS in rats [21-23]. Interestingly, the addition of fructose to a high fat diet in rats for two weeks accelerate the deleterious effects of metabolic alterations [24]. Also, excess of fat and/or fructose has been associated with increased liver lipid accumulation and oxidative stress which may predispose the development of insulin resistance [24-26]. The liver plays an important role in substrate metabolism and is a primary target of insulin action. In the present study it was observed that HFF diet led to increased liver triglyceride content and NADPH oxidase activation, measured by both enzyme activity and NADPH subunit p67phox expression. NADPH oxidase is a major source of cell oxidant production, playing a key role in inflammation and insulin resistance [27]. The liver response to insulin was also impaired in HFF rats. Supplementation with GP and GPE to HFF rats attenuated NADPH oxidase activity and partially restored liver insulin signaling.

On the other hand, increased visceral adipose tissue is one of the most important features in the onset of MetS [1]. We observed that HFF diet leads to increased adipose (epididymal) tissue weight. One of the primary signal causing adipose tissue dysfunction in obesity is the hypertrophy of adipocytes, since enlarged adipocytes produce a pro-inflammatory adipocytokine pattern contributing to adipose insulin resistance [28,29]. Resistin has been associated with adiposity,

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inflammation and insulin resistance [30], while adiponectin correlates with insulin sensitivity and with anti-inflammatory properties that have an important role in the prevention of MetS [3]. In concordance, in the present work it was found that adipocytes from HFF rats were enlarged and presented higher pro-inflammatory adipocytokine expression (resistin) and decreased adiponectin. Moreover, the *in vivo* response to insulin by adipose tissue was impaired in the HFF rats as evidenced by altered insulin-mediated phosphorylation of IRS1, Akt and ERK1/2. Previous reports have shown that animals fed for a long time with high fructose and/or fat diet increase adiposity associated with chronic low grade systemic inflammation and/or insulin resistance [24, 31, 32]. In the present study, supplementation with GPE and GP prevented the increase of epididymal fat weight, adipocyte size, and altered adipocytokine expression. Also, GPE in a greater extent than GP prevent the impairment of adipose tissue response to insulin. The anti-inflammatory properties of GPE have been observed in some studies. For example, Rodriguez-Morgado et al.[33] has shown that grape pomace extract (obtained by an enzymatic process) reduce inflammation in microglia cells stimulated with lipopolysaccharide. Hogan et al. [18] found that supplementation with Norton GPE in a dose of 250 $mgKg^{-1}$ d^{-1} in mice fed with a high fat diet during 12 weeks attenuate inflammation through reduced plasma CRP levels with no differences in plasma parameters of oxidative stress. Also, rats supplemented with red GP (5% in the diet) reduces lipopolysaccharide/galactosamine-induced inflammation through suppression of NF-κB activation and expression of inducible nitric oxide synthase and cyclooxygenase-2 [34].

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Administration of HFF diet to rats during 6 weeks induced insulin resistance as evidenced by HOMA-IR (elevated fasting insulin with normal glucose levels). In spite of we did not measure the glucose tolerance test, these results suggest that in the HFF rats the increased insulin levels are still capable to compensate/maintain comparable glucose concentration. Similar results, with normal glucose levels, higher plasma of insulin and HOMA-IR together with altered glucose tolerance test has been reported in high fat-high fructose diet in rats [24]. Supplementation with GPE and GP to HFF rats significantly and partially attenuated insulin resistance, respectively. According to these results, supplementation with GPE was more effective in attenuating insulin signaling cascade in liver, adipose and muscle tissue compared with GP.

In the present work the chemical identity of the major polyphenols was assessed. We found that GP and GPE have important amounts of polyphenols, 4.2% and 19.5%, respectively. HFF rats supplemented with GP and GPE receive approximately 42 mg Kg^{-1} d^{-1} and 58 mg Kg^{-1} d^{-1} of total polyphenols, respectively. Probably these differences are related with the major effectiveness of GPE observed. Dietary polyphenols have been proposed to have beneficial effects in the prevention of obesity and obesity-related diseases [12, 13]. Accordingly, we have previously shown that (-)-epicatechin, (+)-catechin and quercetin, the main flavonoids found in grapes, ameliorate adipose inflammation and insulin resistance in 3T3-L1 adipocytes and in high fructose-fed rats through modulation of pro-inflammatory and redox signaling [32, 35, 36].

Also, dietary fiber from GP has been related with important benefits on human health [37]. Hence, it was found that GP was more effective to prevent the

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increased SBP and triglycerides plasma levels than GPE and these differences are probably due to the higher dietary fiber content in GP (53.5%). Interestingly, GPE had similar or even greater effect on attenuating insulin resistance, and adipose liver, and muscle parameters related with insulin signaling. As far as we know, Malbec GP and its extract (GPE) have never been investigated simultaneously on alterations related with MetS in rats. Our results provide evidences of the beneficial effects of GP and GPE, a by-product of the winemaking process, in the prevention of alterations associated to high fat-fructose induced MetS.

CONCLUSION

The results of the present study show that GPE and GP supplementation attenuate altered adipose, liver and muscle insulin signaling in HFF diet induced-MetS, at least in part through i) modulation of adiposity and adipocytokine expression, and ii) reducing liver triglyceride content and NADPH-oxidase activity. These findings emphasize the possible nutritive value of GP and GPE with a wide range of applications as ingredients for functional foods to prevents/mitigates MetS.

EXPERIMENTAL

Materials

Folin-Ciocalteu reagent was purchased from Merck (São Paulo, Brazil). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman)-2-carboxylic acid, $NaH_2PO_4.2H_2O$,

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$Na_2HPO_4.12H_2O$, fluorescein, AAPH (2,2′-azobis-(2-methylpropionamidine) dihydrochloride), were supplied from Sigma-Aldrich (St. Louis, MO, USA). Standards of gallic acid (99%), (+)-catechin (≥ 99 %), (−)-epicatechin (≥ 95%), caffeic acid (99 %), syringic acid (≥ 95 %), coumaric acid (99%), ferulic acid (≥ 99%), trans-resveratrol (≥ 99%), quercetin hydrate (95%) and cinnamic acid (99%) were purchased from Sigma-Aldrich. HPLC-grade MeOH and formic acid were purchased from Mallinckrodt Baker (Inc. Pillispsburg, NJ, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Fructose was purchased from Saporiti Labs (Buenos Aires, Argentina) and Bovine Fat was from Recreo Refrigerating Industries S.A.I.C. (Santa Fe, Argentina), HDL, and triglyceride concentrations were determined using commercial kits from GTLab (Buenos Aires, Argentina).

Antibodies for p-AKT (#06248) was purchased from Cell Signaling Technology (Danvers, MA, USA); AKT (sc-8312), p-ERK (sc-7383) and ERK (sc-93) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); IRS1 (#06-248), p-IRS1 (#07-247) and resistin (AB 3371P) were from Millipore (Billerica, MA, USA). The antibody for adiponectin (ADI-905-714-100) was obtained from Stressgen (Farmingdale, NY, USA). Nitrocellulose membranes were obtained from BIO-RAD (Hercules, CA, USA). The ECL Western blotting system was from GE Healthcare (Piscataway, NJ, USA). VAS-2870 was obtained from Enzo Life Sciences, Inc. (NY, USA). Unless otherwise noted, reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

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Grape pomace sampling

This study was performed with GP obtained from *Vitis vinifera* L. cv. Malbec, provided by Catena Institute of Wine from the Adriana vineyard located in Gualtallary, Mendoza, Argentina, and harvested in 2013. The vinification procedure was conducted with mechanical daily pumping over and contact of the skins and seeds with the juice for 11 days. After that, must was pressed, fresh GP samples were collected and placed in ice cooled boxes during transportation to the laboratory, and then, stored at -20 °C until processing. To obtain lyophilized GP, fresh GP were freeze-dried and then kept at –20 °C in dry atmosphere and darkness prior use or analysis. The GPE was obtained by solid-liquid extraction according to the method of Antoniolli et al. [14].

To further analysis 500 mg of lyophilized GP, 5 mL of methanol were added with vigorously hand shaking, placed in an ultrasonic bath 30 min (stirring every 5 min), and centrifuged 2 min. From the supernatant TPC, ORAC and phenolic analysis were performed. For GP analysis 5 mg of GPE was dissolved in 5 mL of ethanol 50% (v/v) aqueous solution.

Total phenolic content

Absorbance measurements were made with a UV–vis spectrophotometer (Cary-50, Varian Inc.). Total phenolic content (TPC) was determined by the Folin–

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Ciocalteu assay according to Alonso et al. [38]. TPC was expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE g^{-1}) by using a calibration curve with gallic acid as standard (three replicates) in a range between 0 - 200 mg L^{-1} (R^2=0,999).

Dietary fiber (AOAC method)

The total, soluble, and insoluble DF content of Malbec GP and GPE samples were determined in quadruplicate according to the AOAC official method 991.43. (AOAC, 1995) [39].

Antioxidant activity

The antioxidant activity of GP and GPE was determined by oxygen radical absorbance capacity (ORAC). The ORAC was determined as follows: re-suspended extracts were diluted 1:750 v/v in 75 mmol L^{-1} potassium phosphate buffer (pH 7.0). Fifty microliters aliquots of diluted samples and trolox standards were added to a 96-well plate. Then, 100 µL of fluorescein solution were added and the mixture was incubated at 37 °C for 7 min before the addition of 50 µL of 140 mmol L^{-1} peroxyl radical generator AAPH. Fluorescence was monitored by using 485 nm excitation and 538 nm emissions at 1 min intervals for 60 min on a fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc, Wilmington, DE). The area under the curve of the fluorescence decay during 60 min was calculated

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and the ORAC was expressed as μmol of trolox equivalents per gram of GPE (μmol TE g^{-1}) as described previously [14, 40].

Low-molecular-weight phenolic analysis

Target polyphenols were determined using a LC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The Chromeleon 7.1 software was used to control all the acquisition parameters of the LC-MWD system and also to process the obtained data. The polyphenols determination was performed according to Fontana and Bottini [41]. LC separations were carried out in a reversed-phase Symmetry C18 column (4.6 mm x 250 mm, 5 μm particle size, Waters, Milford, MA, USA. Ultrapure water with 0.1% FA (A) and MeOH (B) were used as mobile phases. Analytes were separated using the following gradient: 0 min, 20% B; 0-2 min, 30% B; 2-8 min, 32% B; 8-14 min, 80% B; 14-22 min, 20% B; 22-32 min, 20% B. The mobile phase flow was 1 mL min^{-1} and the column temperature 35° C. The injection volume for standards and sample extracts was 10 μL. The identification and quantification of the target polyphenols in GP was achieved by comparing of the retention times (tR) and maximum absorbance value of detected peaks in samples of interest with those obtained with the injection of pure standards.

Standard solutions were prepared at 6 concentration levels by diluting appropriate volumes of the working standard solution. For the calibration curve,

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linear ranges between 0.15 to 50 μg mL^{-1} were obtained, with coefficient of determination (R^2) higher than 0.9961 for all the studied polyphenols.

Animal studies

All animal studies were conducted in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL, Protocol approval Nº 36/2014). Eight weeks-old male Wistar rats (n=24) were used for this 6-weeks supplementation study. Rats weighing 250-260 g were housed in cages (3 rats/cage) in a room under conditions of controlled temperature (21 - 25 ºC) and humidity with a 12 hour light/dark cycle with access to standard rats chow (Gepsa-Feeds, Buenos Aires, Argentina) and water *ad libitum.* Rats were randomly assigned to the following 4 groups (6 rats per group): Control diet (Ctrl), high-fat-fructose diet (HFF) (20% fat and 20% fructose w/w) added to the standard chow, and HFF supplemented with GPE in a dose of 300 mg Kg^{-1} d^{-1} (HFF+GPE), and GP in a dose of 1g Kg^{-1} d^{-1} (HFF+GP) approximately. The dose of GPE and GP was incorporated into a pellet mixture, as well as fat and fructose. Pellets were re-made and stored at 4 °C, every two-three days pellets were weighed and dispensed. Body weight was recorded weekly. Water and food daily consumption was estimated by dividing the total consumed in each cage by the number of animals housed in it. This estimation was performed three times a week and the supply amount was adjusted weekly according to the variation in body weight. Systolic blood pressure (SBP) was measured at the

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beginning, middle (3 weeks) and at the end (6 weeks) of the protocol by tail-cuff method and determined with a plethysmography Koda2® (Kent Scientific Corporation, USA). After 6 weeks on the dietary treatments, and after an overnight fast, rats were weighed and animals were intraperitoneally injected with insulin (10 mU g^{-1} body weight human insulin (Humulin® Eli Lilly and Company)) or saline and 10 minutes after were anesthetized with ketamine (50 mg Kg^{-1}) and acepromazine (1 mg Kg^{-1}) and blood was collected from the abdominal aorta into EDTA tubes. Plasma was obtained after centrifugation at 3000 rpm for 15 min at 4 ºC. Epididymal adipose tissue, liver and soleus muscle were isolated and flash-frozen in liquid nitrogen and then stored at -80 ºC until assayed. Epididymal adipose tissue and liver were previously weighted. A piece of epididymal adipose tissue collected was immediately fixed in 10% neutral formalin solution for 24 h for histological analysis.

Biochemical determinations

Plasma triglycerides (TG) and HDL cholesterol concentrations were determined by enzymatic colorimetric methods using commercial kits (GTLab, Buenos Aires, Argentina). Glucose was measure in blood collected from the tail using a glucometer (Accu-ChekPerforma, Roche, Buenos Aires, Argentina). Hepatic TG was extracted with chloroform/methanol and the extracts were assayed for TG content as described previously [42] and assayed for TGs content as described above. Insulin was measured using the Ultra Sensitive Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA), and insulin resistance was assessed

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using the homeostasis model assessment (HOMA-IR) parameter originally described by Mathew et al.[43] using the following formula: HOMA-IR (mg dL^{-1} × μU/mL) = fasting glucose (mg dL^{-1}) × fasting insulin (μU/mL)/405.

Plasma C-reactive protein (CRP) levels were assessed using a Cobas® c311 series autoanalyzer (Roche Diagnostics, Mannheim, Germany).

NADPH oxidase activity

The NAD(P)H oxidase activity in liver membrane fractions was determinate using the lucigenin-derived chemiluminescence assay as we previously described [36]. Briefly, liver tissue (1:10 w/v) was homogenized in ice-cold Krebs buffer and then centrifuged at 800 g, at 4 ºC for 10 min. The supernatant was collected and then centrifuged at 100,000 g for 1 h at 4 ºC. The supernatant (cytosolic fraction) was used to determine the NOX subunit p67phox by western blot as described below, and the pellet (membrane fraction) was resuspended in Krebs buffer and protein concentration measured. Aliquots containing 10 mg of protein were added to Krebs buffer containing NADPH (500 μM) and lucigenin (5 μM). Each sample was measured in the absence and presence of VAS-2870, a NADPH oxidase inhibitor. Light emission was measured for 10 min at 30 s intervals using a Victor 1420 multilabel counter (Wallac Oy, Turku, Finland), and the area under the curve was calculated. Results are expressed as the difference between AUC in the absence and in the presence of VAS-2870, and referred to control values.

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Western blots

Liver, muscle and adipose tissue were homogenized in radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% w/v sodium dodecylsulfate, 1% w/v Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors) as previously described [32]. Homogenates were centrifuged at 13,000 rpm for 30 min, the supernatant was collected, and protein concentration measured using the Bradford method. Aliquots of homogenates containing 25-40 µg protein were denatured with Laemmli buffer, separated by reducing 10–12.5% (w/v) polyacrylamide gel electrophoresis, and electroblotted to nitrocellulose membranes. Membranes were blotted for 2 h in 5% (w/v) non-fat milk, and subsequently incubated in the presence of the corresponding primary antibodies (1:1000 dilution for all the antibodies) overnight at 4 °C. After incubation for 90 min at room temperature in the presence of the corresponding secondary antibody (either HRP or biotinylated antibody followed for 1 hour with streptavidin) the conjugates were visualized and quantified by chemiluminescence detection in a Luminescent Analyzer Image Reader (LAS-4000) Fujifilm. Densitometry analysis was performed using the US National Institute of Health Image 1.66 software (Schneider C. A., et al., Division of Computer Research and Technology NIH, Bethesda, Maryland, USA).

Histological analysis

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Adipocyte hypertrophy degree was evaluated in epididymal adipose tissue sections prepared from paraffin-embedded samples and stained with hematoxylin and eosin. Images were analyzed using a CCD camera (Nikon, Japan) 20 x magnification and adipocyte area was measure using the Image J program.

Statistical analysis

Data from GPE and GP analysis were mean ± standard deviation. Data from animal study were expressed as mean ± standard error media (SEM). The statistical significance was assessed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. Differences were considered significant at $p<0.05$.

CONFLICT OF INTEREST

None.

ACKNOWLEDGMENTS

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Table 1. Total phenolic content, antioxidant activity, and dietary fiber content in Malbec GPE and GP.

	GPE	GP
Total Dietary Fiber (DF)	8.3 ± 0.1	53.5 ± 0.6
Soluble Dietary Fiber	2.1 ± 0.1	2.7 ± 0.0
Insoluble Dietary Fiber	6.2 ± 0.1	50.8 ± 0.5
TPC	195.1 ± 30.0	41.6 ± 2.1
ORAC	2445.8 ± 324.1	258.1 ± 31.9

DF: % dry matter; TPC: mg of GAE g^{-1} GP or GPE; ORAC: μmol TE g^{-1} GP or GPE. Mean value of determinations ± standard deviation.

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Table 2: Levels of target polyphenols in Malbec GPE and GP.

Analyte	GPE	GP
Gallic acid	444.8 ± 45.8	62.8 ± 5.7
Caffeic acid	963.3 ± 38.5	184.2 ± 18.4
Syringic acid	1471.1 ± 57.2	125.3 ± 13.9
p-coumaric acid	322.7 ± 14.1	55.2 ± 5.9
Ferulic acid	115.4 ± 10.7	11.6 ± 1.2
(+)-catechin	4285.2 ± 136.5	450.8 ± 37.7
(−)-epicatechin	3298.5 ± 95.8	598.8 ± 54.5
***trans*-resveratrol**	321.8 ± 16.4	55.9 ± 5.1
Quercetin	1546 ± 79.1	604.8 ± 74.4

Average concentrations (μg g^{-1} lyophilized GP or GPE) with their standard deviations, n = 3 replicates.

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Table 3. Effects of grape pomace extract (GPE) and grape pomace (GP) supplementation on metabolic parameters in high fat-fructose fed rats (HFF).

Parameter	Ctrl	HFF	HFF + GPE	HFF + GP
Food intake (g d^{-1})	23.5 ± 1.1	23.1 ± 0.6	20.6 ± 0.5	20.3 ± 0.8
Water intake (mL d^{-1})	40.3 ± 2.1	35.3 ± 0.9	33.7 ± 0.9	34.1 ± 1.59
SBP mmHg	115 ± 5^{a}	137 ± 6^{b}	121 ± 3^{a,b}	119 ± 4^{a}
Body weight (g)	335 ± 5^{a}	382 ± 13^{b}	341 ± 10^{a,b}	352 ± 15^{a,b}
Glucose (mg dL^{-1})	92.7 ± 4.7	88.2 ± 3.3	88.7 ± 2.5	95.8 ± 1.6
Insulin (ng mL^{-1})	1.11 ± 0.04^{a}	1.75 ± 0.02^{b}	1.05 ± 0.23^{a}	1.23 ± 0.15^{a,b}
HOMA:IR	3.6 ± 0.2^{a}	5.5 ± 0.2^{b}	3.3 ± 0.3^{a}	4.3 ± 0.4^{a,b}
Triglycerides (mg dL^{-1})	120 ± 4^{a}	154 ± 5^{b}	137 ± 4^{a,b}	132 ± 8^{a}
HDL cholesterol (mg dL^{-1})	30.4 ± 2.6^{a,b}	23.3 ± 1.7^{b}	36.3 ± 1.7^{a}	33.7 ± 2.6^{a}
CRP mg mL^{-1}	55 ± 10^{a}	99 ± 8^{b}	60 ± 10^{a}	54 ± 5^{a}

Metabolic parameters from rats fed for 6 weeks without (Ctrl) or with high fat-fructose diet (HFF 20% each w/w), in the absence (HFF) or presence of GPE (HFF+GPE 300mg Kg BW^{-1} d^{-1}), or GP (HFF+GP 1g Kg BW^{-1} d^{-1}). HOMA:IR: homeostatic model assessment of insulin resistance (mg dL^{-1} x μU/mL). Values are shown as means ± SEM (n = 6 per group). Values having different superscripts are significantly different ($P < 0.05$, one way ANOVA). SBP: systolic blood pressure, CRP: c- reactive protein.

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FIGURE LEGENDS

Figure 1. Effect of dietary GPE and GP on systolic blood pressure (SBP) and body weight gain evolution in HFF rats. SBP was measured at week 0, 3 and 6 and body weight was measured weekly in eight weeks old rats fed control diets without (Ctrl) or with high fat-fructose diet (HFF 20% each w/w), or HFF supplemented with GPE 300 mg Kg^{-1} body weight d^{-1} and GP 1 g Kg^{-1} body weight d^{-1}. * Significantly different from Ctrl and HFF + GP (n = 6 per group); ** significantly different from HFF.

Figure 2. Effect of dietary GPE and GP supplementation on liver weight, triglycerides, NADPH oxidase activity and p67phox subunit expression.
(A) Liver weight, (B) triglycerides content, (C) membrane NADPH oxidase activity, and (D) NOX subunit p67 expression in cytosolic fraction were measured in rats fed control diets without (Ctrl) or with high fat-fructose diet (HFF 20% each w/w), or HFF supplemented with GPE 300 mg Kg^{-1} body weight d^{-1} and GP 1 g Kg^{-1} body weight d^{-1} (n = 6 per group). Values having different superscripts are significantly different, $p < 0.05$, one-way ANOVA test.

Figure 3. Effect of dietary GPE and GP on liver insulin signaling in HFF rats.
After 6 weeks on the corresponding diets, rats were fasted overnight and then injected with saline or insulin (10 mU/g body weight) and then sacrificed after 10 min. Phosphorylation of IRS1, Akt, and ERK1/2 is shown for liver tissue. Results were expressed as the ratio of phosphorylated/total protein level. Bands were

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quantified, and referred to control group values (Ctrl). Results are shown as the mean ± SEM of four animals/treatment.

Figure 4. Effect of dietary GPE and GP supplementation on adiposity in HFF rats. (A) Epididymal fat weight, (B) representative histological images of epididymal adipose tissue stain with Hematoxylin and eosin, (C) mean adipocyte diameter and, (D) frequency distribution of adipocyte diameter from rats fed control diets without (Ctrl) or with high fat-fructose diet (HFF 20% each w/w), or HFF supplemented with GPE 300 mg Kg^{-1} body weight d^{-1} and GP 1 g Kg^{-1} body weight d^{-1} (n = 6 per group). Values having different superscripts are significantly different, $p < 0.05$, one-way ANOVA test.

Figure 5. GPE and GP supplementation regulates adipocytokine expression and enhances insulin signaling in epididymal adipose tissue. (A). Proteins involved in metabolic regulation and inflammation (adiponectin, and resistin) were determined in epididymal adipose tissue in rats fed control diets without (Ctrl) or with high fat-fructose diet (HFF 20% each w/w), or HFF supplemented with GPE 300 mg Kg^{-1} body weight d^{-1} and GP 1 g Kg^{-1} body weight d^{-1}. Bands were quantified and results were expressed as the ratio protein/β-actin protein levels, and referred to control group values (Ctrl). (B) Phosphorylation of IRS1, JNK, Akt, and ERK1/2 is shown for epididymal adipose tissue. After 6 weeks on the corresponding diets, rats were fasted overnight and then injected with saline or insulin (10 mU/g body weight) and then sacrificed after 10 min. Results were expressed as the ratio of phosphorylated/total protein level, and referred to control

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group values (Ctrl). Results are shown as the mean ± SEM of four animals/treatment.

Figure 6. Effect of dietary GPE and GP on muscle insulin signaling in HFF rats. Phosphorylation of IRS1, Akt, and ERK1/2 is shown for muscle tissue. After 6 weeks on the corresponding diets, rats were fasted overnight and then injected with saline or insulin (10 mU/g body weight) and then sacrificed after 10 min. Bands were quantified and results were expressed as the ratio of phosphorylated/total protein level, and referred to control group values (Ctrl). Results are shown as the mean ± SEM of four animals/treatment.

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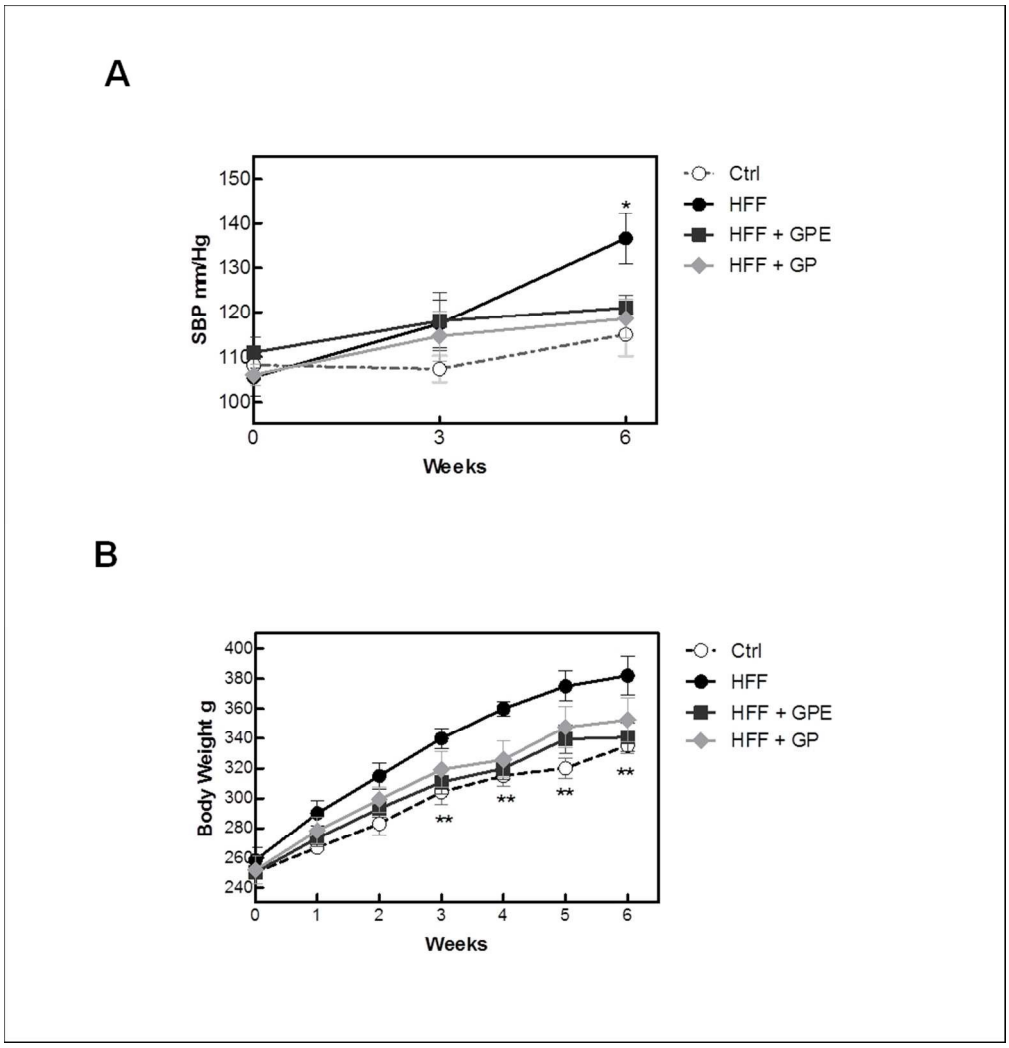

Figure 1. Effect of dietary GPE and GP on systolic blood pressure (SBP) and body weight gain evolution in HFF rats.
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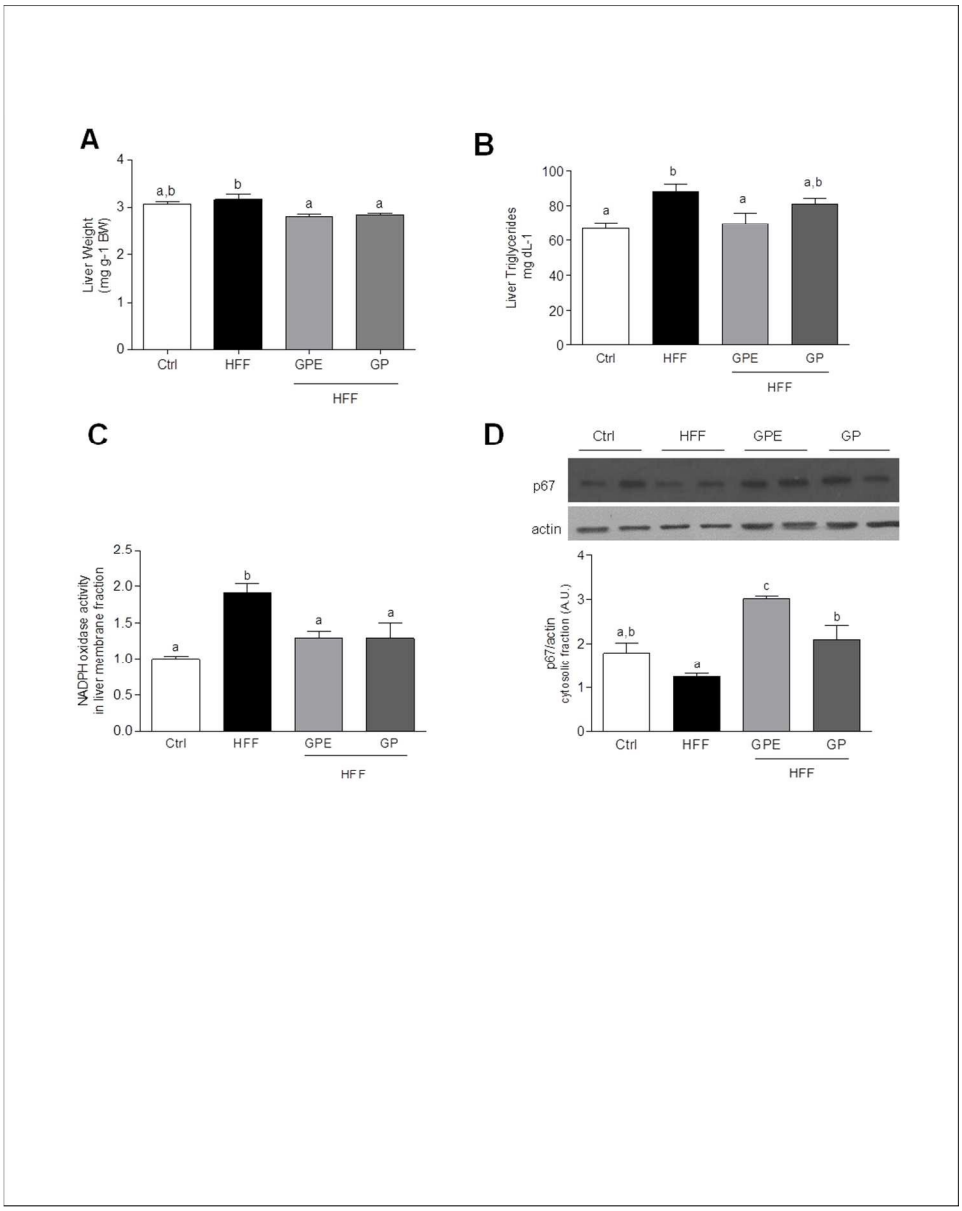

Figure 2. Effect of dietary GPE and GP supplementation on liver weight, triglycerides, NADPH oxidase activity and p67phox subunit expression.
195x245mm (150 x 150 DPI)

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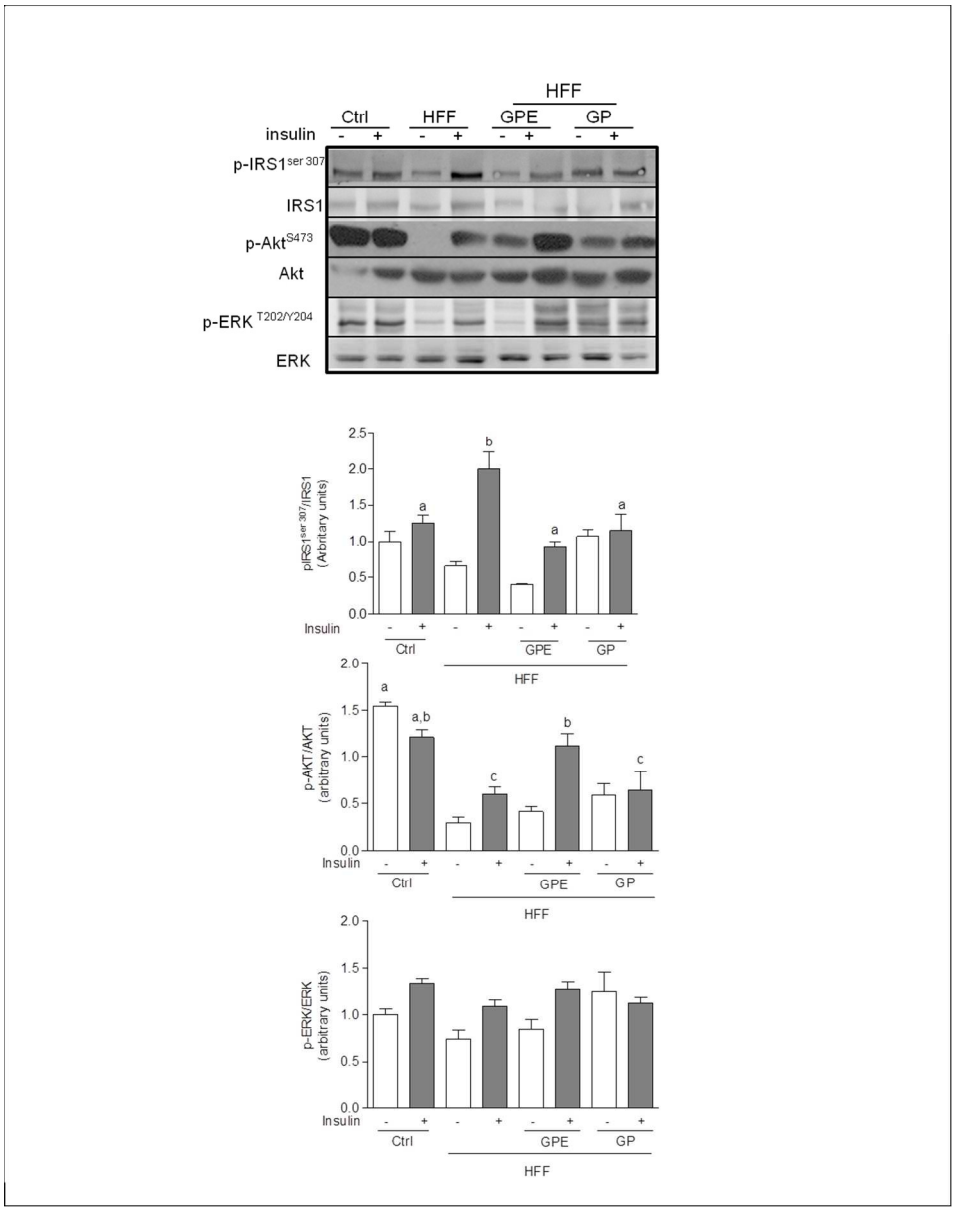

Figure 3. Effect of dietary GPE and GP on liver insulin signaling in HFF rats.
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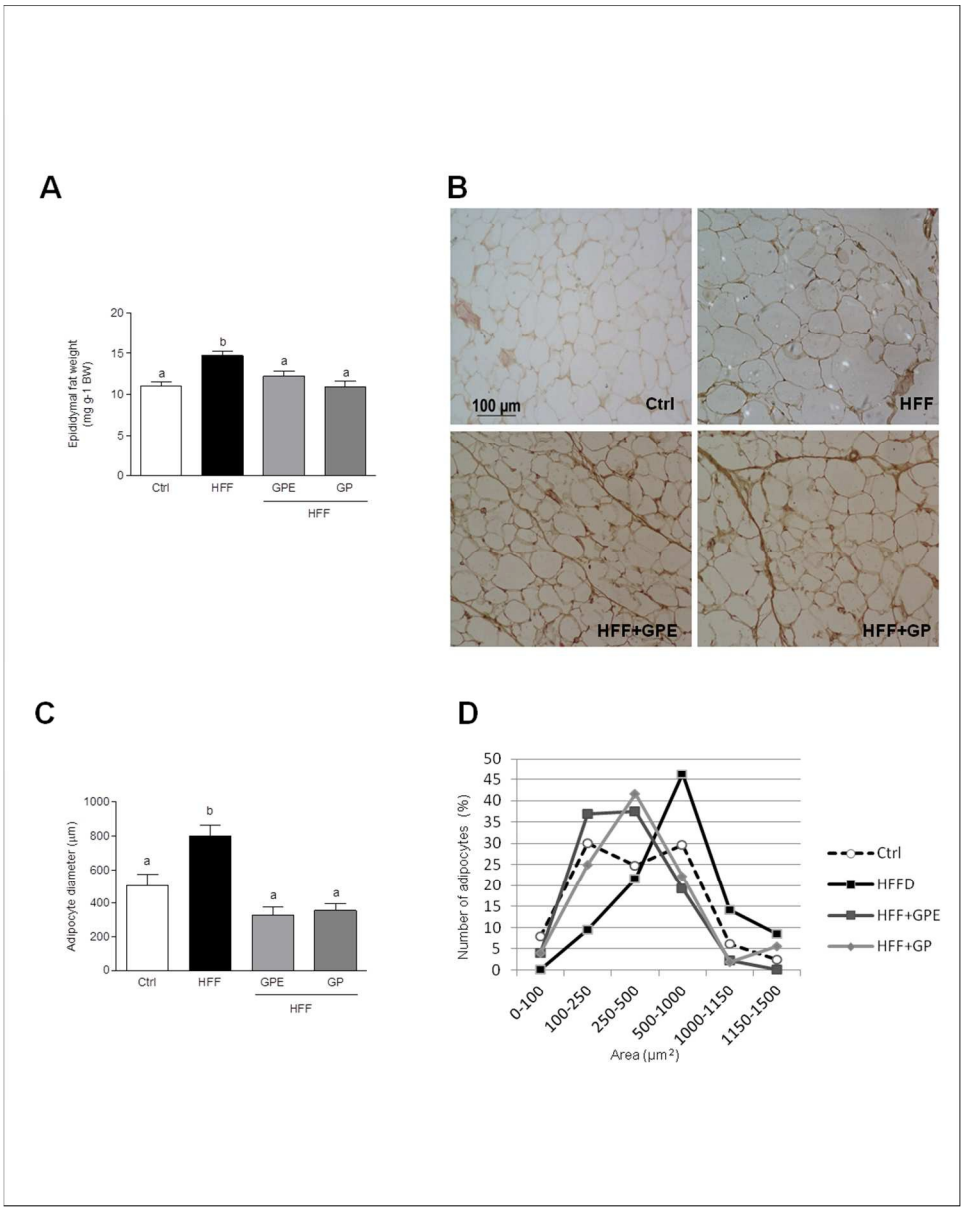

Figure 4. Effect of dietary GPE and GP supplementation on adiposity in HFF rats.
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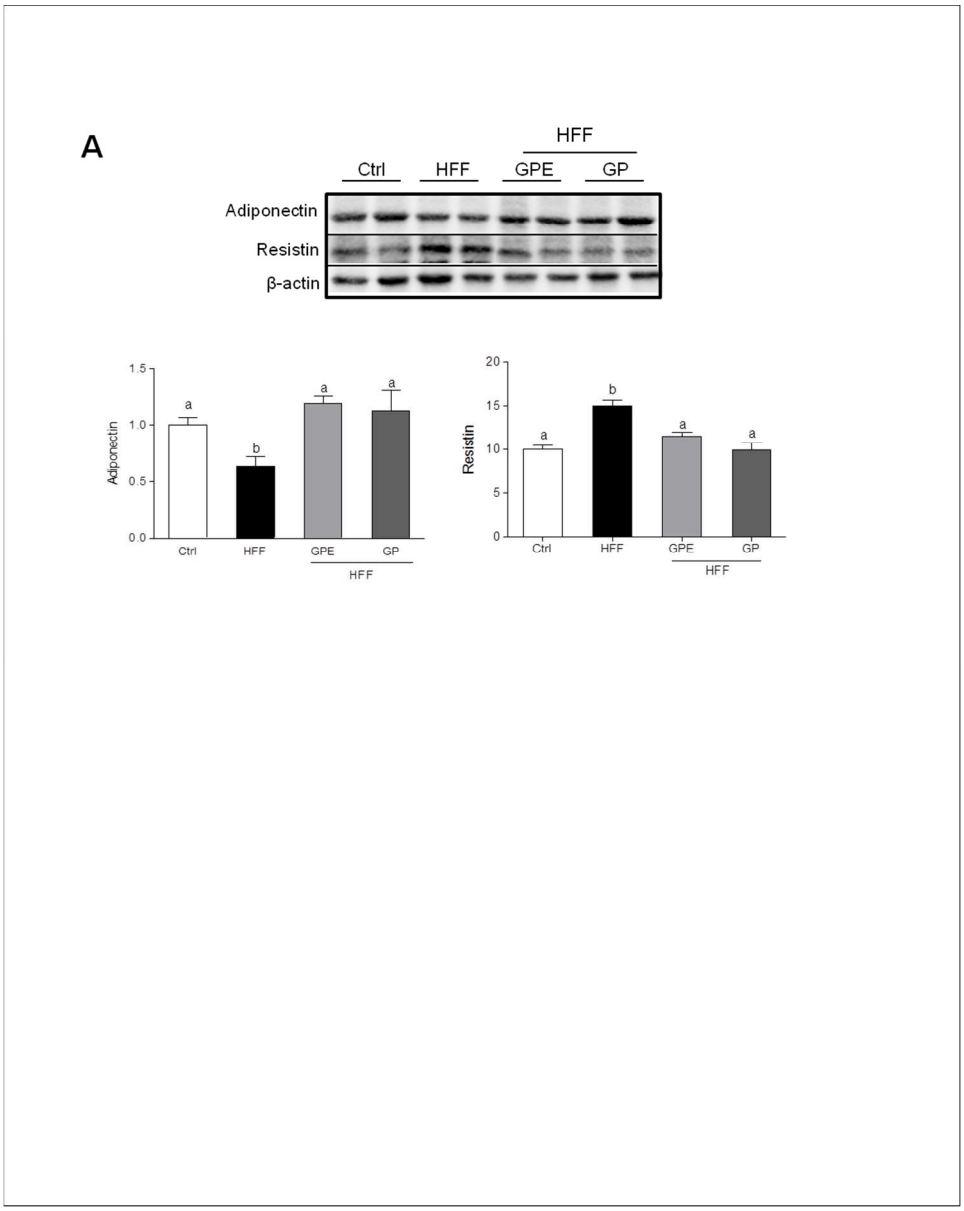

Figure 5. GPE and GP supplementation regulates adipocytokine expression and enhances insulin signaling in epididymal adipose tissue.
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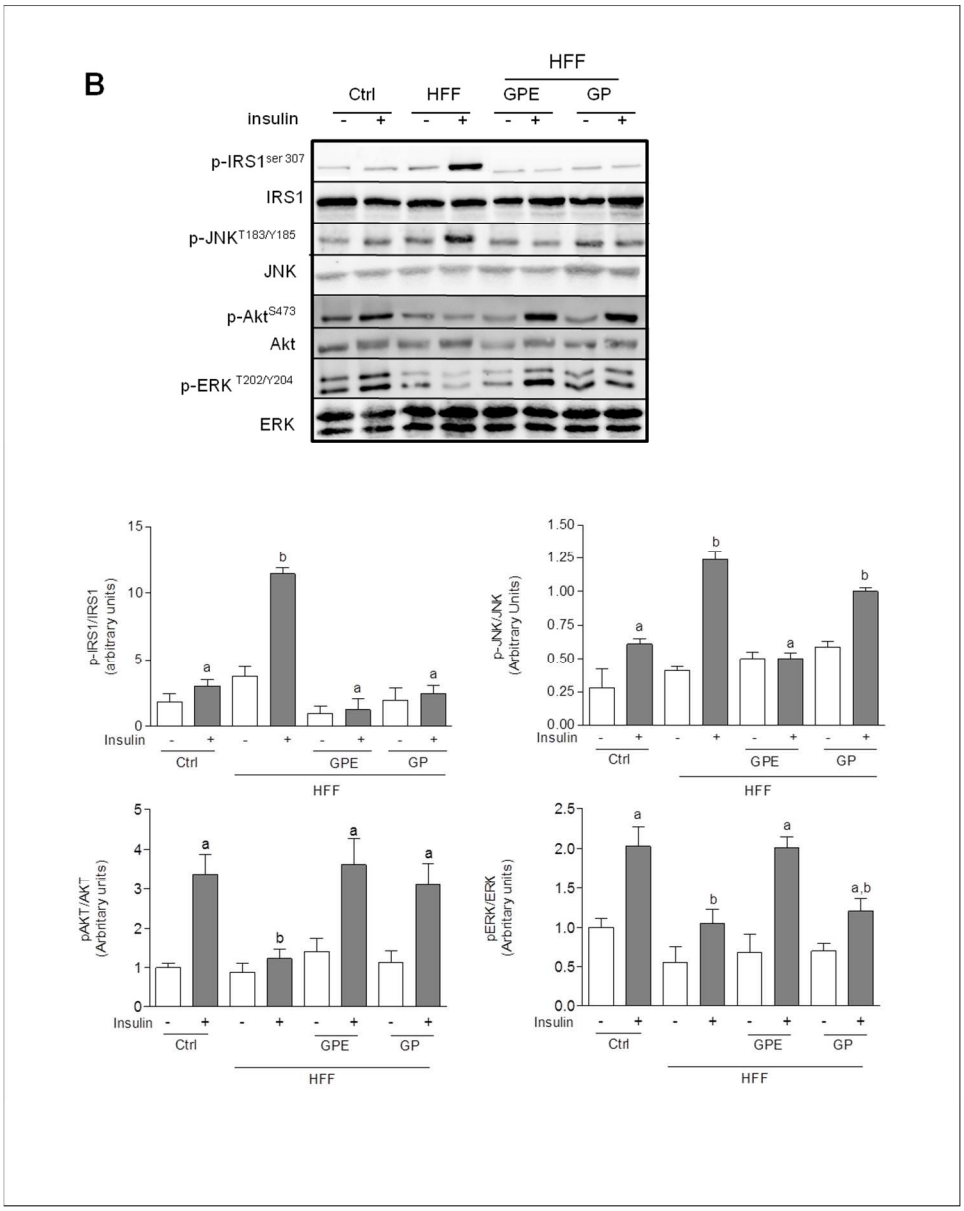

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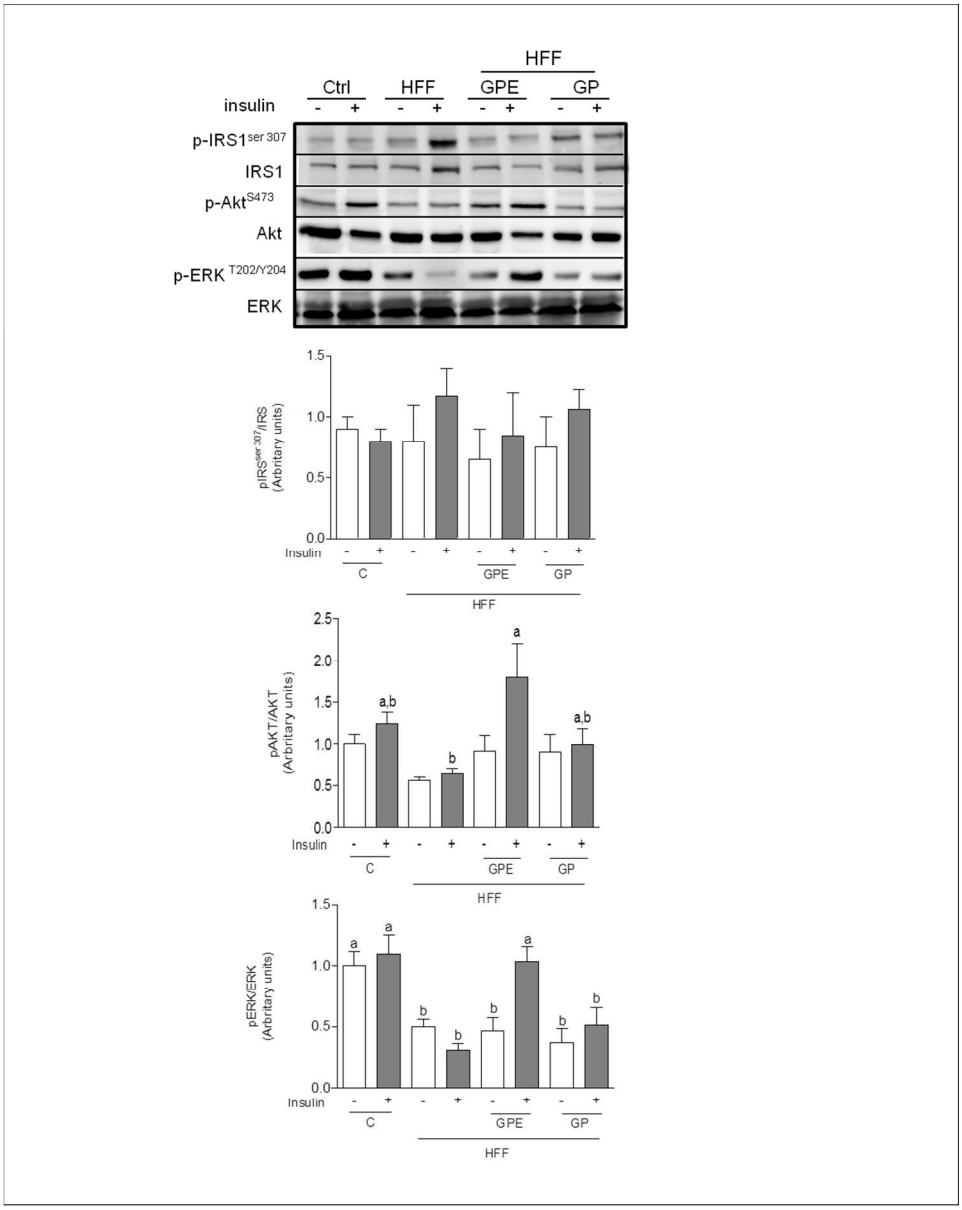

Figure 6. Effect of dietary GPE and GP on muscle insulin signaling in HFF rats.
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