# Anthocyanins from purple corn ameliorated TNF-α-induced inflammation and insulin resistance in 3T3-L1 adipocytes via activation of insulin signaling and enhanced GLUT4 translocation

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Abbreviations: PCW, anthocyanins extracted from purple corn pericarp; C3G, cyanidin-3-*O*-glucoside; Pr3G, pelargonidin-3-*O*-glucoside; P3G, peonidin-3-*O*-glucoside.

Keywords: Anthocyanins / Inflammation / Obesity / Polyphenols / Purple corn

**Scope:** The aim was to compare the effect of an anthocyanin-rich extract from purple corn pericarp (PCW) and pure anthocyanins on adipogenesis, inflammation and insulin resistance in 3T3-L1 adipocytes on basal and inflammatory conditions.

**Methods and results:** Preadipocytes (3T3-L1) were treated during differentiation with or without PCW. Differentiated adipocytes were treated either individually or in combination with tumor necrosis factor TNF-α and PCW, or pure C3G, Pg3G, P3G. PCW reduced preadipocyte differentiation ($IC_{50}$ = 0.4 mg/mL). PCW and pure anthocyanins including C3G

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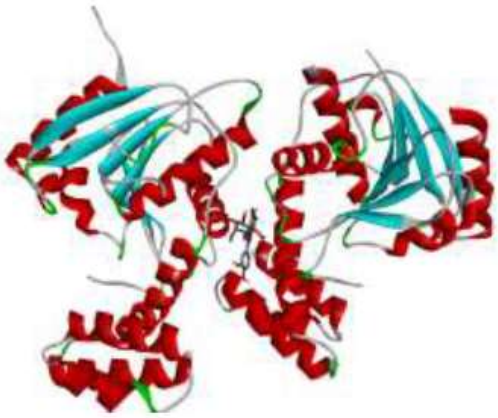
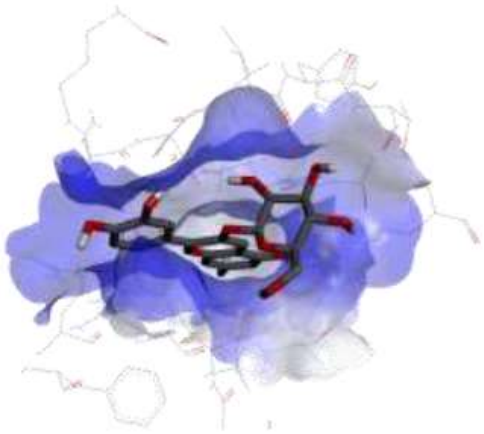
reduced fatty acid synthase enzymatic activity. PCW reduced TNF-α-dependent inflammatory status increasing adiponectin (39%), and decreasing leptin (-79%). PCW and C3G increased glucose uptake and reduced reactive oxygen species generation in insulin resistant adipocytes. An increase in phosphorylation was observed in AKT, IKK, and MEK, and a decrease in IRS and mTOR activating the insulin receptor-associated pathway. PCW (7.5-fold) and C3G (6.3-fold) enhanced GLUT4 membrane translocation compared to insulin resistant adipocytes.

**Conclusion:** Anthocyanins from colored corn prevented adipocyte differentiation, lipid accumulation and reduced PPAR-γ transcriptional activity on adipocytes in basal conditions. Ameliorated TNF-α-induced inflammation and insulin resistance in adipocytes via activation of insulin signaling and enhanced GLUT4 translocation suggesting a reduce hyperglycemia associated with the metabolic syndrome.

Obesity and diabetes are becoming more and more prevalent around the world. Obese individuals have far more adipose tissue than a healthy individual. Highly colored fruits and vegetables contain flavonoid compounds such as anthocyanins. Anthocyanin rich foods clearly illustrate the potential health benefits they contain through their impact in obesity and insulin-resistance diseases. The results of this research suggest, for the first time, that the anthocyanins present in purple corn can promote beneficial effects against obesity and its complications.





## 1 Introduction

Obesity and diabetes are becoming more and more prevalent in modern America. Obesity affects 1 in 3 adults in the United States, and 9.3% of the population has diabetes. Obese individuals have far more adipose tissue than a healthy individual. Adipose cells, or adipocytes, release chemical signaling factors known as adipokines [1]. Adipokines are involved in the regulation of appetite, energy homeostasis, vascular homeostasis, blood pressure, inflammatory responses, and metabolism. Overexpression of adipokines can predispose a patient to the development of insulin resistance and inflammation. Adipocyte differentiation plays an important role in the development of these diseases. Furthermore, reactive oxygen species (ROS) promote obesity and insulin resistance [2]. Build-up of oxidative stress in adipose tissue is one of the early events in the onset of obesity [3]. Antioxidant compounds such as anthocyanins can be used to eliminate ROS and maintain homeostasis.

Highly colored fruits and vegetables contain flavonoid compounds such as anthocyanins [4]. The chemical nature of these compounds is quite complex. Anthocyanins naturally occur as glycosides of flavylium (phenylbenzopyrylium) salts. There are six common anthocyanins in foods: pelargonidin (Pg3G), cyanidin (C3G), peonidin (P3G), delphinidin, petunidin, and malvidin [5]. Anthocyanins differ with regard to the position and number of hydroxyl groups, degree of methylation, type and number of sugar molecules, and type and number of aliphatic or aromatic acids [6]. This diversity in structure allows the compounds to react differently when in contact with other molecules, yielding a variety of possible targets for these compounds.

A previous study demonstrated that mulberry anthocyanin extract (MAE) ameliorated HepG2 cells insulin resistance and increased glucose consumption, glucose uptake, and

glycogen concentration [7]. These researchers also administered db/db mice with MAE and observed decreasing fasting blood glucose, serum insulin, leptin, triglyceride, and cholesterol levels. The results showed that treatment with MAE improved mice ability to uptake insulin in tissues compared to mice that did not receive the treatment. Another study has portrayed the *in vivo* effect of a berry postamberlite extract (PAE) treatment in mice by decreasing percent fat mass, mean adipocyte diameters, and plasma triglycerides and cholesterol [8]. These researchers also observed a decrease in fasting blood glucose levels in mice treated with PAE compared to mice treated with the control. Anthocyanin rich foods clearly illustrate the potential health benefits they contain through their impact in obesity and insulin-resistance diseases. Recent studies have shown that a blue maize extract improved blood pressure, lipid profiles and adipose tissue in high-sucrose diet-induced metabolic syndrome in rats [9]. Furthermore, different anthocyanin extracts from different dietary sources such as red orange, cherry juice, strawberry, black currant have been used in clinical trials with diabetic or overweight patients and a reduction in plasma LDL-cholesterol, triglycerides, and body weight have been observed [10-13].

It is known that purified anthocyanins might exert different biological activities due to their specific chemical structures. Therefore, it is important to characterize and test individual anthocyanins from dietary extracts to elucidate their biological potential. The combination of different anthocyanins can exert synergistic or antagonistic effects; therefore, there is a need to understand the mechanism of action of individual anthocyanins as well as complex mixtures of anthocyanins from different foods in models of non-transmissible diseases. In this study, we provide a comprehensive evaluation using pure anthocyanins, and an anthocyanin-rich extract with a unique chemical profile from pericarp purple corn, under basal and inflammatory conditions related to obesity. Therefore, the aim was to compare the effect of an anthocyanin-rich extract from purple corn pericarp, and pure anthocyanins, on

adipogenesis, inflammation and insulin resistance in 3T3-L1 adipocytes on basal and inflammatory conditions. Therefore, our hypothesis was that anthocyanins from PCW would ameliorate the inflammatory status of TNF-α-treated adipocytes through different mechanisms of action involving decrease levels of reactive oxygen species (ROS), adipokine secretion and modulation of the insulin pathway. We demonstrated, for the first time, the protective effect of anthocyanins from purple corn pericarp on inflammation-associated events induced by TNF-α in 3T3-L1 adipocytes in comparison to basal conditions through mechanistic analysis.

## 2 Materials and methods

### 2.1 Materials

The 3T3-L1 cells (ATCC CL-173) were obtained from ATCC (Manassas, VA). A well characterized anthocyanin-rich purple corn pericarp water extract was used. The anthocyanin profile of the material is shown in **Supplementary Figure 1**. Primary antibody PPAR-γ (sc-7273) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pure anthocyanin standards cyanidin-3-*O*-glucoside (C3G), pelargonidin-3-*O*-glucoside (Pg3G), and peonidin-3-*O*-glucoside (P3G) were obtained from ChromaDex® (Irvine, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

### 2.2 Sample preparation

The method of extraction of anthocyanins used was performed following a previous methodology [14] using an ASE 300 Accelerated Solvent Extraction System (Thermo Scientific). The extract was characterized and called purple corn water extract (PCW). The proanthocyanidins present in the purple corn pericarp were extracted using a gel filtration column (30 cm x 10 mm x 13 µm average particle size) packed with Sephadex LH-20. Peaks

were detected using a GE AKTA prime plus system (GE Healthcare Life Sciences, Pittsburgh, PA) at 280 nm. The sample was eluted with 30% methanol followed by 70% acetone. This material was called proanthocyanidin fraction (PF)

### 2.3 Evaluation of anthocyanins from purple corn in 3T3-L1 adipocytes during the differentiation process

#### 2.3.1 Cell culture and adipocyte differentiation

Dulbecco's modified Eagle's medium (DMEM) with 1.5 g/L sodium bicarbonate was supplemented with 10% newborn calf serum (NCS) and antibiotics. The 3T3-L1 cells (ATCC CL-173) were grown at 37 °C in a humidified atmosphere with 5% $CO_2$. The cells were subcultured at a density of $6 \times 10^3$ viable cells/$cm^2$. To differentiate the cells to adipocytes, the method described by Zebisch et al. [15] was used. After 48 h, cell differentiation was induced by changing the medium to DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone, 1 µg/ml insulin and 2 µM rosiglitazone. After 48 h, the medium was changed to DMEM containing 10% FBS and 1 µg/ml insulin for 48 h. On day 7, the medium was changed to DMEM containing 10% FBS and the cells were kept in this culture media until further use.

#### 2.3.2 Determination of cellular lipid accumulation

As a differentiation parameter, Oil Red O lipid staining was performed. 3T3-L1 cells were seeded in 6-well plates and induced to differentiation as described in section 2.3.1. Different concentrations of PCW (0.125 mg of dry extract/mL to 1 mg/mL; 15 µM eqC3G to 125.6 µM eqC3G) were added to the culture media during the differentiation process, and lipid staining was performed at days 2, 4, 6, 8, and 10. An Oil Red O stock solution was prepared dissolving 0.35% w/v Oil Red O in isopropanol overnight. Cells were fixed with formalin and

a working solution of Oil Red O was added to each well; pictures were taken and Oil Red O staining was eluted with 100% isopropanol for detection at 500 nm in a plate reader.

#### 2.3.3 Peroxisome proliferator-activated receptor gamma (PPAR-γ) protein levels in adipocytes by western blot

To further evaluate the potential of PCW on protein expression in 3T3-L1 adipocytes, the levels of PPAR-γ (sc-7273) were evaluated. Cells were treated with 0.4 mg of dry sample/mL PCW (50.3 μM eqC3G) at day 10 of differentiation. The cells were lysed using the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Santa Cruz, CA), scraped using a plastic cell scraper, and centrifuged at 10,000 x g for 10 min to remove cell debris. To load equal amounts of protein concentration in the SDS-PAGE gels, the protein was quantified in the cell lysates through the DC protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as standard. The cell lysates separated in an SDS-PAGE gel and transferred to PVDF membranes; primary and secondary antibodies were used at manufacturer's recommended dilutions. Protein expression was detected using 1:1 chemiluminescent reagents of ECL Prime Western Blotting kit (GE Healthcare, Buckinghamshire, UK) and visualized using a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY, USA). The intensity of each band was normalized to GAPDH (sc-47724), and the results were expressed as expression level relative to a control.

### 2.4 Effect of the anthocyanins from purple corn in 3T3-L1 mature adipocytes

#### 2.4.1 Lipolysis assessment in adipocytes

3T3-L1 cells were cultured in 6-well plates according to the conditions in section 2.3.1. When adipocytes were matured, they were treated for 24 h with 0.4 mg/mL PCW, or with the main anthocyanins found in the extract (50 μM of either C3G, Pg3G, or P3G). To simulate

postprandial lipolysis, adipocytes were incubated for extra 2 h with high-glucose DMEM and the different treatments. The culture media was tested for glycerol quantification using a glycerol cell-based assay kit (Cayman Chemical Item No. 10011725) and the cell lysates were assayed using a triacylglycerol colorimetric assay kit (Cayman Chemical Item No. 10010303).

### 2.4.2 Fatty acid synthase activity inhibition

Fatty acid synthase (FAS) activity inhibition from 3T3-L1 cells was determined through NADPH oxidation as described by Martinez-Villaluenga et al. [16]. 3T3-L1 cells were cultured using the conditions mentioned in section 2.3.1. Cell lysates were added to the reaction mixture that contained 1 mM EDTA, 1 mM dithiothreitol, 30 µM acetyl-CoA, 0.15 mM NADPH, and different concentrations of PCW (0 – 0.4 mg/mL) or pure anthocyanins C3G, Pg3G, or P3G (0 – 75 µM) in a final volume of 300 µL. The reaction was monitored at 340 nm for 3 min to determined background NADPH oxidation. The reaction was initiated by adding 50 µM malonyl-CoA, and the decrease of absorbance at 340 nm was monitored for 20 min. The results of the FAS activity were expressed as µmol/min/mL. A non-linear regression was performed to calculate the inhibitory concentration 50 ($IC_{50}$).

### 2.4.3 Lipase activity inhibition

Lipase activity inhibition potential of the anthocyanins present in purple corn pericarp was determined using a lipase activity assay kit (Cayman Chemical Item No. 700640). Briefly, 3T3-L1 cells were cultured using the conditions mentioned in section 2.3.1. When the cells were fully differentiated and mature, cell lysate supernatants were assayed using the manufacturer's instructions and different concentrations of PCW (0 – 0.5 mg/mL) or pure anthocyanins C3G, Pg3G or P3G (0 – 75 µM) were tested. The results of the lipase activity

were expressed as nmol/min/mL. A non-linear regression was performed to calculate the inhibitory concentration 50 ($IC_{50}$).

### 2.4.4 Determination of PPAR-γ transcriptional activity

The PPAR-γ inhibitory activity of the anthocyanins was determined through an immunological approach. The 3T3-L1 cells were seeded in 6-well plates and induced to differentiation as described in Section 2.3.1. Fully differentiated adipocytes were washed with PBS twice, and the nuclear fractions were separated using the NE-PER nuclear extraction kit (ThermoScientific, Pierce, IL). The PPAR-γ DNA-binding activity was assessed using the PPAR-γ transcription factor assay kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. Different concentrations of the pure anthocyanins (0 to 100 µM) or the PCW (0 to 0.5 mg/ mL) were added to the wells pre-coated with the double-stranded DNA sequence that contained the PREP (peroxisome proliferator response element). Then, 5 µg of adipocyte nuclear extract was added to the wells and the reaction was incubated during 2.5 h. The primary antibody was added followed by the HRP-conjugated antibody. The developing reagents were added, and bound PPAR- γ was detected at 450 nm.

## 2.5 Assessment of potential mechanisms of inhibition of fatty acid synthase (FAS) and lipases by molecular docking

The 3D crystal structure of peroxisome proliferator-activated receptor gamma (PPAR-γ) (5DSH), monoacyl-glycerol lipase (3PE6), phospholipase-A2 (5I8P), pancreatic lipase (1LPB), FAS domains thioesterase (2PX6), enoyl-acyl carrier-protein reductase (4W9N), β-ketoacyl reductase (5C37), and β-ketoacyl synthase I (1FJ8) were acquired from the Protein Data Bank website (www.rcsb.org/pdb/home/home.do). The human sequence of lipoprotein lipase was acquired from the PubMed database (accession number EAW63764.1;

www.ncbi.nlm.nih.gov/entrez). The homology-modelling of the 3D structure was performed in Swiss-Model [17] using as a template of the structure of the pancreatic triacylglycerol lipase (1N8S), and the binding pockets were determined using metaPocket 2.0 [18]. Flexible torsions, charges and grid size were assigned using Autodock Tools [19]. Docking calculations were performed using AutoDock Vina [20], and the binding pose with the lowest binding energy was selected as representative to visualize in the Discovery Studio 2016 Client (Dassault Systèmes Biovia Corp®).

### 2.5 Evaluation of anthocyanins from purple corn on insulin resistant 3T3-L1 adipocytes

#### 2.5.1 Induction of insulin resistance to 3T3-L1 adipocytes

3T3-L1 cells were cultured and differentiated as mentioned in section 2.3.1. On day 5 after full differentiation, culture media was added with mTNF-α (10 ng/mL) for 6 days replacing the culture media prepared fresh daily.

#### 2.5.2 Effect of anthocyanins from purple corn in adipokine secretion by insulin-resistant adipocytes

The profile of adipokines protein secretion to the culture media was performed and analyzed using a mouse adipokine array kit (ARY013, R&D Systems, Minneapolis, MN) according to the manufacturer instructions. Briefly, 3T3-L1 cells were seeded according to the conditions mentioned in section 2.3.1 and induced to insulin resistance as mentioned in section 2.5.1. Then the cells were treated with 0.4 mg/mL of PCW for 24 h along with the TNF-α treatment. A provided membrane containing immobilized antibodies was blocked with blocking solution reagent for 1 h at room temperature, and treated with equal amounts of cell lysates overnight at 4 ºC. The membrane was washed with the provided washing buffer and incubated subsequently with detection antibody cocktail and streptavidin horseradish

peroxidase-conjugated for 1 h at room temperature each. Protein expression was detected using 1:1 provided chemiluminescent reagents A and B and visualized using a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY).

### 2.5.3 Assessment of glucose uptake in insulin-resistant adipocytes

To evaluate the effect of the anthocyanins present in purple corn pericarp on 3T3-L1 glucose uptake, the 2-NBDG assay was performed according to the method Alonso-Castro et al. [21]. 3T3-L1 cells were differentiated in a black 96-well plate with clear bottom using the conditions mentioned in section 2.3.1. Two approaches were followed: **1**) pretreatment of the mature adipocytes with TNF-α for 6 days followed by a 24 h treatment of PCW (0.4 mg/mL) or pure anthocyanins (50 µM of C3G, Pg3G or P3G), and **2**) simultaneous treatment of the adipocytes with PCW (0.4 mg/mL) or pure anthocyanins (50 µM of C3G, Pg3G or P3G) and TNF-α for a total of 7 days. After treatment, the cells were incubated for 1 h with glucose-free DMEM added with 100 µM 2-NBDG. Then the cells were washed with PBS and the fluorescence was detected at an excitation/emission wavelength of 485 nm/535 nm.

### 2.5.4 Detection of intracellular reactive oxygen species in insulin-resistant adipocytes

3T3-L1 cells were differentiated in a black 96-well plate with clear bottom. Two approaches were followed: **1**) pretreatment of the mature adipocytes with TNF-α for 6 days followed by a 24 h treatment of PCW (0.4 mg/mL) or pure anthocyanins (50 µM of C3G, Pg3G or P3G), and **2**) simultaneous treatment of the adipocytes with PCW (0.4 mg/mL) or pure anthocyanins (50 µM of C3G, Pg3G or P3G) and TNF-α for a total of 7 days. After treatment, the cells were incubated for 1 h with DMEM and added with DCFDA (45 µM). Then the cells were washed with PBS and the fluorescence was detected at an excitation/emission wavelength at 485 nm/535 nm, respectively.

### 2.5.5 Evaluation of the effect of anthocyanins from purple corn on phosphorylation pattern of insulin resistant adipocytes

3T3-L1 adipocytes were cultured, differentiated and induced to insulin resistance as mentioned in sections 2.3.1 and 2.5.1, respectively. Insulin resistant adipocytes were then treated with 0.4 mg/mL of PCW for 24 h along with TNF-α. After treatment, the cells were serum starved for 30 min followed by 10 min stimulation with 10 ng/mL of insulin. Cell lysates were biotinylated following the manufacturer's instructions, and applied to each one of the slides. An antibody array was used following the instructions of the protocol of Full Moon BioSystems® (Insulin Receptor Phospho Antibody Array, PIG219, Sunnyvale, CA). The arrays were labeled with a Cy3-labeled streptavidin solution (0.5 mg/mL) (Sigma Aldrich, S6402). The slides were scanned on a GenePix4000B scanner and the images were analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnivale, CA). Fluorescence signal (*I*) of each array spot was quantified; the mean value and the standard deviation of the replicates were calculated. The phosphorylation signal ratio induction by PCW on insulin resistant adipocytes (Δ) was calculated as follows:

$$\Delta = \frac{I_{p-pcw} / I_{pcw}}{I_{p-TNF} / I_{TNF}}$$

Where $I_{p\text{-}pcw}$ and $I_{pcw}$ are fluorescence signals of the phosphorylated and non-phosphorylated/total protein from the PCW treatment, respectively. Parameters $I_{p\text{-}TNF}$ and $I_{TNF}$ are respective fluorescence signals of the phosphorylated and non-phosphorylated/total protein from the TNF-α control samples.

### 2.6 Fixed-cell immunostaining and confocal laser-scanning microscopy

3T3-L1 cells were suspended in phenol red-free DMEM medium and were seeded in ibiTreat microscopy chambers (ibidi, Munich, Germany) using the conditions mentioned in 2.3.1. The cells were differentiated and treated with either pure anthocyanins C3G, Pg3G, or P3G (50 µM) or PCW (0.4 mg/mL). Cells were fixed with 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences) and permeabilized with 0.1% Triton X 100 in PBS. Cells were blocked with Image-iT FX Signal Enhancer (Life Technologies), and incubated with GLUT4 antibody overnight. Cells were washed with PBS and incubated with Alexa Fluor 568 Goat Anti-Rabbit IgG (Life Technologies) secondary antibody (1:200). Cells were cured with ProLong Gold antifade reagent with DAPI (Life Technologies). The microscopy chamber plate was stored at 4°C in the dark until analysis. Samples were imaged using a 63×/1.4 Oil DIC M27 objective with a Zeiss LSM 880 laser-scanning confocal microscope (Carl Zeiss AG, Germany). To evaluate GLUT4 translocation to the membrane, the fluorescence intensity, or GLUT4 expression, was measured in the membrane of the cells and normalized using the same laser gain (650) for all the samples. For the total GLUT4 measurement, the intensity of the whole picture was taken in consideration and normalized to the nuclear DAPI staining. All of the image panels were resized and consolidated, and the brightness of the final collage displayed was increased by 20% as a whole.

**2.7 Statistical analysis**

The results were expressed as the mean ± SD of at least two independent experiments with three repetitions each and analyzed through ANOVA. Statistical significance was determined using Tukey's test for multiple mean comparisons in the software JMP version 7.0 (SAS Institute, Cary, NC). A two-step cluster analysis was performed to determine associations among the different treatments considering a silhouette measure of cohesion and separation higher than 0.85 using the software SPSS version 22 (IBM, Chicago, IL).

## 3 Results

### 3.1 Anthocyanin-rich purple corn water extract inhibited 3T3-L1 adipocyte differentiation

Purple corn pericarp water extract (PCW) inhibited 3T3-L1 adipocytes differentiation in a dose-dependent manner as depicted in **Figure 1A**. It was determined that 0.4 mg/mL of the extract was needed to reduce 50% of the differentiation ($IC_{50}$) (**Figure 1B)**. Along with lipid staining, the protein level of peroxisome proliferator-activated receptor gamma (PPAR-γ) was also considered as a differentiation parameter in 3T3-L1 adipocytes. In **Figure 2A** it can be observed that PCW reduced ($p < 0.05$) the expression of PPAR-γ in a dose dependent manner after the pre-adipocytes differentiation process to adipocytes compared to untreated cells. Furthermore, PCW, as well as pure anthocyanins, reduced the PPAR-γ transcriptional activity in a dose-response manner, being the $IC_{50}$ 38.4 µg/mL for PCW, and 5.3 µg/mL (10.9 µM) for C3G, which had the lowest $IC_{50}$ among the pure anthocyanins (**Table 1**). To discard the possibility that the reduction of adipocyte differentiation was caused by toxic effects, a viability test was performed in either the pre-adipocytes or fully mature adipocytes. No toxicity of PCW was detected in 3T3-L1 cells before or after the differentiation process with the concentrations used in this study (**Supplementary Figure 2**).

### 3.2 Anthocyanin-rich purple corn water extract reduced lipid concentration in mature adipocytes through potential modulation of lipolytic and lipogenic enzymes

**Figure 2** shows the effect of PCW on lipolysis of 3T3-L1 at different physiological status, basal conditions and glucose-dependent lipolysis. First, as can be observed in **Figure 2B** and **2C**, in basal conditions compared to the untreated adipocytes, the $IC_{50}$ of differentiation of PCW (0.55 mM) caused a lower ($p < 0.05$) triglyceride (TG) concentration after 24 h of

treatment. In contrast with PCW, only P3G (0.88 mM) but not C3G, or Pr3G had a significant difference compared to the control, increasing TG concentration. On the other hand, in agreement with the TG reduction, PCW (108.1 µg/mL) caused an increase of glycerol release to the culture media compared to the control (41.7 µg/mL) after 24 h. The three pure anthocyanins C3G, Pr3G and P3G had higher glycerol release values compared to the control. The difference of the behavior of PCW and pure anthocyanins could be due to a synergistic activity of the different bioactive compounds present in PCW.

The second approach evaluated was the effect of PCW on the glucose-dependent lipolysis in 3T3-L1 adipocytes to mimic a post-prandial status. After glucose-starvation, 3T3-L1 adipocytes were treated with high-glucose media and the different treatments. In this scenario, the treatment with 0.4 mg/mL of PCW promoted lipid accumulation caused by an increase in TG concentration and concomitantly, a significant reduction of glycerol release compared to the control (**Figures 2D** and **2E**). In contrast, the pure anthocyanins C3G and Pr3G reduced significantly TG concentration and increased glycerol released, suggesting their lipolytic effects on glucose-treated adipocytes. These results suggest that the efficacy of PCW reducing lipid concentration is greater when used in basal conditions.

Since one of the mechanisms of action related to lipolysis is the regulation of the activity of lipogenic and lipolytic enzymes, the ability of PCW, pure anthocyanins, and specific inhibitors reducing the lipases and fatty acid synthase activity were evaluated using biochemical analysis (**Table 1**). These treatments reduced the enzymatic activity in a dose-response manner for both enzymes, and PCW had the highest ($p < 0.05$) $IC_{50}$ value (23.1 µg/mL), followed by the proanthocyanidin fraction PF (15.4 µg/mL), and the pure anthocyanins C3G, Pr3G and P3G (18.3 µg/mL, 8.3 µg/mL and 0.5 µg/mL, respectively). The drug Orlistat, specific inhibitor of lipase activity, was used as a control on this analysis, which $IC_{50}$ value (1.9 µg/mL) fell within the range of the inhibitory spectrum of the different

treatments. For FAS activity, PCW had an $IC_{50}$ value of 65.2 µg/mL, followed by P3G and PF (29.7 µg/mL and 28.4 µg/mL, respectively), however, C3G and Pr3G $IC_{50}$ values were greater than 50 µM. These results contrasted with the activity of TVB-3166, a specific inhibitor of FAS, which $IC_{50}$ value was 1.1 µg/mL.

According to the computational modeling, C3G, Pr3G and P3G could potentially interact with several FAS domains and the catalytic cavity of important lipases expressed in 3T3-L1 adipocytes (**Figure 3**). All the interaction energies were lower than -6 kcal/mol indicating high probabilities of chemical interaction.

### 3.3 Anthocyanin-rich purple corn water extract ameliorated TNF-α-dependent inflammatory status in insulin-resistant adipocytes

From a total of 38 adipokines, 10 molecules were differentially ($p < 0.05$) secreted to the media of adipocytes after a 6 days of TNF-α induction followed by simultaneous treatment of PCW and TNF-α for 24 h (**Figure 4A**). PCW caused an increase of adiponectin (39.1%) and a reduction in leptin (-79%) compared to the TNF-α control. Besides, PCW reduced the expression of endocan, fetuin A, insulin-like growth factor-binding protein 3 (IGFBP-3) among others, and increased leukemia inhibitory factor (LIF) and VEGF. Interestingly, PCW promoted an important overexpression (368% higher than the control) of interleukin 6 (IL-6).

Additionally, the reduction of TNF-α-dependent lipolysis in adipocytes was evaluated (**Figure 4B**). This figure shows the different concentrations of TG and glycerol as a response to the treatments. TNF-α induced triglyceride accumulation compared to the untreated adipocytes; however, only C3G decreased statistically intracellular TG concentration. On the other hand, TNF-α increased the glycerol release to the culture media compared to the untreated cells by almost 3-fold. PCW reduced TNF-α dependent lipolysis to basal levels, and

PF reduced it by 50% in insulin resistant adipocytes. No significant differences were observed for C3G, P3G and Pr3G anthocyanins with the TNF-α treatment.

Using the DCFDA reagent, it was possible to detect intracellular ROS level in 3T3-L1 adipocytes. In **Figure 5A,** it can be observed how the treatment with TNF-α significantly increased ($p < 0.05$) the levels of intracellular ROS compared with untreated adipocytes by 3.7-fold. However, the treatment with PCW reduced the intracellular ROS to basal levels of adipocytes treated with TNF-α regardless of the treatment strategy, either as a pretreatment with TNF-α followed by treatment with PCW, or a simultaneous treatment with TNF-α and PCW. On the other hand, the efficacy of C3G, P3G and Pr3G anthocyanins depended on the treatment strategy. When the adipocytes were first treated with TNF-α, the three pure anthocyanins reduced approximately 25% of the TNF-α dependent ROS increase. However, the simultaneous treatment with anthocyanins and TNF-α caused a reduction of approximately 80% on the TNF-α dependent ROS increase. No significant differences ($p > 0.05$) were found among pure anthocyanins in this evaluation **(Figure 5A)**.

### 3.4 Anthocyanin-rich purple corn water extract improved insulin sensitivity in TNF-α-treated 3T3-L1 adipocytes through modulation of the phosphorylation pattern of insulin pathway and GLUT4 membrane translocation

As depicted in **Figure 5B**, PCW increased by approximately 35% the glucose uptake compared to insulin resistant adipocytes, suggesting an increase in insulin sensitivity. No statistical differences ($p > 0.05$) were found for both approaches (pretreatment with TNF-α or simultaneous treatment) in the ability of the anthocyanins to improve glucose uptake. In agreement with these results C3G, the major anthocyanin present in PCW, also improved glucose uptake in the same way ($p < 0.05$) than PCW. In a lesser extent P3G and Pr3G also improved glucose uptake in insulin resistant adipocytes.

In order to better understand the mechanism of action by which PCW improved the insulin sensitivity in adipocytes, an insulin receptor phosphor array was used to determine the effects of PCW on that pathway. In general, PCW reduced ($p < 0.05$) the phosphorylation status in serine residues of the insulin receptor substrate 1 (IRS-1) (Ser312: -6.3, Ser307: -1.8, Ser794: -1.6, Ser612: -1.5) compared to TNF-α. A total of 20 out of 60 proteins had a differential phosphorylation pattern compared to insulin resistant adipocytes (**Table 2**). Among these are eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), protein kinase B (AKT), Bcl-2-associated death promoter (BAD), RAF proto-oncogene serine/threonine-protein kinase (c-Raf), extracellular signal-regulated kinase (ERK), forkhead box O protein (FOXO), inhibitor of nuclear factor kappa-B kinase (IKK), glycogen synthase kinase 3 (GSK3), and mechanistic target of rapamycin (mTOR).

As determined by confocal microscopy, PCW, C3G and PF increased (2.9, 1.9, and 2.2 fold-change, respectively) the total GLUT4 expression (**Figure 6**) compared to TNF-α-treated cells. More importantly, PCW promoted a greater (7.5-fold) membrane translocation of GLUT4 from its typical nuclear and perinuclear localization in insulin resistant adipocytes. Comparing the fluorescence intensity among the treatments in the cell membrane region, the adipocytes treated with insulin had the highest (3645 AU) ($p < 0.05$) GLUT4 fluorescence emission, followed by PCW (2947 AU), C3G (2475 AU), PF (1758 AU), and in a lesser extent Pr3G (756 AU), P3G (451) and the last being TNF-α (389 AU).

## Discussion

Obesity is defined as an excessive accumulation of fat in adipose tissue that increases the risk of non-transmissible diseases such as diabetes, cardiovascular events, and certain types of cancer [22, 23]. In an obese condition, it is desirable to reduce adipocyte differentiation rate in order to reduce fat accumulation. For the first time, the effects of a well-characterized

anthocyanin-rich extract from purple corn in 3T3-L1 adipocytes differentiation was evaluated in comparison to pure anthocyanins. PCW reduced lipid accumulation and PPAR-γ protein levels, considered as differentiation parameters. Several reports have suggested the ability of anthocyanin-rich extracts from other food sources such as black soybean, purple sweet potato, cranberries, bilberry, and sweet cherry, among others [24-28] to reduce adipogenesis in 3T3-L1 cells and to reduce PPAR-γ protein levels. PPAR-γ has been considered as the 'master regulator' of adipogenesis. Induced expression of PPAR-γ is sufficient to promote adipocyte differentiation in fibroblasts [29]. Although the specific mechanism of action of PPAR-γ in the adipocyte differentiation is not fully understood, there are a great number of reports in the literature indicating its importance not only in *in vitro* studies. In animal studies, it has been stated that a lack of PPAR-γ decreased fat accumulation in high fat diet feed mice compared to normal obese mice with the same diet [30]. Even though several flavonoids have been identified as PPAR-γ agonists [30], this effect was not observed in the present study. PCW and the pure anthocyanins lowered the PPAR-γ transcriptional activity, thus avoiding the adipocyte differentiation. Additionally, since PCW impaired the adipocyte differentiation process at very early stages, it potentially avoided PPAR-γ activation and expression. Since high lipid concentration in adipocytes promotes inflammatory status, the lipolysis of mature adipocytes was assessed to investigate the effect of PCW on cellular lipid concentration by increasing lipolysis. PCW promoted lipolysis in mature adipocytes. The ability of anthocyanin-rich extracts from other food sources such as black soybean, to promote lipolysis in mature adipocytes has been reported [31]. Furthermore, in *in vivo* studies and using rodents fed with high-fat diet and in clinical trials, some authors have concluded that the antiobesity mechanism of action of anthocyanins from blueberry and black soybean involve an increase of lipolysis levels through regulation of lipolytic and lipogenic markers [32, 33]. In the present study, we demonstrated for the first time, PCW as well as the major

anthocyanins present in PCW had the ability to reduce lipase and fatty acid synthase enzymatic activity. From the docking output, it was determined that it was through interaction with amino acid residues of the catalytic cavity of lipases and different domains of FAS. Even though the treatments inhibited the enzymatic activity, in previous studies it has been determined that anthocyanins from different berry blends and purple sweet potato increase the expression of lipolytic genes [26, 34] in 3T3-L1 adipocytes. Therefore, the lipolysis increase in the adipocytes in response to PCW and the pure anthocyanins could be due to modulation of the expression and activity of lipogenic and lipolytic markers.

It has been reported that a sustained obesity status leads to inflammatory processes, increasing the risk to develop insulin resistance in metabolic tissues [35]. In this study, TNF-α was used to promote an inflammatory state in 3T3-L1 cells. Through the mechanistic study, it was determined that PCW increased adiponectin and reduced leptin. These markers are considered as inflammatory modulators, known to play a crucial role in insulin sensitivity and body energy balance [36, 37]. Adiponectin is predominately produced by adipocytes and directly sensitizes cells to insulin, and its levels are lower in insulin resistance and diabetes than in healthy individuals [38-40]. On the other hand, circulating leptin levels are directly related to body fat amount due to its ability to regulate energy homeostasis [41]. PCW also suppressed endocan and the insulin-like growth factor binding protein (IGFBP-3), both proteins related to inflammation, lipid metabolic dysregulation and insulin resistance [42, 43]. PCW also increased IL-6 levels. Although IL-6 has been related to insulin resistance, the role of IL-6 in the etiology of obesity and its complications is not fully understood [44]. Indeed, the question about whether IL-6 plays negative or positive roles in adipocytes metabolism is still in controversy since IL-6 is considered an anti- and pro-inflammatory interleukin [45]. Given the conditions and the overall adipokine modulation, IL-6 could be playing and anti-inflammatory role in the PCW-mediated effects. This mechanism of action could be related to

the effects reported by Carey et al. [46], whom observed that IL-6 increased insulin-stimulated glucose in a human clinical study, and glucose uptake in 3T3-L1 adipocytes via AMP-activated protein kinase (AMPK). In another study performed by Stouthard et al. [47], it was also concluded that IL-6 may enhance glucose uptake in 3T3-L1 adipocytes by increasing glucose-transporters intrinsic pathways. Other effect related to inflammatory status in adipocytes is the increase in reactive oxygen species (ROS), known to cause oxidative stress, which plays an important role in peripheral insulin resistance [48]. Therefore, the antioxidant properties of PCW and the pure anthocyanins in adipocytes can be considered as an alternative protective mechanism of action against insulin resistance and inflammation.

One of the main common inflammation-associated complications in obese patients is the insulin resistance in different tissues of the body. Insulin resistance arises from the poor ability of insulin to regulate nutrient metabolism in peripheral tissues, and several evidences using *in vivo* and *in vitro* studies have correlated chronic inflammation and insulin resistance [49]. Impairment in the insulin receptor pathway usually leads to an increase of circulating glucose, condition commonly known as hyperglycemia, which in turn increases the risk of several diseases such as diabetes, cancer progression and cardiovascular events among others [50-52]. Extensive literature has shown that generally insulin binds to its receptor and promotes a tyrosine phosphorylation of the insulin receptor substrate (IRS), which in turn induces the activation of phosphoinositide 3-kinase (PI3K) and AKT. As a consequence, it leads to the translocation of glucose transporter type 4 (GLUT4) and glucose uptake [53-55]. In the present study, treating adipocytes with TNF-α caused an inflammatory status on the cells and subsequently insulin resistance. PCW reduced TNF-α-dependent insulin resistance in 3T3-L1 adipocytes, possibly by inhibiting the phosphorylation in different serine residues of IRS-1. It has been reported that TNF-α induce insulin resistance through IRS-1 phosphorylation of several serine residues by c-Jun N-terminal kinase (JNK), ERK, mTOR

and protein kinase C theta (PKC-θ) mainly [56-58]. This insulin resistance induction involves also the activation of the ribosomal protein S6 kinase (p70S6K) through the induction of the unfolded protein response [59]. It has been shown that C3G, the main anthocyanin present in PCW, suppresses the activation of mTOR targets such as JNK in TNF-α-treated 3T3-L1 adipocytes [60]. To the best of our knowledge, there is no evidence so far of the inhibition effect of dietary anthocyanins on the phosphorylation of PKC-θ and its target in IRS-1 (Ser1101). In this study, it was demonstrate that PCW reduced the TNF-α-dependent phosphorylation of mTOR, p70S6K, PKC-θ, and the serine residues 301, 312, 612, 794 and 1101 of IRS-1. These results suggest that insulin-sensitizing effect of anthocyanins present in PCW could be partly associated to an inhibition of mTOR and PKC-θ. It has been also reported that several cytokines such as TNF-α can induce adipocytes apoptosis [61]. Even though it has been proposed that apoptosis of adipocyte can be a mechanism of obesity prevention [33], given an inflammatory status, a greater apoptosis rate in adipocytes can cause liver steatosis due to the increasing circulating levels of lipids [61]. In this study, a potential protective effect of PCW on apoptosis induction of insulin resistant adipocytes was observed. According to the phosphorylation status, an increased activation of PI3K/AKT signaling and decrease of some of its inactivation targets, such as BAD, FOXO3 and GSK3α/β, known to be pro-apoptotic markers, was observed. A concomitant mechanism of insulin sensitivity of PCW on the adipocytes could be the reduction in TNF receptor-1 levels, thus reducing the stimulation with TNF-α in the cells and subsequently decreasing the further signaling. The effect of the modulation of the phosphorylation status in the insulin receptor pathway was further confirmed with the membrane translocation of GLUT4 by confocal microscopy. Under glucose- or serum-starvation status, GLUT4 was located in the perinuclear region of 3T3-L1 adipocytes, and it can be translocated to the membrane by

hormone-stimulation [62]. In spite of being statistically different, PCW and C3G were the most effective treatments promoting the translocation of GLUT4 to the membrane. This result is in agreement with a previous study where the authors used a plasma membrane extract and immunoblot to determine that pure C3G promoted GLUT4 membrane translocation in insulin resistant adipocytes [60]. These results support the hypothesis that attributes partially to C3G the efficacy of PCW reducing inflammatory conditions in adipocytes.

In summary, insulin binds to its receptor and promotes phosphorylation of IRS, which in turn induces the activation of PI3K and AKT. This leads to the translocation of GLUT4 and glucose uptake. In the present study, treating adipocytes with TNF-α caused an inflammatory status on the cells and subsequently insulin resistance. We demonstrated that PCW reduced TNF-α-dependent phosphorylation of the serine residues 301, 312, 612, 794 and 1101 of IRS-1. According to the phosphorylation status, an increased activation of PI3K/AKT signaling was observed. Furthermore, the modulation of the phosphorylation status in the insulin receptor pathway was confirmed with the membrane translocation of GLUT4 by confocal microscopy. Under glucose- or serum-starvation status, GLUT4 was located in the perinuclear region of 3T3-L1 adipocytes, and then translocated to the membrane by PCW and C3G treatments. An integration of the biological potential of PCW in the TNF-α-dependent effects in adipocytes, including the effect on the activation of IRS or Akt involved in the insulin signaling cascade is shown in **Figure 7**.

## 4 Concluding remarks

For the first time a mechanistic study of the effect of anthocyanins from purple corn reducing obesity complications *in vitro* was presented. According to a two-step clustering analysis including the results where all the different treatments were evaluated, PCW covariated with

cyanidin-3-*O*-glucoside, which is the major anthocyanin present in PCW. Therefore, the efficacy of PCW reducing obesity complications *in vitro* can be partially attributed to C3G. However, the other anthocyanins present, or the PF, could also be playing important roles either by individual action, or as a synergistic system of bioactive compounds. The anthocyanins from purple corn had different effects on 3T3-L1 adipocytes depending on the physiological status. It demonstrated to have obesity prevention potential, as well as protective effect in inflammatory/insulin resistance status. Our results suggest that the effects of anthocyanins from purple corn in 3T3-L1 adipocytes under different physiological conditions have different mechanisms of action. Further *in vivo* studies on the efficacy of anthocyanins from purple corn pericarp water extract in obesity-diabetes crosstalk complications are needed. This study contributes with the knowledge and strategies on the evaluation of the mechanism of action of anthocyanins from a food source, with food pigment potential such as colored corn, under basal and inflammatory conditions related to obesity. In conclusion, our results suggest that the anthocyanins present in purple corn can promote beneficial effects against obesity and its complications.

**Author contributions**

DL-V and EDM proposed the project and designed the experiments; DL-V and MW performed the experiments, developed and wrote the manuscript. EDM provided scientific guidance throughout the research and revised and edited the manuscript. Authors read and approved the manuscript.

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*The authors have declared no conflict of interest.*

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## Figure Legends

**Figure 1.** Oil Red O lipid staining of 3T3-L1 adipocytes treated with different concentrations of anthocyanin-rich purple corn pericarp water extract (PCW) at different days of differentiation. Cells with no treatment, but differentiated, were used as a control. **A**) A representative image of the plates, and **B**) Oil Red O absorbance at 500 nm. A non-linear regression was performed to obtain the $IC_{50}$ value.

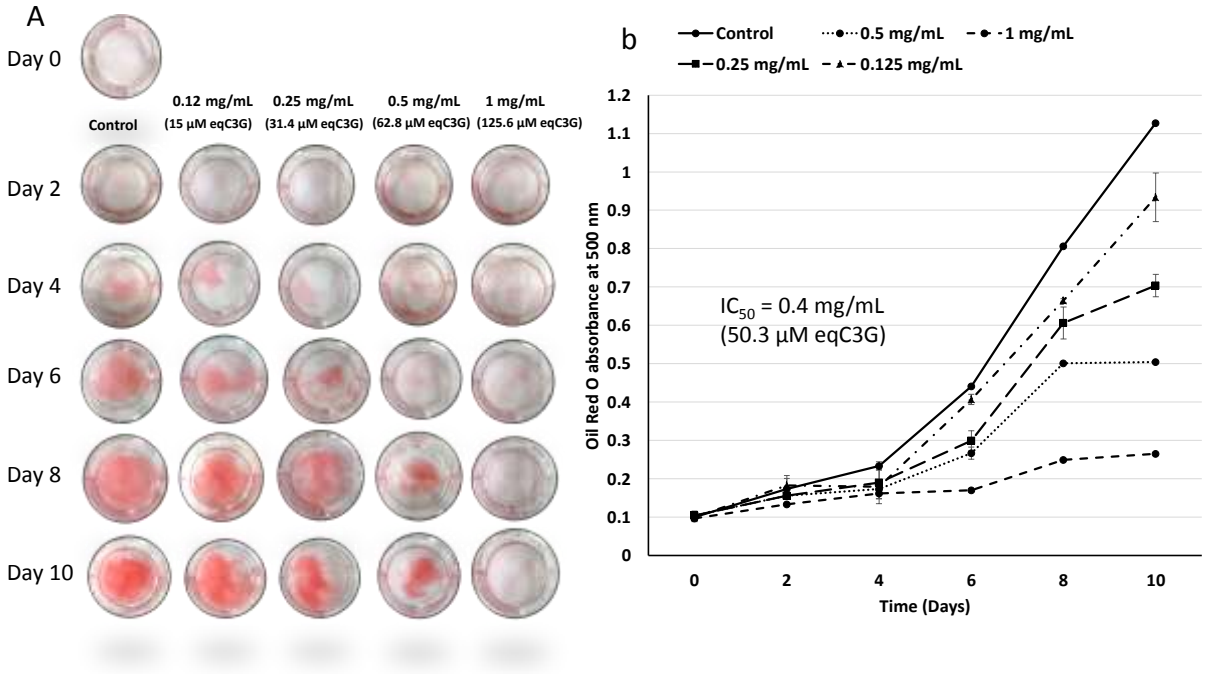


**Figure 2**. **A**) Peroxisome proliferator-activated receptor gamma (PPAR-γ) protein levels of 3T3-L1 adipocytes after differentiation with or without treatment with different concentrations of the anthocyanin-rich purple corn pericarp water extract (PCW). A representative image is shown of the western blot membranes. Also, the lipolysis of mature 3T3-L1 adipocytes expressed as quantification of intracellular triglyceride (**B**) and glycerol (**C**) concentrations in culture media after 24 h of treatment with the different materials. **D**) and **E**) after 24 h of treatment with the different materials **plus** 4 h of treatment with the materials and high-glucose media. The results are expressed as the mean ± SD. Bars with different letters indicate statistical difference ($p < 0.05$) according to Tukey's test. PCW: anthocyanin-rich purple corn pericarp water extract, PF: proanthocyanidin fraction of the purple corn pericarp, C3G: cyanidin-3-*O*-glucoside, Pr3G: pelargonidin-3-*O*-glucoside, P3G: peonidin-3-*O*-glucoside, GS: glucose starved, HG: high-glucose media.

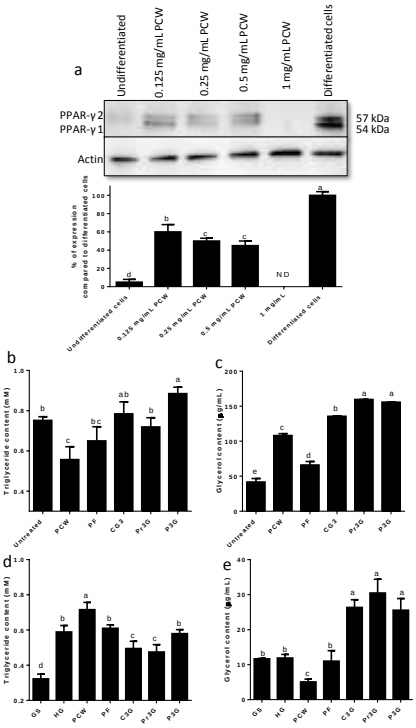
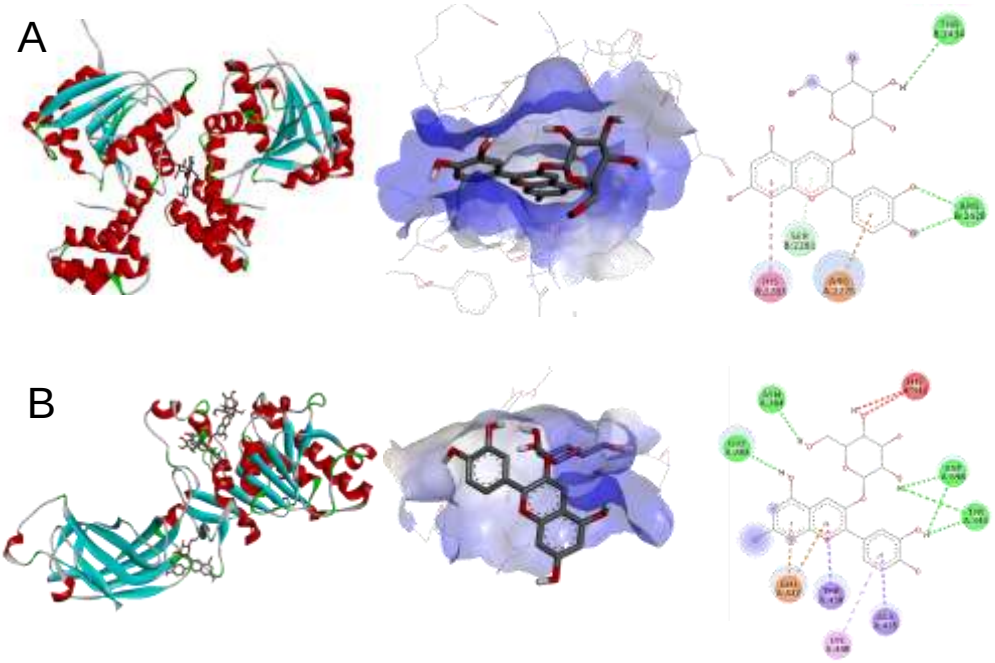


**Figure 3.** Example of best pose with the whole protein, the catalytic site, and potential chemical interactions of cyanidin-3-*O*-glucoside with **A**) thioesterase domain of fatty acid synthase and **B**) the three identified pockets of lipoprotein lipase as determined by molecular docking. **C**) Binding energies of anthocyanins present in the purple corn pericarp water extract with important enzymes related to lipid metabolism. *Crystal structures were acquired from the Protein Data Bank (www.rcsb.org/pdb/home/home.do). C3G: cyanidin-3-*O*-glucoside, Pr3G: pelargonidin-3-*O*-glucoside, P3G: peonidin-3-*O*-glucoside.




C

| Enzymes related to lipid metabolism | PDB ID | Binding energy (kcal/mol) | | | Interacting amino acid residues in common |
|---|---|---|---|---|---|
| | | C3G | Pr3C | P3G | |
| Monoacyl-glycerol lipase | 3PE6 | -8.9 | -7.4 | -8.3 | Ala51, Leu205, Leu241 |
| Phospholipase-A2 | 5I8P | -7.7 | -7.3 | -7.9 | Gln231, Asp286, Arg288 |
| Pancreatic lipase | 1LPB | -7.8 | -7.5 | -8.0 | - |
| Lipoprotein lipase pocket 1 | -- | -6.4 | -6.0 | -6.4 | Glu89, Ser90, Asp259, |
| Lipoprotein lipase pocket 2 | -- | -6.3 | -5.3 | -5.6 | Met336 |
| Lipoprotein lipase pocket 3 | -- | -8.9 | -7.2 | -6.7 | Glu283, Asp398 |
| Thioesterase domain of FAS | 2PX6 | -9.3 | -7.6 | -8.0 | Lys2436, Arg2275, His2283 |
| Enoyl-acyl carrier-protein reductase domain of FAS | 4W9N | -8.1 | -8.4 | -9.0 | Arg1666, Arg1765, Ala1805, Glu1809 |
| β-ketoacyl reductase domain of FAS | 5C37 | -8.7 | -7.7 | -8.9 | Lys1239, Leu2053 |
| β-ketoacyl synthase I domain of Fas | 1FJ8 | -7.1 | -7.8 | -8.2 | Gln37, Leu54, Leu59 |

**Figure 4.** Effect of the anthocyanin-rich purple corn pericarp water extract (PCW) on **A**) TNF-α-dependent adipokine secretion to the culture media of 3T3-L1 adipocytes; TNF-α-dependent lipolysis expressed as **B**) glycerol concentration in culture media and **C**) intracellular triglyceride content. The results are expressed as the mean ± SD. In the adipokine results, only the proteins that were statistically different ($p < 0.05$) according to Dunnet's test are shown. Bars with different letters indicate statistical difference ($p < 0.05$) according to Tukey's test. PCW: anthocyanin-rich purple corn pericarp water extract, PF: proanthocyanidin fraction of the purple corn pericarp water extract, C3G: cyanidin-3-*O*-glucoside, Pr3G: pelargonidin-3-*O*-glucoside, P3G: peonidin-3-*O*-glucoside.

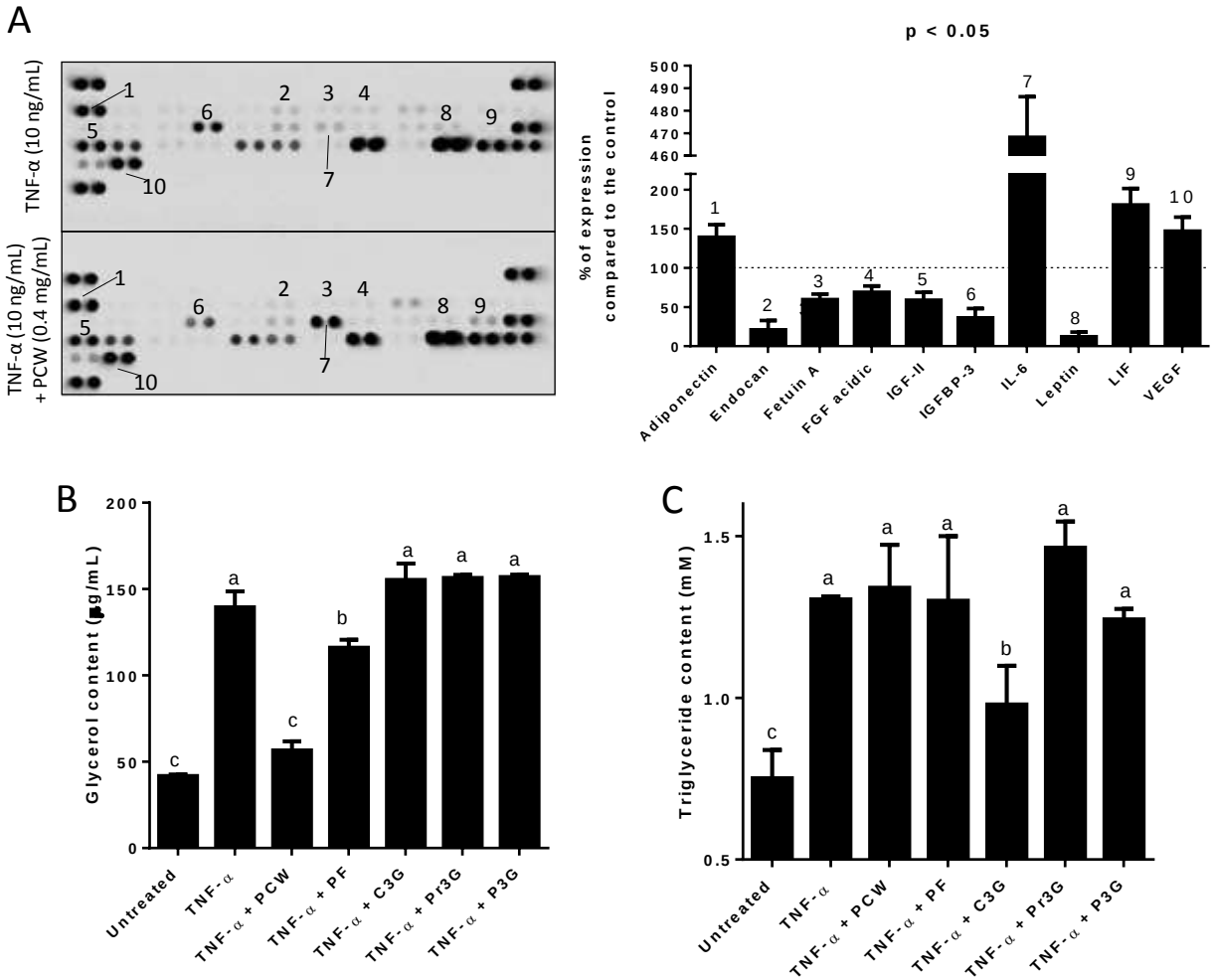


**Figure 5**. Effect of the anthocyanins present in PCW in 3T3-L1 insulin resistant adipocytes regarding **A**) reactive oxygen species (ROS) detection using two different treatment approaches and **B**) glucose uptake. The results are expressed as the mean ± SD. Bars with different letters indicate statistical difference ($p < 0.05$) according to Tukey's test. PCW: anthocyanin-rich purple corn pericarp water extract, PF: proanthocyanidin fraction of the purple corn pericarp, C3G: cyanidin-3-*O*-glucoside, Pr3G: pelargonidin-3-*O*-glucoside, P3G: peonidin-3-*O*-glucoside

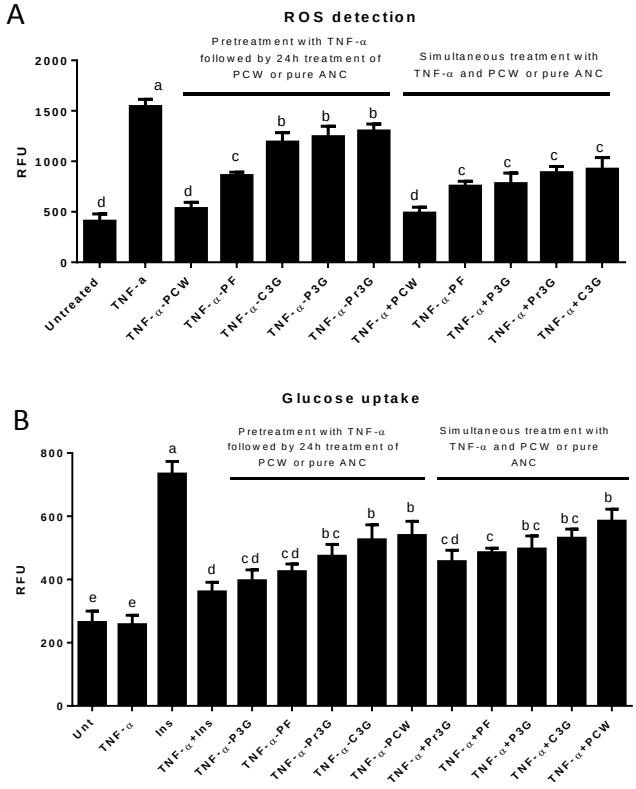


**Figure 6. A)** Confocal laser scanning microscopy depicting two-dimensional fluorescence detection and quantification, determined by the intensity (AU) over area ($\mu m^2$) of glucose transporter type 4 (GLUT4) in TNF-α-induced insulin resistant 3T3-L1 adipocytes after different treatments with PCW (purple corn anthocyanin extract, 0.4 mg/mL), PF (proanthocyanidin fraction of purple corn, 0.1 mg/mL), cyanidin-3-O-glucoside (C3G, 50 μM), pelargonidin-3-O-glucoside (Pr3G, 50 μM), or peonidin-3-O-glucoside (P3G, 50 μM). Insulin in basal adipocytes and insulin resistant adipocytes with no treatment were used as positive and negative controls, respectively. **B**) The fluorescence intensity was quantified in the membrane region of the adipocytes and normalized using the same laser gain for all the samples to evaluate GLUT4 membrane translocation. **C**) Total GLUT4 expression was determined considering the total GLUT4 intensity normalized to the nuclear fluorescence intensity. The data represents the mean ± SD of three independent fields of view from two independent cellular replicates. Means with different letters are significantly different ($p < 0.05$) according to Tukey's test.

**Figure 6.** Confocal laser scanning microscopy depicting two-dimensional fluorescence detection and quantification, determined by the intensity (AU) over area ($\mu m^2$) of glucose transporter type 4 (GLUT4) in TNF-α-induced insulin resistant 3T3-L1 adipocytes after different treatments with PCW (purple corn anthocyanin extract, 0.4 mg/mL), PF (proanthocyanidin fraction of purple corn, 0.1 mg/mL), cyanidin-3-*O*-glucoside (C3G, 50 μM), pelargonidin-3-*O*-glucoside (Pr3G, 50 μM), or peonidin-3-*O*-glucoside (P3G, 50 μM). Insulin in basal adipocytes and insulin resistant adipocytes with no treatment were used as positive and negative controls, respectively. The fluorescence intensity was quantified in the membrane region of the adipocytes to evaluate GLUT4 membrane translocation. The data

represents the mean ± SD of three independent fields of view from two independent cellular replicates. Means with different letters are significantly different ($p < 0.05$) according to Tukey's test.

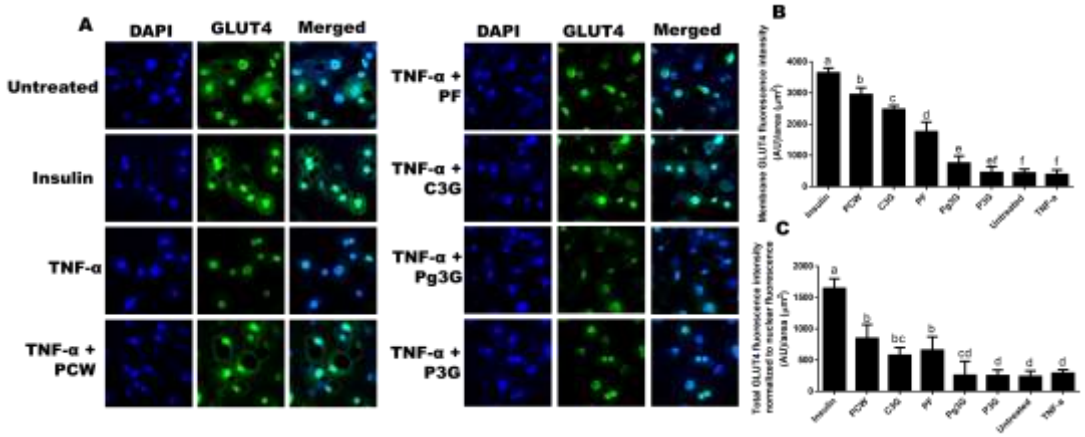


**Figure 7**. Pathway showing the effect of anthocyanins from purple corn pericarp (PCW) on 3T3-L1 adipocytes. PCW activated the insulin receptor-associated pathway, which downstream signaling promoted the membrane translocation of GLUT4 and the subsequent increase in glucose uptake. Besides, PCW ameliorated the inflammatory status through modulation of leptin/adiponectin levels and the reduction in the intracellular levels of reactive oxygen species. Items in red indicate activation ($p < 0.05$). Items in blue indicate inactivation ($p < 0.05$). Items in green indicate a reduction ($p < 0.05$) in the level, and items in purple indicate an increase in the level ($p < 0.05$) of the molecular event or protein.

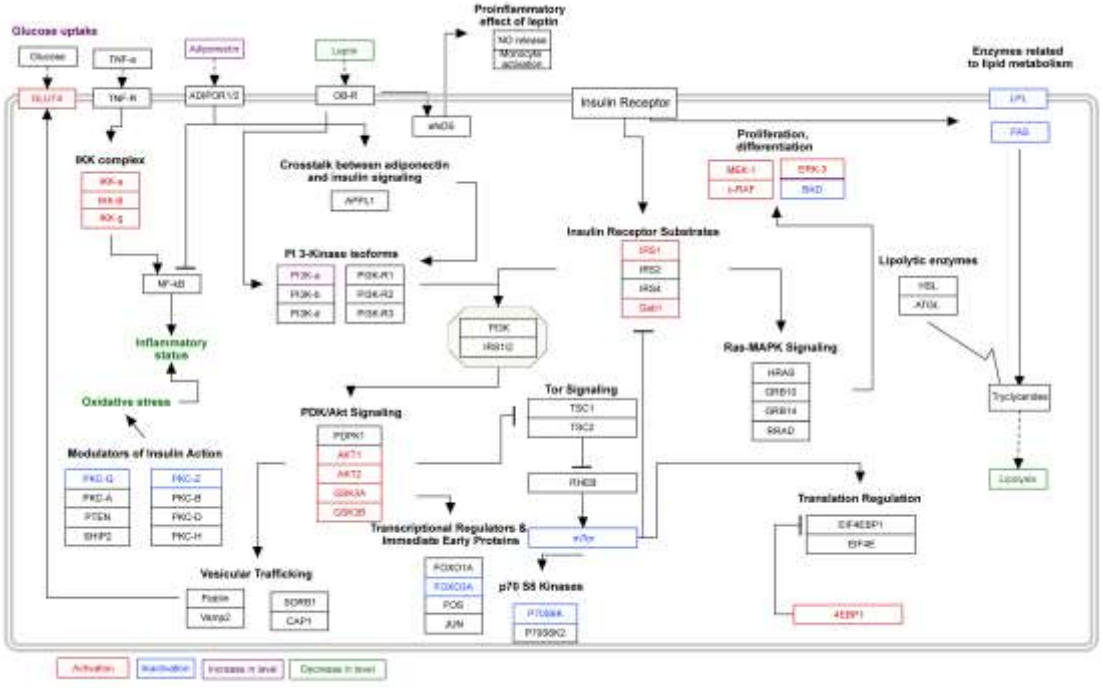


**Table 1. Inhibition of enzymatic activity of lipase, fatty acid synthase and transcriptional activity of PPAR-γ, by the purple corn water extract, pure anthocyanins and inhibitors.**

| Treatment | Lipase activity $IC_{50}$ (µg/mL) | Fatty acid synthase activity $IC_{50}$ (µg/mL) | PPAR-γ transcriptional activity $IC_{50}$ (µg/mL) |
|---|---|---|---|
| **Purple corn pericarp extract (PCW)** | 23.1a | 65.2a | 38.4b |
| **Proanthocyanidins fraction (PF)** | 15.4c | 28.4c | 41.2a |
| **Cyanidin-3-*O*-glucoside** | 18.3b (37.8 µM) | 37.8b (78.1 µM) | 5.3d (10.9 µM) |
| **Pelargonidin-3-*O*-glucoside** | 8.3d (17.8 µM) | 35.9bc (74.3 µM) | 16.4c (34.9 µM) |
| **Peonidin-3-*O*-glucoside** | 0.5f (1.1 µM) | 29.7c (59.7 µM) | 12.5e (26.9 µM) |
| **Orlistat** | 1.9e (4.03 µM) | 40.5b (81.7 µM) | ND |
| **TVB-3166 (FAS inhibitor)** | 8.2d (21.4 µM) | 1.1d (3 µM) | ND |
| **GW9962 (PPAR-γ inhibitor)** | ND | ND | 0.2f (0.8 µM) |

The results are expressed as the mean of three independent replicates. Values with different letters indicate statistical difference ($p < 0.05$) by column according to Tukey's test. ND: not determined.

**Table 2. Differential phosphorylated/non-phosphorylated ratio ($p < 0.05$) of proteins related to the insulin pathway of insulin resistant 3T3-L1 cells in response to treatment with anthocyanins from purple corn**

| Protein code and phosphorylation site | Phosphorylated/non phosphorylated ratio | | |
|---|---|---|---|
| | TNF-α | TNF-α + PCW | TNF-α + PCW / TNF-α |
| **BAD (Ser136)** | 1.2 | 9.4 | **7.8** |
| **c-Raf (Ser43)** | 4.0 | 25.7 | **6.4** |
| **Gab1 (Tyr659)** | 1.1 | 6.1 | **5.5** |
| **IKK-β (Tyr199)** | 8.4 | 34.2 | **4.0** |
| **IKK-γ (Ser85)** | 1.1 | 4.6 | **4.1** |
| **AKT1 (Ser124)** | 4.0 | 11.6 | **2.9** |
| **BAD (Ser155)** | 1.7 | 4.7 | **2.8** |
| **Gab1 (Tyr627)** | 2.0 | 4.8 | **2.4** |
| **IKK-α (Thr23)** | 1.7 | 4.1 | **2.4** |
| **4E-BP1 (Thr45)** | 1.9 | 4.4 | **2.3** |
| **AKT2 (Ser474)** | 1.5 | 3.1 | **2.1** |
| **PI3K (expression only)** | 1.1 | 2.2 | **2.0** |
| **AKT (Tyr326)** | 1.5 | 3.0 | **2.0** |
| **MEK1 (Thr286)** | 26.7 | 51.2 | **1.9** |
| **c-Raf (Ser296)** | 4.1 | 7.3 | **1.7** |
| **FOXO3 (Ser253)** | 2.4 | 4.2 | **1.7** |
| **4E-BP1 (Ser65)** | 12.5 | 20.8 | **1.6** |
| **AKT1 (Thr72)** | 2.4 | 3.4 | **1.4** |
| **ERK3 (Ser189)** | 1.1 | 1.4 | **1.3** |
| **IRS-1 (Ser312)** | 12.2 | 1.9 | **-6.3** |

| | | | |
|---|---|---|---|
| **IRS-1 (Ser1101)** | 5.3 | 1.0 | **-5.3** |
| **mTOR (Ser2448)** | 13.2 | 2.6 | **-5.1** |
| **GSK3-β (Ser9)** | 6.1 | 2.1 | **-3.2** |
| **PKC-θ (Ser676)** | 3.8 | 1.6 | **-2.4** |
| **P70S6K (Thr229)** | 6.08 | 3.2 | **-1.9** |
| **TNF receptor-1 (expression only)** | 3.6 | 1.8 | **-1.9** |
| **GSK3-α (Ser21)** | 4.1 | 2.3 | **-1.8** |
| **P70S6K (Ser411)** | 3.9 | 2.2 | **-1.8** |
| **IRS-1 (Ser307)** | 4.3 | 2.3 | **-1.8** |
| **GSK3-α/ β (Tyr216/279)** | 4.9 | 3.1 | **-1.6** |
| **PKC-ζ (Thr410)** | 2.0 | 1.3 | **-1.6** |
| **IRS-1 (Ser794)** | 12.8 | 8.0 | **-1.6** |
| **IRS-1 (Ser612)** | 4.9 | 3.3 | **-1.5** |
| **P70S6K (Ser418)** | 1.9 | 1.4 | **-1.4** |