Promoting stone fruits for Protection against the Metabolic Syndrome

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Abstract

In this report we studied the effects of stone fruit extracts against Type 2 Diabetes using insulin resistance and glucose insensitivity models as part of the events that take place in the metabolic syndrome. We used cell models including hepatic and muscle cells to determine the effects in insulin resistance representing events in hepatic and muscle tissue. In addition, we studied the effect of stone fruits on glucose insensitivity in pancreatic beta cells representing pancreatic tissue.

Initially, we chemically characterized stone fruit extracts (nectarine, peach and plum) using TLC and HPLC finding that the powder extracts contained only phenolics with profiles of 4 major phenolic groups including chlorogenic acid derivatives, anthocyanins, quercetin derivatives and catechins. These powders were tested in the biological assays.

Results indicated that stone fruit extracts have multiple functions and could potentially work against insulin resistance and glucose insensitivity in different fronts simultaneously. For example, all stone fruit extracts reversed the glucose insensitivity in pancreatic beta cells induced by palmitic acid. These extracts, by possible antioxidant (AOX) and AOX-independent mechanisms, increase insulin secretion. On the other hand, only plum extracts reversed the insulin resistance in hepatic cells induced by palmitic acid. Plum extracts reversed the effect by a mechanism that increased glucokinase activity. Similarly, nectarine and peach extracts reversed the insulin resistance in muscle cells induced by palmitic acid. Both extracts reversed the effect in muscle cells in a similar fashion as the drug metformin by an increase in glucokinase activity and a possible increase in glucose uptake. These results are very promising and indicate that stone fruit extracts have potential health promoting properties against Type 2 Diabetes, which is an important event of the metabolic syndrome.

Introduction

Metabolic syndrome is the name for a group of risk factors linked to overweight and obesity that increase the chance for heart disease and other health problems such as diabetes and stroke. It is estimated that 47 million Americans have metabolic syndrome of which two of its root causes are obesity and physical inactivity (1). The exact mechanism of the complex pathways of this combination of medical disorders or metabolic syndrome is only partially understood. It is estimated that ~75% of people with Type 2 Diabetes (impaired glucose tolerance) and ~ 50% of people with coronary heart disease has metabolic syndrome.

For the past few years the US population has been considered among the highest in the world in regards to overweight and obesity with 64% of adults being overweight and 26% obese. Furthermore, it is estimated that 75% of adults in the United States are projected to be overweight and 41% obese by 2015. These numbers plus the fact that in the U.S., cardiovascular diseases have increasingly become the leading cause of mortality, justify the need for studying the metabolic syndrome. Consequently, in recent years the U.S. market has seen an increase in the consumption of healthier products with the idea of protection against these degenerative diseases.

It is thought that as obesity (visceral fat accumulation) takes place, adipose tissue secretes various cytokines and chemokines some of which are undesirable including TNF-α (causes

diabetes), PAI-I (causes arteriosclerosis, thrombogenesis), angiotensinogen (angiotensin II, hypertension), interleukin-6 (IL-6, inflammation and causes diabetes) and MCP-I (chemotaxis). These undesirable disease-causing adipocytokines, like TNF-α causing insulin resistance and PAI-I associated with clot formation, may be exacerbated in the adipose tissue as macrophages invade them producing even more formation of TNF-α, MCP-1, IL-6 and NO in a feedback loop and causing a state of chronic inflammatory reactions (activation of inflammatory signaling molecules such as JNK, NFK-β, others) resulting in further exacerbation of the metabolic syndrome. Many of these circulating chemicals will affect the vascular endothelial tissue as well, causing inflammation which can be exacerbated by LDL oxidation and infiltration initiating atherosclerosis and thrombosis mediated by platelet aggregation (2).

Under this complex scenario, targeting obesity-related inflammatory components could be a useful strategy for the prevention and amelioration of the development of obesity related pathologies. Our first year report focused on inhibiting inflammatory events in fat cells, endothelial cells (HUVEC cells) and macrophages. That report confirmed that stone fruit phenolics show the ability to inhibit inflammation (please see annual report year 1, December 2010). Another possible strategy could be targeting Type 2 Diabetes components, by reverting insulin resistance and glucose insensitivity states in different tissues which can ameliorate the obesity related pathology.

In this report (second year) we studied stone fruits against Type 2 Diabetes as part of the events taking place in the metabolic syndrome. We hypothesized that stone fruit extracts may have multiple functions and will work against Type 2 Diabetes in different fronts simultaneously either by increasing the insulin sensitivity of target organs (muscle, hepatic tissue), and /or by increasing the amount of insulin secreted by the pancreas (β cells) reverting a glucose insensitivity state.

In this annual report (2nd year) we accomplished the following objectives:

a) Chemical characterization of stone fruit extracts of selected varieties of nectarine, plum and peach fruit.
b) Study of the anti-diabetic properties of stone fruit extracts using pancreatic beta cells, hepatic cells and muscle cells.

The present annual report is divided in 4 sections including 1) the chemical characterization of stone fruit extracts of selected varieties of peach, plum and nectarine fruit, 2) the study of extracts on glucose insensitivity of pancreatic beta cells 3) the study of extracts on insulin resistance of hepatic cells and 4) the study of extracts on insulin resistance of muscle cells.

References

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2) Kawada T, Goto T, Hirai S, Yu R and Takahashi N. 2009. Obesity and nuclear receptors: Effective genomic strategies in functional foods. In: Nutrigenomics and Proteomics in Health and Disease. Ed, Mine, Miyashita and Shahidi. Willey-Blakwell. Chapter 4, pp:47-58.

1) Chemical Characterization of Stone Fruit Extracts of Selected Varieties of Nectarine, Plum and Peach fruit

### A) Stone Fruit Extracts and TLC Characterization

Materials and Methods

Plant Material. Commercial varieties of Black Splendor plum, Rich Lady peach and Spring Bright nectarine were grown in California and collected at a mature stage and stored at 2-4 ^{o}C. Upon arrival at Texas A&M University, the fruits were frozen after removal of the pits and kept at -80 ^{o}C until use.

Extraction and Purification. 60 g of fruit from flesh and skin was blended with 180ml of methanol (ultra-turrax homogenizer) and left at 4 ^{o}C overnight. Extracts were filtered through 4 layers of cheesecloth and then centrifuged at 15000 RPM for 15 min at 4 ^{o}C. Supernatants were collected and methanol was evaporated at 45 ^{o}C using a rotavapor (Buchi, Switzerland). The remaining aqueous extracts were purified of sugars and organic acids through solid phase extraction using a SEP Pack C18 cartridge (Figure 1) as reported by Noratto et al (1). Briefly, the aqueous extracts were adjusted to pH 7.0 with 5 N NaOH. A repeated batch system of 20 mL of concentrated extract was loaded in a SEP Pack C18 cartridge (55-105 µm, Waters Corp., Milford, MA) previously conditioned to pH 7.0 with 200 mL of 100% methanol and 200 mL of nanopure water (pH 7.0). The sugars and some phenolic acids were not retained on the cartridge (A). The cartridge was washed with 300 mL of water (pH 7.0). The water from the wash was combined with the phenolics that were not adsorbed in the cartridge and adjusted to pH 2.0. This mixture was loaded into a second cartridge previously conditioned at pH 2.0 with 200 mL of 100 % methanol and 200 mL of nanopure water at pH 2.0. The cartridge was washed with 300 mL of water (pH 7.0) where sugars and organic

acids were released from the C18. The remaining compounds were eluted with 300 mL of 100% methanol (pH 7) (B). The remaining compounds on the first C18 cartridge were eluted with 300 mL each of 16% acetonitrile (pH 2) (C), ethyl Acetate (D), and 100% methanol (E) to improve recovery of all compounds with polarity and pH differences. All solutions (B-E) were combined and solvent evaporated at 45 °C. The aqueous remains were frozen at – 80 °C and freeze-dried (FTS Systems, INC. Stone Ridge, NY) at -50 °C and 200 mg HG of pressure until powder consistency. The cleaned powders were stored at -20 °C until use.
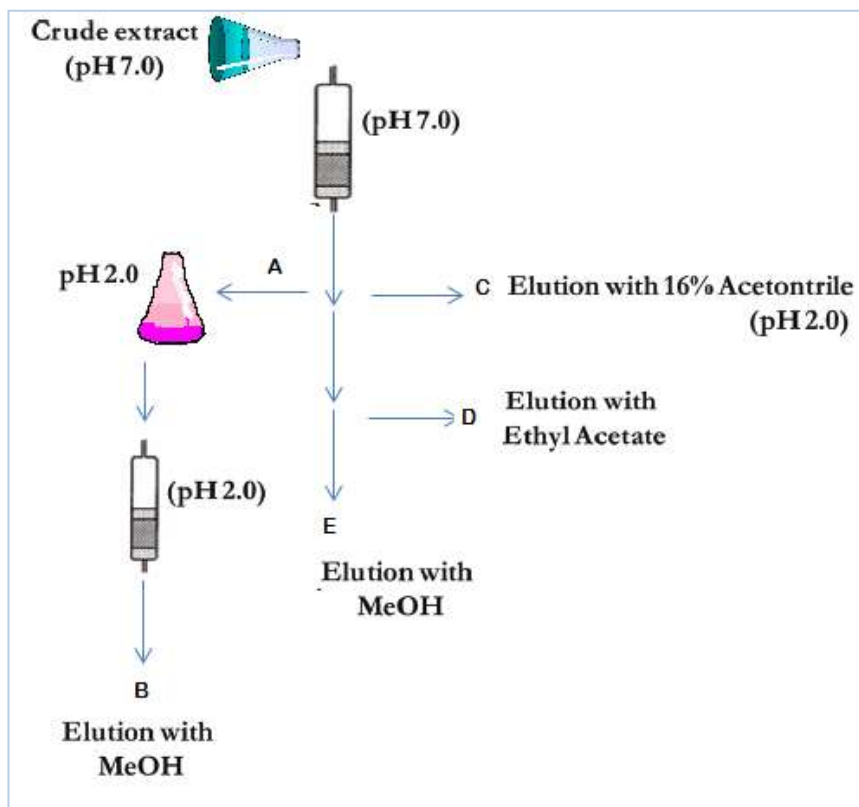

Figure 1: SPE C18 cartridge purification of stone fruit extracts

Thin Layer Chromatography. All TLC assays were done as reported or adapted from Wagner et al (2) except carbohydrate detection reported from Toba and Adach (3). All TLC assays were run on silica gel $60F_{254}$ pre coated plates (Merck, Darmstadt). Powdered stone fruit samples were solubilized in methanol as a 3% solution and 10 µL solutions were used for plating. 5-10 µL of reference solutions were used for plating. For flavonoid detection an ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) mobile phase was used. Plates were sprayed with natural products reagent (NP/PEG) and fluorescence was observed under UV-365. Reference solutions of chlorogenic acid, quercitin and rutin were prepared as 0.05% solutions in methanol. For carbohydrate detection a propanol: water (85:15) mobile

phase was used. Plates were sprayed with DAAP reagent and the plate heated to 80 $^{\circ}$C for 5 min. Sugars were observed vis. Reference solutions of D-glucose, sucrose, L-rhamnose, and D-galactose were used. For sterol determination a hexane: ethyl acetate (1:1) mobile phase was used. Plates were sprayed with Liebermann's reagent and heated to 110 $^{\circ}$C for 10 min and viewed under UV 365 nm. A reference solution of beta sistosterol in chloroform was used. For coumarin detection ethyl acetate was used as a mobile phase. Plates were sprayed with 5% ethanolic KOH and viewed under UV 365nm. A reference solution of Angelica Root (*Dang Gue Wei*) in methanol was used. For terpene detection an n-butanol: ethyl acetate: ammonia: water (60:40:1:10) mobile phase was used. Plates were sprayed with an anisaldehylde: sulfuric acid reagent and heated to 100 $^{\circ}$C for 10 min then observed under UV 365nm. A reference solution of *C. Asiatica* extract was used. For saponin detection a chloroform: methanol: water (64:50:10) mobile phase was used. The plates were sprayed with Vanillin-Sulfuric Acid reagent and heated to 110 $^{\circ}$C for 10 min and observed vis. A reference solution of *Quillaja saponaria* (molina) extract was used. For anthocyanin detection an n-butanol: glacial acetic acid: water (40:10:20) mobile phase was used. Plates were observed vis. Reference solutions of cyaniding 3-glucoside; delphinidin chloride, leutonin chloride, and pelargonidin chloride were used. For Arbutin detection an ethyl acetate: formic acid: water (88:6:6) mobile phase was used. Plates were sprayed with 5% ethanolic KOH and observed vis. A reference solution of hydroquinone was used.

Results and Discussion
Thin Layer Chromatography. The detection of sugars, sterols, coumarins, terpenes, and arbutins were not found in the peach, plum or nectarine powders. The main compounds detected in the stone fruit powders were polyphenolics. All three powders when tested for flavonoids showed orange fluorescence under UV light. With fluorescence behavior being structure dependent, orange TLC results suggests glycosides of quercetin, myricetin or luteolin (2). Peach, plum, nectarine, and the chlorogenic acid standard showed bright fluorescent blue characteristic of chlorogenic acid derivatives. The TLC plates for saponins showed some spotting for the three powders with similar TLC behavior but different R_f values from the saponin standard. As the vanillin-sulfuric acid spray reagent is not specific for saponin compounds, some phenolics could also have similar colors. It has been reported that quercetin and rutin derivatives, hyperoside and myricetin have been reported with this assay previously when looking for saponins, especially from plant extracts (2). This information as well as the negative terpene TLC tests tells us that most likely the spotting on the saponin TLC is from the phenolic flavonoids. Peach and nectarine powders were negative for phenolic anthocyanin compounds; the purified plum powder showed anthocyanins, compositely, cyanidin 3-glucoside (figure 2). Also the TLC results for flavonoids and anthocyanins comparing the original fruit extracts to the purified fruit powders confirmed that the detectable phenolics in the original fruit extracts were also present in the purified powders (figure 3).
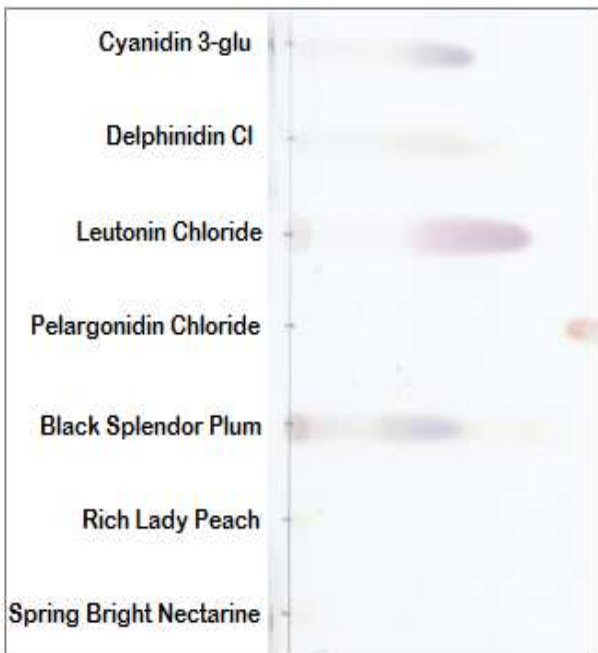
Figure 2: TLC of anthocyanins in stone fruit powders. Black Splendor plum shows presence of cyanidin 3-glucoside and perhaps other anthocyanins not tested on this TLC. Rich Lady peach and Spring Bright nectarine did not show anthocyanins on this TLC. Rich Lady peach can develop red pigments on its skin, but in the powder the amount is too small to detect vis.
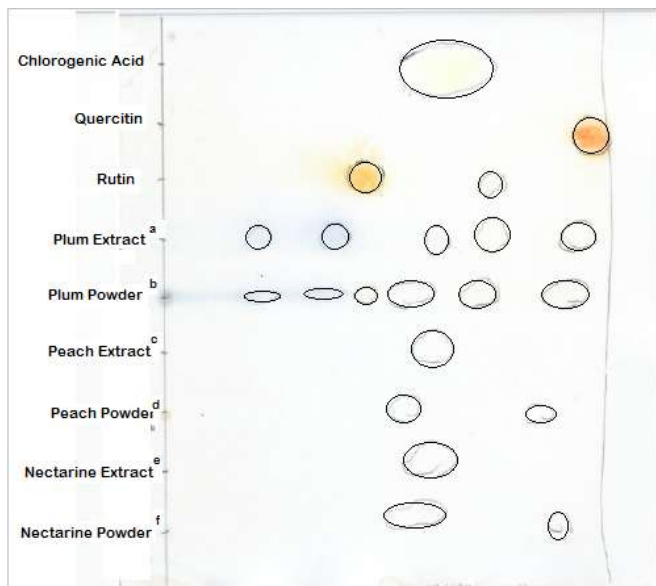
Figure 3: Flavonoid detection TLC of stone fruit extracts and purified powders. [a] Black Splendor plum extract shows presence of anthocyanins (vis. blue), flavonoid derivatives (orange UV 365nm), and chlorogenic acid derivatives (light blue UV 365nm). [b] Black

Splendor plum powder shows the retention of the phenolics from the extract. [c] Rich Lady peach extract show presence of chlorogenic acid derivatives (light blue UV 365nm). [d] Rich Lady peach powder show chlorogenic acid derivatives and a flavonoid derivative. [e] Spring Bright nectarine extract shows chlorogenic acid derivatives (light blue UV 365nm). [f] Spring Bright nectarine powder shows chlorogenic acid derivatives and a flavonoid derivative.

References:

1. Noratto G. Porter W. Byrne D. Cisneros-Zevallos C. Identifying Peach and Plum Polyphenols with Chemo preventive Potential against Estrogen Independent Breast Cancer Cells. *J. Ag. Food Chem*. **2009** 57, 5219-5266
2. Wagner H. Bladt S. Zgainski EM. Plant Drug Analysis A Thin Layer Chromatography Atlas. **1984** Springer-Verlag New York, NY
3. Toba T. dachi S. Specific color reaction for the detection of 1, 2-linked reducing disaccharides on paper and thin-layer chromatograms. *J. of Chromatography*. **1978** 154, 110-112

B) *Stone Fruit Extracts and HPLC Phenolic Profile Characterization*

Materials and Methods

HPLC analysis of stone fruit phenolic profiles. Crude extracts were used to identify the phenolic compounds. They were prepared following the procedure of extraction and purification described previously. 20 µL of filtered sample solution was injected after passing through a 0.22 µm nylon membrane.

Chromatographic separations were performed on a High-Performance Liquid Chromatography_Diode Array Detection (HPLC-DAD). The HPLC system was composed of two 515 binary pumps, a 717-plus autosampler, and a 996-photodiode array detector (Waters Corp., Mildford, MA). Phenolic compounds were separated on a 4.6mm×150 mm, 5 µm, C-18 reverse-phase Atlantis column (Waters Corp.) that was maintained at 25 ℃ by a SpectraPhysics SP8792 column heater. The mobile phases consisted of 1% formic acid water (phase A) and acetonitrile (phase B) at a flow rate of 1 mL/min. Separations were achieved by a linear gradient with A and B: 0 min 85% A, 5 min 85% A, 30 min 0% A, 35 min 0% A. The injection volume was 10 µl. Data was processed with the Millennium software v3.1. The identification of individual phenolics was based on their UV spectra and retention time.

HPLC analysis of purified powder phenolic profiles. The identification was performed by HPLC using 3.0 mg powder dissolved in 1200 uL DMSO and filtered with a 0.22 µm nylon filter. 20 µL of filtered sample solution was injected to the chromatograph system. HPLC conditions used are those described above.

Results and Discussion

Identification of Phenolics in Black Splendor plum, Rich Lady peach and Spring Bright nectarine with High-Performance Liquid Chromatography Diode Array Detection (HPLC-DAD). HPLC analysis showed 13 compounds in Black Splendor plum (dark red skin, yellow flesh), including 2 chlorogenic acid derivates, 2 anthocyanins, 6 flavonoids and 3 procyanidin derivates (Table 1, Figures 1). In Rich Lady peach, 11 compounds were identified, including 2 chlorogenic acid derivates, 4 flavonoids and 3 procyanidin derivates (Table 2, Figures 2). For Spring Bright nectarine, we found 5 compounds, including 2 chlorogenic acid derivates and 2 procyanidin derivates (Table 3, Figures 3).

These results indicate all stone fruits tested have procyanidin derivates. It also shows large variation of the phenolic profiles with plums having more categories of phenolics than peach and nectarines. Only plum had anthocyanins.

Identification of phenolic profile of purified powder extracts with High-Performance Liquid Chromatography Diode Array Detection (HPLC-DAD). Using HPLC we identified 14 compounds in Black Splendor purified powder extracts, including 2 chlorogenic acid derivate, 2 anthocyanins, 6 flavonoids and 4 procyanidin derivates (Table 4, Figure 4). Interestingly, in Rich Lady peach purified powder extract, no compound was detected (Figures 5). For Spring Bright nectarine purified powder there were 4 compounds, including 2 chlorogenic acid derivates and 2 procyanidin derivates (Table 5, Figures 6).

In general, for Black splendor plum, the profile of the purified powder closely resembles that of the crude extract. The only difference is that the flavonoid derivates are enriched in the purified powder. This was also confirmed by the color of the purified powder solutions which had a lighter color (pink) compared to the color of crude extract (light purple).

The Rich lady peach crude extract showed some peaks of chlorogenic acid derivates, flavonoids and procyanidin derivates while the chromatogram of the purified powder did not have any peak. One possible reason to this difference could be the solubility of the powder, which despite using DMSO it still showed insoluble clusters of powder after centrifugation. It is likely that the freeze drying step created an artifact enhancing the insolubility of the purified phenolics.

In Spring Bright nectarine, a few peaks were detected in the crude extract and the purified powder solution showed peaks with higher intensity. It seems that the phenolics in Spring Bright nectarine fruit were enriched in the purified powder.

Despite that in plums and nectarines phenolics were detected in the powder extracts, we also observed insoluble clusters of powder in DMSO at higher concentrations after centrifugation.

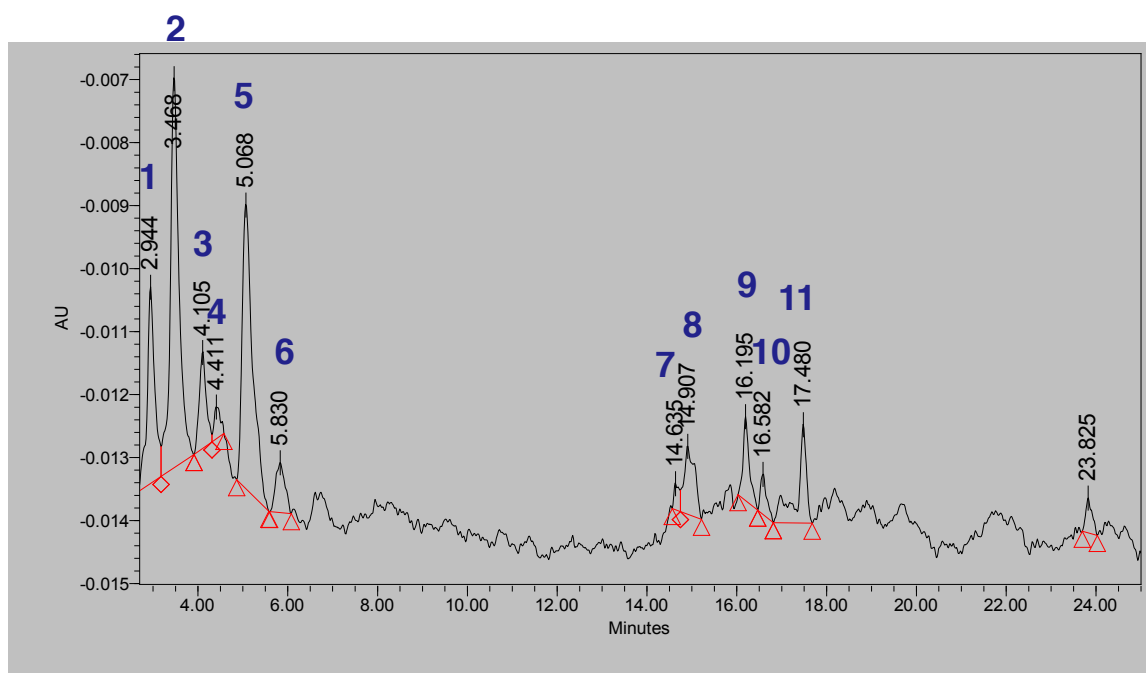**Table 1.** Phenolic profile in Black Splendor plum crude extract

No.	t_R	λ_{max}	Compound
1	2.869	232, 278	procyanidin
2	3.471	232, 280, 328	Neo-chlorogenic acid
3	4.604	233, 278	catechin
4	5.260	233, 279, 330	chlorogenic acid
5	6.339	233, 279, 517	cyanidin 3-*O*-glucoside
6	7.138	233, 279, 519	cyanidin 3-*O*-rutinoside
7	7.756	233, 280	*epi*-catechin
8	14.458	237, 352	rutin
9	14.826	238, 352	quercetin 3-*O*-glucoside
10	15.416	239, 347	quercetin-*O*-pentoside
11	15.789	239, 347	quercetin *O*-pentosylepentoside
12	16.077	239, 351	quercetin-*O*-pentoside
13	16.548	240, 353	quercetin-*O*-rhamaose
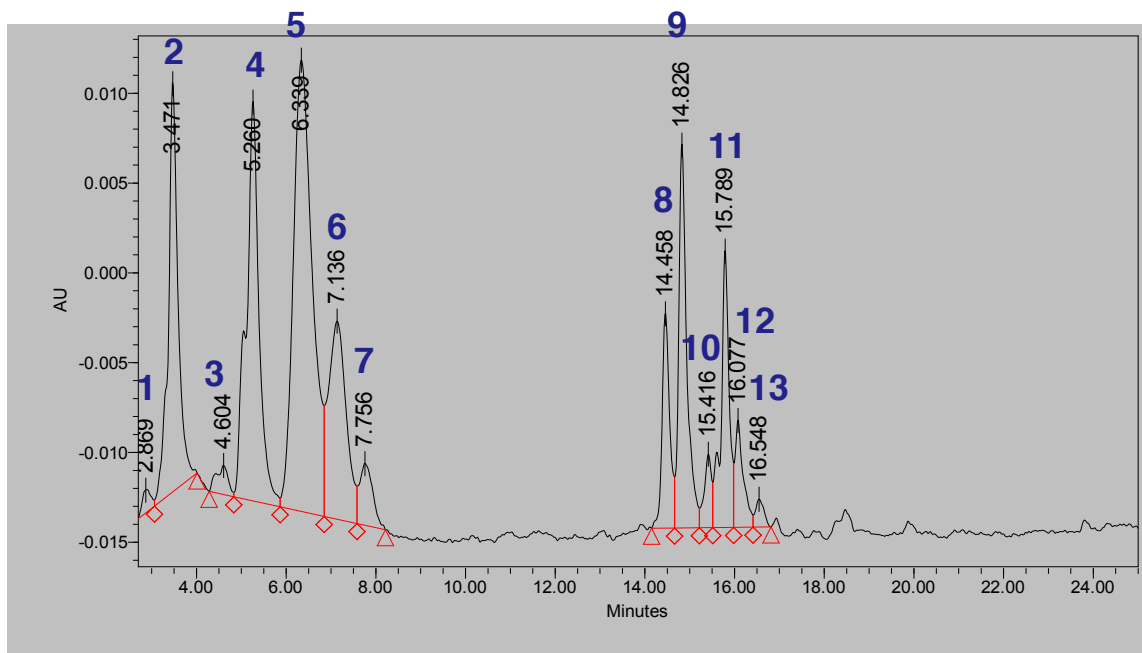
Figure 1. HPLC Chromatogram of Black Splendor plum crude extract at 320 nm

**Table 2.** Phenolic profile in Rich Lady peach crude extract

No.	t_R	λ_{max}	Compound
1	2.944	232, 278	procyanidin
2	3.468	232, 280, 328	Neo-chlorogenic acid
3	4.105	233, 278	catechin
4	4.111	232, 278	procyanidin
5	5.068	233, 279, 330	chlorogenic acid
6	5.830	232, 278	procyanidin
7	14.635	237, 352	rutin
8	14.907	238, 352	quercetin 3-*O*-glucoside
9	16.195	239, 351	flavonoid
10	16.582	240, 353	flavonoid
11	17.480	240, 353	flavonoid

Figure 2. HPLC Chromatogram of Rich Lady peach crude extract at 320 nm

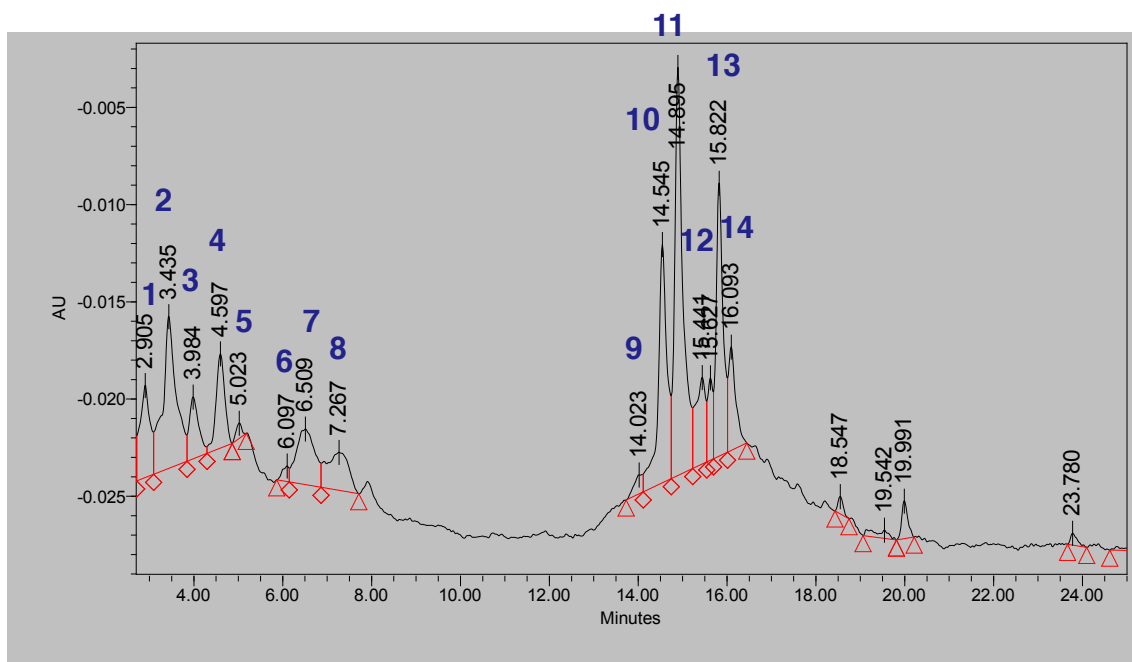**Table 3.** Phenolic profile in Spring Bright nectarine crude extract

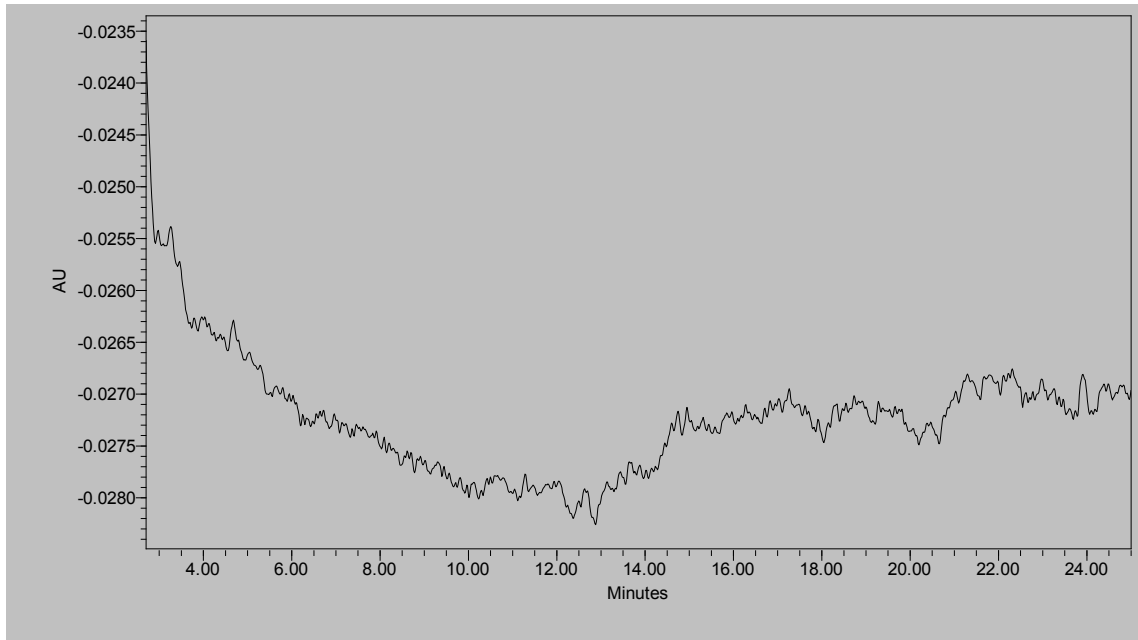| No.	t_R	λ_{max}	Compound
1	2.450	232, 278	procyanidin
2	3.474	232, 280, 328	Neo-chlorogenic acid
3	4.475	233, 278	catechin
4	4.673	232, 278	procyanidin
5	5.066	233, 279, 330	chlorogenic acid
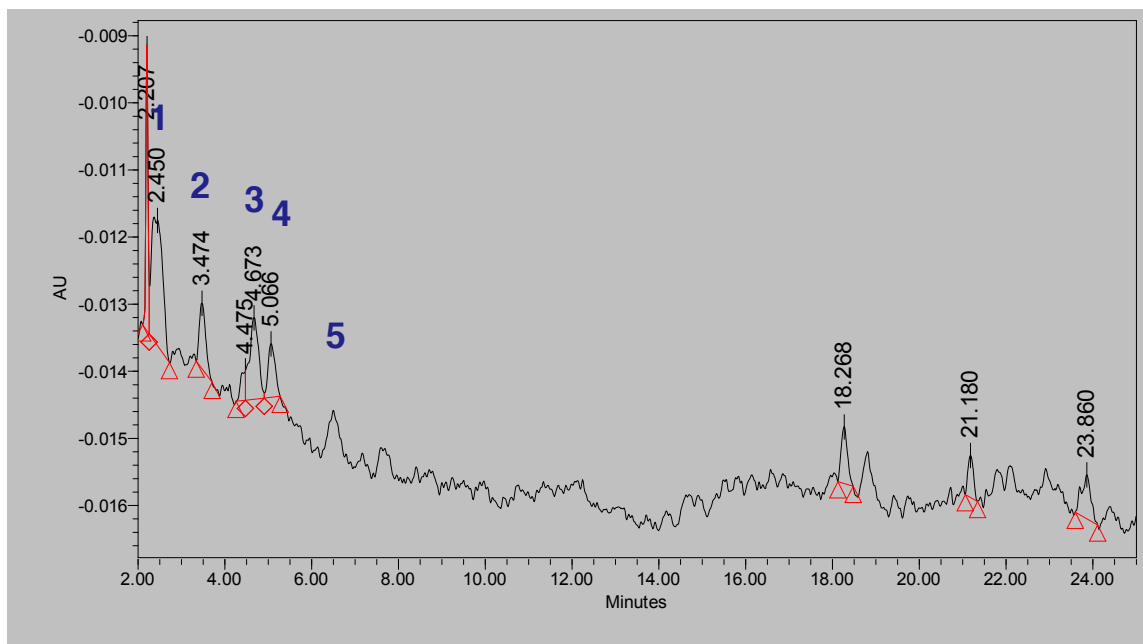
Figure 3. HPLC Chromatogram of in Spring Bright nectarine crude extract at 320 nm

Table 4. Phenolic profile in Black Splendor plum powder in DMSO

| No.	t_R	λ_{max}	Compound
1	2.905	232, 278	procyanidin
2	3.435	232, 280, 328	Neo-chlorogenic acid
3	3.984	233, 278	catechin
4	4.597	233, 279, 330	chlorogenic acid
5	5.023	232, 278	procyanidin
6	6.509	233, 279, 517	cyanidin 3-*O*-glucoside
7	7.267	233, 279, 519	cyanidin 3-*O*-rutinoside
8	7.925	233, 280	*epi*-catechin
9	14.545	237, 352	rutin
10	14.895	238, 352	quercetin 3-*O*-glucoside
11	15.441	239, 347	quercetin-*O*-pentoside
12	15.627	239, 347	quercetin *O*-pentosylepentoside
13	15.822	239, 351	quercetin-*O*-pentoside
14	16.093	240, 353	quercetin-*O*-rhamaose

Figure 4. HPLC Chromatogram of Black Splendor plum powder in DMSO at 320 nm

Figure 5. HPLC Chromatogram of Rich Lady peach powder in DMSO at 320 nm

Table 5. Phenolic profile in Spring Bright nectarine powder in DMSO

No.	t_R	λ_{max}	Compound
1	3.522	232, 280, 328	Neo-chlorogenic acid
2	4.481	233, 278	catechin
3	4.737	232, 278	procyanidin
4	5.141	233, 279, 330	chlorogenic acid
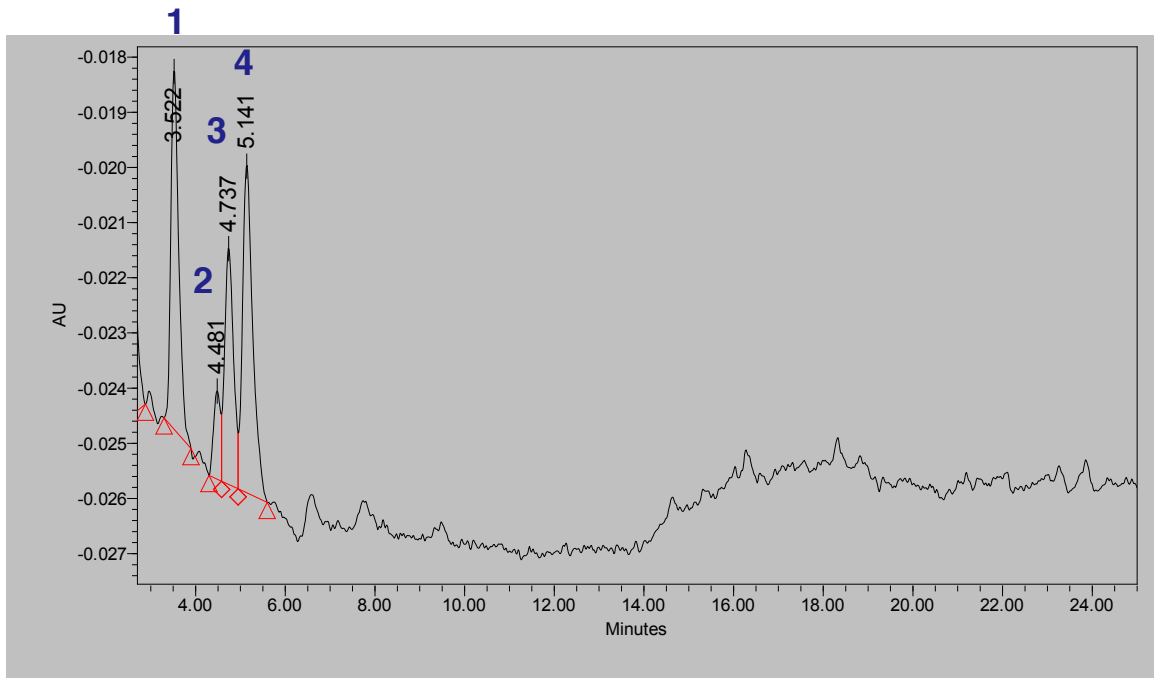
Figure 6. HPLC Chromatogram of in Spring Bright nectarine powder in DMSO at 320 nm

2) The effect of Stone Fruit Extracts on Glucose Insensitivity of Pancreatic beta cells (beta-TC6)

Materials and Methods

Materials: The following chemicals were used in the experiments: Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), D-glucose, trypsin-EDTA, DMSO were acquired from SIGMA (St. Louis, MO), and sodium bicarbonate from Mallinckrodt Chemicals (Phillipsburg, NJ). Beta-TC6 pancreatic cells were acquired from the American Type Culture Collection (ATCC) (Manassas, VA). Penicillin-Streptomycin was purchased from Invitrogen (Carlsbad, CA),

Cell viability assay: Cytotoxicity effects of plum, peach and nectarine extracts were evaluated in beta-TC6 pancreatic cells using the MTS assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. The assay was performed in a 96-well plate with a density 30,000 cells/well. The pancreatic cells were left with DMEM 1mM glucose and 15% FBS medium for 24 h before starting the treatments. The treatments were added at concentrations of 0 to 300 μg/mL, and the cell viability was measured at 24h. Solutions of every powder extract were dissolved in DMSO 100%.

Measurement of reactive oxygen species production: 30,000 beta-TC6 cells were placed in 96-well black and clear bottom plates (Costar) with growth medium (DMEM, 1mM glucose and 15% FBS). Cells were cultured for an additional day. For the reactive oxygen species (ROS) assay, cell culture was washed with warm PBS and then incubated with 5 μM DCFA in DMEM 1mM glucose phenol red-free medium at 37 C for 30 min. At the end of the incubation period, the pro-inflammatory cytokine TNF-α was added at 2 ng/mL (final concentration) and incubated at 37 °C for 90 min. At this point of the protocol the fluorescence (initial time) was measured and the different extracts added at 37 °C for 120 min (Final time). At the end of the incubation period the fluorescence was measured in a microplate reader.

Amount of %ROS = ((Sample $_{Final\ time}$- Sample $_{initial\ time}$) / (control $_{final\ time}$-control $_{initial\ time}$)) x 100

Quantification of insulin secretion: 30,000 beta-TC6 cells were placed in 96-well plates with growing medium (DMEM, 1mM glucose and 15% FBS). Cells were cultured for an additional day. For glucose insensitivity induction, medium was replaced with fresh medium and 0.5 mM palmitic acid was added and incubated for 48 h. After this incubation period plum, peach and nectarine extracts were added during 16 additional hours in the growing medium. In all cases, the medium was removed on the day of the experiment, and the cells washed twice with warm KRB medium (119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl$_2$, 1.19 mM MgS0$_4$, 1.19 mM KH$_2$P0$_4$, 25 mM NaHC0$_3$, 10 mM HEPES [pH 7.4], 0.1 g BSA). The cells were then incubated with 200 μL of KRB at 37 °C for 60 min. At the end of the incubation period, cells were washed with KRB medium. The cells were then incubated with KRB containing 20 mM glucose for 1 h at 37 °C. The supernatant was collected and insulin

was measured by ELISA using mouse insulin as a standard (Alpco Diagnostics, Windham, New Hampshire, United States) following the manufacturer's indication.

Statistical analysis: The data were analyzed using one-way analysis of variance (ANOVA) followed by t-test, using the software JMP v9.0. Results are expressed as means ± standard deviations (S.D) of at least 8 replicates. Different letters show significant differences ($P < 0.05$).

Results and Discussions

Effect of plum, peach and nectarine extracts on pancreatic beta cell cytotoxicity.
To determine the effects of stone fruit extract on cell viability in pancreatic beta cells, the MTS assay was used and performed as described in materials and methods. The screening with plum extract determined significant cytotoxicity effects at concentrations higher than 200µg/mL (Fig. 1). While in peach and nectarine extract, the reduction of the cell viability on pancreatic cells was found at concentrations of 200 and 300µg/mL (Fig. 1). Based on these results the range of concentrations selected to continue the studies of ROS and Insulin secretion assays were those showing no differences with the control.
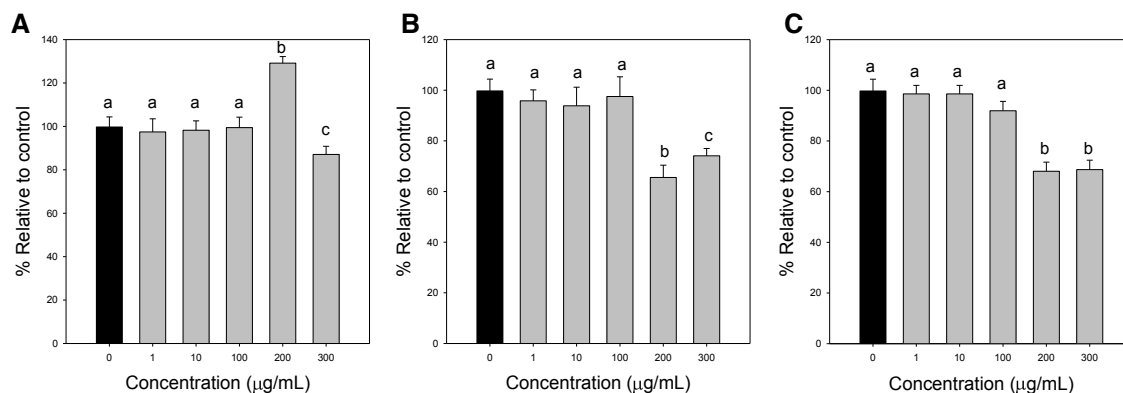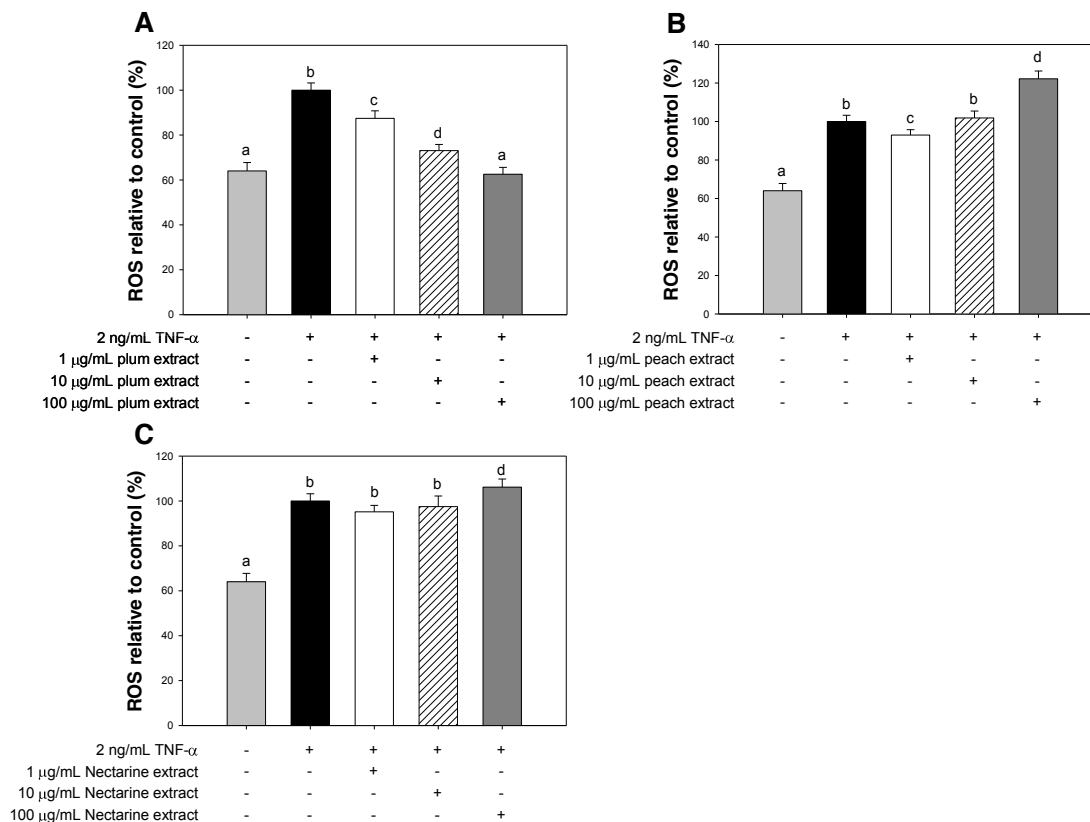

Figure 1. Effect of Plum (A), peach (B) and nectarine (C) extract on cell cytotoxicity in pancreatic beta cells. Cells were incubated with each extract between 0-300 µg/mL for 24 h at 37 °C in a humidified 5% CO_2 incubator. Reported values are relative means ± S.D. compared to the control (n= 8). Different letters show significant differences ($P < 0.05$).

Effect of plum, peach and nectarine extract on ROS generation in pancreatic beta cells.
Tumor necrosis factor-α (TNF-α) is a major mediator of inflammation and is an inductor in the amount of intracellular reactive oxygen species in different kind of cell cultures (1, 2, 3). We investigated whether these extracts have an antioxidant potential on pancreatic beta cells and determined a significant decrease ($P<0.05$) in the amount of intracellular ROS induced

by TNF-alpha (2 ng/mL) in pancreatic cells exposed to plum extract in all of the concentrations (Fig. 2). However we did not find changes in the amount of intracellular ROS in pancreatic cells exposed to peach and nectarine extracts.
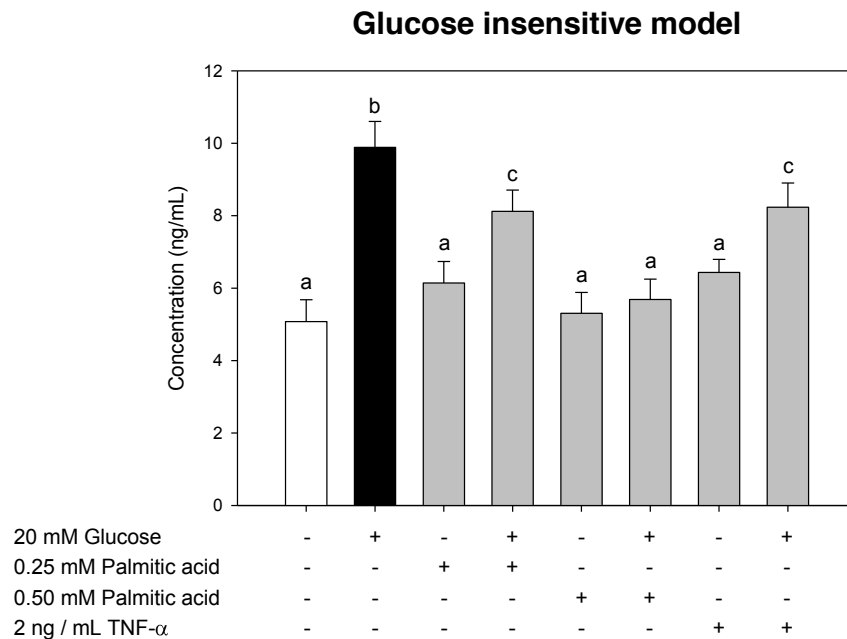

Figure 2. Effect of plum (A), peach (B) and nectarine (C) extract on ROS generation in pancreatic beta cells. Pancreatic cells were pretreated with 5 μM DCFH for 30 min at 37 °C. Cells were then treated with TNF-α for 90 min and then incubated with different concentrations of each extract for 120 min. Reported values are relative means ± S.D. (n= 8). Different letters show significant differences (P < 0.05).

Induction of a glucose insensitivity model on pancreatic beta cells

Type 2 Diabetes is associated with obesity and is characterized by insulin resistance and pancreatic β-cell dysfunction (glucose insensitivity). It is accepted that prolonged exposure to circulatory free fatty acids (FFA) and the accumulation of lipids at sites other than adipose tissue, contributes to diabetes development (4, 5). Our first step was to determine a glucose insensitive model on pancreatic cells and its insulin secretion. For this objective, pancreatic beta cells were incubated with palmitic acid and TNF-α, both diabetes inductors, during 48 h

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(Fig. 3) and then stimulated with glucose, the principal stimulus for insulin secretion. Our results showed that beta cells exposed to 0.5 and 0.25 mM of palmitic acid and 2ng/mL of TNF-α reduce significantly its response to glucose, decreasing the insulin secretion (Fig. 3). Among the treatments tested, 0.5 mM palmitic acid was selected because it did not show major differences between beta cells stimulated with or without glucose recreating a glucose insensitivity state in the cell. This model was used to determine if plum, peach and nectarine extracts could reverse the glucose insensitivity state.
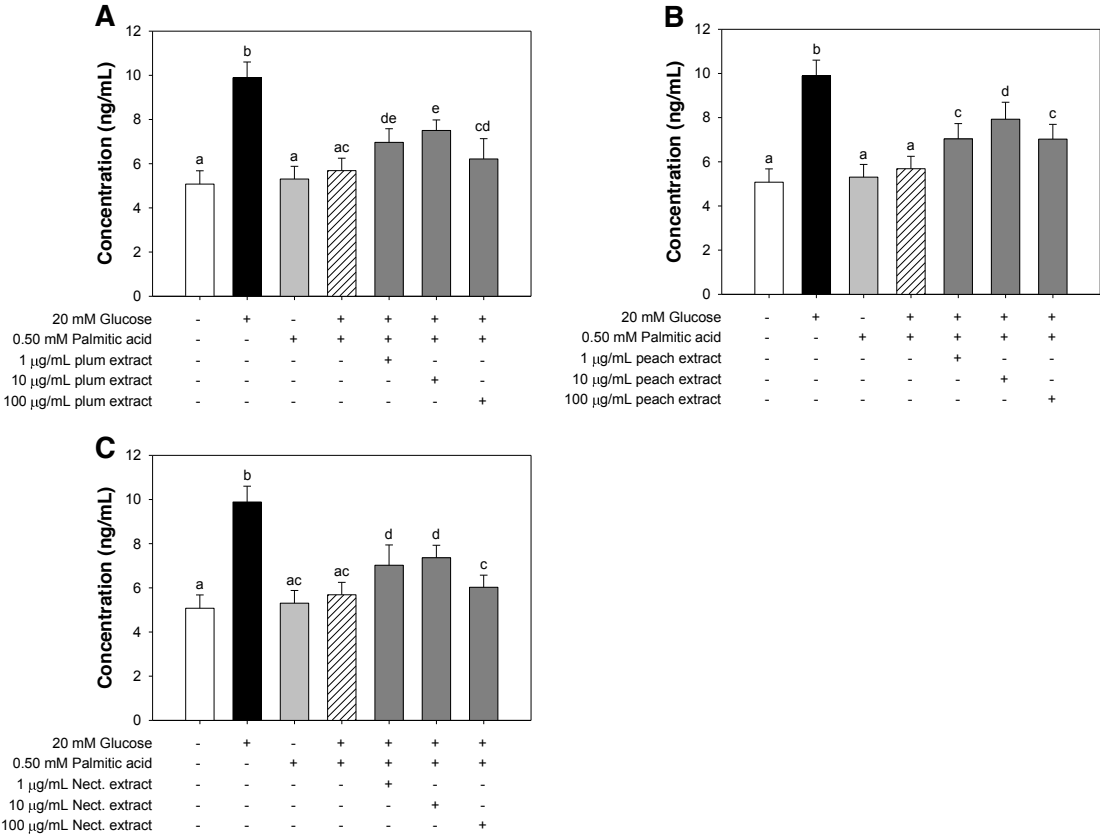
Glucose insensitive model

20 mM Glucose	-	+	-	+	-	+	-	+
0.25 mM Palmitic acid	-	-	+	+	-	-	-	-
0.50 mM Palmitic acid	-	-	-	-	+	+	-	-
2 ng / mL TNF-α	-	-	-	-	-	-	+	+

Figure 3: Effect on insulin secretion of palmitic acid and TNF-α and generation of the glucose insensitive model. Pancreatic cells were treated with both diabetes inductors for 48 h at 37 °C and then incubated with 20mM glucose for 60 min. Reported values are relative means ± S.D. (n= 8). Different letters show significant differences (P < 0.05).

Effect of plum, peach and nectarine extract on insulin secretion in glucose insensitive pancreatic beta cells

To investigate whether the stone fruit extracts could revert on pancreatic beta cells the glucose insensitivity model generated in the study, we analyzed the insulin secretion, the main marker in beta cell dysfunction. Our results demonstrated that after 48 h with palmitic acid and a subsequent incubation for 16 h with palmitic acid plus each stone fruits extract, there was a significant increase in the insulin secretion at concentrations of 1 and 10μg/mL for plum and peach extract relative to beta cells exposed to palmitic acid and glucose (Control glucose insensitive model). However, the treatment with higher concentration for any stone fruit extract (100μg/mL) did not show significant changes relative to the glucose insensitive control (Fig. 4). These results suggest a concentration response-specific for each

stone fruit extract used. It also confirms that the extracts improve the insulin secretion in glucose insensitive pancreatic beta cells as observed in Type 2 Diabetes (Fig. 5).

Figure 4. Effect of plum (A), peach (B) and nectarine (C) extracts on insulin secretion in glucose insensitive pancreatic beta cells. Pancreatic cells were pretreated with 0.5 mM palmitic acid for 48 h at 37 °C. Cells were then treated with palmitic acid and stone fruit extracts for 16 h and then incubated with 20mM glucose for 60 min. Reported values are relative means ± S.D. (n= 8). Different letters show significant differences (P < 0.05).

The insulin secretion in normal and glucose insensitive pancreatic beta cells are described in Fig 5. The models allows to propose possible mechanisms by which stone fruit extracts reverse the glucose insensitivity state of pancreatic beta cells.
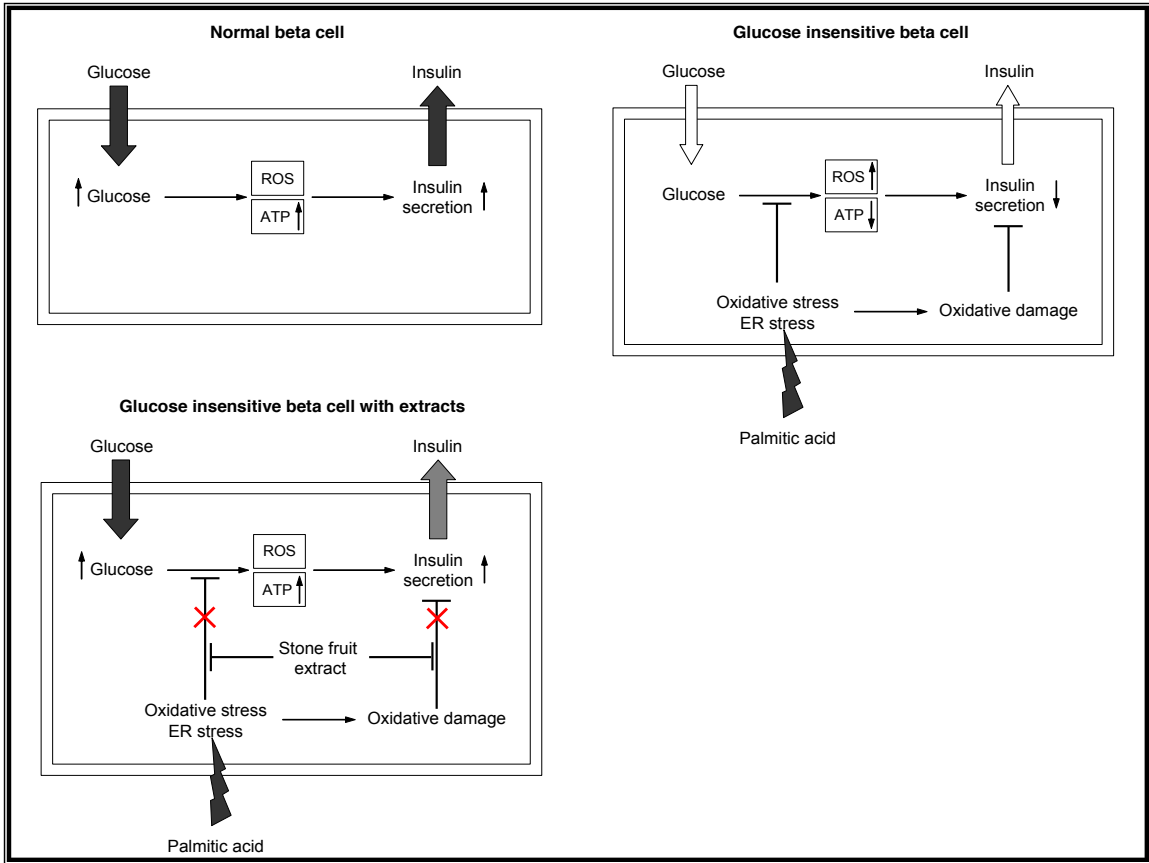
Figure 5. Diagram of insulin secretion of normal, glucose insensitive and reversed pancreatic beta cells presented in this study.

In Figure 5 we observe how insulin secretion is activated in normal beta cells, where glucose the main physiological stimulus for insulin secretion, produces ROS slightly and ATPs (6, 7). In the glucose insensitive beta cell induced by palmitic acid there is increasing oxidative stress and ROS, this process induces the endoplasmic stress and oxidative damage inhibiting the ATP synthesis and the insulin secretion (8, 9, 10). When stone fruit extracts are used in the glucose insensitive beta cells, the insulin secretion is partially recovered (Fig. 4), reversing the glucose insensitivity process generated with palmitic acid. However, the processes by which each stone fruit extract elicits its reversal effect may be different. For example, only the plum extract decreased the amount of intracellular ROS (Fig. 2), while peach and nectarine did not show an antioxidant potential. This would suggest that plum extracts increase the insulin secretion through an antioxidant (AOX) mechanism, while peach and nectarine extracts increase insulin secretion through an AOX- independent mechanism.

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3) The Effect of Stone Fruit Extracts on Insulin Resistance of Hepatic cells (AML 12)

Materials and Methods

Materials. The following chemicals were used in the experiments: palmitic acid, dexamethasone, insulin, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, penicillin/streptomicyn mixture, trypsin EDTA, DMSO and Fetal Bovine Serum (FBS) were purchased from Sigma (St. Louis, MO), glucose from Acros Organics (Fair Lawn, NJ) and sodium bicarbonate from Mallinckrodt Chemicals (Phillipsburg, NJ). Murine AML12 cells were acquired from the American Type Culture Collection (ATCC) (Manassas, VA). Insulin-transferrin-selenium supplement was purchased from Gibco (Grand Island, NY).

Cell viability assay. Cytotoxic effects of extracts from peach, plum and nectarine were evaluated in AML12 hepatic cells using the MTS assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. The quantity of formazan product was measured at 490 nm and is directly proportional to the number of living cells in culture. The assay was performed in a 96-well plate with a density of 21,000 cells/well. AML12 cells were left with DMEM-HAM'S F12/10% FBS medium supplemented with ITS mixture for 24 h before starting the treatments with the stone fruits extracts. Previously to their use, the extracts from peach, plum and nectarine were diluted in 100% DMSO. The concentrations used were in the range of 0 to 300 µg/mL, and the cell viability was measured after 24 h of exposure to the extracts. The final percentage of DMSO per well was 0.5%.

Quantification of intracellular lipid content. Intracellular lipid content was measured using a commercially available kit (AdipoRed, LonzaWakersville, Inc., Wakersville, MD). AdipoRed is a solution of the Nile Red stain, which fluoresces and enables the quantification of intracellular lipid droplets. To evaluate the intracellular fat accumulation, AML12 cells were cultured in 12-well plates using DMEM-HAM'S F12/10% FBS medium supplemented with ITS mixture, until 100% confluency. Medium was replaced every 2 - 3 days. Once cells were confluent, 0.5 mM palmitic acid and three different concentrations (0, 1, 10 and 100 µg/mL) of extracts from peach, plum and nectarine were added and co-incubated during 48 h. After this period of time, Adipored assay was performed following the manufacturer's instructions.

Generation of an insulin-resistance model and treatment with stone fruits extracts. AML12cells were seeded at a density of 41,000 cells/well in 6-well plates and cultured with DMEM-HAM'S F12/10% FBS medium supplemented with ITS mixture for several days until they reached 100% confluency. Medium was replaced every 2-3 days. When cells reached confluency, 0.5 mM palmitic acid was added for 48 h to induce insulin-resistance. After exposure to palmitic acid, the culture medium was replaced with fresh serum free-DMEM-HAM'S F12 medium supplemented with ITS, and the extracts from peach, plum and nectarine were added separately in concentrations of 0, 1, 10 and 100 µg/mL, and co-incubated in presence of 0.5 mM palmitic acid for 16 h. DMSO was added to control cells

until 0.5%. After the treatments with stone fruit extracts and palmitate, the cells were exposed to 100 nM of insulin for 60 min, and cell samples were recovered on ice using G6P assay buffer from PicoProbe™ Glucose-6-phosphate assay kit (BioVision. Catalog #K687-100). Proteins were precipitated with TCA and all the samples were centrifuged at 14,000 g for 2 min. After centrifugation, supernatants were recovered and the pH was neutralized with NaOH until pH $6.5 - 7.5$.

Intracellular Glucose-6-phosphatequantification in AML12 cells. Cells were cultured and treated with palmitic acid and stone fruit extracts under the conditions described above. To measure the intracellular amount of Glucose-6-phosphate, the PicoProbe™ Glucose-6-phosphate assay kit (BioVision. Catalog #K687-100) was used, following the manufacturer's instructions. Briefly, neutral pH samples were placed in duplicated in a 96-well plate (black body, clear bottom, COSTAR) and incubated at 37°C, protected from light, with the reaction mix for 30 min. A glucose-6-phosphate standard curve was measured together with the samples, under the same conditions. Once the incubation time was over, the amount of glucose-6-phosphate was measured in a microplate reader at Ex/Em = 485/560. Data was calculated according the manufacturer's instructions.

Statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by t-test, using the software JMP v9.0. Results are expressed as means ± standard deviations (S.D) of at least 3 replicates. Different letters denotes significant differences ($p < 0.05$).

Results and Discussions

### Effect of stone fruits extracts on cell viability
To carry out the experiments to measure the intracellular fat accumulation and quantify intracellular concentrations of glucose-6-phosphate, it is necessary to determine the range of concentrations to use with the stone fruits extracts, without altering the cell viability. To reach this goal, MTS assay was performed as described in materials and methods. Results showed after 24 h of treatment with the stone fruits extracts, a significant increase in the cell numbers (or alternatively higher mitochondrial activity), confirming that peach and plum extracts do not exhibit a cytotoxic effects in AML12 cells (Figure 1, A, C respectively). On the other hand, nectarine extract exhibited a cytotoxic effect at 300 µg/mL (Figure 1), reducing the cell viability in 35.6% respect to the control.

Effect of stone fruits extracts over intracellular fat accumulation
Non-alcoholic fatty liver disease (NAFLD) is becoming an important health problem in recent years, affecting different populations around the world. This pathology usually is associated with obesity, Type 2 Diabetes, and metabolic syndrome (1, 2, 3), probably as a result of contemporary lifestyle and nutritional habits (4). Several studies have shown potential properties of natural extracts and compounds to reduce the incidence of this disease and even reverse it (5, 6, 7, 8, 9). Based on that evidence, the present study was focused in evaluating a possible effect of stone fruits extracts against NAFLD or Type 2 Diabetes. To evaluate a possible effect against NAFLD, AML12 cells were treated with palmitic acid and

peach, plum and nectarine extracts, and then, the intracellular fat accumulation was measured using Adipored assay. Results showed that control cells exposed to palmitic acid for 48 h accumulate ~ 8 times more intracellular fat compared to control cells non-exposed to palmitic acid (Figure 2, 3). Treatments with stone fruits extracts at concentrations of 1-10 µg/mL did not alter the intracellular fat accumulation induced by palmitic acid, however at 100 µg/mL there was a significant increase for all extracts indicating that these stone fruit extracts do not reverse NAFLD (Figure 2).
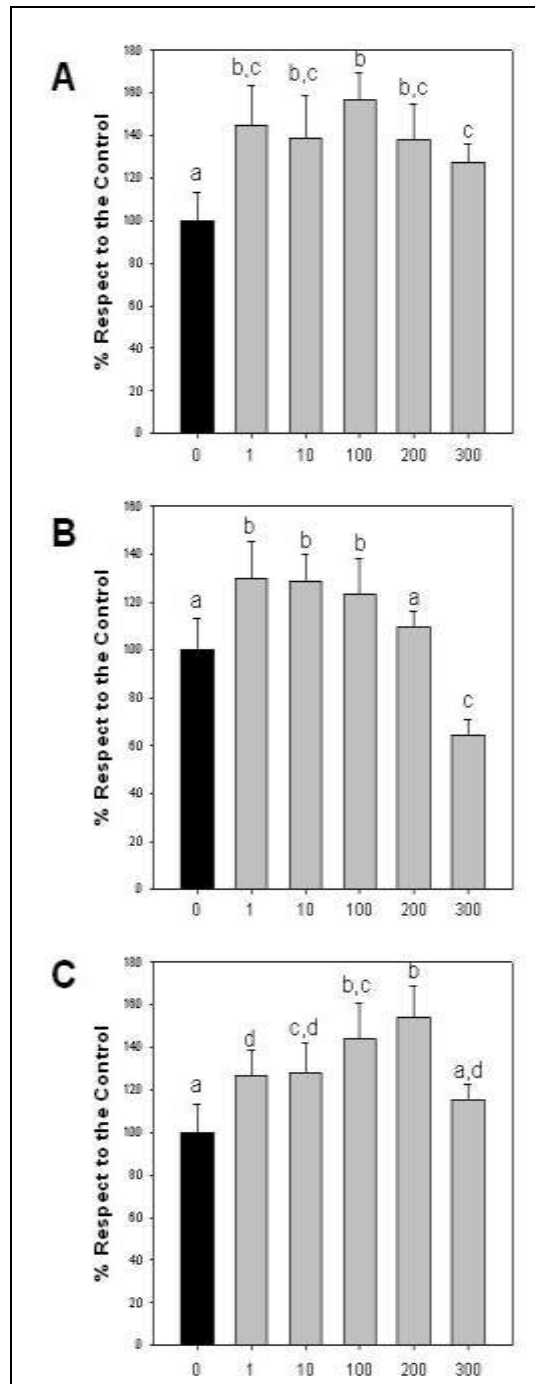
Figure 1. Evaluation of cytotoxic effects of stone fruit extracts on hepatic cells. Cells were exposed during 24 h to extracts from peach (A), nectarine (B) and plum (C), and the cytotoxic effect was measured using the MTS assay. Data are means ± S.D. (n= 8). Different letters denotes significant differences by ANOVA (p < 0.05).
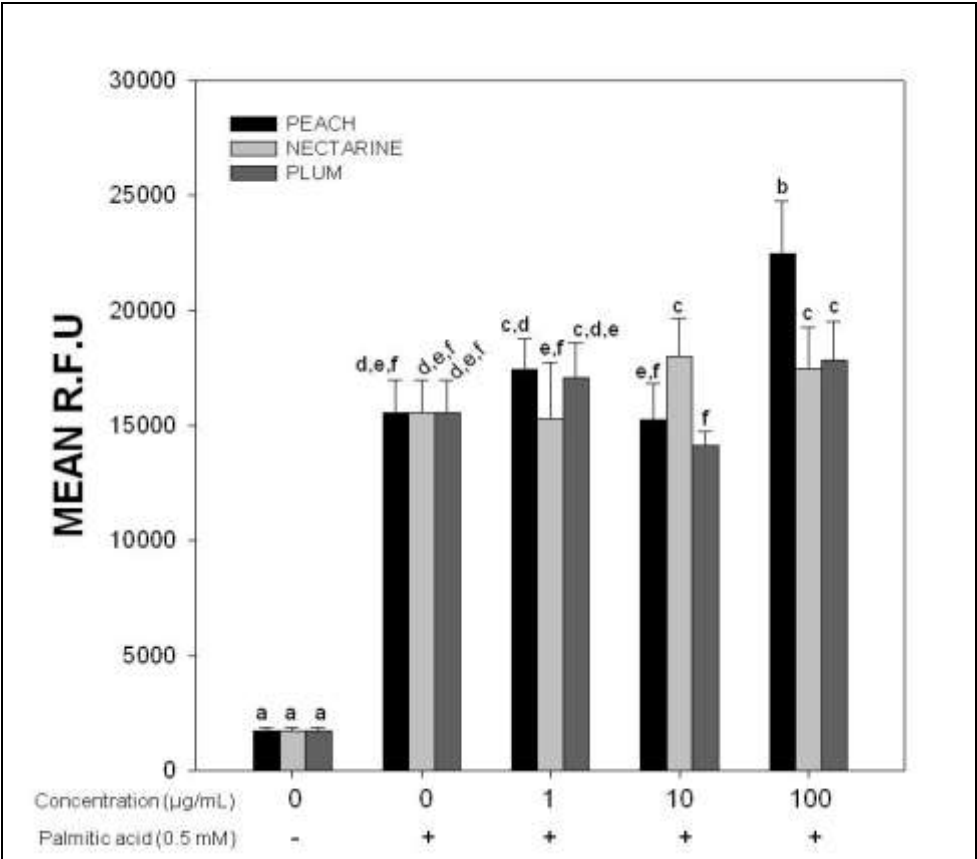
Figure 2. Evaluation of the effect of stone fruits extracts on intracellular fat accumulation on AML12 hepatic cells. Cells were co-incubated with peach, nectarine and plum extracts for 48 h in presence of 0.5 mM of palmitic acid. Intracellular fat accumulation was determined using the Adipored assay. Data represent means ± S.D. (n= 6). Different letters denotes significant differences by ANOVA (p < 0.05).
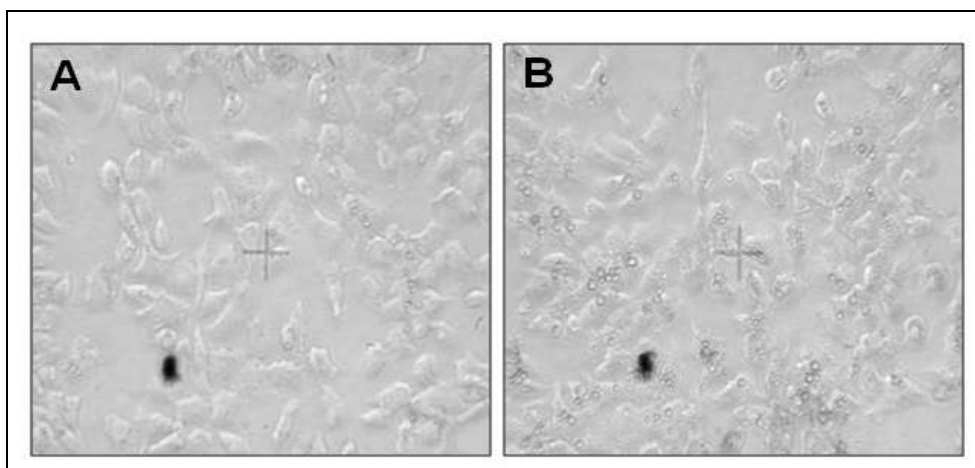
Figure 3. Intracellular fat accumulation in hepatic cells (AML12). Hepatic cells non-exposed to palmitic acid contain small amounts of intracellular lipid droplets (A). After 48 h of incubation with 0.5 mM of palmitic acid (B), large lipid droplets are clearly visible in an optical microscope.

Effects of stone fruits extracts on insulin-resistant AML12 hepatic cells

One of the objectives of this study was to evaluate the effects of stone fruits extracts against Type 2 Diabetes. The approach used to evaluate this effect, was the measurement of the intracellular concentration of glucose-6-phosphate (G-6-P), which is the direct product of the activity of glucokinase (GK), a key enzyme in charge of the first reaction of the glucose metabolism: the ATP-dependent phosphorylation of glucose into glucose-6-phosphate. This glucose-6-phosphate could be used in different pathways, but in liver, it will be mainly utilized for the formation of glycogen. The convertion of glucose into glycogen, is one of the key pathways by which the liver removes glucose from the portal vein after a meal (post-prandial state) (10). In the post-absorptive state, the hepatic glucose 6-P content is ~ 0.1 nmol/mg of protein and it increases 2-fold in a post-prandial state (11, 12). This increase reflects the balance between increased production by glucose phosphorylation and decreased production by suppression of glycogenolysis. In Type 2 Diabetes conditions, the mechanism to keep the glucose homeostasis is working deregulated, and previous studies have shown that GK activity is decreased in morbidly obese subjects with Type 2 Diabetes (13), as well as glycogen synthesis, which is parallel to GK activity (14). On the other hand, several studies suggest that activators of GK could be used as a new alternative for the treatment of Type 2 Diabetes (15, 16, 17, 18).

The first challenge for the present study was the generation of a model in hepatic cells, able to mimic the conditions in Type 2 Diabetes, for a subsequently evaluation of the effects of stone fruit extracts on the insulin-resistant hepatic cells. To do so, normal hepatic cells were treated with palmitic acid, to induce an insulin-resistance state in these cells, based on previous studies that have shown that several saturated fatty acids inhibit the insulin action in hepatic cells (19, 20, 21, 22). With the purpose of testing the insulin-resistant hepatic cell model, cells were treated with insulin to determine if they are able to generate a response. The effect of insulin in these cells was evaluated through the quantification of the intracellular concentration of G-6-P, which depends directly of the amount and activity of GK. Despite that insulin does not stimulate glucose uptake in hepatic cells (22), insulin is the

primary up-regulator of the GK gene promoter, and its effect is mediated by the phosphatiodylinositol-3-kinase (PI3K)/protein kinase B (PKB or AKT) pathway, in a glucose concentration independent manner (23). Moreover, specific activation of the GK gene could be as early as 30 min after insulin exposure (24).

Our results show that in control cells exposed to insulin, the intracellular concentration of G-6-P was increased ~ 3 times, compared with control cells non-exposed to insulin, while in cells treated with palmitic acid, the levels of G-6-P remain similar to control cells non-exposed to insulin (Figure 4). These results suggest that the increase on the concentration of G-6-P could be a consequence of the increase in the amount of GK present in the cells, triggered by insulin and moreover, confirm that exposure to palmitic acid of hepatic cells induce an insulin resistance state. Once the cellular model was developed, the extracts from peach, plum and nectarine were tested to evaluate a possible "insulin sensitizing" effect. Our results showed that only the plum extract was able to significantly increase the intracellular concentration of G-6-P in a concentration dependent manner, reaching similar levels to control cells exposed to insulin (Figure 5). This suggests that this extract is able to increase either the amount of GK or its activity, and for that reason, this extract could be useful as a potential treatment to revert insulin resistance in Type 2 Diabetes patients.
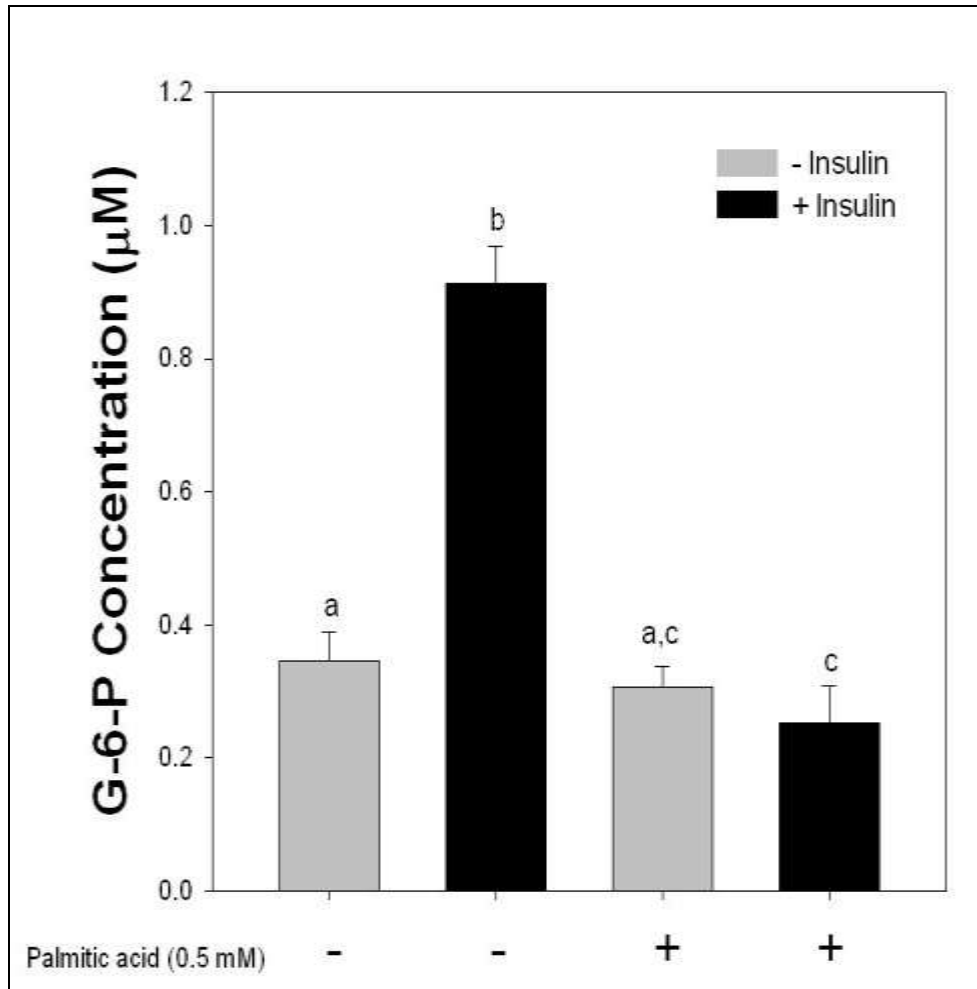
Figure 4. Effect of Palmitic acid on intracellular glucose-6-phosphate accumulation on hepatic cells. Cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and exposed to 100 nM of insulin for 60 min. Control cells were not exposed to palmitic acid. Glucose-6-phosphate was determined using a commercial kit. Bars are means ± S.D. (n= 3). Different letters denotes significant differences by ANOVA ($p < 0.05$).
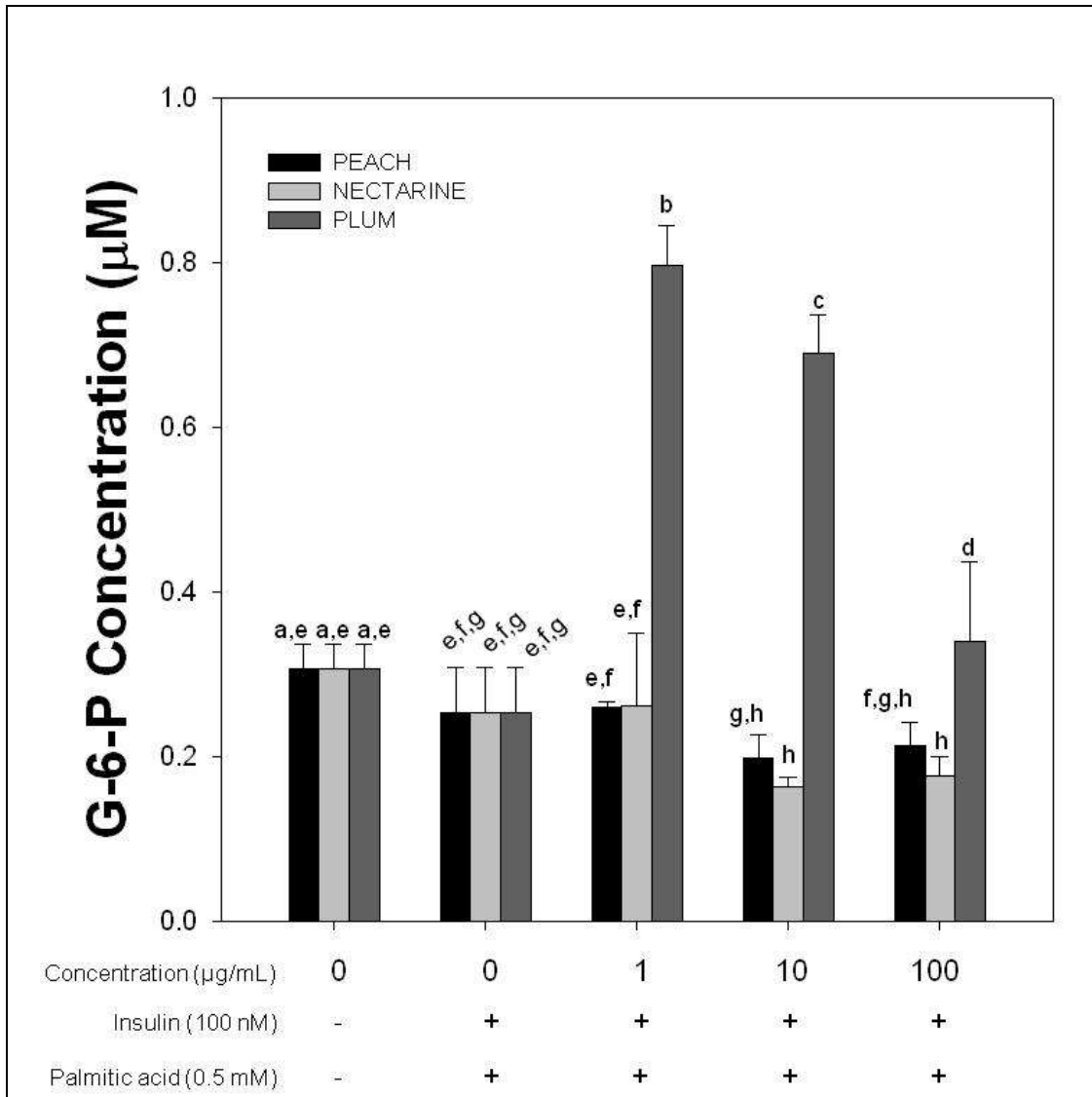
Figure 5. Effect of stone fruits extracts on intracellular glucose-6-phosphate accumulation on hepatic cells. Cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and treated with extracts from peach, plum and nectarine for aditional 16 h, and exposure to 100 nM of insulin for 60 min. Samples were collected inmediatly after exposure to insulin. Glucose-6-phosphate was determined using a commercial kit. Bars are means ± S.D. (n= 3). Different letters denotes significant differences by ANOVA ($p < 0.05$).
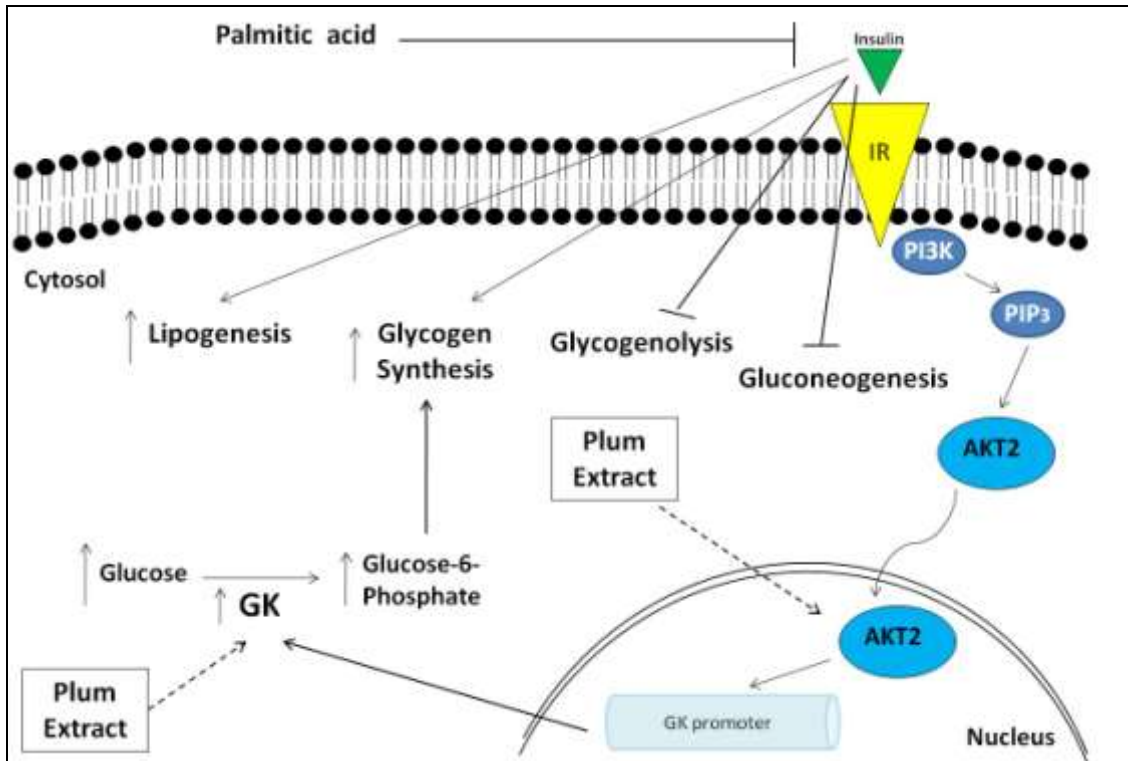
Figure 6. Mechanism of action of insulin in hepatic AML12 cells. In hepatic cells, insulin is able to stimulate lipogenesis and glycogen synthesis, besides it inhibits glycogenolysis and gluconeogenesis thus regulating fasting glucose levels. Moreover, insulin activates AKT and this transcription factor is able to induce the gene expression of the glucokinase (GK), increasing its levels quickly. GK phosphorylates glucose generating glucose-6-phosphate, which will be stored in the form of glycogen. Accordingly, the plum extract could act directly over the activity of GK or it could induce the gene expression of this enzyme, and both changes lead to an increase in the concentration of intracellular glucose-6-phosphate.

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4) The Effect of Stone Fruit Extracts on Insulin Resistance of Muscle cells (C2 C12)

Materials & Methods

Materials. The following chemicals were used in the experiments: palmitic acid, insulin,**1,1-Dimethylbiguanide hydrochloride** (metformin), Low Sugar-Dulbecco's Modified Eagle's Medium, penicillin/streptomicyn mixture, trypsin EDTA, DMSO, BSA and Fetal Bovine Serum (FBS) were purchased from Sigma (St. Louis, MO), glucose from Acros Organics (Fair Lawn, NJ) and sodium bicarbonate from Mallinckrodt Chemicals (Phillipsburg, NJ). Murine C2C12 cells were acquired from the American Type Culture Collection (ATCC) (Manassas, VA).

Cell viability assay. Cytotoxic effects of extracts from peach, plum and nectarine were evaluated in C2C12 muscle cells using the MTS assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. The quantity of formazan product was measured at 490 nm and is directly proportional to the number of living cells in culture. The assay was performed in a 96-well plate with a density of 7,500 cells/well. C2C12 cells were left with Low sugar DMEM/10% FBS medium for 24 h before starting the treatments with the stone fruits extracts. Previously to their use, extracts from peach, plum and nectarine were diluted in 100% DMSO. The concentrations used were in the range of 0 to 300 µg/mL, and the cell viability was measured after 24 h of exposure to the extracts. The final percentage of DMSO per well was 0.5%.

Generation of an insulin-resistance model and treatment with stone fruits extracts. C2C12 cells were seeded at a density of 120,000 cells/well in 6-well plates and cultured with Low sugar DMEM/10% FBS medium until they reached around 70% confluency. Medium was replaced every 2-3 days. Once the cells were confluent, they were cultured with low sugar DMEM/10% Horse serum medium during four days, to induce myoblast formation. To the myoblast, 0.5 mM palmitic acid was added for 48 h to induce insulin-resistance. After exposure to palmitic acid, the culture medium was replaced with fresh serum free-2% BSA-Low sugar-DMEM medium, and the extracts from peach, plum and nectarine were added separately in concentrations of 0, 1, 10 and 100 µg/mL, and co-incubated in presence of 0.5 mM palmitic acid for 16 h. DMSO was added to control cells until 0.5%. 2.0 mM of **1,1-**

Dimethylbiguanide hydrochloride (metformin) was used as a positive control. After the treatments with stone fruit extracts, metformin and palmitic acid, the cells were exposed to 100 nM of insulin for 30 min, and cell samples were recovered on ice using G6P assay buffer from PicoProbe™ Glucose-6-phosphate assay kit (BioVision. Catalog #K687-100). Proteins were precipitated with TCA and all the samples were centrifuged at 14,000 g for 2 min. After centrifugation, supernatants were recovered and the pH was neutralized with NaOH until pH 6.5 – 7.5.

Intracellular Glucose-6-phosphatequantification in C2C12 muscle cells. Cells were cultured and treated with palmitic acid and stone fruit extracts under the conditions described above. To measure the intracellular amount of Glucose-6-phosphate, the PicoProbe™ Glucose-6-phosphate assay kit (BioVision. Catalog #K687-100) was used, following the manufacturer's instructions. Briefly, neutral pH samples were placed in duplicated in a 96-well plate (black body, clear bottom, COSTAR) and incubated at 37°C, protected from light, with the reaction mix for 30 min. A glucose-6-phosphate standard curve was measured together with the samples, under the same conditions. Once the incubation time was over, the amount of glucose-6-phosphate was measured in a microplate reader at Ex/Em = 485/560. Data was calculated according the manufacturer's instructions.

Statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by t-test, using the software JMP v9.0. Results are expressed as means ± standard deviations (S.D) of at least 3 replicates. Different letters denotes significant differences ($p < 0.05$).

Results and Discussions

**Effect of stone fruits extracts on cell viability**
Before determining the effect of the stone fruit extracts on the accumulation of intracellular G-6-P, it was necessary to evaluate if the extracts could elicit a cytotoxic effect in muscle cells. After 24 h of exposure to stone fruits extracts, the cell viability was determined using the MTS assay. Results showed that peach extract slightly increased the cell number at 1 and 10 μg/mL, while at concentrations > 100 μg/mL it induced cell cytotoxicity (Figure 1 A). For nectarine extracts, concentrations > 100 μg/mL showed a cytotoxic effect, reducing cell viability by about 40% (Figure 1 B). On the other hand, plum extract did not exhibit a cytotoxic effect until a concentration of 300 μg/mL (Figure 1 C). Based on the data obtained, the extract concentration range determined to use in this cell line, without altering cell viability was from 0 to 100 μg/mL.
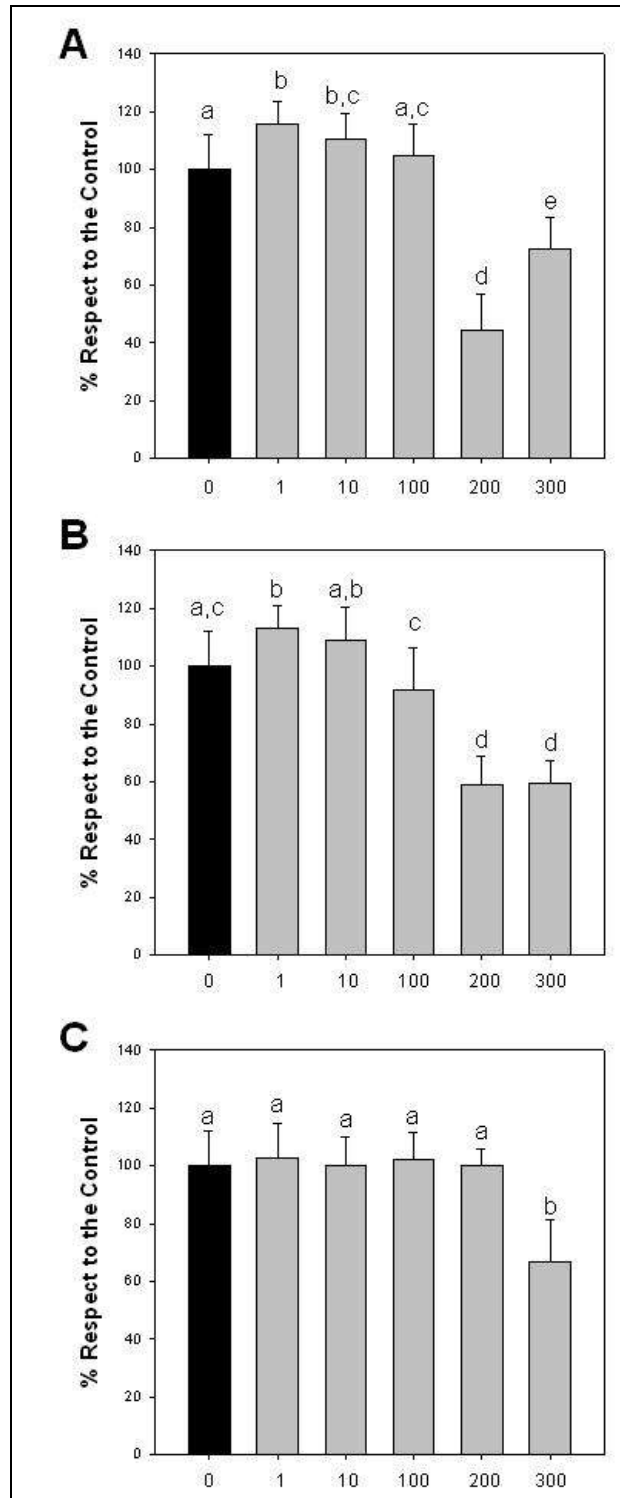
Figure 1. Evaluation of cytotoxic effects of stone fruit extracts on muscle cells. Cells were exposed during 24 h to extracts from peach (A), nectarine (B) and plum (C) and the cytotoxic effect was measured using the MTS assay. Data are means ± S.D. (n= 8). Different letters denotes significant differences by ANOVA (p < 0.05).

Differentiation of C2C12 cells into myoblast and development of a model of insulin-resistant muscle cells

The first step in the experiments with muscle cells, is to induce the differentiation of the cells into myotubes, trying to emulate the behavior of a real skeletal muscle. After four days of treatment with horse serum, the formation of myotubes is complete (Figure 2), and the cells are ready to be used in further experiments.
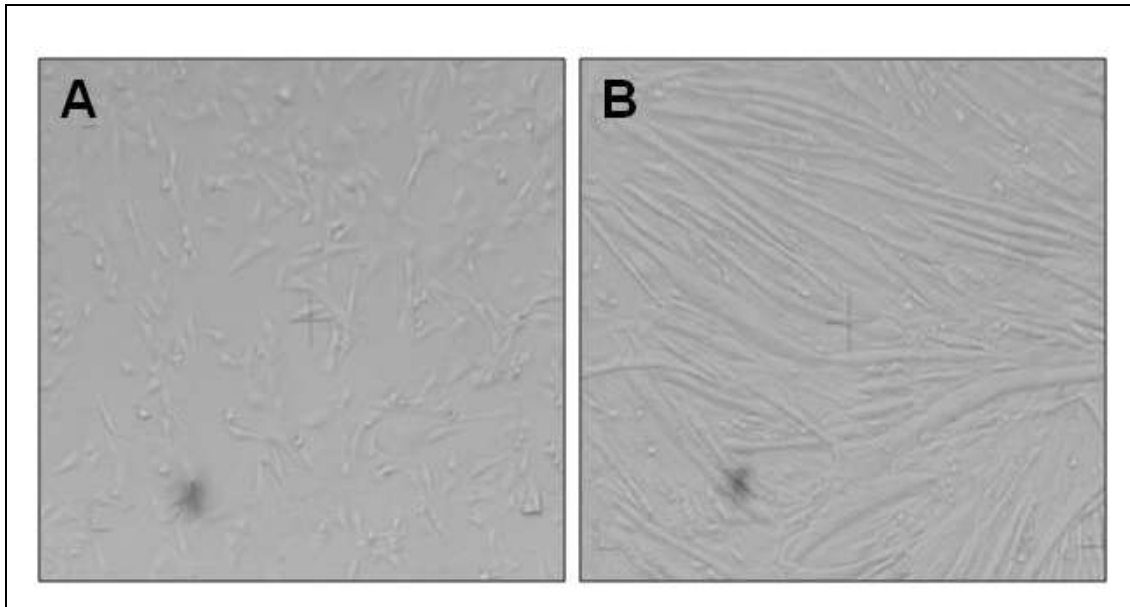
Figure 2. Differentiation of C2C12 cells into myotubes. C2C12 cells were cultured in medium supplemented with FBS until 70% confluency (A), then the cells were cultured during 4 days in medium supplemented with horse-serum to induce the fusion of the cells and formation of the myotubes (B).

Once C2C12 cells complete their differentiation into myotubes, the induction of an insulin-resistance state was done by using palmitic acid. Similar to the AML12 hepatic cells, previous studies have shown that different free fatty acids are able to induce insulin resistance in muscle cells (1, 2, 3, 4, 5), reducing its ability to perform glucose uptake. The main objective of this study was to evaluate a possible anti-insulin-resistant effect of stone fruits extracts, with the purpose of finding a possible alternative treatment for Type 2 Diabetes by using these extracts.

1,1-Dimethylbiguanide hydrochloride or metformin, is a well studied compound, used as a treatment for Type 2 Diabetes, because is able to induce the glucose uptake in skeletal muscle cells via AMPK pathway (6, 7, 8). The idea to incorporate the use of metformin in the experiments of quantification of intracellular concentration of G-6-P, was to test if the insulin-resistant model generated in the myotubes was working properly, in addition to obtaining a reference value for the stone fruits extracts. Our data obtained in relation to the development of an insulin-resistant condition in the myotubes, showed that there is an increase in the basal intracellular concentration of G-6-P in myotubes treated with palmitic

acid, at levels comparables to control cells after 30 min of exposure to insulin. Despite this result was unexpected, there are previous reports indicating that palmitic acid acutely induce the activation of AKT, and consequently an increase in the glucose uptake (9). This extra-glucose present on the cells could be rapidly transformed into G-6-P, by activation of the glucokinase, increasing its intracellular concentration. Nevertheless, palmitic acid treated cells (insulin-resistant cells) exposed to insulin are not able to increase the glucose uptake and therefore the intracellular concentration of G-6-P remains unchanged compared to palmitic acid treated cells non-exposed to insulin (Figure 3). On the other hand, metformin is able to partially reverse the inhibitory effect of palmitic acid in the myotubes, increasing by 22.3% the intracellular concentration of G-6-P, which is dependent of an increase of the glucose uptake (Figure 3). As expected, control cells exposed to insulin showed an increase (44.69%) of the intracellular concentration of G-6-P compared to control cells non-exposed to insulin (Figure 3). Based in all this data, the possible insulin-sensitizing effect of the stone fruits extracts was tested using this cellular model.

**Effect of stone fruits extracts on muscle cells**
The stone fruits extracts were tested in the insulin-resistant myotubes described above. Results showed that plum extract had no effect on insulin-resistant myotubes, maintaining the concentration of G-6-P similar to the control cells (Figure 4). On the other hand, nectarine extract showed a similar effect to metformin at 10 µg/mL, increasing the concentration of G-6-P (Figure 4). Similarly, the peach extract at 1 µg/mL also showed an insulin-sensitizing effect (Figure 4). The fact that nectarine extract at 10 µg/mL and peach extract at 1 µg/mL are able to increase the intracellular concentration of G-6-P at levels comparables with metformin, a known anti-diabetes compound, suggest that these extracts have the potential to be used as the basis for developing anti-diabetes drugs in the future. However, the mechanism of action for these extracts is not well elucidated and should be studied in more detail. The observed results for both extracts could be consequences of an increase of the glucose uptake or an increase in the activity or amount of the enzyme glucokinase, which is responsible of the transformation of glucose into glucose-6-phosphate. In figure 5, is depicted a model of the possible interaction between palmitic acid, insulin, peach and nectarine extracts in insulin-resistant myotubes.
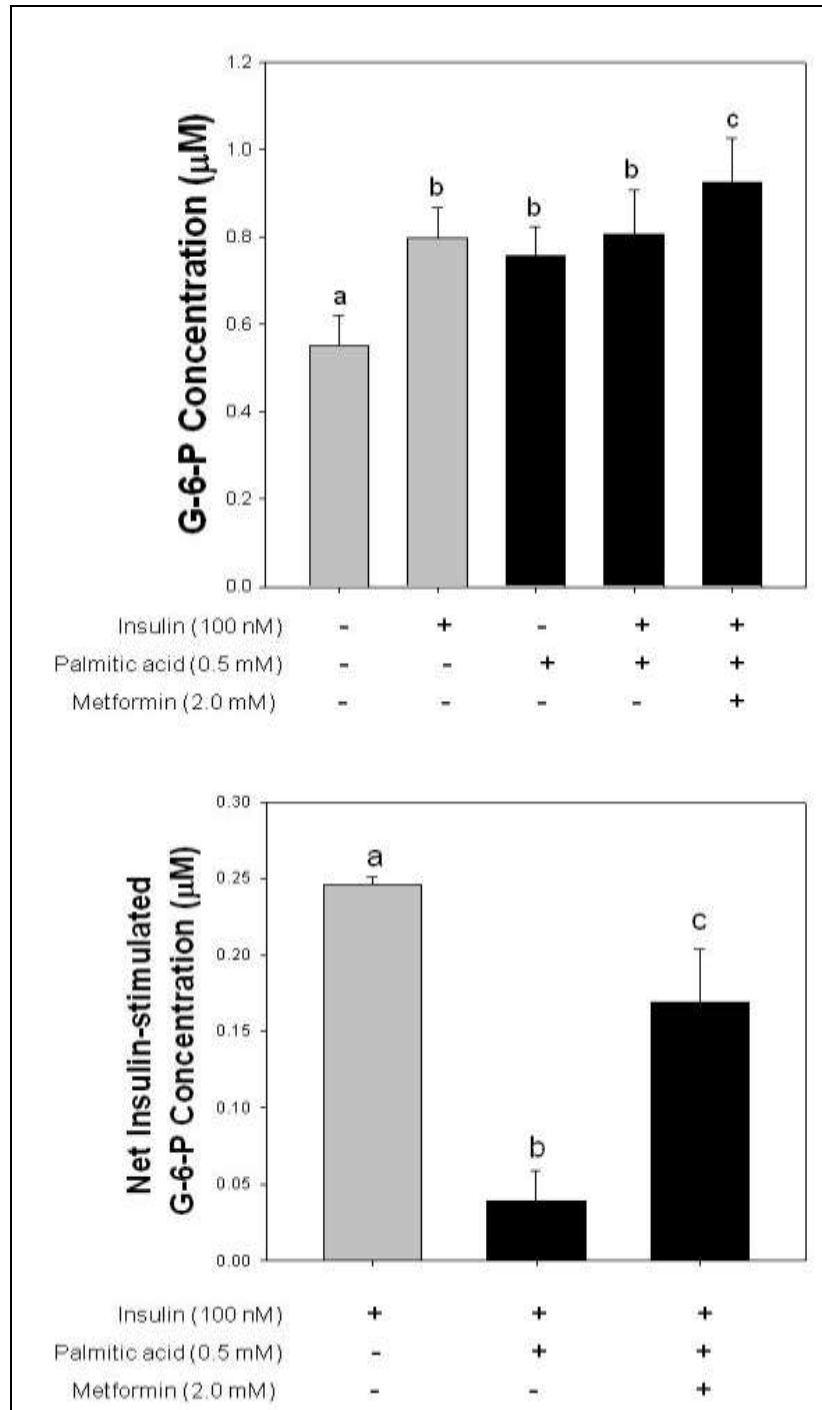
Figure 3. Effect of Palmitic acid and insulin on glucose-6-phosphate accumulation in muscle cells. (A) Cells were cultured in absence or presence of 0.5 mM of palmitic acid during 48 h and exposed to 100 nM of insulin for 30 min to determine the intracellular concentration of G-6-P. In (B) is depicted the increase in G-6-P concentration using as reference control cells non-exposed to insulin. Bars represent means ± S.D. (n = 3). Different letters denotes significant differences by ANOVA (p < 0.05).
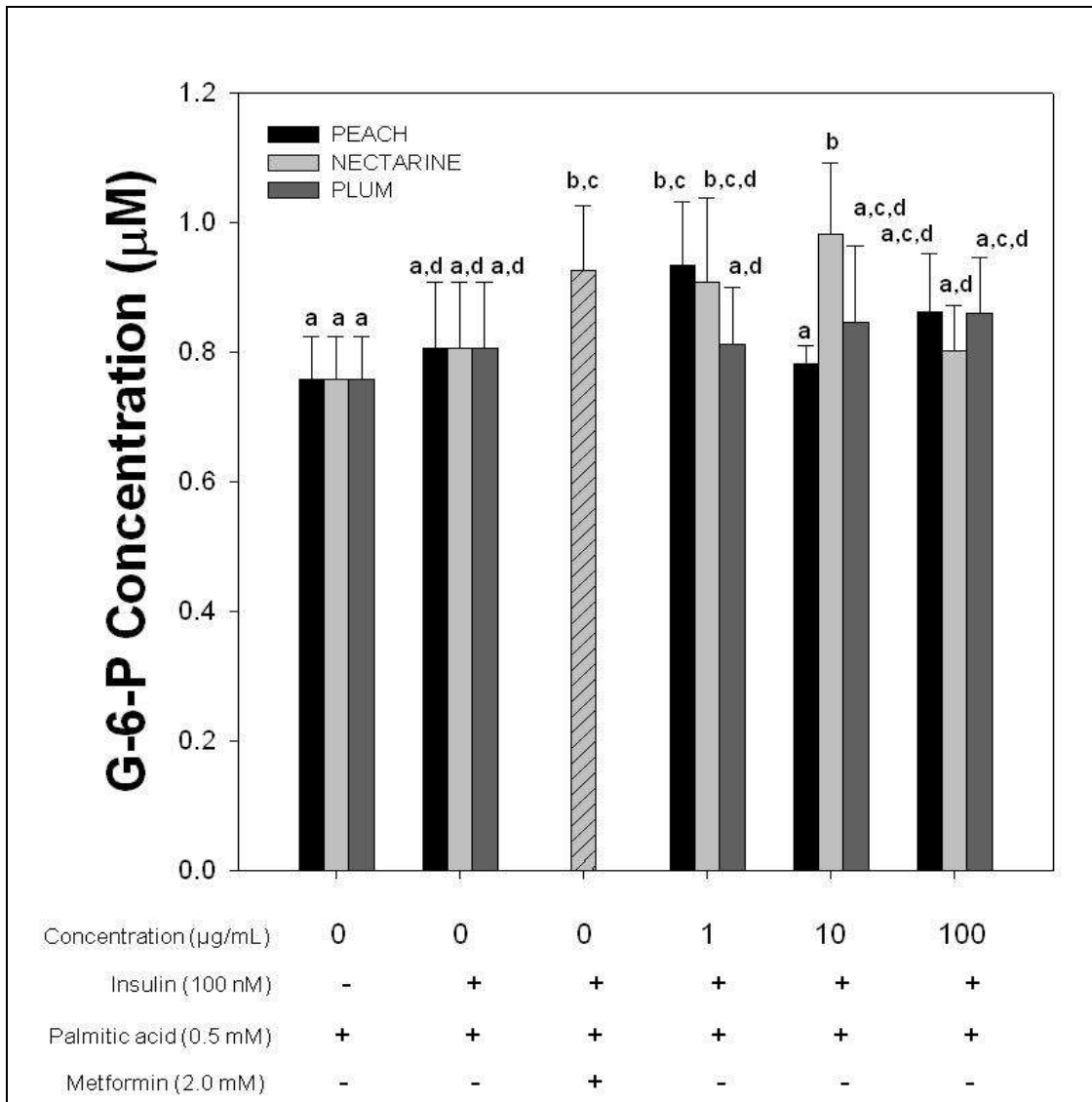
Figure 4. Effect of stone fruits extracts on intracellular glucose-6-phosphate accumulation in muscle cells. Cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and treated with extracts from peach, plum and nectarine for aditional 16 h, before exposure to 100 nM of insulin for 30 min. Samples were collected inmediatly after exposure to insulin. Glucose-6-phosphate was determined using a commercial kit. Bars are means ± S.D. (n= 3). Different letters denotes significant differences by ANOVA (p < 0.05).
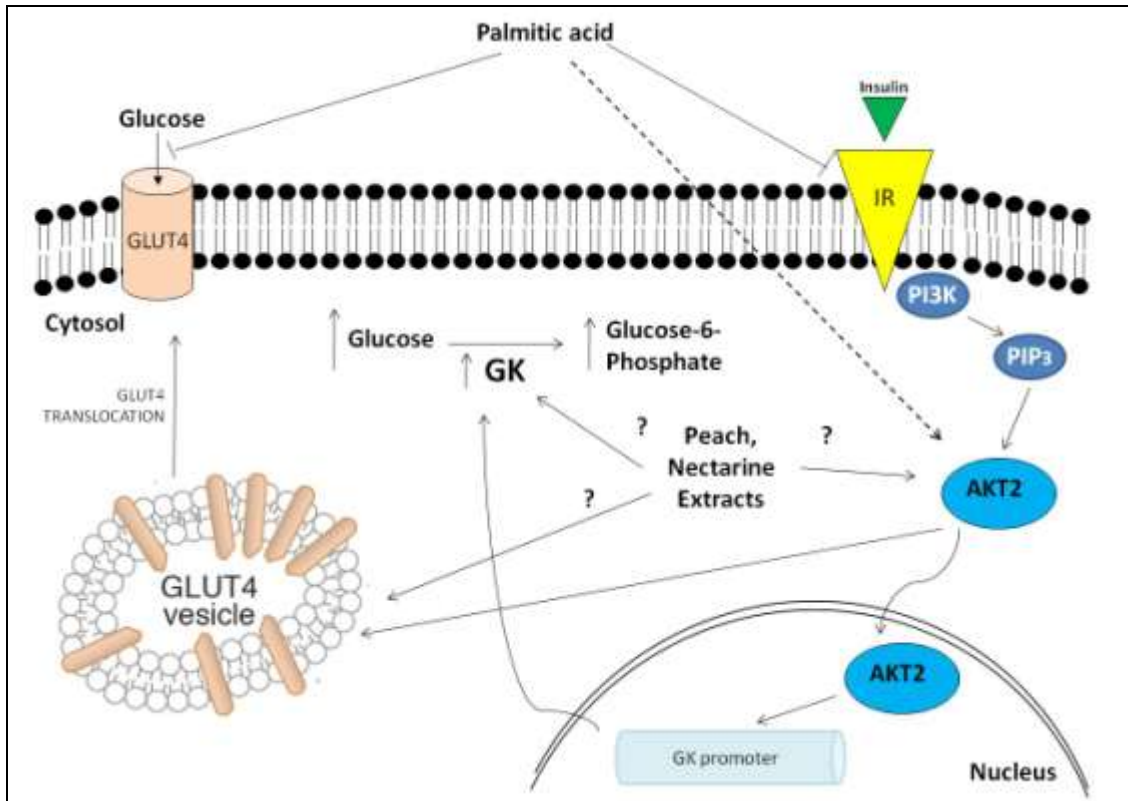
Figure 5. Diagram of possible mechanisms of action of peach and nectarine extracts in insulin-resistant myotubes. Since the exposure to palmitic acid is inhibiting the proper activation of the insulin receptor (IR), this leads to a reduction in the rate of glucose uptake and therefore a reduction in the concentration of intracellular glucose-6-phosphate (G-6-P). Peach and nectarine extracts could be acting at two different levels, by increasing the glucose uptake as a consequence of an increase in the translocation of GLUT-4 transporters to the surface of the cell; or through an increase in the amount or activity of the enzyme glucokinase (GK), probably due to the activation of AKT. Both posibilities may lead to an increased concentration of G-6-P, indicating a reversion of the insulin-resistance state.

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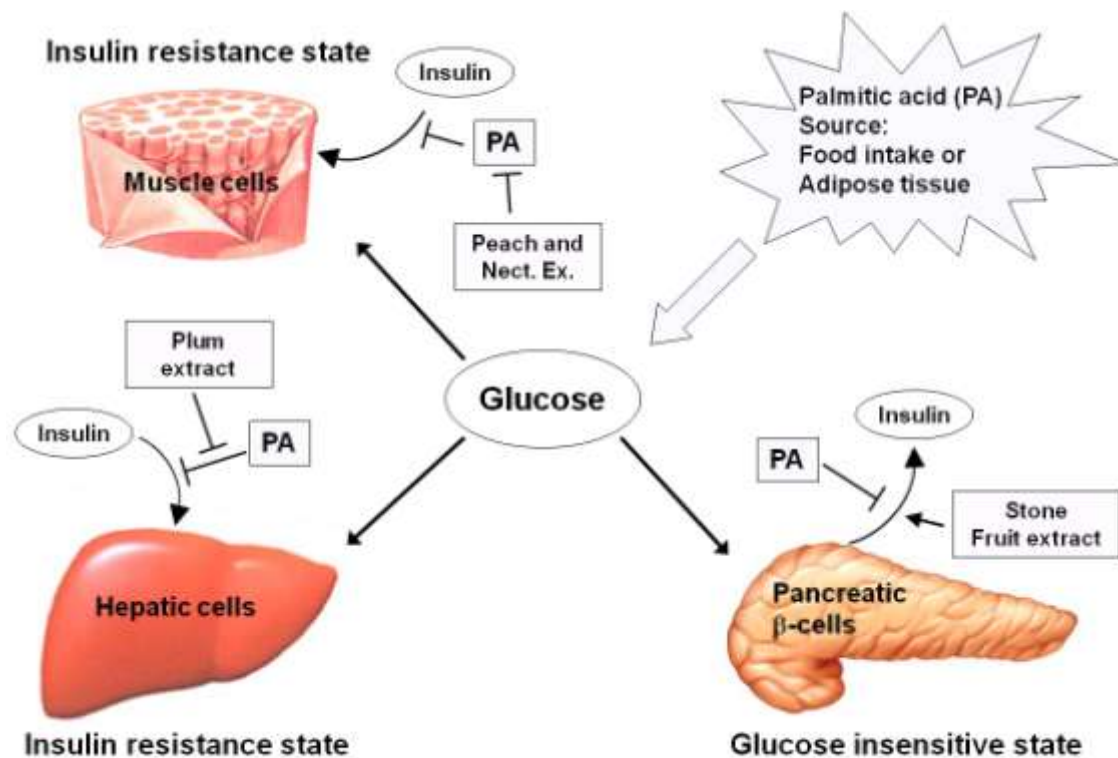
Figure 6. Diagram summarizing the multiple functions stone fruit extracts exert in reversing insulin resistance in hepatic and muscle cells as well as reversing glucose insensitivity in pancreatic beta cells.

Summary

In this report we studied the effects of stone fruit extracts against insulin resistance and glucose insensitivity as part of the events of the metabolic syndrome. We used cell models including hepatic and muscle cells to determine effects in insulin resistance representing events in hepatic and muscle tissue. In addition, we studied the effects of stone fruits in glucose insensitivity in pancreatic beta cells representing pancreatic tissue. Initially, we chemically characterized stone fruit extracts (nectarine, peach and plum) using TLC and HPLC finding that the powder extracts contained only phenolics with profiles of 4 major phenolic groups including chlorogenic acid derivatives, anthocyanins, quercetin derivatives and catechins. These powders were tested in the biological assays. Results indicated that stone fruit extracts have multiple functions and could potentially work against insulin resistance and glucose insensitivity in different fronts simultaneously (Figure 6). For example, all stone fruit extracts reversed the glucose insensitivity in pancreatic beta cells induced by palmitic acid. These extracts, by possible AOX and AOX-independent mechanisms, increase insulin secretion. On the other hand, only plum extracts reversed the

insulin resistance in hepatic cells induced by palmitic acid. Plum extracts reversed the effect by a mechanism that increased glucokinase activity. Similarly, nectarine and peach extracts reversed the insulin resistance in muscle cells induced by palmitic acid. Both extracts reversed the effect in muscle cells in a similar fashion as the drug metformin by an increase in glucokinase activity and a possible increase in glucose uptake. In general, these results are very promising and indicate that stone fruit extracts have potential health promoting properties against Type 2 Diabetes, which is an important event of the metabolic syndrome.