BIOACTIVE POLYPHENOLICS FROM STONE FRUIT CULTIVARS ON C2C12 INSULIN RESISTANT SKELETAL MUSCLE CELLS

A Thesis

by

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ABSTRACT

Skeletal muscle insulin resistance is a common precursor to type II diabetes. Phytochemicals in fruits have shown to have bioactive properties that could alleviate some of the symptoms and causes of insulin resistance in peripheral tissues. In this thesis, stone fruits of peach, plum, and nectarine were viewed for their bioactive properties against insulin resistant murine C2C12 myotubes *in vitro*. Fruits were characterized using HPLC-DAD and TLC, removed of sugars and organic acids by SPE reverse phase C18 silica gel, and tested on a palmitic acid induced insulin resistant skeletal muscle model for cytotoxicity levels and glucose 6-phosphate accumulation. Peach extracts investigated in depth analysis in this study. Using SPE the peach extract was fractionated into major phenolic compound categories, identified with HPLC-DAD, and quantified with wet based chemistry assays. The standardized peach fractions were tested on the cell model for cytotoxicity levels, glucose uptake, glucose 6-phosphate, ROS accumulation, and immunochemistry.

Isolated and identified extracts were used at 0-300 μg/mL for cytotoxicity testing. From these results, stone fruit phenolic levels used for glucose 6-phosphate quantification were 1, 10, and 100 μg/mL. Of the stone fruits, peach and nectarine had the most pronounced effect on glucose 6-phosphate concentration at 1 and 10 μg/mL. Further isolated peach extract was separated using SPE, identified using HPLC-DAD and quantified with a wet based chemical assay. These peach phenolic fractions were tested in the murine insulin resistant model for their effect on glucose uptake, with the

most effective doses in the cell used for further assays (phenolic acid fraction: 1µg/mL; anthocyanin and flavonoid fractions: 0.1µg/mL; catechin fraction: 0.5 µg/mL). All fractions were able to restore intracellular glucose 6-phosphate to levels comparable to normal and control cells with the exception of F3 which showed no difference from IR induced cells. The fractions had no significant effect on ROS accumulation but were able to increase AKT phosphorylation and decrease phosphorylation of negative regulators of insulin signaling (JNK and IRS1ser307). Further research is needed to view if these compounds act directly on insulin receptors, or work through the AMPK mechanism similar to the positive control, metformin.

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NOMENCLATURE

T2DM	Type II Diabetes Mellitus
ADA	American Diabetes Association
CVD	Cardiovascular Disease
IR	Insulin Resistance
HGP	Hepatic Glucose Production
FFA	Free Fatty Acids
cAMP	Cyclic Adenosine Monophosphate
IRS	Insulin Receptor Substrates
PI3K/PKB	Phosphoinositide 3-Kinase/Protein Kinase B
MAPK	Ras-Mitogen Activated Protein Kinase
IRS1	Insulin Receptor Substrate 1
SH2	Src Homology Region 2
AKT	Protein Kinase B
PDKs	3-Phosphoinositide-Dependent Proteins
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
PIP2	Phosphatidylinositol-4,5-Bisphosphate
PH	Pleckstrin Homology
PDK1	3-Phosphoinositide-Dependent Protein Kinase 1
Thr	Threonine
Ser	Serine
GLUTs	Glucose Transport Vesicles

TBC1D4	TBC1 Domain Family Member 4
GAP	GTPase-Activating Protein
G6P	Glucose 6-phosphate
F16BP	Fructose 1,6-bisphosphate
AcCoA	Acetyl Coenzyme A
ROS	Reactive Oxygen Species
ER	Endoplasmic Reticulum
AGEs	Advance Glycation End Products
TNFα	Tumor Necrosis Factor Alpha
SOD	Superoxide Dismutase
AOX	Antioxidant
JNK	c-Jun N-Terminal Kinase
F6P	Fructose 6-Phosphate
DAG	Diacylglycerol
AMPK	5-Adenosine Monophosphate-Activated Protein Kinase
TZDs	Thiazolidinediones
PPARγ	Peroxisome-Proliferator-Activated Receptor Gamma
KATP	Adenosine Triphosphate-Dependent Potassium
SM	Secondary Metabolites
HFD	High Fat Diet
NFkβ	Nuclear Factor Kappa Beta
PPO	Polyphenol Oxidase

SPE	Solid Phase Extraction
DMEM	Dulbecco's Modified Eagles' Medium
EDTA	Ethylene Diamine Tetra Acetic Acid
DMSO	Dimethyl Sulfoxide
BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
ATCC	American Type Culture Collection
TCA	Trichloroacetic Acid
TFA	Triflouroacetic Acid
CAE	Chlorogenic Acid Equivalents
HBS	Horse Bovine Serum
SDS	Sodium Dodecyl Sulfate
TEMED	N.N.N', N'-Tetramethylethylenediamine
APS	Ammonium Persulfate
PVDF	Polyvinylidene Fluoride
DCFA	2′7′-Dichlorofluorescein Diacetate
TBST	Tris-Buffered Saline with Tween® 20 Surfactant
HRP	Horseradish Peroxidase
ECL	Enhanced Chemiluminescence
GK	Glucokinase

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Type II diabetes mellitus is a chronic metabolic disorder that currently affects the US population, with over 6% diagnosed as diabetic. In 2011 the American Diabetes Association (ADA) reported that there are currently over 18 million Americans diagnosed with diabetes and almost 80 million with pre-diabetes. Type II diabetes mellitus results from a genetic mixture of predisposition, behavioral, and environmental risk factors and is characterized by β-cell dysfunction from the pancreas, excessive glucose production from the liver and tissue insulin resistance from the adipose and skeletal muscle.[1, 2] Over time, type II diabetes mellitus can often lead to other health complications including cardiovascular disease (CVD), high blood pressure, kidney disease and neuropathy.

Insulin resistance (IR), a common precursor to type II diabetes, can be developed as far as 10 years prior to diabetes diagnosis due to normal β-cell function[3] as pancreatic insulin secretion overcompensates for the reduced insulin tissue response.[4] IR occurs in insulin sensitive tissues (adipose, skeletal muscle, and liver) when the body develops a decreased response to insulin release most likely caused by problems in the insulin signaling pathway, inflammatory pathways, accumulation of free fatty acids in the plasma, and/or decreased mitochondrial function in the cells.[5] Therefore, treatments that have an insulin sensitizing effect on the body prove significant importance in type II diabetes.

It has been stated many times in the past decade that having a diet rich in fruits and vegetables may reduce the occurrence of non-communicable diseases such as CVD, certain types of cancer, and type II diabetes. Specifically, the phytochemicals in fruits and vegetables have attracted great interest since the 1990s due to growing evidence of their beneficial effects on human health. Phytochemicals are plant secondary metabolites that largely contribute to plant fitness by antibiotic, antifungal, and antiviral mechanisms as well as protect the plants against pathogens and damage from UV light.[6] Phenolic compounds are a class of phytochemicals defined by at least one or more phenol group structure and are very widely dispersed in the plant kingdom. There are over 8000 phenolic compounds that have been identified in the plant kingdom and many have shown antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic effects in different disease states *in vitro, in vivo*, and in human clinical trials.

Stone fruit cultivars contain a mixture of diverse phenolic compounds in their flesh and peel including hydroxycinnamates, chlorogenic and neo-chlorogenic acids, anthocyanidins, flavonols, quercitin derivatives, and procyanidin dimers and trimers.[7] There has been research on some of these individual compounds effect on insulin resistance such as chlorogenic acids from coffee consumption on insulin sensitivity[8] and blueberry anthocyanins on glucose absorption and lipid metabolism in vivo and in vitro.[9] Current work will focus on phenolics isolated from stone fruit to view any potential positive effect on cellular insulin sensitivity.

1.2 Literature Review

1.2.1 Type II Diabetes Mellitus

Type II diabetes mellitus occurs when there is an inability of insulin to promote sufficient glucose uptake in insulin sensitive tissues and prevent glucose output from liver. In healthy individuals there is a constant plasma glucose homeostasis due to an intrinsic feedback loop regulated by insulin and glucagon; both of these hormones function to maintain normal blood glucose levels. Normally, plasma glucose levels increase postprandial, which triggers the release of insulin from β-cell from the islets of the pancreas stimulating glucose uptake into adipose and skeletal muscle and promoting the storage of sugars as triglycerides and glycogen. Tissue insulin resistance plays a major role in the development of diabetes and can be acutely present for years before diagnosis occurs. Issues arising from tissue insulin resistance include uncontrolled fasting hepatic glucose production (HGP) from the liver and increased free fatty acid (FFA) release from the adipose, leading to further hindering of glucose uptake and glycogen synthesis in the skeletal muscle[4]. In this stage of insulin resistance, plasma glucose levels may remain normal as the secretory β-cells of the pancreas will overcompensate and release more insulin to the tissues to try to overcome the decreased sensitivity. Eventually, the pancreatic β-cells will fail to compensate for the insulin resistance over a period of time leading to hyperglycemia. Chronic high blood glucose levels then progress the disease by further impairing insulin sensitivity and worsening β-cell exhaustion. Some known risk factors that increase the odds of being diagnosed are environmental, including age and central obesity mainly due to a high calorie, high fat

diet, decreased energy expenditure, and genetic predisposition, considering factors such as ethnicity and immediate family history such as a parent with type II diabetes or gestational diabetes[1]. There are several current treatment methods available for type II diabetes, but almost all of them have side effects or downsides to their prescriptions.

1.2.2 Glucose Homeostasis

In the post absorptive state (overnight fasting), the main source of plasma glucose comes from the liver, with a small amount being produced by the kidneys. The liver is the most important site of insulin-mediated basal glucose release, with about half of hepatic glucose production (HGP) from glycogenolysis and half from gluconeogenesis[10.] Most of this glucose from the liver is used by insulin independent tissues, approximately 50% of all glucose disposals occurs in the brain and around 25% in the splanchnic area (gastrointestinal tract and the liver). The remaining 25% of glucose uptake occurs in the insulin dependent tissues, primarily skeletal muscle. Skeletal muscle lacks glucose 6-phosphatase so there is no glycogenolysis present in muscle to provide glucose release, but instead the skeletal muscle will export amino acids and other substrates to the liver for gluconeogenesis[11].

In the absorptive state, there is a rise in blood glucose levels from ingested food, which rapidly stimulates insulin release into the portal vein from the β-cells of the pancreas, which then directs the body to store fuel by increasing glucose transport through the insulin signaling pathway in sensitive tissues. Insulin promotes the deposition of glycogen in the liver, triglycerides in the adipose, and activates glucose transport and glycogen synthesis in the skeletal muscle. Insulin also inhibits glucagon

secretion and lowers serum FFA concentrations which contribute to the decline in HGP. Skeletal muscle is the major site of insulin-mediated dietary glucose uptake through stimulation of the insulin-sensitive glucose transport system. After a meal, approximately 80-85% of glucose disposal in insulin stimulated tissues occurs in the skeletal muscle. Disposal of glucose in the muscle tissue is essential in preventing hyperglycemia. Insulin will initiate the uptake of glucose into the skeletal muscle and enhance the glycogen synthase activity through the regulation of a cyclic adenosine monophosphate (cAMP) mediated cascade that converts glucose into glycogen in the cells[4]. After glucose enters the skeletal muscle it can be converted into glycogen (70% of glucose uptake) or enter the glycolytic pathway (90% glucose oxidation and 10% anaerobic glycolysis).[12] Although fat account for a small amount of glucose disposal (around 5%) it plays an important role in the maintenance of total body glucose homeostasis by regulation of lipolysis with subsequent release of glycerol and FFAs into the bloodstream.

1.2.3 The Insulin Signaling Pathway

Insulin is a hormone that regulates many pathways including aiding in the synthesis of protein, glucose homeostasis, and glycogen storage in muscle. Released insulin travels to the muscle and adipose for glucose uptake and to the liver to suppress HGP. Adipose accounts for only a small amount of total body glucose uptake but is a major regulator of glucose homeostasis. Released insulin inhibits lipolysis and prevents the release of FFAs from adipocytes; the decline in plasma FFA concentrations leads to an increase in glucose uptake in muscle and also contributes to the inhibition of

HGP.[13]The tissue responsible for the major site of glucose disposal in the body is the skeletal muscle. The insulin signaling cascade in myofibers initiates with the post prandial release of insulin from the β-cells. Insulin travels to the muscle tissue and binds to its receptor on the muscle cell surface which initiates a signaling cascade of events. The insulin receptor is a tetrameric protein than consists of two extracellular α-subunits containing a ligand binding site and two trans-membrane β-subunits which have a tyrosine protein kinase catalytic domain. The two α and β subunits are held together by disulfide bonds and the receptor is dimeric in its inactive state. The insulin receptor signals the insulin receptor substrates (IRS) through docking proteins.[14] Insulin binds to the α-subunits of the receptor, activating one of the β-subunits which lead to the insulin receptor's auto phosphorylation. This leads to the activation of the Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/PKB) pathway, which is responsible for most metabolic actions of insulin, and the activation of the Ras-Mitogen Activated Protein Kinase (MAPK) pathway, which regulates expression of some genes and cooperates with PI3K to control cell growth and differentiation.[15]

The insulin receptor tyrosine kinase activity promotes the tyrosine phosphorylation of the Insulin Receptor Substrate 1 (IRS1) protein. These phosphotyrosine residues act as docking sites and from complexes with many Src homology region 2 (SH2) domain-containing proteins, including the p85 regulatory subunit of PI3K. PI3K is a heterodimeric enzyme composed of a p85 regulatory subunit and a p110 catalytic subunit. Once p85 on PI3K associates with IRS1 it activates protein kinase B (AKT) through 3-phosphoinositide-dependent proteins (PDKs). Class 1A PI3K

catalyzes the formation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) from plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2). Signaling proteins with Pleckstrin homology (PH) domains can bind directly to PIP3 and become activated, including serine threonine kinases like AKT and 3-phosphoinositide-dependent protein kinase 1 (PDK1)[16]. Association with PIP3 at the membrane brings the two proteins in close proximity and PDK1 can then phosphorylate AKT16. AKT contains an N-terminal PH domain and a C-terminal catalytic domain. The first terminal 100 amino acids possess the PH domain that can bind to phospholipids. There is a short glycine rich region bridging the PH domain to the catalytic domain. The carboxy-terminal 70 amino acid tail contains the putative regulatory domain[17]. AkT2 is the main regulator of glucose uptake in the insulin signaling pathway and has a high concentration in the insulin sensitive tissues.[18, 19] AKT is phosphorylated by PDK1 at threonine 308 (Thr308) and at serine 473 (Ser473) by PDK2 or auto phosphorylation. This promotes the full activation of AKT which then detaches from the plasma membrane and translocates to the cytosol and the nucleus. Thr308 resides within the activation loop of the linase domain and Ser473 lies in the carboxy-terminal tail. The activation of AKT leads to the translocation of intracellular glucose transport vesicles (GLUTs) to the cell membrane to uptake free glucose into the cell. GLUT1 is involved in skeletal muscle basal glucose uptake and GLUT4 is the primary insulin responsive glucose transporter in muscle. In the absence of insulin over 90% of the vesicles are found intracellularly. In the presence of insulin they translocate to the plasma membrane and transverse tubules in the muscle for glucose uptake into the

cell[13]. The exact molecular mechanism of how AKT activation leads to GLUT4 translocation is still being researched, however the TBC1D4 (TBC1 domain family member 4), also known as Rab-GAP AS160, has been discovered as a direct target of downstream AKT activation[20]. The model is that AKT-mediated phosphorylation of some sites on AS160 inhibits GAP (GTPase-Activating Protein) activity and thus allowing a Rab-family GTPase to become GTP loaded and stimulate GLUT4 vesicles translocation to the membrane surface.[21]At the cell surface, GLUT4 permits facilitated diffusion of glucose down a concentration gradient into the insulin sensitive tissue. Once within the cells, glucose is rapidly phosphorylated by hexokinase to glucose 6-phosphate (G6P) which then is polymerized into glycogen, or enters the glycolysis cycle.

1.2.4 Glucose Metabolism

Glucose enters the cells via GLUT transporters from the insulin signaling pathway. That glucose is immediately converted into G6P by hexokinase in skeletal muscle. G6P is further converted into fructose 1,6-bisphosphate (F16BP) which converts to glyceraldehyde 6-phosphate and then to 1,3-phosphoglycerate which finally gets converted into pyruvate which will end up as acetyl-coenzyme A (AcCoA) and oxaloacetate which will enter the mitochondria where they produce Reactive Oxygen Species (ROS) due to the Kreb's cycle production of NADH, H+, and FADH2. During hyperglycemia, elevated glucose levels induce the TCA cycle electron donor overproduction which causes the increase of the mitochondrial protein gradient.[22]

1.2.5 Oxidative Stress and Reactive Oxygen Species

Reactive Oxygen Species (ROS) are naturally occurring in living organisms and are found as either waste products from biological processes or that which is produced intentionally in a signaling pathway, cell defense system, or during synthesis or breakdown. The mitochondria are the largest source of ROS in the body, due to oxidative phosphorylation and the electron transport chain processes. Major known sources for overproduction of ROS in the cells include endoplasmic reticulum (ER) stress, advance glycation end products (AGEs), nitric oxide synthase and lipid peroxidases, lipotoxicity, glucose metabolism, and Tumor Necrosis factor alpha (TNFα).[23] Overproduction of ROS has been known to be associated with T2DM and insulin resistance.[24] To alleviate the toxic buildup of ROS from the cellular processes, the body produces enzymes such as superoxide dismutase (SOD) to repair the oxidative damage to the cell. Oxidative stress occurs in cells when there is an imbalance between the antioxidant (AOX) available for reactions and the oxygen radicals, such as ROS, present in the cell system. Studies have shown that an increase in mitochondrial oxidative stress may play a role in type II diabetes and insulin resistance. [25-34]

Oxidative stresses such as ROS are known to activate c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase family member. JNK may also be activated by ER stress, free fatty acids, MAPKs, MAP2Ks, MAP3Ks, as well as many other cellular events.[35] ROS accumulation in the cell promotes prolonged activation of JNK by inactivating JNK phosphatases. A strong activation of JNK has been observed in liver,

fat and muscle tissue in mice placed on a high fat diet, and genetically (ob/ob) obese mice.[36-37]

JNK is important for the regulation of cell death and survival, differentiation, proliferation, ROS accumulation, metabolism, insulin signaling, and many other cell functions. Free fatty acid accumulation in the cell can lead to the activation of oxidative stress, which leads to ER stress lipid peroxidation which may activate JNK1 leading to the disruption of the insulin pathway through the activation of IRS1 serine 307 phosphorylation. Mitochondrial stress will activate JNK and p38 MAPK in a Ca^{2+} dependent manner and represses IRS1threonine activation through ATF3 in a Ca^{2+} and JNK dependent manner.[38] The JNK specific phosphorylation site in the insulin signaling pathway is IRS1 serine 307 phosphorylation. Ser307 phosphorylation of IRS1 by JNK decreases the threonine phosphorylation capabilities, therefore inhibiting the insulin signaling pathway, as threonine phosphorylation is necessary to activate AKT in the PI3K pathway.[39-41] Mitochondrial dysfunction will increase intramyocellular lipid metabolites which may activate ser/thr kinases and suppress insulin signaling before the phosphorylation of IRS1.[42-45]

1.2.6 Insulin Resistance

Insulin resistance is a fundamental predictor of diabetes development and is also linked to other complications such as hypertension, hyperlipidemia, insulin resistance syndrome (syndrome X), renal failure, gestational diabetes and atherosclerosis. Insulin resistance refers to the relationship between insulin release and its ability to stimulate glucose uptake in peripheral tissues (fat and muscle).[46] Insulin resistance occurs when

higher than normal insulin concentrations are needed to achieve a normal metabolic response, or when normal insulin concentrations fail to establish a normal metabolic response.[12] In the basal state, insulin resistance occurs in the liver initiated by too much hepatic glucose production (HGP). An excess amount of HGP is the main cause of fasting hyperglycemia and is caused by increases in gluconeogenesis and glycogenolysis. In the insulin stimulated state, skeletal muscle is the primary site of insulin resistance and is responsible for an increase in plasma glucose concentrations above the baseline following ingestion.[47]While there are many contributing factors to the development of insulin resistance and eventually, diabetes, the accumulation of plasma FFAs is a large contributor to the development of insulin resistance. Previously it was thought that the cellular mechanisms behind this connection were due to a competition between fatty acids and glucose oxidation known as the Randle cycle. Briefly, an increase in fatty acid concentration results in an elevation of the intra-mitochondrial acetyl CoA/CoA and NADH/NAD+ ratios with the inactivation of pyruvate dehydrogenase which leads to an increase in citrate concentration and therefore inhibiting phosphofructokinase. FFAs could also decrease muscle insulin sensitivity by increasing the flux of G6P to the hexosamine biosynthetic pathway; with an increase in UDP-N-acetyl glucosamine, G6P would be converted to F6P, resulting in the elevation of intramuscular hexosamines, which have been shown to decrease glucose transport and GLUT4 translocation.[48] In reality, a more complex effect of fatty acids on insulin signaling exists. One theory is that FFAs interfere with insulin signaling through PKC induced serine phosphorylation of IRS-1, resulting in decreased PI3K activity and

decreased glucose transport. Another concept of the effect on fatty acids on the insulin signaling cascade involve an increase in intracellular fatty acid metabolites like diacylglycerol (DAG), fatty acyl CoA, and ceramides that can lead to the phosphorylation of serine/threonine sites on IRS-1 that affect PKB and other downstream proteins in the pathway.[1, 49] FFAs also increase HGP, by stimulating key enzymes and by providing energy from gluconeogenesis while the glycerol released during triglyceride hydrolysis serves as a substrate for gluconeogenesis.[12] Many papers have reported that saturated FFAs do initiate an insulin resistant response in cellular tissues, but that unsaturated FFAs do not.[50-51] Specifically, hexadecanoic acid (palmitic acid, C16:0), one of the most comply found fatty acid in nature, has been known to induce insulin resistance in vitro.

1.2.7 Current Treatments for Type II Diabetes Mellitus

In a study of lifestyle changes (keeping food and exercise logs, increasing fiber and vegetable consumption while decreasing fat, sugar, salt and alcohol consumption as well as increasing exercise and changing the type of fats consumed in the diet) development of diabetes among subjects with impaired glucose tolerance was 58% lower in the intervention group than the control group.[52]

During bouts of exercise, there is an increase in GLUT4 translocation to the sarcolemmal membrane and the t tubules where glucose uptake occurs in pathways independent from insulin stimulation. The glucose uptake is activated by muscle contractions, exercise induced production of nitric oxide and bradykinin. Skeletal muscle has high levels of bradykinin receptors which stimulate GLUT4 translocation.[53] Calcium

ions (Ca^{2+}) also play an important role in insulin-independent glucose uptake. 5 – adenosine monophosphate-activated protein kinase (AMP kinase) is activated by a decrease in cellular energy charge during exercise. The enhanced glucose uptake from exercise in type 2 diabetic patients shows to persist into the immediate post exercise period for up to 20 hours after a workout and endurance exercise training also leads to improvement in insulin action on skeletal muscle glucose metabolism. After six weeks of exercise training by insulin resistant offspring of diabetic subjects, there was an enhancement of insulin stimulated glucose transport and phosphorylation in skeletal muscle.[54]

Outside of clinical trials, there is an uncertainty that real populations can maintain diet and exercise interventions for long term period of time. Also, in some diabetic or high risk individuals such as the elderly and the disabled, it is difficult to add an exercise regime. This is where the use of pharmaceutical aids begins. Current and past drugs used for the prevention and treatment of T2DM include biguanides (metformin), thiazolidinediones (TZDs) (rosiglitazone, troglitazone), sulfonyurea derivatives, exogenous insulin, α-glucosidase inhibitors (acarbose), and lipase inhibitors (orlistat).

The biguanide metformin is one of the most thoroughly studied drug used for diabetes prevention. Metformin has an exceptional safety record and a low cost affordability and so it is a popular option for diabetes prevention.[55] Metformin works independently of the pancreas. It improves insulin sensitivity in the liver by decreasing HGP, increases muscle insulin sensitivity and has been shown to have an effect on

cardiovascular options. It is not as effective on its effects on insulin resistance, inflammation, and vascular function as compared to TZDs.[56] Metformin has also been shown to cause adverse gastrointestinal disturbances and, rarely, lactic acidosis and therefore should not be used in patients with impaired renal or hepatic function.[4]

Thiazolidinedione drugs have also been shown to enhance insulin sensitivity as well as enhance vascular function of type 2 diabetic patients. TZDs are synthetic selective ligands of the nuclear transcription factor peroxisome-proliferator-activated receptor γ (PPARγ) in adipose tissue.[57] TZDs activate PPARγ which alters the distribution of triglycerides from storage which reduces FFAs in the plasma. They also reduce the circulating concentrations of pro-inflammatory cytokines such as TNFα and IL-6, which are associated with insulin resistance development through inflammatory pathways.[56] There are signs of cross talk between TZDs effect on adipose to skeletal muscle, liver and pancreatic β-cells which could explain the overall improvement of insulin action and secretion when taking TZDs. These drugs are better tolerated than metformin without GI tract effects; however these drugs do show to increase weight gain and fluid retention in patients. There is also a concern for some patients on TZDs of congestive heart failure, although the cases reported are quite rare. From a clinical case of rosiglitazone treatment, the frequency of congestive heart failure increased from the placebo, but with only few cases reported.[55] In another case study where the risk of congestive heart failure was higher in diabetic patients given TZD over controls they determined that the patients, because they did not have previous history of congestive heart failure, the TZD-related heart failure may be due to TZD-related fluid retention

and diastolic dysfunction in the patients.[57] There are also some rare risks of hepatic toxicity and anemia with the use of these drugs.[58]

Sulfonylurea derivatives act by closing the pancreatic islet cell potassium channels to increase insulin secretion. They bind directly to cell surface receptors that induce the closure of adenosine triphosphate-dependent potassium (KATP) channels which leads to membrane depolarization and an influx of extracellular calcium which prompts insulin secretion. With the insulin stimulating action that sulfonylurea derivatives possess, there is often a chance of extreme hypoglycemia.[4]

Other preventative drugs include α-glucosidase inhibitors, mainly acarbose, and intestinal lipase inhibitors, such as Orlistat. Both of these drugs have a high drop-out rate and limited acceptability as potential oral treatment drugs. There has been current research to find other pharmaceutical and natural products that can aid in the prevention and reversal of insulin resistance. There have been many claims about the effects of insulin sensitivity from many natural sources that are worth looking into for potential therapeutic effects.

1.2.8 Plant Secondary Metabolites

Primary plant metabolites are essential to plant growth, development and homeostasis and are ubiquitous in all plants. Primary metabolites include carbohydrates, lipids, proteins, chlorophyll and nucleic acids. In addition to these key roles in plants, some of these metabolites are pre-cursors to secondary metabolite synthesis. Phytochemicals are secondary plant metabolites that are variously distributed among the plant kingdom and have specific functions to the plants they are synthesized in.

Phytochemicals are mainly synthesized from carbohydrates through the shikimate and phenylpropanoid pathways; however some secondary metabolites such as alkaloids are synthesized from amino acids. Most water soluble secondary metabolites (SM) can be found in the plant cytosol and vacuoles while most lipophilic compounds are found in the endoplasmic reticulum. SM synthesis can also be found in the chloroplasts (the alkaloid caffeine and the monoterpenes). Phytochemicals serve many purposes in plant tissues including protection from abiotic stresses, defense against herbivores, pathogens, and inter-plant competition, and attraction from beneficial organisms such as pollinators. Phytochemicals also exert beneficial effects through synergistic actions of several chemical compounds acting at target sites associated with a physiological process.[59] In some ways phytochemicals could act for potential human health benefits include being an inhibitor, substrate or cofactor for some cellular enzymatic action, a ligand for cell surface receptors, or as scavengers for toxins or free radicals in the body.[60]

There are four major classes of phytochemicals: terpenoids, polyphenolics, alkaloids, and fiber. Terpenoids, also known as isoprenoids, are the largest group of plant secondary metabolites in green foods, soy plants and grains. The terpenoids class of compounds include monoterpenes such as limonene in citrus peel and menthol in peppermint, sesquiterpenoids and their lactones, diterpenoids including kahweol and cafestol in coffee beans, triterpenoids including limonoids, phytosterols such as β-sitosterol and its glycoside, saponins and their derivatives, and carotenoids including carotene and xanthrophiles.[61] Terpenes have some properties unique to other phytochemicals including the ability to fix carbon through photosynthetic reactions

using photosensitizing pigments. Terpenes have a unique antioxidant activity mechanism when interacting with free radicals. Terpenes partition themselves into fatty membranes by their long carbon side chains. Carotenoids are extensively studied for their AOX activity and appear red, orange, and yellow in foods.[60]

Alkaloids are nitrogen containing compounds produced mainly from amino acids. Some alkaloid rich plants including many flowering and herbaceous plants found in Ranunculaceae, Rubiaceae, Papaveraceae, Fumariaceae, and Solanaceae families. The legume/bean family, Leguminosae, is known to have high alkaloid concentrations in the plants and roots. The rose family, Rosaceae, containing the large genus *Prunus* (plums, peaches, cherries, almonds etc.) is very low in alkaloids, as is the mint family (Graminaceae).[62] Many alkaloid containing plants are toxic for human consumption in high concentrations. Some alkaloids found in small doses in foods include glucosinolates in cruciferous vegetables such as cabbage. Glucosinolates contain sulfur and nitrogen and are derived from amino acids and glucose. In plant cells, glucosinolates can transform into isothiocyanates and sulfuraphanes.

The SM class of fiber has the most publicity associated with it for its multiple health benefits in humans and functions in foods. Fiber is classified as plant material that is resistant to digestive enzymes that is neither degraded nor absorbed but pass through the upper GI into the large intestine. Fiber can be classified as insoluble or soluble. Insoluble fiber is found in mostly cereals and legumes. It is slowly and incompletely fermented by the colon and has a pronounced effect on bowel habit. Typical plant sources of insoluble fiber include cellulose, lignin and chitin. Soluble fiber mainly is

found in fruits and vegetables and undergoes colonic degradation. It has high ferment ability and is also known to slow glucose absorption. Some sources of soluble fiber include pectin, β-glucans, arabinoxylans, and galactomannans.[63]An important function that fiber has in T2DM high risk individuals is protection by increasing fecal bulk, which dilutes the increased colonic bile acid concentration that occurs with a chronic high fat diet (HFD).

Lastly, the polyphenolic SMs are an integral part of the human diet. They are produced mainly through the shikimic pathway in plants, and malonic acid pathways in fungi and bacteria. The shikimic pathway begins with carbohydrate intermediates from the pentose phosphate pathway which is used to form phenylalanine (F) and tyrosine amino acids. Phenylalanine ammonia lyase will transform F into cinnamic acid for benzoic acid derivatives, and transformation into p-coumaric acid which forms caffeic acids and other simple phenylpropanoids and coumarins. Synthesis can continue to produce chalcones, flavonones, flavones, isoflavones, flavonols, anthocyanins and tannins. Flavonoids are the largest class of phenolic compounds in plants. Ring A on the flavonoid skeleton is derived from the acetic acid (via acetyl CoA) and malonic pathways, while ring B is a product of the shikimic pathway.[61] Some major phenolic compounds found in plants include hydroxybenzoic and hydroxycinnamic acids, coumarins, napthoquinones, xanthones, stilbenes, flavonoids, isoflavonoids, and tannins.[64]

1.2.9 Polyphenolic Compounds

There are over 8000 phenolic compounds currently described from the literature ranging from a simple one carbon ring phenolic acid structure to very complex procyanidin dimer and trimer structures. Phenolic compounds are categorized by their chemical structure, with all phenolics containing a benzene ring and one hydroxyl group (Figure 1). More often in nature, phenols will be attached to a sugar moiety or an organic acid. Phenolic compounds are classified as either flavonoid or non-flavonoid.

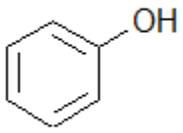

Figure 1. Phenolic compound backbone structure. Phenolic compounds are classified by their chemical composition of containing one or more phenol structures (at least one benzene ring attached to at least one hydroxyl side chain group).

Flavonoids are the largest class of phenolic compounds in plants and are known for their contribution to the quality of some fruit's color, aroma and texture. Flavonoids are found in high concentrations in the epidermis of leaves and skin of fruits. Flavonoids are consumed frequently by humans and are found ubiquitously in fruits, vegetables, tea, wine, cereals and other grains, roots, coffee and chocolate. Flavonoids contain 15 carbon

atoms arranged in a C_6-C_3-C_6 skeleton (Figure 2). Hydroxyl groups are usually found on the 4', 5 and 7 positions. In nature, flavonoids exist as glycosides. The major flavonoid subclasses are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones. Minor flavonoids (from a dietary perspective) include the flavan-3,4-diols, chalcones and aurones.[65]

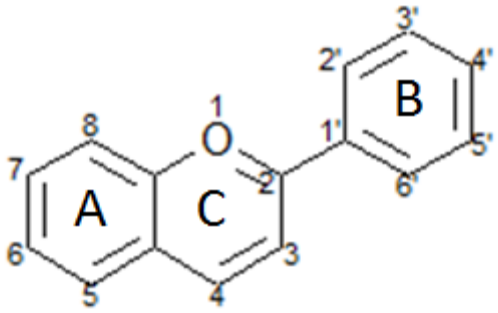

Figure 2. Basic flavonoid skeleton backbone (C_6-C_3-C_6). Flavonoids contain a 15 carbon atom backbone with hydroxyl groups usually found on the 4', 5 and 7 positions

The flavonols are the most widespread of all the flavonoids with over 200 aglycones reported. The most commonly found flavonols include kaempferol, quercetin, myricetin, and isohamnetin. These mainly occur as *O*-glycosides in the outer tissues of plants as their synthesis is stimulated by sunlight. Flavonol bioavailability to humans depends on the attached sugar moiety. Flavones are structurally similar to flavonols but

are not widely dispersed in nature. The only sources in plants are in parsley, celery, and a few herbs. There are some reports of polymethoxylated flavones in citrus fruits, such as tangeretin and nobiletin. Flavones occur as 7-*O*-glycosides. Flavan-*3*-ols (flavanols) are complex flavonoids that range in structure of monomers of (+) catechin and (-) epicatechin to polymeric and ogliomeric proanthocyanidins (condensed tannins). In plants, common proanthocyanidins can have up to 50 monomer units and are primarily made up of epicatechin (procyanidins). Not so common proanthocyanidins include those made up of (-) epiafzelechin, (+) afzelechin, and epi-gallocatechin (EGC), namely propelargonidins and prodelphinidins. Catechins are widespread in nature, with most dietary sources coming from tea, wine and chocolate. Flavan-*3*-ols are easily transformed with food processing, as seen with the fermentation of green tea catechins to black tea theaflavins, theacitrins, and thearubigins.[65]

Anthocyanins are ubiquitous in nature, mostly occurring in fruits and flower tissues. Main sources of anthocyanins in the diet are from the families Vitaceae (grapes) and Rosaceae (cherry plum raspberry strawberry blackberry apple peach etc.) other families include Solanaceae (tamarillo and eggplant), Saxifragaceae (red and black currants), Cruciferae (red cabbage) and Ericaceae (blueberry and cranberry). Anthocyanins are responsible for the red, blue and purple pigments that attract pollinators to the plants. Anthocyanins are also found in leaves, stems, seeds and root tissues and protect plants from excessive light by shading leaf mesophyll cells.[65]In fruits, anthocyanin concentration will usually increase with plant ripening. Anthocyanin aglycones that are responsible for the majority of color in fruits are cyanidin (most

commonly found), delphinidin, peonidin, pelargonidin, petunidin, and malvidin. These compounds are monoglycosylated at position 3 on the *O*-glycosidic bond with glucose, arabinose, and galactose. Cyanidin glucosides are the most common anthocyanins in fruits. Anthocyanins can also be chemically bonded to acids (acylated anthocyanins) as in the case of some grape species.[6]The flavanones are present in high concentrations in citrus fruits with the most common flavanones glycoside being hesperetin-3-*O*-rutinoside (hesperidin) which is found in citrus peels along with narirutin. These flavanones rutinosides are tasteless, while their conjugates neohesperidin (bitter orange) and naringin (grapefruit peel) are intensely bitter.[65] Isoflavones are found mainly in leguminous plants with the highest concentrations coming from soybean. Isoflavones, although are classified as flavonoids, are also sometimes known as phytoestrogens because of their estrogenic activity, mainly daidzein, genistein, coumestrol and coumestan.[66]

Non-flavonoid compounds are, by definition, all other phenolic compounds that do not have the flavonoid skeleton backbone. This category includes simple phenols such as volatiles like eugenol from cloves, 4-vinylguaiacolin from coffee roasting, and tyrosol and hydroxytyrosol in virgin olive oil, the phenolic acids including hydroxybenzoic acids and hydroxycinnamic acids, hydrolysable tannins, acetophenones, phenylacetic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones and secoiridoids, such as oleuopein, the bitter principle in black olives.[67] The main difference in the phenolic acid classifications is that hydroxybenzoic acids contain (C_6-C_1) backbone skeletons and hydroxycinnamic acids contain (C_6-C_3) backbones (Figure 3).

The most common hydroxybenzoic acid in food is gallic acid, found mostly in an ester bond to sugar compounds. All hydroxybenzoic acids are derived directly from benzoic acid. Hydroxycinnamic acids are derived from cinnamic acid and are present as caffeic acid, ferulic acid, p-coumaric acid and their conjugates with caffeic acid being the most ubiquitous in fruits and ferulic acid being the most common in cereal grains.[65] Hydroxycinnamic acids are found in all parts of fruits, with the highest concentrations in the outer parts but decrease in abundance upon ripening. Usually, these phenolics are rarely found as free acids but rather are ester-bonded between the carboxylic acid group of the phenolic and a hydroxyl group of an organic compound such as quinic acid, as in chlorogenic acids. Coumarins and lactones are mainly derived from *o*-hydroxycinnamic acids by cyclization. The main coumarins in foods are found as essential oils in foodstuff such as chicory, green teas, bitter orange, and grapefruit.

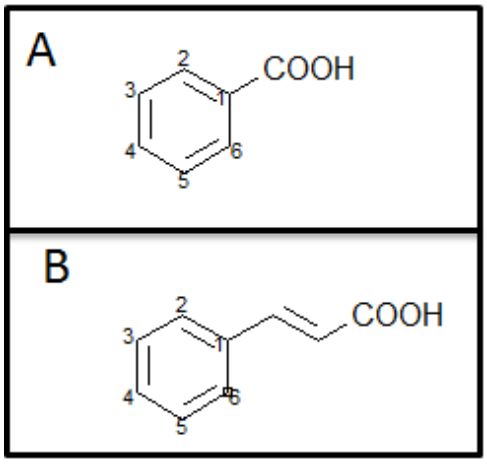

Figure 3. Structural differences between phenolic acid backbones.
A. hydroxybenzoic acids (C_6-C_1) B. hydroxycinnamic acids(C_6-C_3)

Benzophenones are scarce in foods and xanthones are only found in mango and mangosteen. Of the other non-flavonoid groups, some commonly known compounds include the stilbene resveratrol and the chalcones naringenin and phloridzin. While these compounds can be found in all foods, some compounds have a high concentration in specific plants. In cereal grain products higher concentrations of flavonoids, phenolic acids, and tannins are present. Legumes contain isoflavonones and vegetables hold mainly flavonoid glycosides. Most roots and tubers hold low concentration of flavonoids. Apples and citrus fruits are dominated by phenolic acids and flavonoids, while berries show high anthocyanin content.[68]

1.2.10 Bioavailability

It is estimated that around 25mg-1g of phenolic compounds are consumed regularly in the diet, with around 50mg–800mg per day from flavonoid sources. Phenolics are transformed before absorption which determines the biological activity of the compounds. The gut bacteria can hydrolyze these compounds, and depending on their structure, this may enhance or decrease availability. Gut bacteria can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones. Glycoside hydrolysis leads to more biologically active compounds, while aglycone hydrolysis leads to more or less active compounds depending on the original substrate and the resulting product.[69] Anthocyanins are consumed in varying concentration in the diet, from 12.5mg/day up to 200mg/day with regular intake of fruits and berries but studies have shown low availability in the body with no outstanding evidence of post absorption or metabolism before excretion with less than 0.1% of quantities ingested found in urine.[70]

Procatechuric acid may be an oxidative degradation product from cyanidin-3-glucoside consumption, but there are few studies on anthocyanin digestion and absorption. The anthocyanin aglycone is based on the flavilium ion (2-phenylbenzopyrilium). The chemically unstable aglycone converted to phenolic acids, for further metabolism by micro flora. Procatechuric acid (3,4-dihydroxybenzoic acid) is the main end product. Other metabolites produced by bacteria from anthocyanins include syrinic acid (3,5-dimethoxy-4-hydroxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde), phloroglucinol acid (2,4,6-trihydroxybenzoic acid) gallic acid (3,4,5-trihydroxybenzoic acid) and 3-*O*-methylgallic acid, depending on the original composition. Other metabolites that are not converted into phenolic acids are unknown in structure.[69]

Flavonols such as quercetin have a low concentration in the diet (about 20-50mg per day) but their bioavailability has been extensively studied.[71] Flavonols are extensively degraded by colonic bacteria. Quercetin metabolites depend on the hydroxylation pattern of the B ring and includes 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, and 3,4-dihydroxybenzoic acid from the B ring and phloroglucinol, 3- (3, 4 dihydroxyphenyl) propionic acid and 3- (3-hydroxyphenyl) propionic acid from the A ring. There have also been reports of 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzoic acid, 2-(3,4-dihydroxyphenyl) ethanol, 3-(3,4-dihydroxyphenyl) benzoic acid methyl ester, 3-methoxy-4-hydroxybenzoic acid (vanillic acid), and 3-(m or p-hydroxyphenyl) propionic acid metabolites. Flavanones, from citrus fruits, contribute more to the total daily intake flavonoids than flavonols and

are more bioavailable than other flavonoid groups as they are less degraded by colon bacteria so more available for absorption.[69]

For hydroxycinnamates a general trend is that from esters, the free acid is released by bacterial esterases, and the free acid is then metabolized to reduce the double bond to give rise to phenylpropionic acid and, then, decarboxylated to produce phenylacetic acids. After the ingestion of chlorogenic acids they are hydrolyzed in the gastrointestinal tract by cytosolic esterase in the gut micro flora. Once broken down available caffeic acid metabolites are absorbed into the vascular system.[72]It has been reported that approximately 33% of ingested chlorogenic acids are absorbed, and 95% of ingested caffeic acids are absorbed. The main microbial metabolites of caffeic acid are 3-hydroxyphenylpropionic acid and benzoic acid. Both metabolites are also obtained from chlorogenic and caftaric acid, suggesting that the esterification has no influence on the metabolism of caffeic acid by the gut Microbiota.[69] Hydrolysable tannins including gallotannins and ellagitannins are hydrolyzed to yield glucose and gallic acid (ellagitannins undergo lactonization to produce gallic acid) While studies from condensed tannins (oligomeric and polymeric procyanidins) show benzoic, phenylacetic, phenylpropionic, and phenyllactic acid derivatives produced. Polymeric procyanidins are not absorbed, however, and pass unaltered into the large intestine where colonic bacteria break down the polymer into phenolic acids that get absorbed into the circulatory system and get excreted into urine.[7] However, epicatechin and catechins are extensively metabolized and are conjugated during transfer from the gut lumen to the serosal surface. Rapid metabolism of catechins in the GI tract and quick distribution to the

tissues may be responsible for some reports of low plasma concentrations in some studies. Any phenolic being ingested must be cleaved of any glycosides before absorption in the colon. The remaining aglycone is absorbed through the epithelium and is conjugated with glucuronic acid or sulfate in the lumen. When 50mg of aglycone is ingested, approx. 0.4μmol/L plasma metabolites will be measured[71]. Main metabolite after intake in urine from catechin and epicatechin are 3-(3-hydroxyphenyl) propionic acid, 5-(3',4'-dihydroxyphenyl)-y-valerolactone, 5-(3'-hydroxyphenyl)-y-valerolactone, and 3-hydroxyhippuric acid.

1.2.11 Phenolics in Insulin Resistance

In the metabolic syndrome and T2DM, some studies on the effect of natural compounds in vivo, in vitro, and clinical trials have been performed with phenolic compounds showing promising results. Flavonoid antioxidants (AOX) have shown inhibition of reactive oxygen species (ROS) production by inhibiting ROS producing enzymes and quercetin has been shown to regulate the gene expression of the pro-inflammatory cytokine, nuclear factor kappa beta (NFkβ).[73] Also, dietary AOX have shown the ability to protect pancreatic β-cells from glucose induced oxidative stress. Anthocyanins are free radical scavengers that increase serum AOX levels after consumption. Anthocyanins were able to decrease high fat diet induced obesity in vitro.[74]In human studies of chokeberry consumption (high anthocyanin content) fasting glucose and serum triglyceride levels of T2DM patients were significantly decreased.[75] Canadian low-bush berry (high anthocyanin content) has been able to illicit an increase in glucose uptake by muscle cells in the presence of insulin, while cyanidin and

delphinidin glycosides may increase insulin secretion in pancreatic β-cells.[76] Oligomers of grape seed procyanidin extracts (GSPE) activate the insulin receptor and key targets of the insulin signaling pathway by activating protein kinase B at Thr308 however at a weaker signal than insulin does, according to the lower insulin receptor activation by procyanidins. On the other hand, they phosphorylate AKT at Ser473 to the same extent as insulin. GSPE also decrease hyperglycemia in diabetic rats and stimulate glucose uptake insulin sensitive cells.[77]In a double blind clinical study, obese, non-diabetic and insulin resistant men and women were put on a diet supplementation with blueberry bioactives to determine their in vivo effects on insulin sensitivity. The study concluded the blueberry bioactives group enhanced insulin sensitivity without significant changes in overall adiposity, energy intake and inflammatory biomarkers in the participants.[78] Caffeic acid was shown to increase glucose utilization and glucose uptake in streptozotocin induced and insulin resistant models of rats.[79] Flavonoids and phenolic acids are able to inhibit adipogenesis in 3T3-L1 adipocytes with *o*-coumaric acid and rutin giving high inhibition of intracellular triglyceride accumulation and on GPDH activity and inhibited PPARγ, C/EBPα and leptin as well as up regulate adiponectin.[80] Caffeic, chlorogenic, and ferulic acids were able to lower glucose production via gluconeogenesis and glycogenesis compared to metformin via in vitro rat hepatoma cells.[81] Ethyl acetate fractions of rice bran high in ferulic acid as well as a ferulic acid standard were able to decrease blood glucose levels and increase plasma insulin levels in type II diabetic mice.[82] In an in vitro study on L6 muscle cells, chlorogenic acid was shown to increase glucose uptake by increasing GLUT4 translocation via PI3K

dependent pathway.[83] Finally, dried plums were tested on insulin resistant and obese Wistar fatty rats and were found to significantly increase plasma adiponectin concentrations and PPARγ mRNA expression in the adipose tissue as well as decrease blood glucose and plasma triglyceride concentrations.[84]

1.2.12 Stone Fruit Prunus Persica *(L.) Batsch*

Stone fruits (drupes) refer to fruits that develop around a hardened stone center (pit) that is derived from the ovarian wall of the plant. These fruits belong in the family Rosaceae under the genus Prunus which includes the fruits of peach, plum, nectarine, cherry, and apricot as their inner pit is large and extremely hard. The fruit crops provide prime fruit from June through September. As these fruits are native to warm climates one main issue of concern of for consumption is chilling injuries due to low temperatures.

Peaches (*Prunus persica*) are one species of the Prunus genus that originated from China and migrated all over the world with main growing sites in Europe (Spain and Greece) the US (California) and South America (Argentina and Chile). The peach falls under the kingdom Plantae (plants); subkingdom Tracheobionta (vascular plants); superdivision Spermatophyta (seed plants); division Magnoliophyta (flowering plants); class Magnoliopsida (Dicotyledons); subclass Rosidae; order Rosales; family Rosaceae (rose family); genus *Prunus* species *Prunus persica* (L.) Batsch (peach); with two taxonomy varieties: *Prunus persica* (L.) Batsch var. *persica* (peaches) and *Prunus persica* (L.) Batsch var. *nucipersica* (nectarines).[85]

Other than the accepted taxonomy order, there are endless ways to categorize peaches being used currently. Peaches are categorized by their fruit shape (round, flat), texture (melting, non-melting, stony hard), flesh adherence to pit (freestone, clingstone), flesh color (white, yellow), flesh acidity (acidic, low acidic), or commercial fruit type: peach (pubescent skin), nectarine (glaborous skin), and canning peach (non-melting flesh).[86] The white and yellow flesh peaches are popular for commercial consumption, with the white having a unique flavor and aroma and yellow able to mask oxidation from bruising and blemishes. Peaches have a soft flesh and are highly perishable commodities. They are composed of approx. 90% water and contain carbohydrates, vitamins, volatiles, antioxidants and trace amounts of proteins and lipids. Immature peaches have little to no starch grains, and with maturation these get converted into soluble sugars. Peach total weight is comprised of around 7-18% of soluble sugars and around 0.3% of fiber. Sucrose is the most predominant sugar found in peaches (40-60%), followed by glucose and fructose which when all combined account for 75% of the peach's soluble sugars. There has also been trace amounts of sorbitol found in peaches as well. Low quality peaches may report higher fructose and sorbitol values, however.[86]

The flavor of peach flesh comes from the fruit's aromatic volatiles, organic acids, phenolics, and sugars. The main volatiles in peaches to add to its flavor include hexanol, trans-2-hexanol, linalool, and decalactones while the main organic acids present are malic (>50%), citric, quinic, and succinic acids. Vitamin C (ascorbic acid) is typically low in most fruit cultivars (<10mg in 100g fresh weight), and Pro-Vitamin A (carotenoids) are also found, more so in yellow flesh varieties. Peaches contain yellow

pigments from xanthophylls that are synthesized by hydroxylation from carotenoids, specifically lutein, zeaxanthin, antheraxanthin, and violaxanthin from β-carotene. Carotenoids in peaches are photosynthetically active, while xanthophylls dissipate light that can disrupt photosynthesis. Both of these compounds can be found in the chloroplasts of the cells.[86] Phenolics in peaches can affect the overall flavor by giving astringent off flavors when are found in high concentrations. Exposed phenolics also undergo reactions by polyphenol oxidase (PPO) enzymes that create quinone browning products, also negatively affecting the fruit.

The polyphenolic compounds that are found in varying peach varieties include hydroxycinnamic acids, anthocyanidins, procyanidins, and flavonol derivatives. Specifically, of a research paper characterizing phenolics in different varieties of peaches, as well as plums and nectarines, the predominant phenolics found in the flesh and skin of these fruits were 3-caffeoylquinic acid (chlorogenic acid), 5-caffeoylquinic acid (neo-chlorogenic acid), procyanidin dimers and trimers, quercetin derivatives and cyanidin conjugates as glycosides, galactosides, rutinosides, and acetyl-glycosides. Only cyanidin-3-*O*-glucosides were found in peach varieties.[7] Anthocyanins such as cyanidin-3-*O*-glucoside are responsible for the production of a red color from the vacuoles of the peach and interestingly, the anthocyanin content in peaches usually occurs in the skin and the flesh nearest to the pit of the fruit. The anthocyanin content is independent of cultivar variety flesh color (yellow or white) and in some part, is dependent on light exposure; with the highest concentration occurring at the point of the fruit's full ripening.[86]

The main objectives of this thesis are to identify peach, plum, and nectarine phenolic compounds in cultivars of Rich Lady, Black Splendor, and Spring Bright, develop an insulin resistant murine skeletal muscle model, and view the effects of the stone fruit on G6P production in the cells. The next objective was to isolate the four major phenolic classes of compound by reverse phase silica cartridge while eliminating unwanted fruit material from original extracts and to identify those compounds. The second was to determine the yield recovery of those phenolic fractions after separation with the silica to determine the feasibility of the method. The last was to test the phenolic fractions on a cell model of insulin resistance; to determine if there is any positive biological effect on insulin sensitivity. Lastly, the conclusions to the findings of the experiments are reported.

2. PEACH, PLUM AND NECTARINE EXTRACTS SCREENING EFFECTIVENESS ON MYOTUBULAR INSULIN RESISTANCE

2.1 Overview

Stone fruit cultivars contain a mixture of diverse secondary metabolites in their flesh and peels, including polyphenolic compounds. Previous research has identified phenolic compounds, carotenoids, and vitamin C content as well as macronutrient composition on various stone fruit cultivars. Peach, plum, and nectarine cultivars show to have hydroxycinnamic acids, anthocyanin, flavonols, and catechins present in their flesh and peels[7]. These compounds of hydroxycinnamates, anthocyanins, procyanidin polymers, and other flavonoids have been reported in the literature to have positive effects against insulin resistance, type II diabetes mellitus (T2DM), and other symptoms of the metabolic syndrome.[8-9, 73-84] A major player in cellular tissue insulin resistance is skeletal muscle; with a majority of the blood glucose from food entering into the muscle and being converted to glycogen storage for future energy usage. Skeletal muscle is the second main source of glycogen storage behind the liver tissue. From Rich Lady peach, Black Splendor Plum, and Spring Bright nectarine the HPLC phenolic profiles were found and fruits were used as potential treatments in palmitic acid induced insulin resistant C2C12 myotubes. The fruit's effectiveness was analyzed by the results of insulin stimulated intracellular G6P production. Extracts were purified from sugars and organic acids by SPE reverse phase C18 silica gel and freeze-dried. Extracts used in cells were dissolved in DMSO and expressed at μg freeze-dried powder/mL. Final DMSO

concentration in the cells was 0.5%. Extracts were tested at levels of 0 – 300 μg/mL for cytotoxicity testing, and were used at levels of 0, 10, and 100 μg/mL for glucose 6-phosphate assay. Myoblasts were fully differentiated into mature myotubes and exposed to palmitic acid (0.5mM) for 48hr to induce insulin resistance and then treated with fruit extracts for 16hr and stimulated with insulin (100nM) for 30 minutes. Peach and nectarine showed to have a significant effect on glucose 6-phosphate accumulation in the cells at 1 and 10 μg/mL, respectively, while plum extracts showed no significant effects on the cells as compared to the positive control, metformin, a known diabetic, insulin sensitizing drug.

2.2 Introduction

There has been recent interest in stone fruits, along with other fruits, for their potential health benefits from certain diseases. For example, Black Splendor plum and Rich Lady peach were tested for their chemo preventative potential against breast cancer in vitro.[87] Stone fruit phenolics have shown similar phenolic flavonoids as apples, but also provide anthocyanin compounds (mostly in the skin) as in various berries. Stone fruit phenolics consist of mainly hydroxycinnamates, procyanidin dimers, flavonols, and anthocyanins. Specifically, of 25 varieties of peaches, plums, and nectarines, the predominant phenolics found in the flesh and skin were 5-caffeoylquinic acid (neo-chlorogenic acid), 3-caffeoylquinic acid (chlorogenic acid), procyanidin dimers and trimers, quercetin derivatives and cyanidin conjugates as glycosides, galactosides, rutinosides, and acetyl-glycosides. Only cyanidin-3-*O*-glucosides were found in peach varieties while some plum varieties contain cyanidin-3-*O*-glicosides, galactosides, and

acetyl-glycosides.[7]As mentioned previously, there has been literature study on these compounds as potential insulin sensitizers in clinical and animal studies as well as cell studies. While literature has shown individual compounds as well as some whole extracts of the same group of compounds having beneficial affects against insulin resistance, few papers report fruit extract of these compounds effect on insulin resistance specifically in muscle tissue. The main uptake and storage of insulin-stimulated glucose occurs in the liver and muscle tissues. As muscle cells present a simple mechanistic pathway from glucose uptake to glycogen storage, it makes this model more suitable for this experiment. While glucose uptake and glycogen storage is also important in liver tissues, there are more complex pathways to consider when looking into insulin sensitivity and resistance, while muscle cells function regarding glucose uptake uses mainly insulin stimulated pathways that are more easily tracked and explained through biomarkers. G6P was chosen as the biomarker of interest because the G6P level is directly related to the amount of glucose uptake into the muscle cells, with an increase of insulin stimulated glucose uptake into the cell, there is a direct increase in G6P intracellular concentration. Unlike the liver, muscle cells contain no G6Pase so it is safe to assume a direct increase in insulin stimulation and sensitivity with a G6P increase in muscle cells. The objectives of this chapter were to extract peach, plum, and nectarine phenolic fractions without significant organic acids or sugars and to test their bioactive potential against a developed insulin resistant muscle cell model, specifically the phenolics effect on G6P .

2.3 Materials and Methods

2.3.1 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.

2.3.2 Extraction and Purification

60 grams of fruit with proportional distributions from flesh and skin in the form of slices was blended with 180ml of 100% methanol (ultra-turrax (IKA. Works, Inc., Wilmington, NC)) and left at 4^{o}C overnight. Extracts were filtered through 4 layers of cheesecloth and then centrifuged at 15000 RPM for 15 minutes 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 reconstituted in distilled water to original volume (180ml) and purified of sugars and organic acids through solid phase extraction using a SEP Pack C18 cartridge as reported by Noratto *et al.*[87] Briefly, the aqueous extracts were adjusted to pH 7.0 with 5 N NaOH. A repeated batch system of 20mL of aqueous extract was loaded in a SEP Pack C18 cartridge (9 cartridges) (55-105 μm, Waters Corp., Milford, MA) previously conditioned to pH 7.0 with 200mLof 100% methanol and 200mLof nanopure water (pH7.0). The sugars and some phenolic acids were not retained on the cartridges. The cartridges were washed with 100ml water (pH 7.0) per batch. The water from the wash was combined with the phenolics that were not adsorbed in the cartridge (900ml); were evaporated in a rotavapor to 20ml and adjusted

to pH 2.0 with 0.01% triflouro-acetic acid (TFA). This mixture was loaded into a second cartridge previously conditioned at pH 2.0 with 200mL of 100% methanol and 200 mL of nanopure water at pH 2.0. The cartridge was washed with 100 mL of water (pH 7.0) per batch where sugars and organic acids were released from the C18. The remaining compounds were eluted with 100ml of 100% Methanol (pH 7). The remaining compounds on the first C18 cartridge were eluted with 100ml per batch each of 16% Acetonitrile adjusted to(pH 2) with 0.01% TFA, Ethyl Acetate, and 100% methanol to improve recovery of all compounds with polarity and pH differences. All solutions from all batches were combined (2.8L) and solvent evaporated at 45^{o}C using the rotavapor (Buchii, Switzerland) using a large volume (400ml) sample flask. Samples were evaporated in a batch system until low volume (10ml) from the large sample flask and collected into a smaller sample flask. The small sample (100ml) flask containing the condensed aqueous remains (70ml) were evaporated until only water remained (10-20ml), frozen at – 80^{o}C and freeze-dried (FTS Systems, INC. Stone Ridge, NY) at -50^{o}C and 200 mg HG of pressure until a powder. The clean powders were stored at -20^{o}C until use.

2.3.3 Total Soluble Phenolics

Peach, plum, and nectarine freeze dried powders were weighed after lyophilization and tested for their amounts of total soluble phenolics by the Folin-Ciocalteau method adapted by Swain & Hillis.[88] Peach, plum, and nectarine freeze-dried powders (4.5mg) were diluted in 1ml of methanol (Sigma, St Louis, MO). 15 µl of sample and 15 µl of chlorogenic acid standard curve were diluted in 240 µl nanopure

water in a 96 well microplate and reacted with 0.25N Folin-Ciocalteau reagent (15µl) for 3 minutes and then 1N Na2CO3 (30µl) for two hours before reading the spectrophotometric value at 725nm. Sample amount of total phenolics were determined from the standard curve developed. Total soluble phenolics were expressed as mg chlorogenic acid equivalents (CAE)/g freeze dried sample.

2.3.4 Thin Layer Chromatography of Stone Fruit Phenolics

All TLC assays were done as reported or adapted from Wagner *et al.*[89] except carbohydrate detection reported from Toba and Adach. [90] All TLC assays were run on silica gel 60F254 pre coated plates (Merck, Darmstadt). Powdered stone fruit samples were solubilized in methanol as a 3% solution (w/w) and 10µl solutions were used for plating. 5-10µl of reference solutions were used for plating. For phenolic 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^{o}C for 5 minutes. Sugars observed visibly. 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^{o}C for 10 minutes and viewed under UV 365 nm. A reference solution of *b*-sistosterol in chloroform was used. For coumarin detections 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^{o}C for 10 minutes then observed 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^{o}C for 10 minutes and observed visibly. 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 cyanidin 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 visibly. A reference solution of hydroquinone was used.

2.3.5 HPLC Analysis of Stone Fruit Phenolic Profiles

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 auto-sampler, and a 996-photodiode array detector (Waters Corp., Mildford, MA). Phenolic compounds were separated on a 4.6mm×150 mm, 5 μm, C18 reverse-phase Atlantis column (Waters Corp.) that was maintained at 25℃ by a Spectra-Physics 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 were processed with the Millennium software v3.1. The identification of individual phenolics was based on their UV spectra, ESI fragmentation patterns from previous work, and retention time. The identification was performed by HPLC using 3.0 mg powder dissolved in 1200uL 100% 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.

2.3.6 Biological Material

The following chemicals were used in the experiments: palmitic acid, insulin, 1,1-Dimethylbiguanide hydrochloride (metformin), Low Sugar-Dulbecco's Modified Eagle's Medium (DMEM), 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 myoblasts were acquired from the American Type Culture Collection (ATCC) (Manassas, VA).

2.3.7 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 dry freeze dried powder weight/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%.

2.3.8 Generation of an Insulin Resistant Cell Model and Treatment with Stone Fruit 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% confluent. Low sugar DMEM/10% FBS 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 in serum free 2% BSA low sugar DMEM media 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 1-5 µl tri-

chloroacetic acid (2N TCA) as enzymes in the cells can consume G6P and manufacture suggests deproteinizing samples; all the samples were centrifuged at 14,000 g for 2 min. After centrifugation, supernatants were recovered and the pH was neutralized with 1N NaOH until pH 6.5 – 7.5 for assay measurement conditions.

2.3.9 Intracellular Glucose 6-Phosphate Quantification 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 by a fluorescence in a microplate reader at Ex/Em = 485/560. Data was calculated according the manufacturer's instructions.

2.3.10 Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Turkey-Kramer HSD 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 ($p < 0.05$).

2.4 Results and Discussion

2.4.1 Analysis of Yield Recovery of Total Soluble Phenolics from Freeze-Dried Peach, Plum, and Nectarine Powder Extracts

Total soluble phenolics were quantified in the freeze dried fruit extracts as well as the methanol extracts. The methanol fruit extract total soluble phenolics in plums was 0.64 mg CAE/ml, while peach methanol extract phenolics was 0.123 mg CAE/ml, and nectarine methanol extract phenolic content was 0.06mg CAE/ml. From the lyophilization process, 392 mg of powder was collected from plum, 499 mg collected from peach, and 221 mg collected from nectarine. Of this powder quantified, the fruit mg CAE/ml was determined, and by extension of weight, the total amount of phenolics standardized by chlorogenic acid was found in the total powder. Each fruit freeze dried powder came from 60g of raw fruit. The final amounts are represented in table 1. Folin results show that the amount of chlorogenic acid equivalent phenolics in the powder is much less than the total powder weight, specifically, 6.3% for plum, 1.2% for peach, and 1.4% for nectarine. However, HPLC and TLC analysis confirm that the phenolic compounds are the main category of compounds found in the freeze-dried powders and possibly the rest of the powder weight come from a carbohydrate source. This reported data implies that the phenolic concentrations at work in the cells are at a much lower level than 1, 10, and 100µg freeze dried powder/ml solution.

Table 1. Total Soluble Phenolics found in Stone Fruit Lyophilized Powder

Fruit	Phenolics In fruit [mg CAE/g fruit]	Methanol Extract [mg CAE/ml]	Total Phenolics [mg CAE/g powder]	Total Powder (grams)	% Phenolics (w/w)	%* Yield Recovery
Plum	3.2	0.64	63.49 ± 0.18	0.3919	6.3	16.2
Peach	0.62	0.12	11.83 ± 0.10	0.4986	1.2	20.5
Nectarine	0.3	0.06	14.23 ± 0.109	0.2214	1.4	21.9

* Calculated based on methanol extract phenolic concentration and loaded volume of 180 ml

From table 1 we can see the recovery of phenolics in the powder. From the original extracts, around 20% of phenolics were recovered after extraction and purification. This shows that in our methodology there are losses occurring. Chapter III will address these yield recovery losses for their test procedures.

2.4.2 Thin Layer Chromatography

The detection of sugars, sterols, coumarins, terpenes, and arbutins was not found in the peach, plum, and 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.[89] 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 the 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.[89] 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 has anthocyanins, compositely, cyanidin 3-glucoside. 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.

2.4.3 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 analyses of samples were identified previously with UV spectra and ESI fragment patterns. Results showed 13 compounds in Black Splendor plum (dark red skin, red flesh), including 2 chlorogenic acid derivatives, 2 anthocyanins, 6 flavonoids and 3 procyanidin derivatives (Table 2, Figures 4). In Rich Lady peach, 11 compounds were identified, including 2 chlorogenic acid derivatives, 4 flavonoids and 3 procyanidin derivatives (Table 3, Figures 5). Other researchers have found that plums have different

types of anthocyanins, while many peach varieties only contain cyanidin 3-glucoside.[7] In black splendor, we found cyanidin 3-glucoside and cyanidin 3-rutinoside.

Table 2. Phenolic profile in Black Splendor plum

No.	$\mathbf{t_R}$	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 3-*O*-pentoside
11	15.789	239, 347	quercetin 3-*O*-pentosylepentoside
12	16.077	239, 351	quercetin 3-*O*-pentoside
13	16.548	240, 353	quercetin 3-*O*-rhamaose

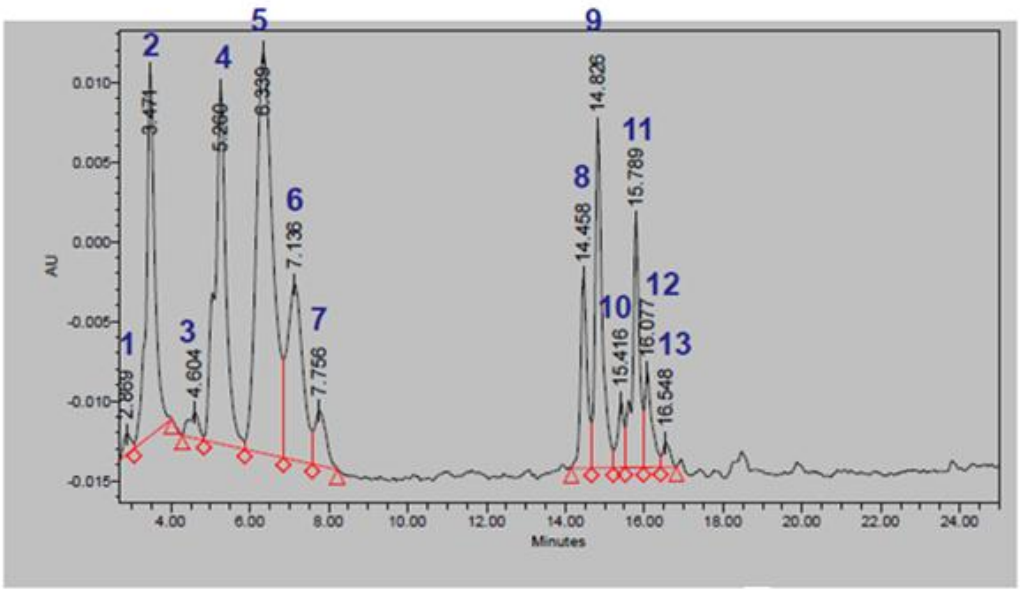

Figure 4. HPLC Chromatogram of Black Splendor plum at 320 nm

Table 3. Phenolic profile in Rich Lady Peach

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, 532	quercetin 3-*O*-glucoside
9	16.195	239, 351	flavonoid
10	16.582	240, 353	flavonoid
11	17.480	240, 353	flavonoid

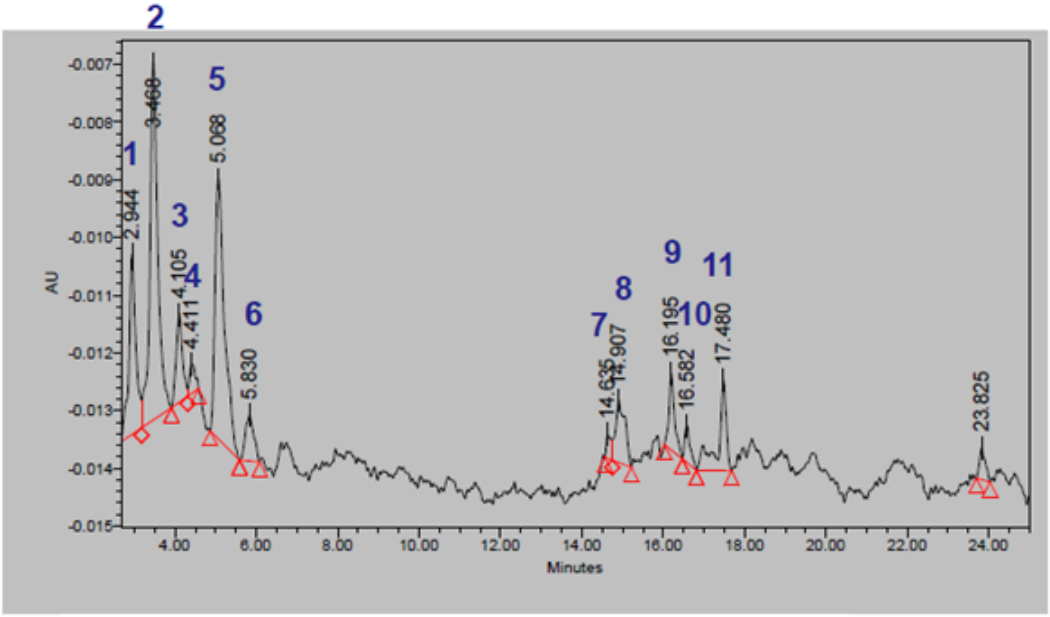

Figure 5. HPLC Chromatogram of Rich Lady peach at 320 nm

In peach, we found neo-chlorogenic and chlorogenic acids, rutin, quercetin 3-*o*-glucoside, three procyanidin polymers, and three flavonoid polymers. For Spring Bright nectarine, we found 5 compounds, including 2 chlorogenic acid derivatives and 2 procyanidin derivatives (Table 4, Figures 6). All stone fruits seem to have similar phenolic classes of compounds present, but in different proportions. Plum has the most anthocyanin content, while peach and nectarine are high in phenolic acids and flavonoid derivatives. Peach seems to have more variety of phenolics than nectarine

Table 4. Phenolic profile in Spring Bright nectarine

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

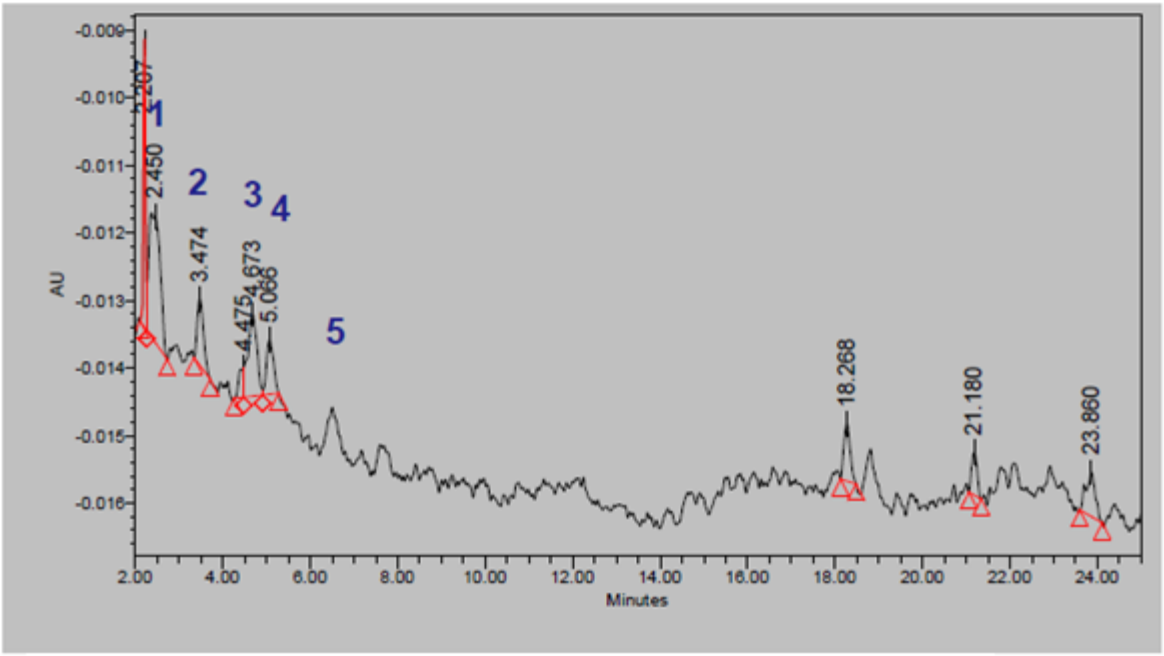

Figure 6. HPLC Chromatogram of Spring Bright nectarine at 320 nm

The data is comparative to other researcher's identifications of stone fruit cultivars.[7] The extraction and cleanup methods used were partially determined by

previous trials of SPE cleanup, where the majority of phenolics were lost in the process to remove sugars by the elution solvent of methanol alone (data not shown). Thus the method of using different solvent systems to play with the polarity and pH of the nature of the phenolic compounds was used. This method was previously proved to be also effective for stone fruit phenolics.[87] Because the aim was to see the holistic effect of the fruit phenolics on cellular glucose 6-phosphate production, the SPE treated phenolic compounds were combined together as one extract (freeze dried to powder) for cell study use. This is acceptable as our TLC work showed us that only phenolic compounds were present in the final extract powders.

2.4.4 Effect of Stone Fruit 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 at concentrations > 100 μg/mL induced cell cytotoxicity (Figure 7 A). For nectarine extracts, concentrations > 100 μg/mL showed a cytotoxic effect, reducing cell viability by about 40% (Figure 7 B). On the other hand, plum extract did not exhibit a cytotoxic effect until a concentration of 300 μg/mL (Figure 7 C). Based on the data obtained, the extract concentration range determined to use in this cell line, without altering cell viability was from 0-100 μg/mL for the peach, plum, and nectarine samples. The true total soluble phenolic content range of 0-100 μg dry powder/ml is 0-6.3 μg CAE/ml for plums, 0-1.2 μg CAE/ml for peach, and 0-1.4 μg CAE/ml for nectarines.

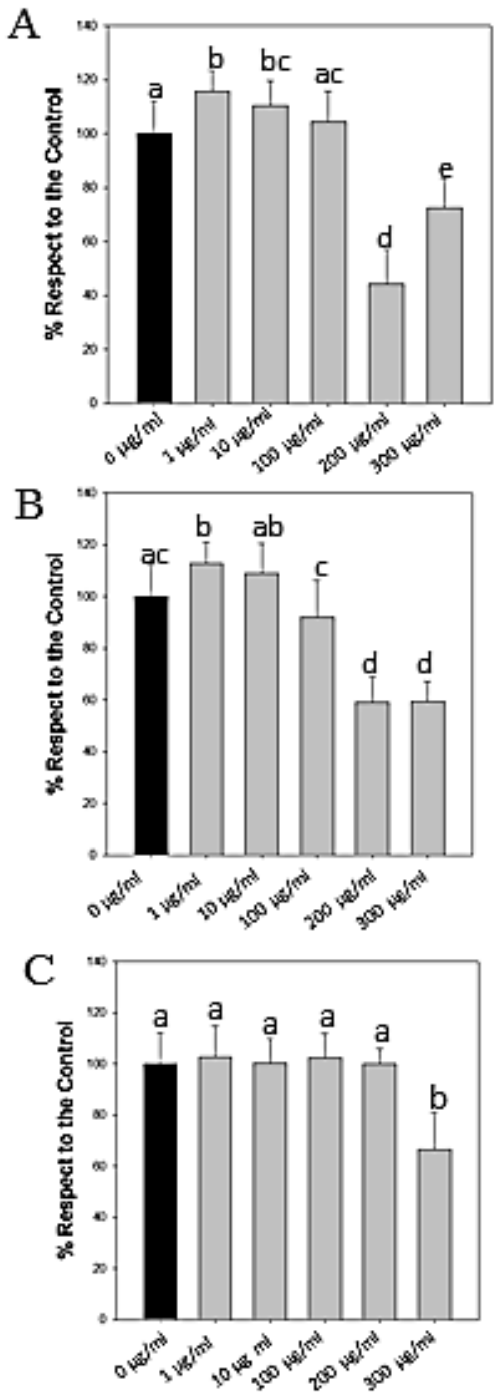

Figure 7. Evaluation of cytotoxic effects of stone fruit extracts on muscle cells. Cells were exposed during 24 h to extracts from peach (A), nectarine (B) and plum (C) and the cytotoxic effect of phenolics from 0-300 µg dry powder/ml was measured using the MTS assay. Data are means ± S.D. (n= 8). Different letters denotes significant differences by ANOVA Turkey-Kramer HSD ($p < 0.05$).

2.4.5 Differentiation of C2C12 Cells into Myoblasts 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. In the process of myogenesis, satellite cells will proliferate into myoblasts which will then differentiate to multi-nucleated myotubes fibers; with maturation, these myotubes become the well-recognized myofibers that make up striated skeletal muscle. After four days of treatment with horse serum, the formation of myotubes is complete (Figure 8), and the cells are ready to be used in further experiments.

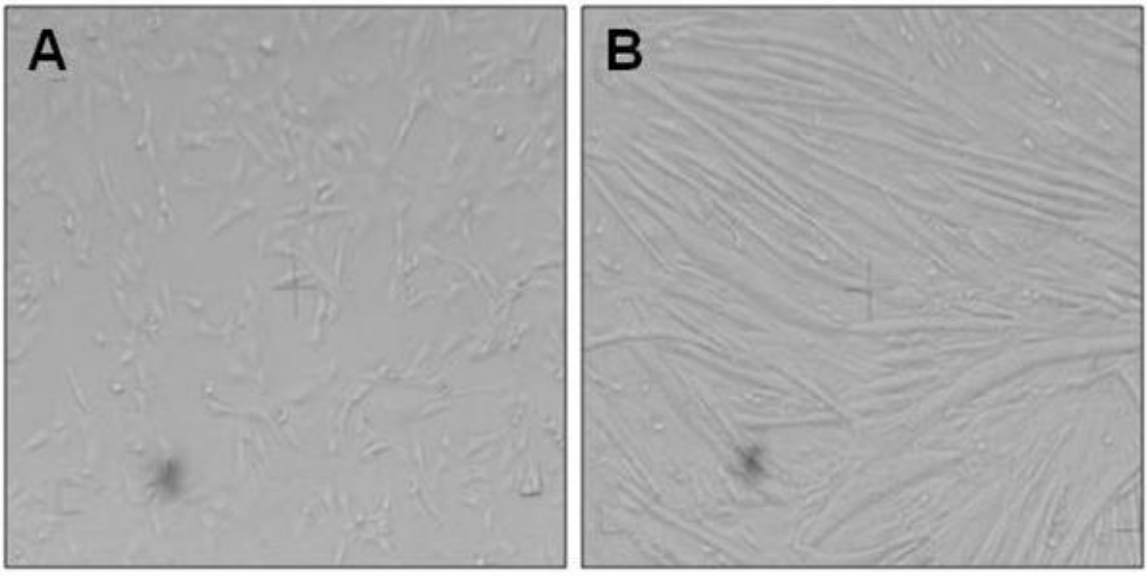

Figure 8. Differentiation of C2C12 cells into myotubes. C2C12 cells were cultured in medium supplemented with FBS until 70% confluent (A), then the cells were cultured during 4 days in medium supplemented with horse-serum to induce the fusion of the cells and formation of the myotubes (B).

Once C2C12 cells complete their differentiation into myotubes, the induction of an insulin-resistance state was done by using palmitic acid. Previous studies have shown that palmitic acid is able to induce insulin resistance in muscle cells, reducing its ability to perform glucose uptake.[91-95] 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.[96-98] The idea to incorporate the use of metformin in the experiments of quantification of intracellular concentration of glucose 6-phosphate, 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 glucose 6-phosphate in myotubes treated with palmitic acid, at levels comparable 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.[99] This extra-glucose present on the cells could be rapidly transformed into glucose 6-phosphate, 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 glucose 6-phosphate remains unchanged compared to palmitic acid treated cells non-exposed to insulin (Figure 9).

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 as compared to the insulin resistant cells. As expected, control cells exposed to insulin showed an increase (44.69%) of the intracellular concentration of glucose 6-phosphate compared to control cells non-exposed to insulin. Figure 9 is represented in two parts, the first being the intracellular glucose 6-phosphate accumulation in the cells as described earlier. The second part of the figure is the data transformed to show the net insulin stimulated values of intracellular glucose 6-phosphate, as insulin is the trigger hormone that activates glucose transporters to the cell membrane for glucose uptake into the cell, which is a crucial step in converting plasma glucose into glycogen, with the first process of this being the enzyme hexokinase converting newly taken glucose into the cell into the intermediate, glucose 6-phosphate. Based in all this data, the possible insulin-sensitizing effect of the stone fruits extracts was tested using this cellular model.

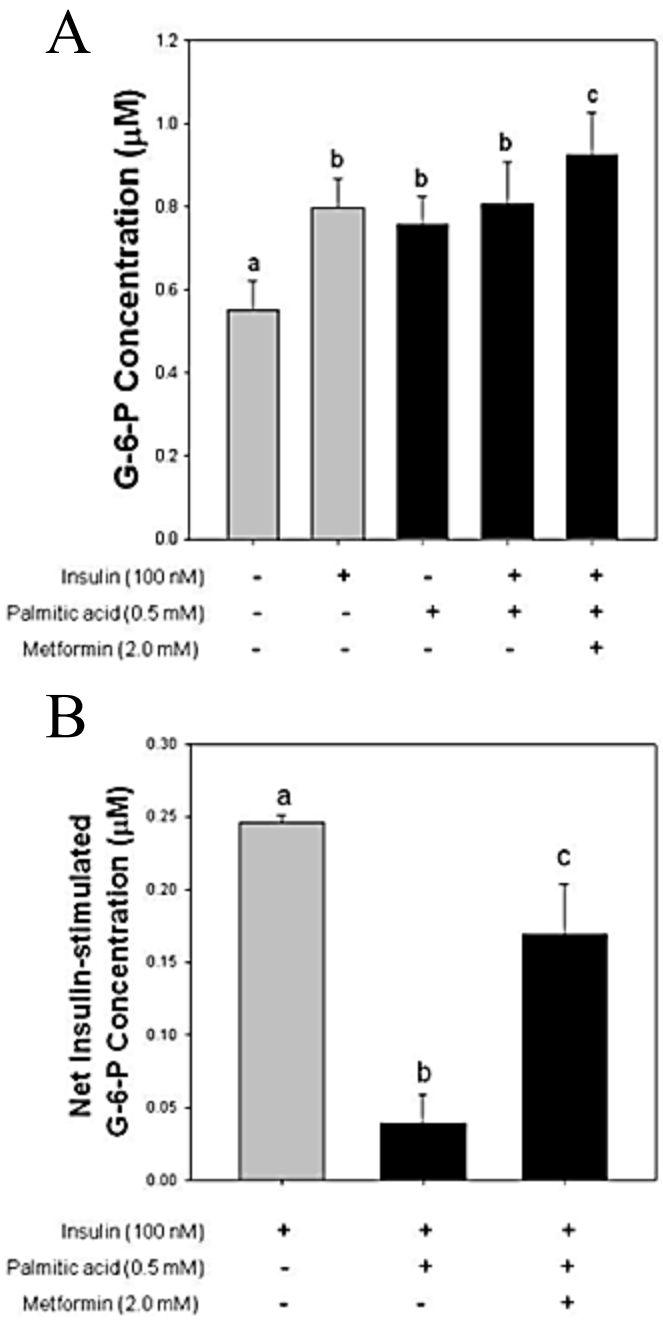

Figure 9. Effect of Palmitic acid and insulin on glucose-6-phosphate accumulation in muscle cells. G6P accumulation (A) and net insulin stimulated G6P accumulation in muscle cells (B). Cells were cultured in absence or presence of 0.5 mM of palmitic acid during 48 h and exposed to 100 nM of insulin for 30 min to determine the intracellular concentration of G-6-P. Bars represent means ± S.D. (n = 3). Different letters denotes significant differences by ANOVA Turkey-Kramer HSD ($p < 0.05$).

2.4.6 Effect of Stone Fruit 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 glucose 6-phosphate similar to the control cells. On the other hand, nectarine extract showed a similar effect to metformin at 10 μg/ml, increasing the intracellular concentration of glucose 6-phosphate. Similarly, the peach extract at 1 μg/mL also showed an insulin-sensitizing effect (Figure 10). 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 comparable 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 11, the data is transformed to show the net insulin stimulated glucose 6-phosphate, showing the visual differences in plum from peach and nectarine.

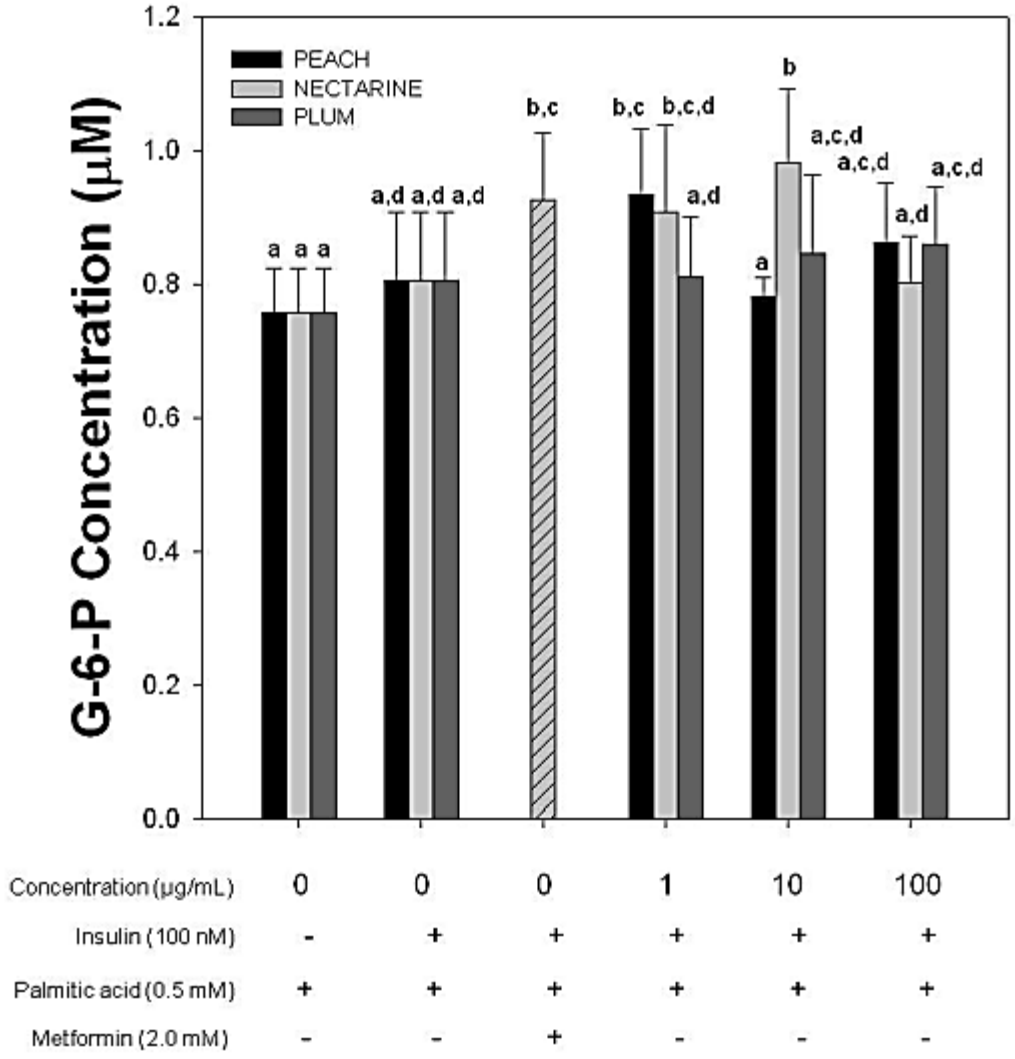

Figure 10. Effect of stone fruits extracts on intracellular glucose-6-phosphate accumulation in muscle cells. Cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and treated with extracts from peach, plum and nectarine co-incubated with palmitic acid for an additional 16 h, before exposure to 100 nM of insulin for 30 min. Samples were collected immediately after exposure to insulin. Intracellular Glucose-6-phosphate was determined using a commercial kit. Bars are means ± S.D. (n= 3). Different letters denotes significant differences by ANOVA Turkey-Kramer HSD ($p < 0.05$).

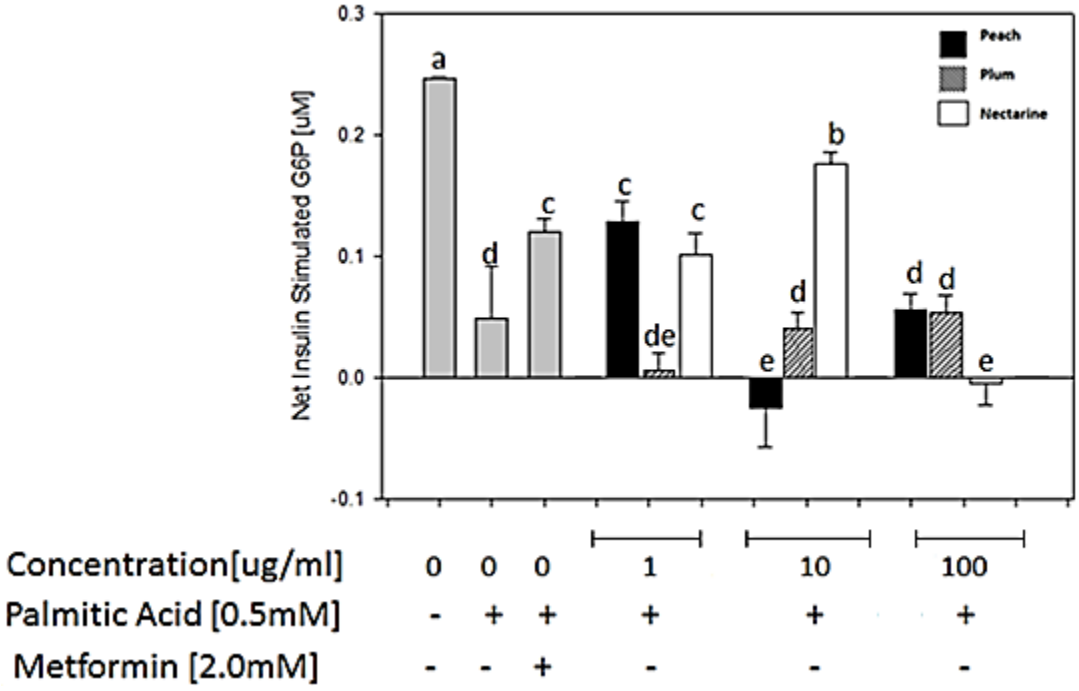

Figure 11. Effect of stone fruits extracts on net insulin stimulated intracellular glucose - 6-phosphate accumulation in muscle cells. Depicts the net insulin stimulated values of figure 10, taken by the difference in the insulin stimulated and control cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and treated with extracts from peach, plum and nectarine co-incubated with palmitic acid for 16 h, before exposure to 100 nM of insulin for 30 min. Samples were collected immediately 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 Turkey-Kramer HSD ($p < 0.05$).

Plum extract treated samples did not have a significant increase G6P concentration in respect to the control cells, however the peach and nectarine treated samples did show an increase. This is interesting as all the stone fruits contain similar phenolic profiles, but their composition varies, with the peach and nectarine phenolic compositions being similar. It is likely that it is either the phenolic acids, flavonoids, or catechin derivatives working in the cells to increase the G6P production, as these are abundant in the peach and nectarine samples. The peach and nectarine extracts could be working through many different ways to increase the G6P concentration including the insulin signaling pathway or the glucose metabolism steps in converting glucose to glycogen (figure 12). From these results we see a quick result of the fruit phenolic compounds on a marker of insulin resistance in muscle, with the next steps to research the separate groups of stone fruit phenolics on more markers of the disease in muscle.

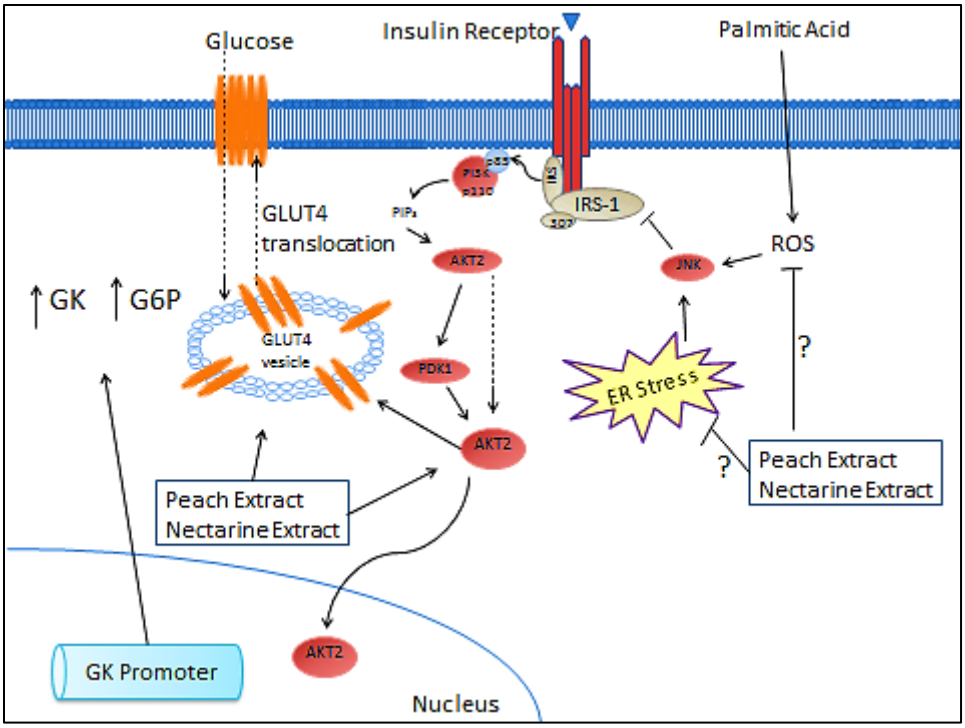

Figure 12. Diagram of possible mechanisms of action of peach and nectarine extracts in insulin-resistant myotubes. The phenolic extracts can be working to increase intracellular G6P through working on GLUT4 translocation, AKT increased phosphorylation, inhibiting ROS accumulation or inhibiting JNK and IRS1 serine phosphorylation. Diagram adapted from cell signaling.

In figure 12, we show a possible mechanism that the fruit phenolics might be targeting. Palmitic acid causes insulin resistance in muscle tissue by decreasing insulin receptor expression and activity as well as reducing downstream insulin signaling pathway activators such as IRS1 and AKT, key regulators in GLUT4 translocation and glucose uptake. Cellular factors to consider include palmitic acid's effect on ROS accumulation and ER stress, affecting JNK and IRS1 receptor signaling as well as the possible pathway components that are directly affected by insulin stimulation. Since the

exposure to palmitic acid is inhibiting the proper glucose uptake into the cells as seen on the control cells and cells treated with palmitic acid in their reduction in the concentration of intracellular glucose-6-phosphate (G6P). 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), in which both pathways are in part due to the activation of AKT, which is partly activated by the insulin receptor substrate activity in the insulin signaling pathway. Both possibilities may lead to an increased concentration of G6P, indicating a reversion of the insulin-resistance state. Another possible area that the stone fruits are working is decreasing the ROS and activated JNK. Intracellular ROS accumulation and JNK activation lead to an inhibition in the insulin signaling pathway, and therefore inhibit G6P intracellular accumulation. Further research will be investigated later in this thesis to determine which phenolic compounds found in the extract are exerting this effect on the insulin resistant C2C12 myotubes.

Since peach and nectarine fruit phenolics are the same compound categories and since peach HPLC chromatograms had identified more individual phenolic compounds present in the samples peach will be studied further into this mechanism to view the specific effects of each category of phenolic compounds (phenolic acids, anthocyanins, flavonoids, and catechin derivatives) on specific sites of the insulin signaling pathway as well as oxidative pathway involvement such as with JNK and ROS production.

3. EFFECTS OF PEACH PHENOLIC ISOLATE FRACTIONS ON MYOTUBE INSULIN RESISTANCE

3.1 Overview

Insulin resistance is a common precursor to T2DM and occurs when there is an inability of insulin sensitive tissues to transport glucose into the cell. In this study, peaches (*Prunus persica* var. Rich Lady) were extracted, purified, and separated into four fractions of polyphenolic compounds (phenolic acids (F1) anthocyanins (F2) catechins (F3) and flavonoids (F4) based on known chemistry reactions of the nature of the compounds with silica gel, pH, and solvent polarities using C18 SPE silica gel cartridges. Studies on the efficacy of the phenolic SPE separation method were done in a small scale trial (85% phenolic yield recovery after purification from extracts) and a scale-up production (48% yield recovery) for use in C2C12 myotube model. It was determined that with the correct solvent and pH system, phenolic acids and anthocyanins can be easily and distinguishably separated using this method; flavonol derivatives and catechins may also be separated, but as their polarity is so similar, complete isolation is difficult. From methods and recovery analysis it was determined that large scale production of clean phenolic extracts incur more losses due to stages involving solvent evaporation equipment, extraction ratio, and separation washing steps. The phenolic fractions were quantified for phenolic content with wet-based chemistry (Folin), compound identified with chromatography (HPLC-DAD), and used in an insulin resistant skeletal muscle cell model to determine potential bioactivity on glucose uptake,

glucose 6-phosphate, intracellular ROS, and immunochemistry. Muscle cells were induced to insulin resistance with 0.5mM palmitic acid and treated with fractions at ranges of 0-10 µg CAE/ml showed increased glucose uptake in cells (F1: 1.0 µg CAE/ml, F2: 0.1 µg CAE/ml, F3: 0.1 µg CAE/ml, F4: 0.5 µg CAE/ml). At these concentrations all fractions were able to increase glucose 6-phosphate intracellular accumulation similarly to control cells, with F3 showing no significant difference from palmitic acid induced cells. All fractions showed no significant effect on intracellular ROS, but were able to increase AKT phosphorylation similarly to control cells, and decrease JNK and IRS1 ser307 phosphorylation to levels of the controls. The fraction phenolics could be working through AMPK to regulate ROS and JNK production as it affects to IRS1 activation as well as through the transport of GLUT4 to the membrane for glucose uptake.

3.2 Introduction

Skeletal myotubes and adipocytes have been previously used as models for insulin resistance in vitro as they are the major sites of insulin stimulated glucose uptake in the body. Skeletal muscle accounts for over 80% of this uptake and therefore is a suitable cell culture model for studying the effects and potential reversals of insulin resistance symptoms. [4,12] Insulin stimulated glucose uptake is regulated primarily by the PI3K/insulin signaling pathway which begins with the insulin binding to its receptor ultimately leading to glucose transport vesicles to move to cell membranes to uptake glucose into the cell for transformation into glycogen for storage. Major players in the insulin signaling pathway include the insulin receptor and insulin receptor substrates,

PI3K and AKT activation, and the glucose transporter vesicles. In cellular insulin resistance the insulin hormone is able to bind to its receptor on cell surfaces, however, the PI3K signaling cascade initiated after insulin: insulin receptor binding is altered by excess amounts of free fatty acids in the body that lead to a decrease in the amount of glucose uptake into the cells, leading to a state of high plasma glucose levels that eventually, along with β-cell dysfunction, leads to type II diabetes mellitus (T2DM).[1, 49]

Phytochemicals have shown to have some beneficial effects on glucose uptake and insulin sensitivity in myotubes, adipocytes, hepatocytes, and pancreatic β-cells. Specifically, flavonoids have been found to inhibit ROS producing enzymes as well as quercetin regulating inflammatory cytokines.[73] Anthocyanins were found to decrease fasting glucose and triglyceride levels and increase muscle cell glucose uptake. [76] Finally, chlorogenic acids were able to increase GLUT4 translocation and glucose uptake, as well as decrease hepatic glucose production more than the common standard diabetic drug metformin. [81, 83]Chapter II in this thesis analyzed the effects of peach, plum, and nectarine phenolic extracts on C2C12 insulin resistant cells and found that peach extracts were able to increase intracellular glucose 6-phosphate concentration more so than nectarine. The peach extract contained a mixture of hydroxycinnamic acids, anthocyanins, flavonols, and procyanidins.

While the acting compounds in the peach fraction are known, and the total peach extract was able to increase intracellular G6P of murine muscle cells after being treated with palmitic acid, more research is needed to investigate what class of phenolic compounds are acting on the cells and where in the insulin signaling pathway are these

compounds having an effect. It is also worthwhile to look into other mechanisms that the phenolics could be acting that affect the insulin signaling pathway, such as the effect on intracellular ROS, as it leads to activation of JNK which inhibits the insulin signaling pathway. Therefore the next objectives will be to separate the phenolic compound classes found in peach and test them on the cells. By previous results, the peach contains four main compound classes: phenolic acids, anthocyanins, flavonoid derivatives and procyanidin polymers. These compounds will be chemically separated using SPE methods to be tested. For insulin resistance markers affected, G6P will be measured again, as well as glucose uptake as it is directly related to G6P. More elaboration in the pathway involves protein expression, specifically, AKT, IRS1, and JNK, as well as a look into intracellular ROS production. AKT is the main signaling protein in the insulin stimulated pathway; its activation stimulates glucose transporters for glucose uptake into the cell. Palmitic acid exposure in cell culture causes an increase in intracellular ROS production which in turn increases phosphorylation if the inflammatory cytokine JNK and IRS1 serine phosphorylation, both negative regulators of insulin signaling cascade. The phenolic compounds in peach may be targeting ROS and thus inhibiting the signaling pathway through negative regulators of insulin signaling. All of these markers will be investigated further to see the potential area the phenolic compounds of interest are activating.

3.3 Materials and Methods

3.3.1 Plant Material

Commercial variety of peaches (*Prunus persica* var. Rich Lady) 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 pits were removed and fruits frozen at -80^{o}C until further use.

3.3.2 Extraction and Purification

3.3.2.1 Rich Lady Peach Recovery Yield Study

13g of fruit cut in slices to preserve proportion between skin and flesh were homogenized (ultra-turrax (IKA. Works, Inc., Wilmington, NC)) with 40ml of methanol and left at 4^{o}C overnight. Extracts were centrifuged at 15000 rpms for 15 minutes at 4^{o}C and supernatants collected. Small aliquots were taken from the extracts for further analysis of methanol extract phenolics. The remaining methanol was evaporated at 45^{o}C using a rotavapor (Buchi, Switzerland) until all solvent was removed and only the plant water content remained. The remaining aqueous extracts were re-diluted in distilled water until original extraction volume (40ml) purified of sugars and organic acids through a solid phase extraction (SPE) C18 cartridge system. Extracts were adjusted to pH 7 with 1N NaOH. A repeated batch system of 20ml extract per cartridge (2 cartridge) was loaded into a C18 (55-105 μm, Waters Corp, Milford, MA) previously conditioned with 100ml methanol (pH 7) and 100ml distilled water (pH 7). The cartridge was washed with 200ml distilled water (pH 7) per batch. The sugars, organic acids, and some phenolics acids were not retained on the cartridge (A). The water from the wash containing the non-absorbed compounds from the fruit was concentrated to 20ml using a

rotavapor (Buchii, Switzerland) and adjusted to pH 2 with 0.01% tri-flouroacetic acid (TFA). This water 20ml sample was loaded onto a new C18 cartridge previously conditioned with 100ml methanol (pH 2) and 100ml distilled water (pH 2). The cartridge was rinsed with 200ml distilled water (pH 7), removing unwanted organic materials. The remaining phenolic compounds were eluted with 200ml 100% methanol (pH 7) (Fraction 1). The remaining compounds attached to the silica on the previous C18 cartridge were collected with 200ml of the following solvents: 16% acetonitrile (pH 2) (Fraction 2); ethyl acetate (Fraction 3); and 100% methanol (Fraction 4). All four fraction solvents (400 ml each) were evaporated in a rotavapor until 50 ml. The recovered 50 ml was transferred and concentrated in the a centrivap concentrator (Labconco, Kansas City, MO) to minimize the losses due to loss of concentrated liquid during transfer from a large evaporating flask with small concentrated volumes. The concentrator tubes contain a maximum of 10ml, so 5 tubes of 10 ml for each fraction were evaporated until ½ their volume (5 ml per tube) and collected into 3 tubes of 10ml and the process was repeated until only one tube was needed per fraction. Each 10ml fraction tube was then evaporated of solvent completely and diluted in 1 ml of 100% DMSO.

There is few literature publications of product recovery after purification procedures and those that are reported vary greatly in recoveries from anywhere to less that 10% to upwards of 80% This step in the methodology was to reduce any losses that may occur to provide a most accurate yield recovery from our chemical separation.

3.3.2.2 Rich Lady Peach Scale-up for Biological Study

100g of fruit cut in slices to preserve proportion between skin and flesh were homogenized (ultra-turrax (IKA Works Inc., Wilmington, NC)) with 400ml of methanol and left at 4^{o}C overnight. Extracts were centrifuged at 15000 rpms for 15 minutes at 4^{o}C and supernatants collected. Small aliquots were taken from the extracts for further analysis of methanol extract phenolics. The remaining methanol was evaporated at 45^{o}C using a rotavapor (Buchi, Switzerland) until all solvent was removed and only the plants water content remained. The remaining aqueous extracts were reconstituted in distilled water to original volume (400ml) and purified of sugars and organic acids through a solid phase extraction (SPE) C18 cartridge system, as described in figure 13. Extracts were adjusted to pH 7 with 1N NaOH. A repeated batch system of 20ml extract per cartridge (20 cartridges) was loaded into a C18 (55-105 μm, Waters Corp, Milford, MA) previously conditioned with 100ml methanol (pH 7) and 100ml distilled water (pH 7). The cartridge was washed with 200ml distilled water (pH 7) per batch. The sugars, organic acids, and some phenolics acids were not retained on the cartridge (A). The water from the wash containing the non-absorbed compounds from the fruit (2L) was concentrated to 20ml using a rotavapor (Buchii, Switzerland) and adjusted to pH 2 with 0.01% tri-flouroacetic acid (TFA). This water 20ml sample was loaded onto a new C18

cartridge previously conditioned with 100ml methanol (pH 2) and 100ml distilled water (pH 2). The cartridge was rinsed with 200ml distilled water (pH 7), removing unwanted organic materials. The remaining phenolic compounds were eluted with 200ml 100% methanol (pH 7) (Fraction 1). The remaining compounds attached to the silica on the previous C18 cartridge were collected with 200ml per batch of the following solvents: 16% acetonitrile (pH 2) (Fraction 2); ethyl acetate (Fraction 3); and 100% methanol (Fraction 4). All four fraction solvents were evaporated (4L each) in a rotavapor flasks for large volumes (400ml capacity) until 10ml which was then placed in a smaller volume evaporation flask (200ml capacity). The process was repeated until all volume was reduced into the smaller evaporation flask (100ml total) which was then evaporated completely until only phenolics remained, which was re-diluted to 1ml in 100% dimethyl sulfoxide (DMSO) and stored at 4^{o}C until further use. Figure 13 describes this process of fractionation in detail.

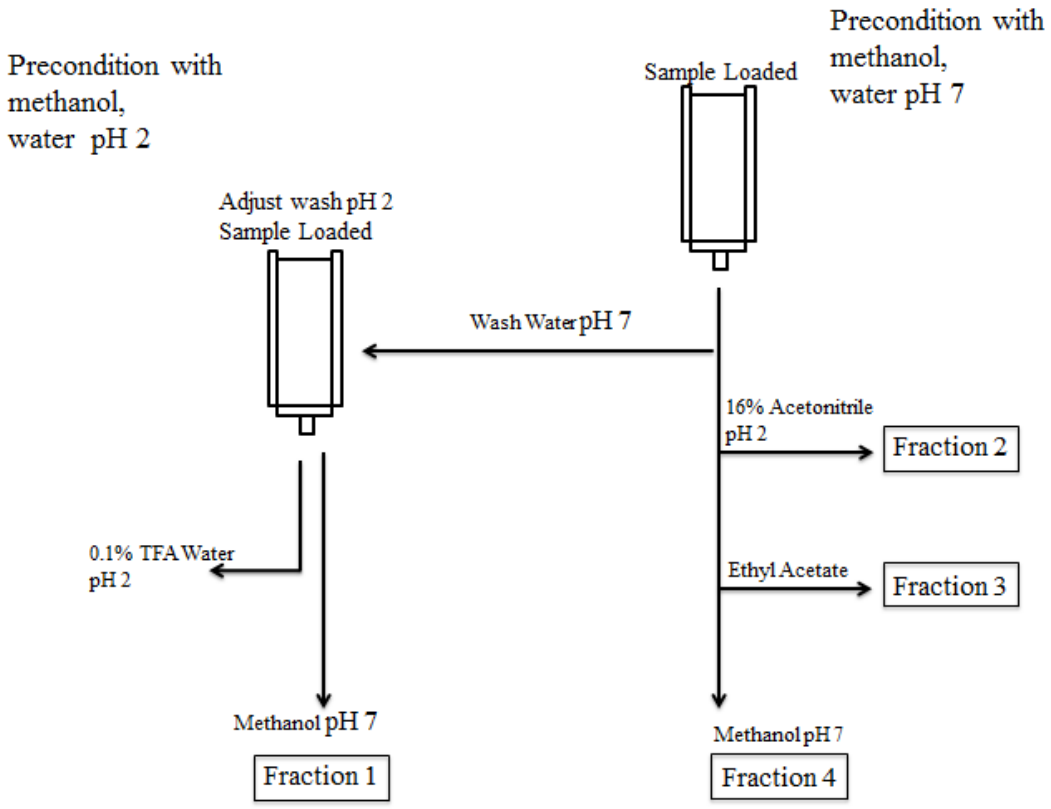

Figure 13. Solid phase C18 silica phenolic separation method. Aqueous pH 7 adjusted extracts (20ml extract to 10g sorbent) to a SEP PAK C18 cartridge previously conditioned with 100ml methanol (pH 7) and water (pH 7) using NaOH. The C18 sorbent is washed with water (pH 7, NaOH) and the wash is collected and concentrated back to original extract volume using a Rotovap (Buchi, Switzerland), adjusted to pH 2, TFA and added to a second C18 cartridge, preconditioned with 100ml methanol (pH 2, TFA) and water (pH 2, TFA). The C18 sorbent is washed with water (pH 2, TFA) and phenolic fraction collected with methanol (pH 7, NaOH) (Fraction 1). The original C18 cartridge is fraction collected with 16% acetonitrile (pH 2) (Fraction 2), ethyl acetate (Fraction 3) and 100% methanol (Fraction 4)

3.3.4 Total Soluble Phenolics

Peach extracts and 4 fractions were tested for their amount of total soluble phenolics by the Folin-Ciocalteau method adapted by Swain & Hillis. [88] Briefly, 15 µl of

sample along with chlorogenic acid standard curve were diluted in 240 µl nanopure water in a 96 well microplate and reacted with 0.25N Folin-Ciocalteau reagent (15µl) for 3 minutes and then 1N Na2CO3 (30µl) for two hours before reading the spectrophotometric value at 725nm. Sample amount of total phenolics were determined from the standard curve developed. Total soluble phenolics were expressed as mg chlorogenic acid equivalents (CAE)/ml sample.

3.3.5 HPLC Analysis of Peach Extract and Fraction Phenolic Profiles

Peach extract and 4 fractions were tested for their phenolic compounds. Briefly, 20 µL of filtered sample solution from peach extracted in methanol (1:4 (w/w)) and SPE separated fractions were injected after passing through a 0.22 µm nylon membrane. Individual compounds were identified based on retention time, UV spectra, and previous ESI fragmentation. Chromatographic separation was 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 auto-sampler, and a 996-photodiode array detector (Waters Corp., Mildford, MA). Phenolic compounds were separated on a 4.6mm×150 mm, 5 µm, C18 reverse-phase Atlantis column (Waters Corp.) that was maintained at 25 ℃ by a Spectra_Physics 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 20 µl. Data was processed with the Millennium software v3.1. The identification of individual phenolics

was based on their UV spectra as compared with authentic standards and former samples identified before with UV spectra and ESI fragment patterns.

3.3.6 Biological Material

The following chemicals were used in the experiments: palmitic acid, insulin,1,1-Dimethylbiguanide hydrochloride (metformin), Low Sugar-Dulbecco's Modified Eagle's Medium (DMEM), trypsin ethylene-diamine-tetra-acetic acid (EDTA), Dimethyl sulfoxide (DMSO), Bovine serum albumin (BSA), Fetal Bovine Serum (FBS), Horse Bovine Serum (HBS) and penicillin streptomycin mixture 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 lysis buffer and antibodies for AKT (pan), IRS-1, p-AKT (thr308), p-AKT (ser473), p-IRS-1 (ser307), p-IRS-1 (ser318), JNK, and p-JNK were obtained from Cell Signaling Technologies (Danvers, MA). 10% sodium dodecyl sulfate (SDS) solution, 30% acrylamide/bisacrylamide solution, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Tween 20, and Precision Plus Protein marker were from Bio-Rad Laboratories (Hercules, CA). Laemmli's loading buffer was acquired from Fermentas Inc. (Glen Burnie, MD). Polyvinylidene fluoride (PVDF) membranes were from Millipore Corp. (Billerica, MA). Goat anti-rabbit-HRP polyclonal secondary antibody (A120-101P) was from Bethyl Laboratories (Montgomery, TX). Water used was nanopure grade.

3.3.7 Cell Viability Assay

Cytotoxicity effects of peach fractions were evaluated in C2C12 myotubes 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 1,500 cells/well. C2C12 cells were left with Low sugar DMEM/10% FBS medium until they reached around 70% confluent. 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. When the cell culture was induced to myoblast peach phenolic fractions were added for 24 h. Previous to use, the peach phenolic fractions were diluted in 100% DMSO. The peach concentrations used were based on the folin results and were in the range from 0 to 80 μg CAE/mL, and the cell viability was measured after 24 hours of exposition to the fractions. The final percentage of DMSO per well was 0.5%.

3.3.8 Measurement of Reactive Oxygen Species Production

1,000 C2C12 cells were placed in 96-well black and clear bottom plates (Costar) with growing medium (DMEM, 1mM glucose and 10% FBS) until they reached around 70% confluent. 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. For ROS assay, the low sugar DMEM/10% HS medium was replaced with low glucose DMEM with serum free 2% BSA and 0.5 mM palmitic acid was added and incubated for 48h. After incubation peach phenolic fractions were

added during 16 additional hours co-incubated with palmitic acid in fresh serum free 2% BSA-low glucose-DMEM medium. In all cases, the serum free 2% BSA-low glucose-DMEM medium was removed on the day of the experiment, and the cells washed two times with warm PBS and then incubated with 5µL M DCFA in DMEM 1mM glucose phenol red-free medium at 37^{o}C for 30 minutes and the fluorescence was measured Ex/Em 485/535in a Microplate reader (Biotek, Vinooski, VT) .

3.3.9 Generation of an Insulin Resistant Model and Treatment with Peach Fractions

C2C12 myoblasts were seeded at a density of 1,000 cells/ well in 24-well plates and cultured with low glucose DMEM with 10% FBS medium until they reached around 70% confluence. Medium was replaced every 2-3 days. Once the cells were confluent, they were cultured with low sugar DMEM with 10% horse serum medium for 4 days, to induce myotubes formation. To the myotubes, 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 glucose-DMEM medium, and the fraction treatments were added and co-incubated in presence of 0.5 mM palmitic acid for 16 h (2.0 mM of 1,1-dimethylbiguanide hydrochloride (metformin) was used as a positive control). After the treatments with the fractions, metformin and palmitic acid the cells were exposed to 100 nM of insulin for 30 minutes, and cells treated for experiments below

3.3.10 Glucose Uptake in C2C12 Cells

Cells were cultured and treated with palmitic acid and peach isolate fractions under the conditions described above. To measure the glucose uptake of the cells a

glucose assay kit (Abcam. Cambridge, MA Catalog #ab65333) was used, per manufacturer's instructions. Briefly, media was collected from the cultured cells and diluted with glucose assay buffer into a 96 well plate and mixed with a glucose reaction mix and incubated for 30 minutes at 37^{o}C. A glucose standard curve was measured together with the samples, under the same conditions. The glucose present in the cell samples reacts with the glucose enzyme reaction mix to create a color compound visible at 570nm. The color produced is proportional to the glucose amount. Once incubation time was over, the amount of glucose in the media was measured in a microplate reader at 570nm for a colorimetric reading. Data was transformed to express the amount of mmole glucose uptake per well in the cells.

3.3.11 Intracellular Glucose-6-phosphate Quantification in C2C12 Cells

Cells were cultured and treated with palmitic acid and peach phenolic fractions under the conditions described above. To measure the intracellular amount of Glucose-6-phosphate, the PicoProbe™ Glucose-6-phosphate assay kit (BioVision. Milpitas, CA, Catalog #K687100) was used, following the manufacturer's instructions. Briefly, cultured cells were lysed and centrifuged to remove insoluble cellular material. The 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 minutes. A glucose-6-phosphate standard curve was measured together with the samples, under the same conditions. The glucose present in the samples and the known standard curve reacted with the G6P reaction mix to create a fluorescence that is directly proportional to the amount of sample G6P. 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 and expressed as mmoles G6P/well.

3.3.12 Immunoblotting

The muscle cells C2C12 were harvested with a cell lysis buffer (Cell signaling Beverly, MA) following the manufacturer's instructions. Each 6 well plate was washed with ice cold PBS (Sigma), added 100 µL lysis buffer, and containing protease inhibitor cocktail (Abcam Cambridge, MA). Cells were then scraped, left incubated at -80 ºC overnight, centrifuged at 14,000 rpm at 4 ºC for 15 min and supernatant was aliquot and stored at -80 ºC. The total protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) and 50 µg of protein were loaded on a gel with a pre-stained, broad-range, molecular weight protein marker (Fermentas). Proteins were fractionated by electrophoresis using 10% polyacrylamide gels made by mixing 9 ml of 22.2% acrylamide/0.6% Bis., 7.5 ml of 1M Tris//HCl (pH=8.8), 3.2 ml of nanopure water, 200 µl of 10% SDS, 100 µl of 10% ammonium persulfate, and 10 µl TEMED. The gels were transferred by wet blotting onto PVDF membranes (Millipore). The membranes were blocked with 5% BSA in Tris-buffered saline with 1% Tween-20 (TBS-T) for 2 h with gentle shaking; five times of 5 min washes with TBST were performed consecutively. Membranes were then incubated with specific primary antibodies against phosphorylated-AKT, AKT, phosphorylated-IRS, IRS, phosphorylated-JNK, JNK and b-Actin following the manufacturer's recommendations (Cell signaling company). The membranes were washed five times with TBS-T and

incubated for 1 h with the secondary antibody conjugated with horseradish peroxidase (HRP) at 1:30000 dilutions. Specific bands were developed using a Super Signal West Femto enhanced chemiluminescence (ECL) Western blotting detection kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) after 60 seconds incubation the signal were captured by CCD Camera (Cascade II:512, Photometrics, Tucson, AZ) using the Win View/32 software (Version 2.5, Princeton Instruments, Trento, NJ). Bands were measured and quantified using densitometry with Image J software (NIH, Bethesda, MD). For JNK isomers, both bands densitometry quantifications were averaged.

3.3.13 Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by tukey-kramer HSD, using the software JMP v9.0. Results are expressed as means ± standard deviations (S.D) of at least 6 independent experiments. Different letters show significant differences ($P < 0.05$).

3.4 Results and Discussion

3.4.1 Quantitative Analysis of the Recovery Yield of Total Soluble Phenolics from Peach Extracts Purified through C18 Cartridge and Peach Fractions Used for Cell Culture Treatments

3.4.1.1Analysis of Recovery Yield of Peach Fractions Total Soluble Phenolics

The total soluble phenolics of the trial peach extract and purified fractions are represented in below. Using the amount of soluble phenolics per gram of fruit, the amount of phenolics loaded onto the C18 cartridge may be predicted, and with the amount of phenolics recovered in the fractions, the loss and yield recovery may be

determined. With a net loss of 1.16 mg CAE, it can be calculated that the yield recovery of phenolic compounds from the sum of the four fractions as compared to the original extract is ~85%, based on the total soluble phenolics wet based chemistry assay. This number shows a relatively efficient process in purifying and separating phenolic compounds from plant materials. It is worth to mention here that there should always be yield losses in any extraction to purification or separation processes. Some ideas as to the loss in phenolics here can be placed to human error, the heating element brought on by concentration of samples, and the chemistry of the silica gel itself but the main reason for losses in this experiment come from samples losses in concentration and equipment used. When comparing results in Chapter II, the yield of recovery was an issue of phenolic solution recovery from the rotavapor sample flask. When drying large amounts of solvents to small volumes final concentrated samples are viscous and never completely recovered from the evaporating flask. After running this controlled yield study experiment with a smaller scale extraction the centrivap concentrator could be used, where the room for losses is much smaller due to fewer losses from transferring solutions and we had an acceptable yield recovery (84.6%). This hypothesis of losses occurring from the evaporating equipment was also confirmed from Chapter II results on obtaining freeze dried powders. The extracts in the Chapter II were left to evaporate until all organic solvent was rotavapor evaporated and all that remains aqueous solution. The aqueous solution was difficult to remove from its container for lyophilization without losses occurring due to the flask walls, and the final phenolics found in the powder obtained in Chapter II were extremely low levels (of 0-100 µg dry powder/ml is 0-6.3 µg

CAE/ml for plums, 0-1.2 µg CAE/ml for peach, and 0-1.4 µg CAE/ml for nectarines) with a phenolic yield recovery around 20% for all fruits. These true levels of phenolic compound exposure to the cell were taken into consideration in this chapter's phenolic dosage and treatments to the cell line.

Overall, SPE C18 methods can be a fast, easy and inexpensive way to separate and purify certain components of natural product matrices, as long as there is knowledge of polarity and acidity and the behavior of the silica and the compounds of interest. Because methanol has good extraction proficiency, a clean-up system is needed after extraction, as unwanted impurities from the fruit matrix are likely to be present also in the extract. Solid phase extraction (SPE) clean-up systems are useful for fairly clean end extracts. For SPE of phenolic compounds the solid phase is usually a C18 sorbent bounded to silica.[100] SPE has 50 times more separation power than a typical liquid-liquid extraction.[101] For C18 extractions, samples are pretreated for the column and eluted from the column with normally 6-10 volumes of solvent load. For reverse phase chromatography, elutions occur from weak to strong organic solvents as weak organic solvents elute slowly down the column and lead to a strong particle retention while stronger organic solvents pass quickly and allow analyte passage. Table 5 contains the fractioned samples are presented as concentrated phenolic solutions compared to the original extract containing phenolics as well as other unwanted materials. Fraction 2 seems to have the highest phenolic amount compared to the other fractions; however these values are arbitrary in their meaning as all of the fractions for the following

biological studies will be standardized to the same concentration based on their chlorogenic acid equivalency for homogeneity and control in the experiments.

Table 5. Yield recovery studies of Rich Lady peach through SPE C18 silica gel

Sample Measured	Total soluble Phenolics [mg CAE/ml]	Final Totals
Rich Lady Peach Extract	0.6* ± 0.03	13 g fruit
Peach Fraction 1	1.07	1 ml collection
Peach Fraction 2	3.65	1 ml collection
Peach Fraction 3	1.17	1 ml collection
Peach Fraction 4	0.70	1ml collection
mg phenolics loaded onto C18 cartridge:	7.80 mg CAE	84.6%
mg phenolics sum of fractions recovered:	6.60 mg CAE	recovery

*Expressed as [mg CAE/g fresh weight]

The yield study samples were evaporated in the rotavapor until 50 ml, an amount experimentally found to be easily transferrable out of the sample flask with minimal losses. The remaining solution was evaporated in a centrivap concentrator, capable of a 10ml sample flask holding capacity. With a smaller sample flask container the final phenolic removal showed minimal losses, as there was less surface area to leave sample residue. Reasoning behind this is the pure size of the experiments. The rotavapor is meant for evaporating solvents at high volumes (up to 1000mL capacity) while the centrivap can hold test tube up to 10ml capacity. It was not plausible to recover any amount of evaporated solvent from a large container containing a small, condensed

amount of sample; therefore, the centrivap was used. For the centrivap study, the 50ml rotavapor reduced solutions had to be placed in 5 10ml tests tubes per fraction into the centrivap. These 10ml were reduced to ½ of their volume and then reduced to three 10ml test tubes and repeated until each fraction was completely evaporated into just one 10ml test tube. Afterwards there is no transfer of small solutions that could lead to high losses, but just a re-dilution in 1ml 100% DMSO for phenolic analysis to confirm the yield.

3.4.1.2 Analysis of Scale-up Peach Fractions Total Soluble Phenolics

From the extraction and purification through SPE from the fruit used for the bioassays presented in table 6, the final phenolic recovery was much lower than what was recovered in the yield study, as depicted in table 5. From the 100 g of fruit with 0.32 mg CAE/g fruit, 32 mg CAE of phenolics were put through the C18 silica gel. From the folin quantification of the fractions 15.7 mg CAE phenolics were recovered. This gives a 49% yield recovery of the phenolic compounds from the 100g of peach fruit Total soluble phenolics for the biological assays were determined as mg CAE for each fraction and the original extract aliquot. The results are presented in table 6. The extract was taken from a mixture of peel and flesh. These values recorded higher phenolic values for the peel extracts than our extract, (0.32 mg CAE/g fruit), and lower values for the flesh extracts than our extract, showing that the concentration is higher in the skin of the fruit rather than the flesh and our extract shows the phenolic content of the peach as a whole.[102] The experiment for the biological study experiments used the same separation procedure as the yield study, however this was done on a much larger scale, and the procedure of removing solvent from the fractionated phenolics differs from the yield

study in that the centrivap concentrator for small volumes was not used, and losses from removing solvent from a larger sample flask could account for the large yield losses from the original fruit. These yield losses are permissible as we verified though our yield study that the methodology works for obtaining high quality yields of phenolic compounds from non-pure extracts and as the phenolics are standardized before treatment of cell culture, the actual phenolic content added to the cells is known, as well as the HPLC chromatograms confirmation that the type and ratio of phenolic compounds remains the same for both experiments, indicating no alterations in the phenolic profiles.

Table 6. Total soluble phenolics of Rich Lady peach extract and fractions

Sample Measured	Total soluble phenolics [mg CAE/ml]	Final Totals
Rich Lady Peach Extract	0.320* $\pm$ 0.21	100 g fruit
Rich Lady Peach Fraction 1	2.580 $\pm$ 0.16	1 ml
Rich Lady Peach Fraction 2	8.610 $\pm$ 0.18	1 ml
Rich Lady Peach Fraction 3	1.450 $\pm$ 0.11	1 ml
Rich Lady Peach Fraction 4	3.020 $\pm$ 0.23	1 ml
Phenolics loaded C18	32 mg CAE	48.9%
Phenolics recovered C18	15.66 mg CAE	Recovery

*Expressed as [mg CAE/g fresh weight]

In the 100g scale up of the procedure more materials such as extraction and collection solvents and reverse phase separation cartridges are needed as well as

different concentration processes. For the 100g fruit processing for the bioassays, the large amount of solvent that was passed though the C18 cartridge for fractionation was evaporated with the rotavapor, as this evaporator can hold large volumes of solvent to evaporate. After evaporation of the solvent much sample residue remained coated the walls of the flask and was unable to be transferred out of the sample flask. There are many other opportunities for product loss when scaling up the phenolic concentration for cell use. From 85% from the 13 g fruit sample, two 10 g silica C18 sorbent phase is used for clean-up and purification. For 100g fruit extraction, batch systems of twenty 10g sorbent cartridges are needed to prevent the risk of overloading any one cartridge with compound. Even so, this is not as effective as large silica packed column with a sorbent weight heavy enough to pass the entire sample through one time. Losses can develop with batch separation of one sample for clean-up and purification, as well as during the transfer from the sample flask after solvent evaporation. This yield study shows that the methodology is efficient at separating phenolic compounds by their chemical structures with minimal losses but if larger phenolic concentrations are desired the technology should be adapted to correct processes and equipment that cause product loss that occurs when scaling up the procedure.

3.4.2 Identification of Phenolics in Rich Lady Peach Extracts and Fractions with High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD)

HPLC analysis showed 7 compounds in Rich Lady crude extract including 2 chlorogenic acid derivatives, 1 anthocyanin, 3 flavonoids and 1 procyanidin derivatives (Table 7, Figures 14, 15). This result is comparable to previously reported data on Rich

Lady peach varieties. In an analysis by HPLC-DAD of a Rich Lady extract done previously by Noratto and others, three peaks were determined: neo-chlorogenic acid, cyanidin 3-*O*-glucoside, and quercetin 3-*O*-glucoside.[87] Another HPLC-DAD analysis done by Tomas-Barberan and others found in the flesh and peel of Rich Lady: neo-chlorogenic acid and chlorogenic acid (main), around equal levels of catechin and procyanidin B1 as well as slightly lower amounts of epicatechin, quercetin 3-*O*-galactoside, glucoside, and rutinosides, as well as cyanidin 3-*O*-glucoside and trace amounts of cyanidin 3-*O*-rutinoside, found only in the peel extracts.[7] Our extract showed the same compounds as listed above, with the exception of cyanidin 3-*O*-rutinoside, which could be due to the fact that we extracted a peel and flesh extract, whereas cyanidin 3-*O*-rutinoside was reported in trace amounts in a peel only extract.

Table 7. Phenolic profile in Rich Lady peach crude extract

No.	$\mathbf{t_R}$	λ_{max}	**Compound**
1	2.64	232, 278	procyanidin
2	3.03	232, 280, 328	Neo-chlorogenic acid
3	4.23	233, 279, 330	chlorogenic acid
4	5.16	233, 279, 517	cyanidin 3-*O*-glucoside
5	14.57	237, 352	Rutin
6	16.00	238, 352	quercetin 3-*O*-glucoside
7	16.31	240, 353	flavonoid

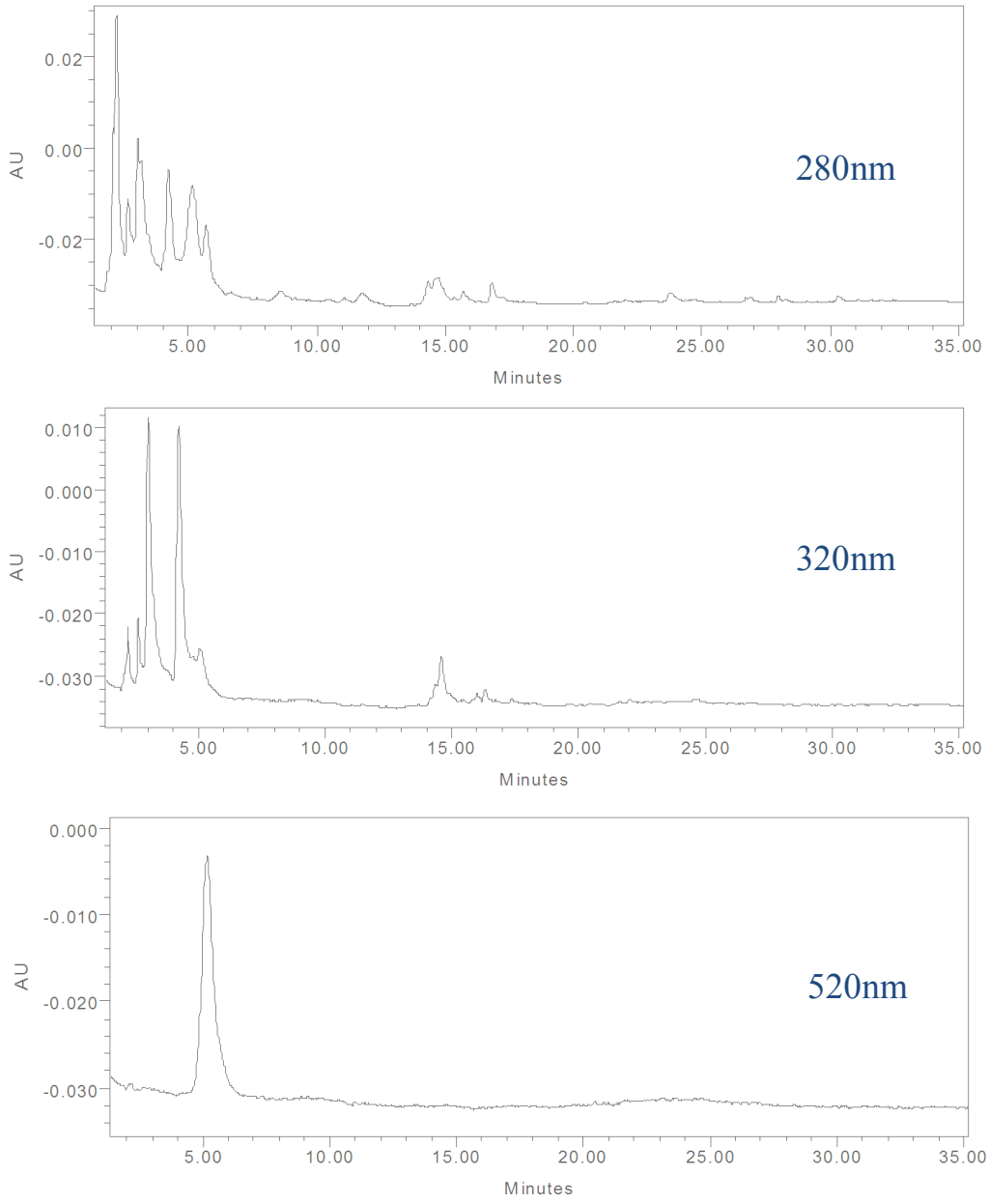

Figure 14. HPLC Chromatogram of crude extract at 280nm, 320nm and 520 nm

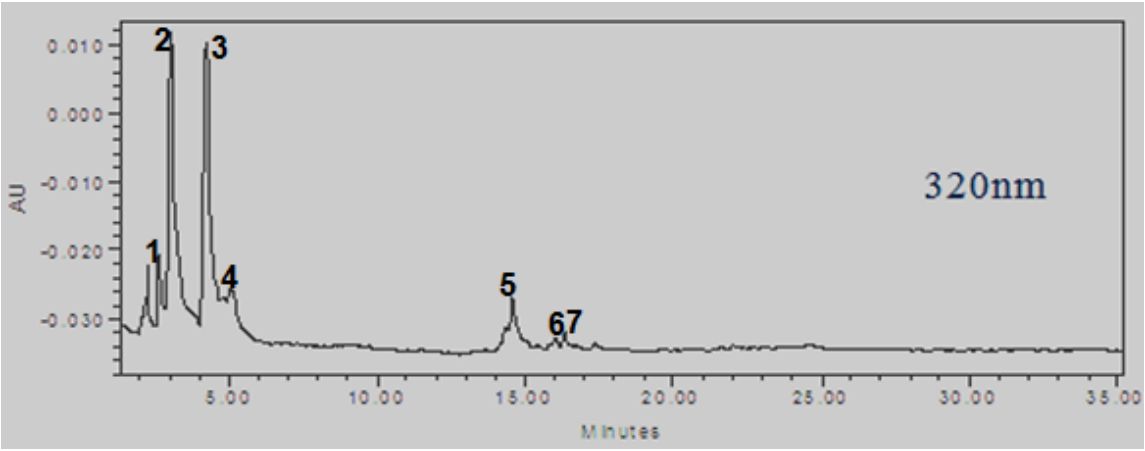

Figure 15. HPLC Chromatogram of crude extract at 320 nm with phenolic compound identification as listed by table 6

In fraction 1, 4 compounds were identified, including 2 chlorogenic acid derivatives, 1 anthocyanin and 1 procyanidin derivatives (Table 8, Figure 16). Previously, the silica C18 separation method was used to isolate phenolic fractions from an original extract from plums and peaches. Previously, Noratto and others separated a phenolic acid fraction from rich lady peach and obtained a fraction containing neo-chlorogenic acid and chlorogenic acid.[87]The same silica and solvent system was used here. The idea of the C18 clean up system for phenolics uses the basis that the organic compounds will stick to the silica sorbent while during washing with water, hydrophilic compounds such as organic acids and sugars will elute off the column, leaving the organic material to be eluted off with organic solvents with similar polarity to the compounds of interest. In the case of phenolic acids, their elution is dependent on pH, as their hydrophobic affinity changes due to association/dissociation. In cases of neutral pH environments, dissociated phenolic acids will show affinity to a water elution. In our

method this water elution was added to a second cartridge but this time at a low pH environment. At a low pH, phenolic acids will absorb to the C18 phase due to its non-polar behavior. Once absorbed the phenolic compounds will have more affinity for a slightly more organic phase over water, thus during washing, the remaining acids will remain on the column until eluted with the correct polarity solvent. By the HPLC chromatogram the methodology worked well, with mostly phenolic acids separated with lower amounts of epi-catechin and cyanidin 3-glucoside recovered as well, most likely due to overloading of the C18 cartridges, and thus these phenolic fell out of the silica with the water washing.

Table 8. Phenolic profile in fraction 1

No.	t_R	λ_{max}	**Compound**
1	3.87	232, 280, 328	Neo-chlorogenic acid
2	4.03	233, 279, 330	chlorogenic acid
3	4.86	233, 279, 517	cyanidin 3-*O*-glucoside
4	5.76	233, 280	*epi*-catechin

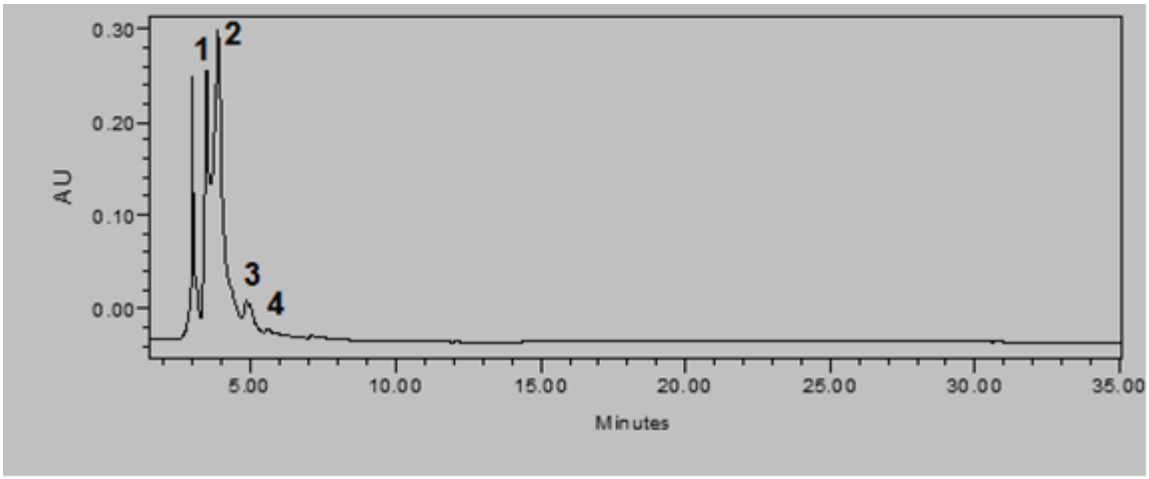

Figure 16. HPLC Chromatogram of fraction 1 at 320 nm

Fraction 2 (Table 9, Figure 17) only has 1 anthocyanin, cyanidin 3-*O*-glucoside. This shows an acceptable purification of anthocyanins from C18 with acidic 16% acetonitrile, as no other compounds were found in this fraction. In the water washing phase, the anthocyanins will remain on the solid phase, allowing interfering compounds to elute, however with an organic polar solvent, the anthocyanins may be eluted. Two methods may be used after this step: first, remove all other polyphenols from the cartridge with ethyl acetate or similar solvent, before eluting the anthocyanins with an acid, more polar solvent such as methanol, or first elute the anthocyanins from the cartridge as proceeded with in this experiment, by selecting a polarity solvent that will only elute anthocyanins (16% acetonitrile) at an acidic pH = 2, anthocyanins are most stable.[100, 103] If methanol or another solvent system is used to separate the anthocyanins first without previously removing all other compounds, the isolation will not be so complete, with other phenolics most likely eluting out with the anthocyanin fraction,

therefore with 16% acetonitrile at an acidic pH, elution of only anthocyanins from the cartridge is possible.

Table 9. Phenolic profile in fraction 2

No.	t_R	λ_{max}	Compound
1	4.23	233, 279, 517	cyanidin 3-*O*-glucoside

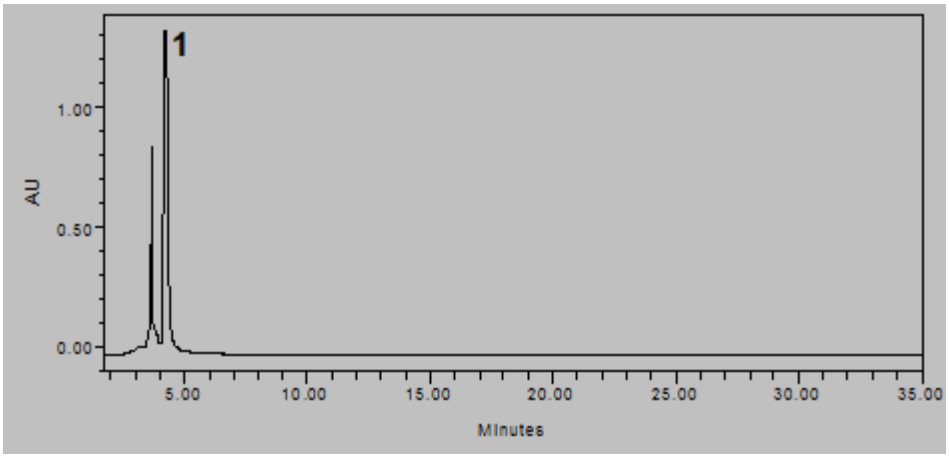

Figure 17. HPLC Chromatogram of fraction 2 at 520 nm. First peak shown on graph is solvent peak of DMSO

In fraction 3 (table 10, figures 18, 19), 9 compounds were identified including 2 procyanidin, 2 chlorogenic acid derivatives and 5 flavonoids. The polarity and structure of flavonols found in stone fruits such as quercetin and catechin derivatives are very

similar and therefore it is difficult to obtain a pure fraction of only flavonols and one of only catechin derivatives. The same is seem with HPLC elutions of these compounds as they have very close peaks, and therefore some flavonoid derivatives that are hard to quantify to a specific structure. In the fractionation procedure it was thought that with ethyl acetate the flavonols would elute, and later with the methanol, the catechins would elute. From the chromatograms and phenolic identification it seems that the third fraction eluted most of the remaining phenolic compounds left on the silica cartridge after elution of phenolic acids and anthocyanins.

Table 10. Phenolic profile in fraction 3

No.	$\mathbf{t_R}$	λ_{max}	**Compound**
1	2.74	232, 278	procyanidin
2	3.46	232, 280, 328	Neo-chlorogenic acid
3	4.44	233, 279, 330	chlorogenic acid
4	5.94	232, 278	Procyanidin
5	15.08	237, 352	Rutin
6	16.10	238, 352	quercetin 3-*O*-glucoside
7	17.10	239.351	Flavonoid*
8	22.12	240.353	Flavonoid*
9	23.81	240.353	Flavonoid*

*denotes flavonoid glycosides, tentatively quercetin derivatives

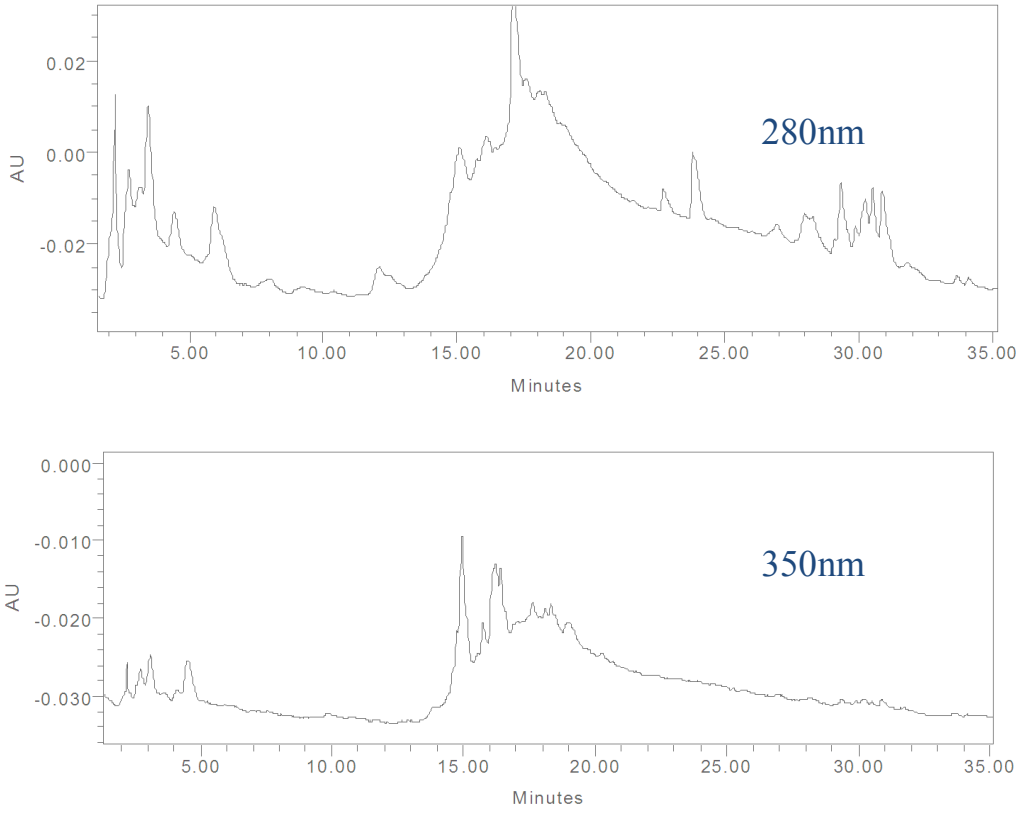

Figure 18. HPLC Chromatogram of fraction 3 at 280nm and 350nm

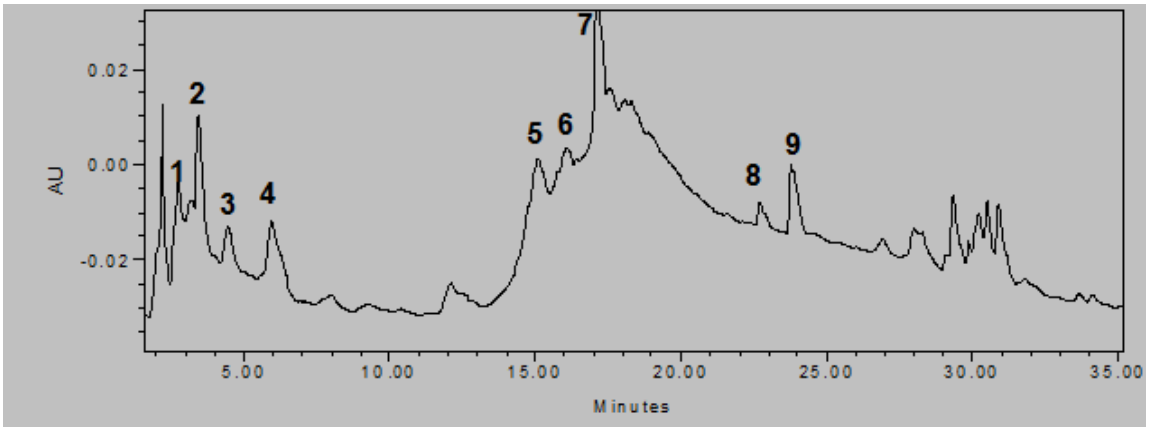

Figure 19. HPLC Chromatogram of Fraction 3 at 280 nm with phenolic compound identification as listed by table 9

Table 11. Phenolic profile in fraction 4

No.	t_R	λ_{max}	Compound
1	8.72	232, 278	procyanidin

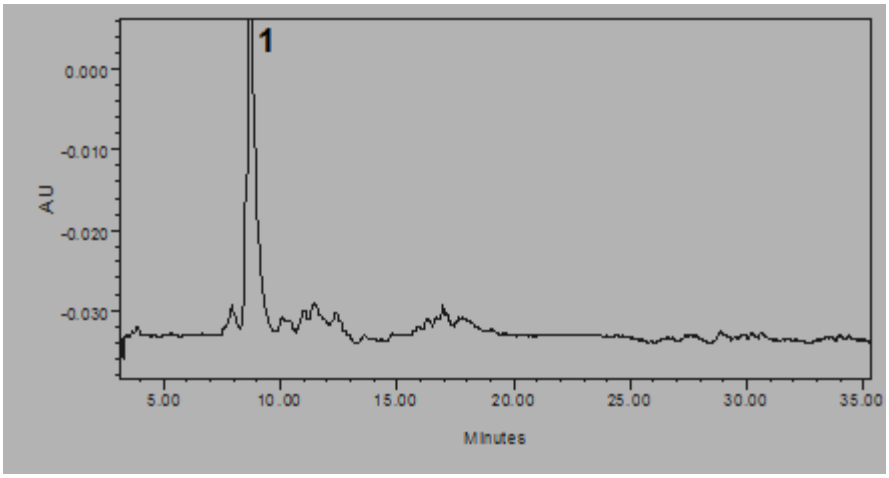

Figure 20. HPLC Chromatogram of fraction 4 at 350nm

For fraction 4, we found 1 procyanidin (Table 11, Figure 20). Fraction 4 did elute one procyanidin polymer, possibly complex in structure and slow to elute down the solid phase with ethyl acetate, and the extra wash with methanol finally released it from the column. Noratto *et. al*. were able to identify quercetin derivatives in their ethyl acetate fraction 3, but in their methanol fraction 4, only small amounts of cyanidin 3-glucoside could be determined.[87] In that study it was most likely that the cartridge was overloaded, and after flavonol elutions with ethyl acetate, the methanol released any remaining compounds in the cartridge, in this case, cyanidins.[87] In our case, in fraction 3, we do have many flavonols to determine a flavonol rich fraction, and in fraction 4, as it only

contains a procyanidin, this by name, is our procyanidin fraction. With the compounds present in rich lady, the C18 methodology of cleaning and isolating the phenolics based on their pH and polarity tendencies worked well, with phenolic profiles similar to that what was theorized in the isolation procedure method. Perhaps with a different column or solvent systems, the separation of flavonoid compounds, mainly flavonols from procyanidins may be achieved with more separation. The separation of anthocyanins is well reported and in this protocol, was achieved with excellent separation. The isolation oh phenolic acids was also achieved in this protocol, but a cleaner isolation would have been possible if there was less of an overload of the C18 cartridge. Overall, C18 separation methods are useful in the separation of phenolic compounds in large volumes for use in biological assays. While there are other methods that are used for cleaner separations, their final yield is low in volume and may only be used for few chemical experiments, but cannot be produced in high enough quantities for further biological experiments like C18 solid phase extraction has shown.

3.4.3 Effect of Peach Fractions on Cell Viability

Before proceeding with any experiment, it was necessary to determine the concentration levels that cause cytotoxicity in the cells. After incubation with the fruit fractions for 24 hours, the cytotoxicity was measured according to the MTS assay. Results showed that after a concentration of 10 μg CAE/ml there is an induction of cytotoxicity for all four fractions.

For fraction 1, there appears to be no drastic effect on cell death based on the results (figure 21A), however statistical differences do appear at higher concentrations.

In fraction 2 and fraction 4, there are similar cell death inductions after 10 µg CAE/ml (figure 21B, D). For fraction 3, there is a clear induction of cell death after 5 µg CAE/ml (figure 21C). The harsh effect of fraction 3 on the cells is based on the chemical composition of the compounds found in the fraction, with many flavonol and catechin derivatives. Based on the data obtained, the concentration range for the remaining experiments will range from 0 – 5 µg CAE/ml, with the exception of fraction 3 (0 – 1 µg CAE/ml) and fraction 4 (0 - 10 µg CAE/ml). In Chapter II, even though higher levels of extracted powder were used on the cell culture, the actual phenolic content of these stone fruits treated was much lower (0.014ug/ml-0.063ug/ml). Know the phenolic range to add to our cells as well as their results in Chapter II we tested our cytotoxicity within the similar phenolic compound range.

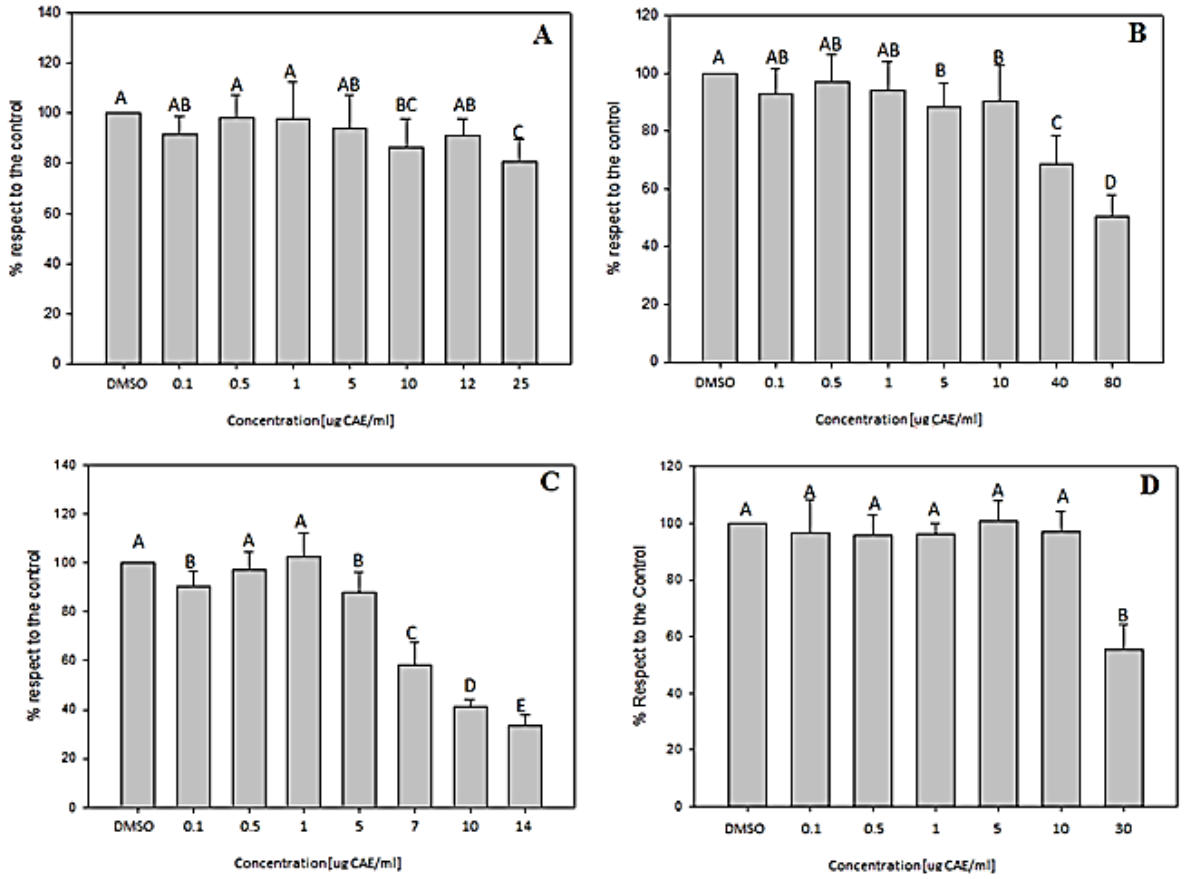

Figure 21. Peach fractions cytotoxic effects on C2C12 differentiated cells. **A**: phenolic acid fraction (fraction 1), **B**: anthocyanin fraction (fraction 2), **C**: flavonoid fraction (fraction 3), **D**: procyanidin fraction (fraction 4). Differentiated cells treated with phenolic fractions for 24 hrs. Bars represent means ± S.D. (n = 3). Different letters denotes significant differences by ANOVA Turkey-Kramer HSD ($p < 0.05$).

3.4.4 Differentiation of C2C12 Myoblasts and Development of an Insulin Resistant Cell Model

In the process of myogenesis, satellite cells will proliferate into myoblasts which will then differentiate to multi-nucleated myotubes fibers; with maturation, these

myotubes become the well-recognized myofibers that make up striated skeletal muscle. For the following assays, the differentiation of C2C12 myoblasts into myotubes must occur. After 4 days of exposure to horse serum, the myotubes are ready for insulin resistance. After differentiation, C2C12 cells were induced with palmitic acid to an insulin resistant state. There have been many references that state saturated fatty acids, namely palmitate, can induce an insulin resistant state in muscle cell culture, inhibiting cellular AKT activation, decreasing GLUT4 translocation, and preventing insulin stimulated glucose uptake and glycogen synthesis.[92-95, 104-108] Metformin was used as a positive control to verify the insulin resistant model's effectiveness as well as provide a reference value for the peach fractions for comparison. With glucose uptake, we found that metformin was able to partially reverse the effects of palmitic acid on the myotubes (figure 22). Based on this data, the C2C12 insulin resistant model was used for the possible insulin sensitizing effect of the peach fractions.

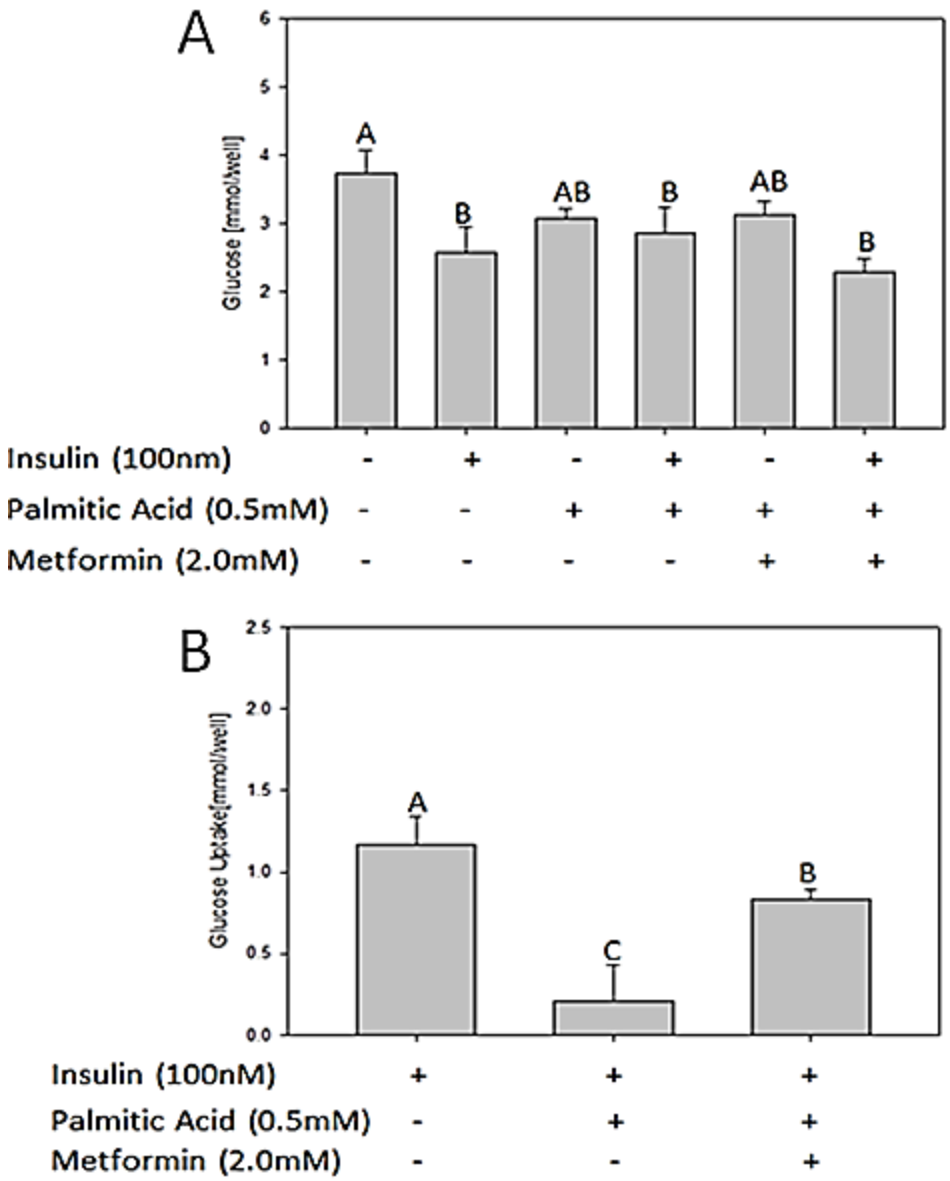

Figure 22. Effect of palmitic acid and metformin on glucose uptake and net insulin stimulated glucose uptake. Cells were cultured in absence or presence of 0.5 mM of palmitic acid during 48 h and exposed to 100 nM of insulin for 30 min to determine the glucose uptake. A._ Depicts glucose levels of insulin and non-insulin stimulated cells. B.) The increase in glucose uptake is depicted using reference control cells non-exposed to insulin. Bars represent means ± S.D. (n = 3). Different letters denotes significant differences by ANOVA Turkey-Kramer HSD ($p < 0.05$).

3.4.5 Effect of Peach Phenolic Fractions on Glucose Uptake in Muscle

The objectives of this study were to evaluate a possible effect of peach phenolic fractions against type 2 diabetes. The approach used to evaluate this effect, was the measurement of the glucose uptake and intracellular concentration of glucose-6-phosphate (G6P), which is the direct product of the activity of glucokinase (GK), a key enzyme that functions to carry out the first reaction of glucose metabolism: the ATP-dependent phosphorylation of glucose into glucose-6-phosphate.[11] Our results might indicate that peach phenolic extracts increase the glucose uptake significantly on muscle cells exposed with palmitic acid an inductor of insulin resistance (Figure 23). These findings coincide with other in vitro reports studying the activity of chlorogenic acids and anthocyanins, with reports that chlorogenic acid stimulates basal and insulin mediated glucose transporters in muscle cell culture as well as activates AKT.[109] The effect of caffeic acid on intramyocellular glucose uptake have also been studied with results noting that Caffeic acid enhances glucose uptake in muscle and adipose cells in a concentration dependent manner.[110-111] Chlorogenic acid's molecular structure is that of caffeic acid esterified to quinic acid, therefore, the caffeic acid molecular structure of chlorogenic acid may be the bioactive site on the molecule.

Anthocyanins have also been reported for their ability to enhance skeletal muscle insulin mediated glucose uptake. Anthocyanin reports include the ability to enhance glucose transporters in skeletal muscle of diabetic rats and increase total body insulin sensitivity in insulin resistant participants.[112-114] Studies have shown that quercetin glycosides enhance glucose uptake, but do so independently of insulin stimulation, most likely by the AMPK pathway.[115] Another study shows that flavonoids such as quercetin and catechin-gallate inhibit glucose uptake in adipocytes over the concentration range of 10-100 uM.[116] These may be possible reasons why fraction 3 does not show any glucose uptake enhancement higher than our positive control (Figure 23). This glucose uptake assay will determine the single concentrations of each fraction for further studies on this cell line, with the highest effect in consideration. For fraction 1, the concentration used for further study is 1µg CAE/mL and for fractions 2 and 3 it is 0.1µg CAE/mL and for fraction 4 it is 0.5µg CAE/mL.

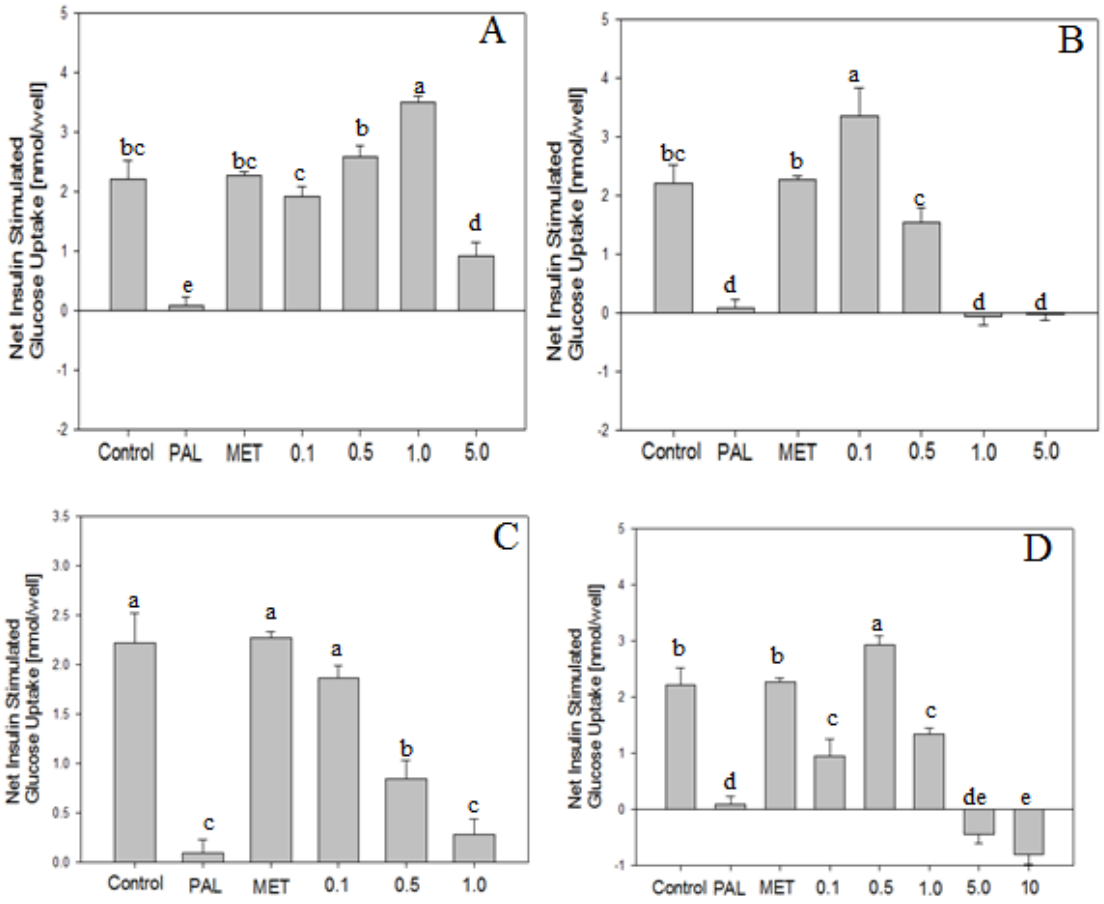

Figure 23. Effects of peach phenolic fractions on insulin stimulated glucose uptake in muscle cells (C2C12). Cells were treated with 0.5 mM of palmitic acid during 48 hours to induce insulin resistance and treated with peach phenolics fractions co-incubated with palmitic acid for additional 16 hours (**A**: Fraction 1**, B:** Fraction 2, **C**:Fraction 3, **D**: Fraction 4) previous to be exposed to 100 nM of insulin for 30 minutes. Palmitic acid (PAL) and metformin (MET) were also measured. Samples were collected immediately after exposure to insulin. Glucose was determined using a commercial kit as described in materials and methods. Bars are means ± S.D. (n= 5). Different letters denotes significant differences using one-way analysis of variance (ANOVA) followed by tukey-kramer HSD ($p< 0.05$)

From figure 23 it shows that all four fractions are successful at increasing glucose uptake into the cell, even some more than the tested level of metformin used. However, it is important to note the fraction concentrations that stimulated glucose uptake above the level of metformin. Metformin is usually given at a dose effective at reverting insulin resistance but too high a concentration can cause excessive glucose uptake into the cells and thus develop cases of hypoglycemia in the blood. Cases of hypoglycemia have been reported even under normal dosing regimen.[117] These results show that the different phenolic types present in Rich Lady peach are effective at reverting the process of insulin resistance like metformin but perhaps for further research purposes it would be better to look at these peach fraction levels at concentrations that do not exceed the tested level of the positive control as to not produce a state of hypoglycemia in the body. Also for future experiments, deoxyglucose could be used as an alternative method to measure glucose uptake as it is accurate and widely used in the literature. Also for future experiments, deoxyglucose could be used as an alternative method to measure glucose uptake as it is accurate and widely used in the literature. Flouro-deoxyglucose assays for studying glucose uptake in cell culture are the most common assays found in the literature.[118] The basis for this assay is that glucose and deoxyglucose are transported in the same manner into the cells and phosphorylated by hexokinase, but unlike glucose it accumulates as it cannot be rapidly transformed into G6P like glucose is in the cell. It measures total glucose uptake, including those transported specifically by GLUT1 and GLUT4 transporter proteins and can be used to

measure GLUT as well as glucose uptake.[118] Another note to mention is that the number of cells needed for deoxy-glucose is less than our current glucose uptake assay.

The negative glucose uptake observed by some higher concentrations of peach fractions two and four could in fact be due to multiple causes. As the cytotoxicity results showed no visual changes at higher concentrations for some samples but a statistical significant difference it could be hypothesized that the negative glucose uptake at these high concentrations could be due to cytotoxicity occurring in the cells. Another explanation for the negative concentrations could be explained by the phenolic compounds at higher concentrations are actually eliciting glucose release from these cells through some mechanistic pathway. Because of this we cannot assume at high concentrations with these compounds that there will not be any detrimental effects. For further research purposes it would be worthwhile to look at these compounds at smaller concentrations to view their activity on glucose uptake and the insulin signaling pathway, as these smaller level seem to increase insulin sensitivity at levels that would mimic normal conditions of homeostasis in the entire system.

3.4.6 Intracellular Glucose 6-Phosphate

After glucose is taken into muscle cells by transport vesicles, most of it is sent though pathways for glycogen synthesis. Intramyocellular glucose is first transformed into an intermediate G6P in the pathway. G6P intracellular concentration was measured as described above for C2C12 myotubes. The results are shown in figure 24. The control cells with palmitic acid show a significant decrease from the control cells without palmitic acid in G6P concentration. This shows again that palmitic acid is

working to create an insulin resistant model. Fraction 2 at a concentration of 0.1 ug CAE/ml showed similar increases in G6P comparable to the positive control, metformin. Fraction 1 at 1 ug CAE/ml and fraction 4 at 0.5 ug CAE/ml also showed increases in G6P, however not as high as an increase as fraction 2. Fraction 3 at 0.1 ug CAE/ml showed the least amount of G6P concentration, showing no significant difference from palmitic acid treated cells. This could be due in part to the large sample variation for fraction 3. When measured with only a standard t-test against palmitic acid control cells, F3 was able to show statistical difference ($p<0.05$). All fractions did increase the amount of intracellular G6P relative to the control. These results shown agree with the results obtained from the glucose uptake assay, in which the highest amount of glucose uptake was found in fraction 2 at 0.1 ug CAE/ml, followed by fractions 1 and 4, and with fraction 3 having the least amount of glucose uptake. As the amount of glucose taken into the myotubes is related to the amount of G6P produced in the cells, these results show a strong correlation and agree that the early steps of the glycogen synthesis pathway remain intact in the model, and is mainly represented by intracellular glucose levels.

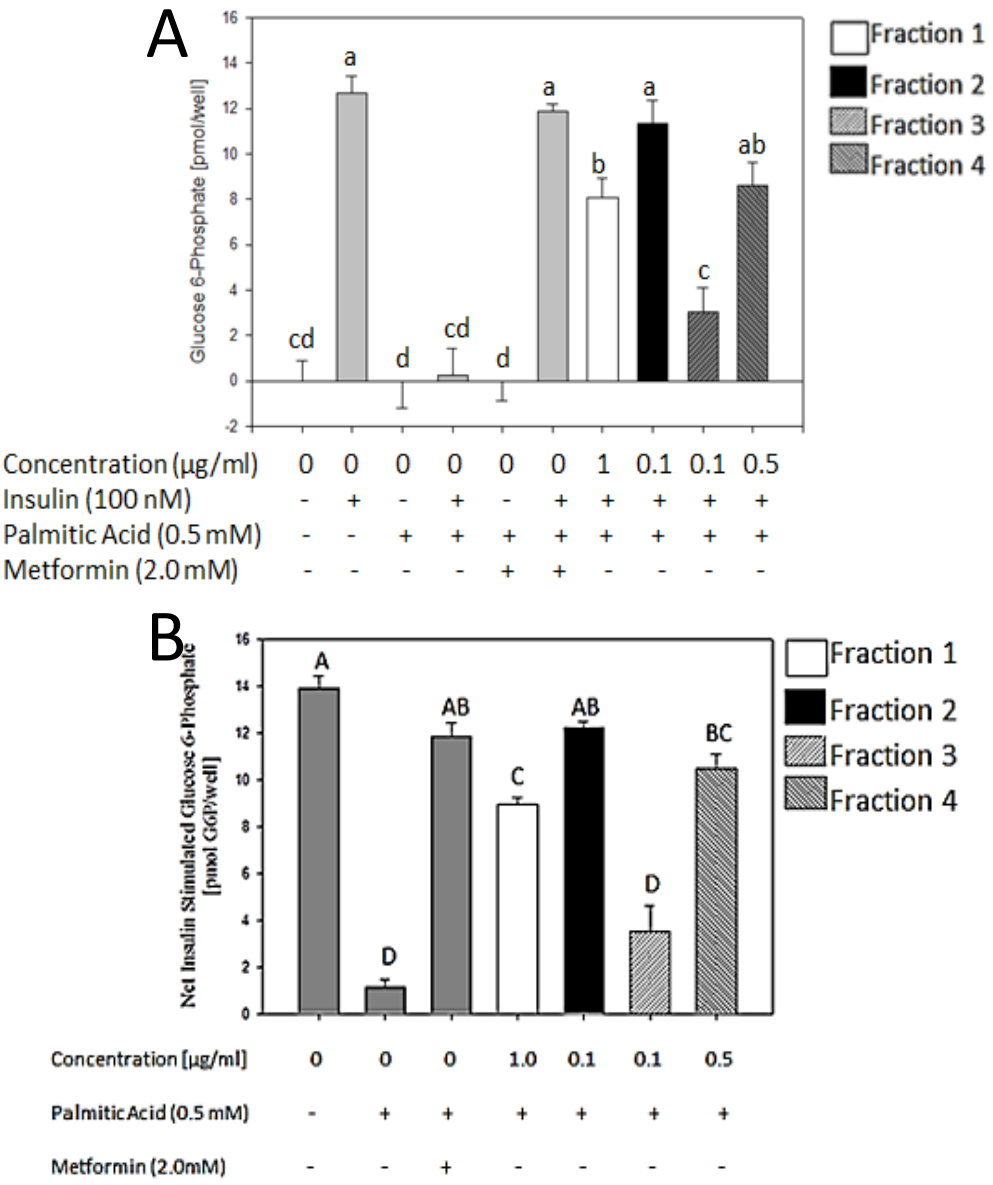

Figure 24. Effect of peach phenolic fractions on intracellular glucose 6-phosphate concentration. G6P (A) and net insulin stimulated G6P (B) were measured. Cells were treated with 0.5 mM of palmitic acid during 48 h to induce insulin resistance and treated with fractions from peach co-incubated with palmitic acid for an additional 16 h, before exposure to 100 nM of insulin for 30 min. Samples were collected immediately after exposure to insulin. Glucose-6-phosphate was determined using a commercial kit. Each fraction dosage was in µl/ml. Bars are means ± S.D. (n= 3). Different letters denotes significant differences by ANOVA Turkey=Kramer ($p < 0.05$).

Here from the results on glucose uptake it is predicted that fractions 1 and fractions 2 would have the highest G6P production. This theory is proven true with fraction 2, with levels comparable to metformin, but not exactly for fraction one. This could be due to the fraction compounds acting not only on the insulin signaling pathway, affecting the amount of glucose uptake into the cell, but also perhaps directly on hexokinase as well. If the intracellular glucose levels and the amount of intracellular G6P seem proportionate than glucose transport is most likely a target for the fractions but if the intracellular glucose levels are high but do not correspond to a proportionate G6P level then hexokinase activity must be reduced.[119] Hexokinase II activity is known to be reduced in insulin resistance[120] and since fraction 2 shows proportionate glucose uptake and G6P it could also be acting on hexokinase activity as well.

3.4.7 Effect of Peach Fractions on Intracellular ROS Production

Palmitic acid is known for its ability to induce ROS in cellular tissues, including muscle.[121-124] ROS production is toxic for cells at certain levels. Intracellular ROS production from palmitic acid can lead to an increase in ER stress that leads to a production in JNK, which disrupts the insulin signaling pathway and, in part, may be a contributor to the occurrence of insulin resistance.

There have been many papers that cite that mitochondrial SOD production precedes insulin resistance with ROS inhibiting IRS and AKT activity while activating JNK phosphorylation. Mitochondrial b-oxidation also leads to increased levels of ROS in the skeletal muscle. Inevitable, oxidative stress due to ROS can cause cross talk and decrease insulin signaling pathways. Excessive ROS overwhelms AOX defenses and increases more oxidative stress in the system. It was determined to view the antioxidant potential of the peach fractions on skeletal muscle cells. Results showed that the four fractions had no statistically significant difference with the control cells with palmitic acid. Prolonged exposure (48 hours to palmitic acid induces a significant increase in the amount of intracellular ROS production; however acute (16 hour) treatments of antioxidants (vitamin E) can reduce ROS, but not drastically enough to return to the original control cell state (figure 25). Future studies should evaluate the effects of polyphenolics on intracellular ROS production during a time trial.

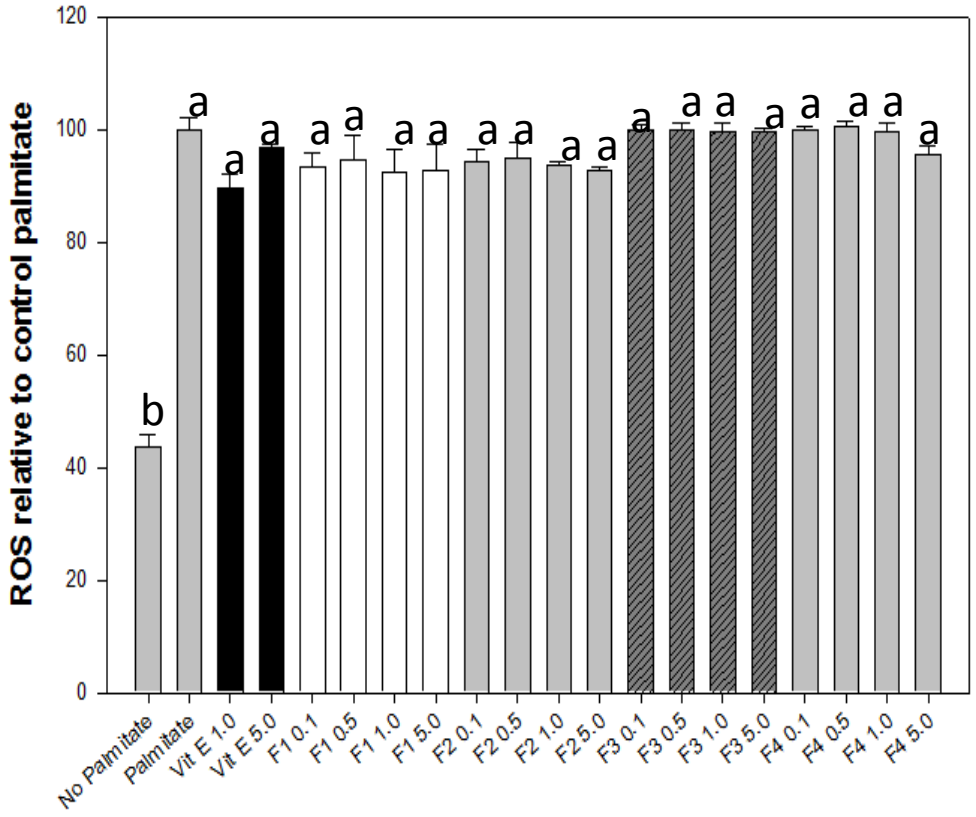

Figure 25. Peach fractions on C2C12 intracellular ROS production. Differentiated cells were exposed to 0.5 mM palmitic acid for 48 hours and treated with peach fractions co-incubated with palmitic acid for an additional 16 hours. Cells were washed two times with warm PBS and then incubated with 5 uM DCFA in DMEM 1mM glucose phenol red-free medium at 37^{o}C for 30 minutes and the fluorescence was measured Ex/Em 485/535. Fraction concentrations are expressed in µg CAE/ml. Vitamin E (tocopherol) was used as a positive control. Reported values are relative means ± S.E. (n= 8). Different letters show significant differences using one-way analysis of variance (ANOVA) followed by tukey-kramer HSD ($P < 0.05$).

While it seems that our model is not working though ROS it is important to point out that after each treatment with palmitate for 48 hours to induce insulin resistance within the cells the treatment is given for 16 hours for each of the bioassays. To keep in the same variables with the other assays the same protocol was followed for intracellular ROS, however, to view the activity that the fractions have on intracellular ROS at 48 hours a treatment should be given after 32 hours of palmitic acid exposure and measurement of the intracellular ROS taken at the time point of 48 hour exposure to ROS (with 16 hour exposure to the treatment). Figure 25 is showing ROS accumulation in the cells after 64 hour exposure to palmitic acid (with 16 hours of treatment). After 64 hours it is likely that the ROS levels intracellularly are so high that any AOX activity that the phenolic compounds possess were depleted moments after initial treatment and therefore we see no change in the ROS levels from any of the treatments, so it is unknown whether our phenolic treatments are acting through a ROS pathway at least partly and a further initial assay done at 48 hour exposure to palmitic acid with treatment should be performed as well as other necessary assay testing to determine if the phenolic antioxidants have any effect on ROS.

3.4.8 Protein Expression of C2C12 Myoblasts

With the determination that the insulin resistant model is affecting the earlier stages of the insulin signalling pathways, protein expression of AKT, IRS1, and JNK were performed as described above. During the insulin signaling pathway, AKT becomes phosphorylated as part of a cascade of events leading ultimately to glucose uptake into the cells. AKT2 is an important signaling protein in the Insulin signaling

pathway. It is required to induce glucose transport. When the muscle cells are incubated with palmitic acid during 48 hours, the insulin signaling pathway is impaired and the AKT activation is inhibited inducing an insulin resistant model.[125-126] therefore, is necessary to find compounds to able of induce the AKT activation as Metformin. In our results we found that only F3 and F4 increase significantly the AKT activation. This result is promissory to find natural compounds able to reduce the effects related to type 2-diabetes. Out of the fraction samples, fraction 3 showed the most increase in serine phosphorylation protein expression, followed by fractions 4, 2, and 1. (Figures 26-27)

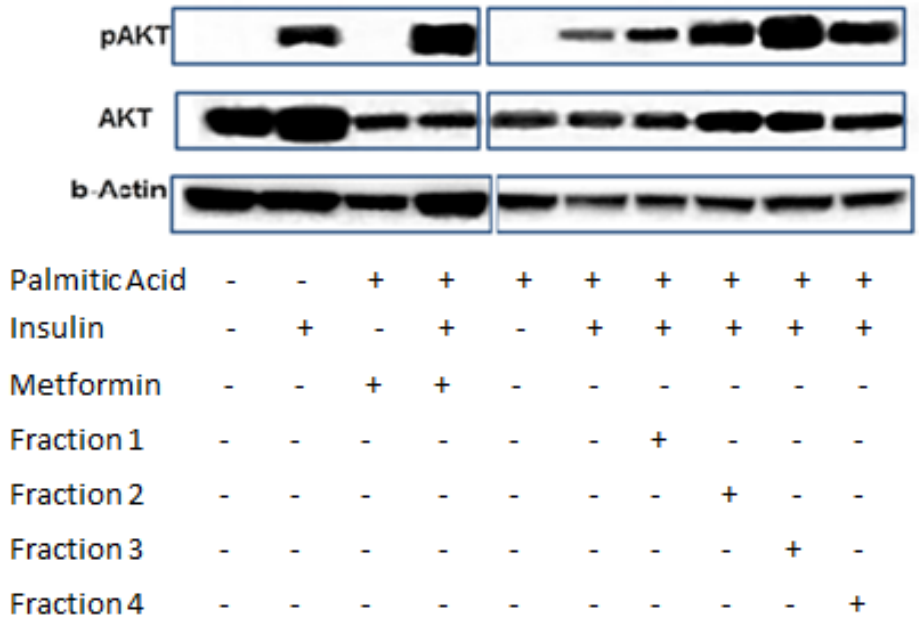

Figure 26. Western Blot of specific peach phenolic fractions on AKT activation in muscle cells (C2C12).

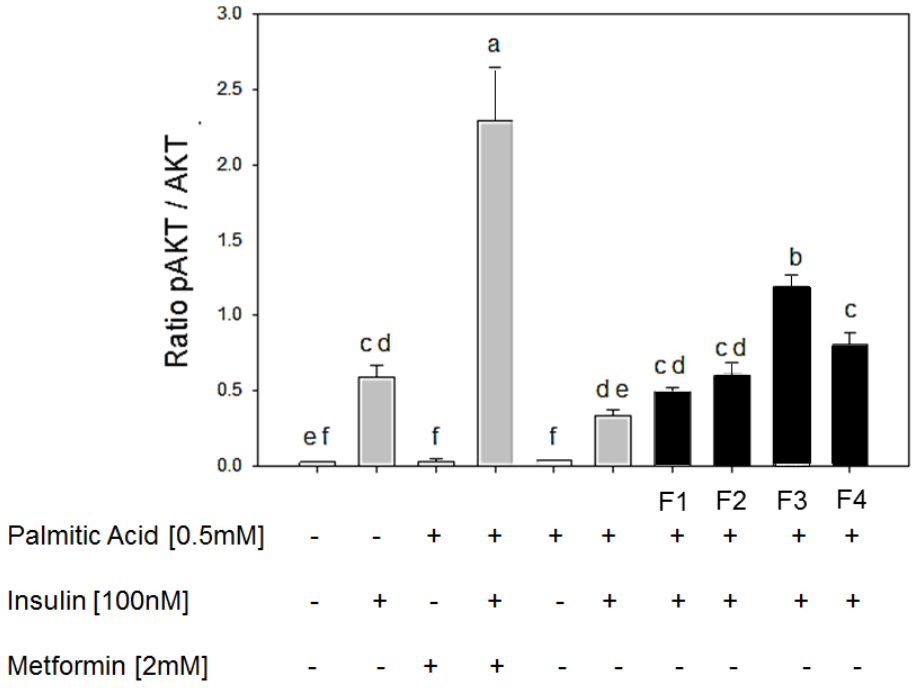

Figure 27. Effect of specific peach phenolic fractions on AKT activation in muscle cells (C2C12). Cells were treated with 0.5 mM of palmitic acid for 48 hours to induce insulin resistance and then treated with peach phenolics fractions co-incubates with palmitic acid for 16 hours, previous to be exposed to 100 nM of insulin for 10 minutes before assay. The western blot images are representative of three independent experiments. Bars are means ± S.E. (3= 5). Different letters denotes significant differences using one-way analysis of variance followed by tukey-kramer HSD .($P < 0.05$)

In comparison with previous results, fraction 3 did not increase the glucose uptake and intracellular G6P concentration as well as the other fractions. Fraction 3 may possibly be working through the serine phosphorylation of AKT to increase glucose uptake into the cell more directly than the other peach fruit fractions, but all fractions

may be working through various other proteins to increase the sensitivity of the pathway to give the resulting increased in glucose into the cell.

Insulin receptor substrate-1 (IRS) plays a key role in transmitting signals from the insulin and insulin-like growth factor-1 receptors to intracellular pathway PI3K/AKT. IRS-1 contains many potential serine phosphorylation sites, such as ser307 and ser318 that are thought to play regulatory roles during insulin signaling. One of these sites, Ser_{307} in rat IRS-1, was originally found to be phosphorylated specifically by JNK. Ser_{307} was later found to be phosphorylates in IRS-1 isolated from cells and tissues stimulated with TNF-α, insulin/IGF-1, or anisomycin, which might contribute to peripheral insulin resistance and type 2 diabetes. [125-128]In our results we found that all the peach phenolic fractions decrease significantly the IRS serine 307 phosphorylation (Figures 28-29) in our muscle cell insulin resistant model. This result is promissory because a reduction in the serine phosphorylation is able to reduce the effects related to type 2-diabetes. IRS ser318 was also measured in our studies as it is referenced as a potential target for JNK and other kinases. In our results we found no band activation on this serine residue (data not shown) displaying that this particular serine site is not activated in our insulin resistant model or with our treatments. In the insulin signaling pathway, IRS1 threonine phosphorylation triggers the downstream activation pathway through AKT, however, serine phosphorylation at ser307 inhibits IRS1 activation by interfering with the phosphor-tyrosine binding (PTB) thus inhibiting the pathway cascade further. [129] The serine 307 phosphorylation (ser307) of IRS1 was increased in the cells treated with palmitic acid over the control cells. Control cells, metformin

treated cells, and fraction treated cells all showed similar decreased ser307 activation compared to palmitic acid control cells. Metformin and the four peach fractions are able to reverse the activation of ser307 induced by palmitic acid in the cells. Under insulin stimulation is it normal to see slight IRS1 serine phosphorylation by control cells, as ser_{307} is a negative regulator of the insulin signaling pathway, however too much serine phosphorylation is detrimental to the signaling pathway (like the activation triggered by palmitic acid). It is important to note that under normal cell conditions without insulin stimulation there is no IRS1 phosphorylation however cells treated with metformin without insulin stimulation do show a slight activation of ser307, and more so with insulin stimulation. The phenolic fractions decrease the amount of activation as compared to cells treated with palmitic acid with fraction 3 showing similar results as the positive control metformin.

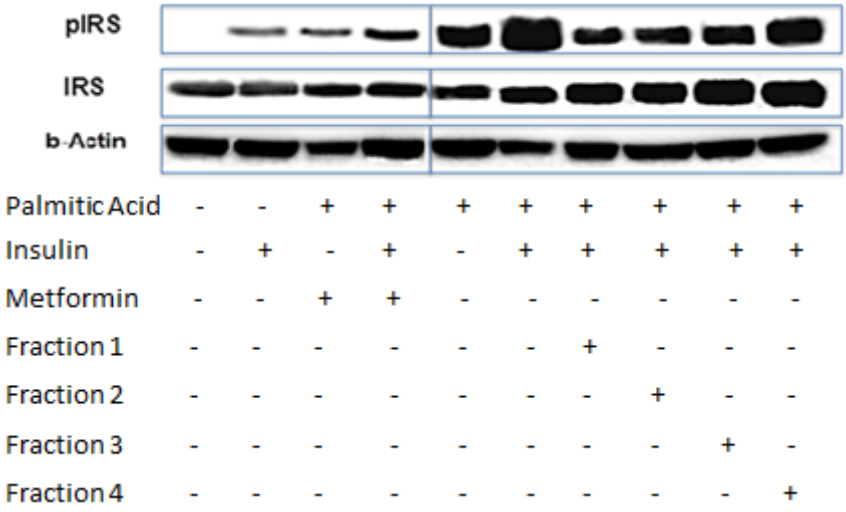

Figure 28. Western Blot of specific peach phenolic fractions on IRS1 activation in muscle cells (C2C12)

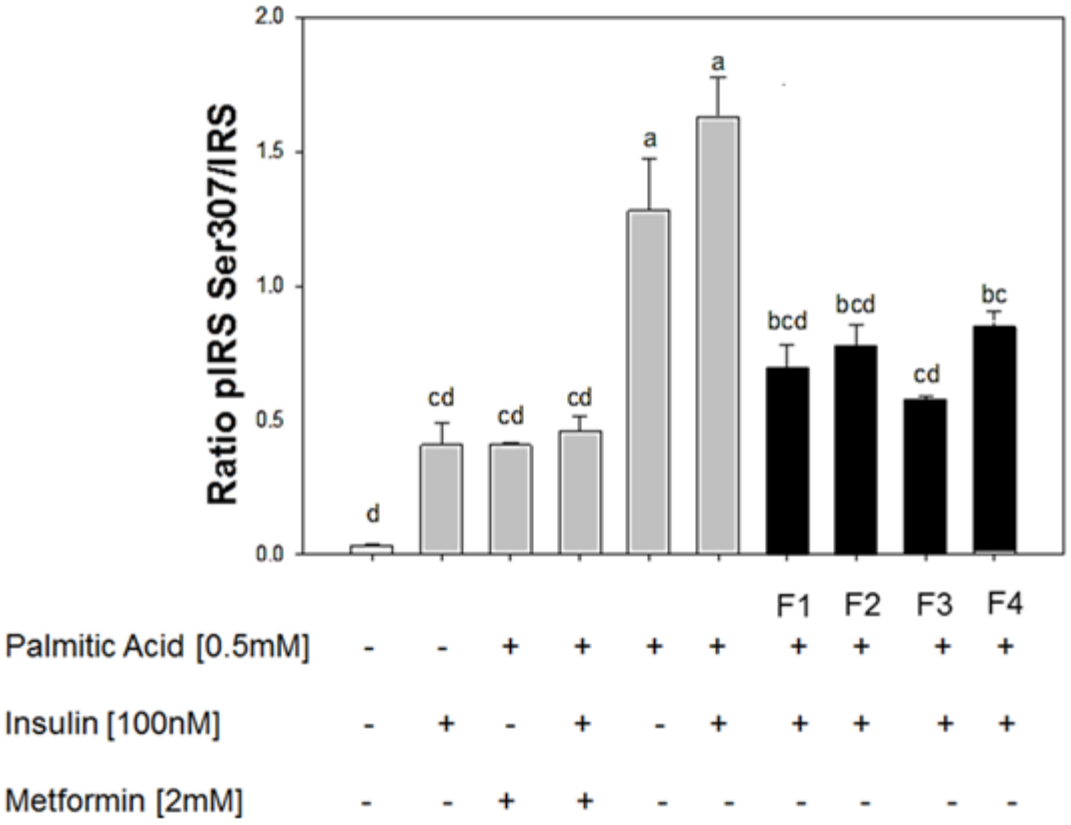

Figure 29. Effect of specific peach phenolic fractions on IRS1 activation in muscle cells (C2C12). Cells were treated with 0.5 mM of palmitic acid during 48 hours to induce insulin resistance and then treated with peach phenolics fractions co-incubated with palmitic acid for 16 hours, previous to be exposed to 100 nM of insulin for 10 minutes before assay. The western blot images are representative of three independent experiments. Bars are means ± S.E. (3= 5). Different letters denotes significant differences using one-way analysis of variance followed by tukey-kramer HSD. ($p < 0.05$)

Inflammatory signals, changes in levels of reactive oxygen species, ultraviolet radiation, protein synthesis inhibitors, and a variety of stress stimuli can activate JNK. One way this activation may occur is through disruption of the conformation of sensitive protein phosphatase enzymes; specific phosphatases normally inhibit the activity of JNK itself and the activity of proteins linked to JNK activation.[130-131] The activation occurs through a dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues within a Thr-Pro-Tyr motif located in kinase subdomain VIII. Activation is carried out by two MAP kinases, MKK4 and MKK7 and JNK can be inactivated by Ser/Thr and Tyr protein phosphatases. It has been suggested that this signaling pathway contributes to inflammatory responses in mammals and insects.[132-133] The oxidative stress-sensitive JNK pathway is known to be activated in diabetic condition and is involved in the progression of insulin resistance.[134-135] The present study was aimed to investigate the effect of peach phenolic fractions on insulin sensitivity and JNK activation in muscle cells insulin-resistant In our results we found that all the peach phenolic fractions reduce significantly the JNK activation (Figures 30, 31). This result is promissory to find natural compounds able to reduce the effects of palmitic acid (free fatty acid) related to type 2 diabetes.

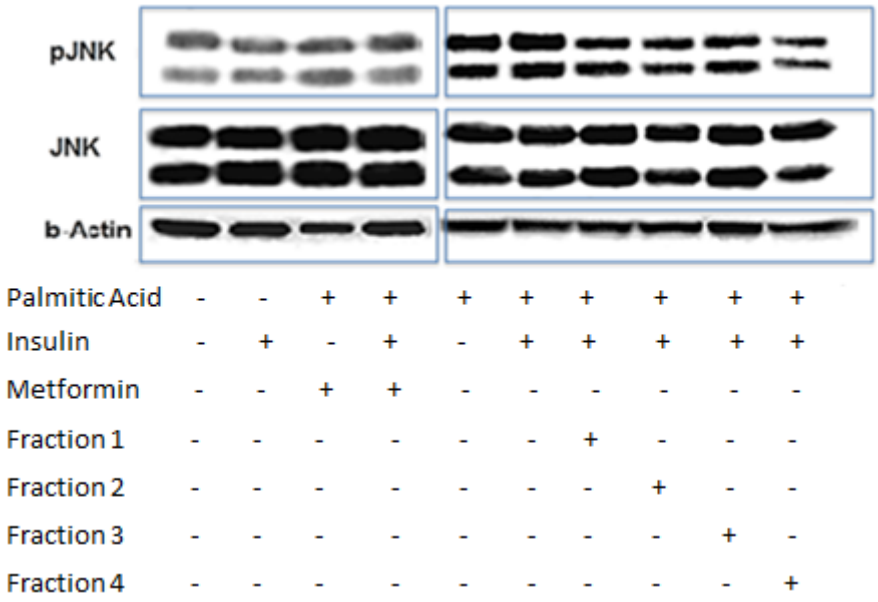

Figure 30. Western Blot of specific peach phenolic fractions on JNK activation in muscle cells (C2C12)

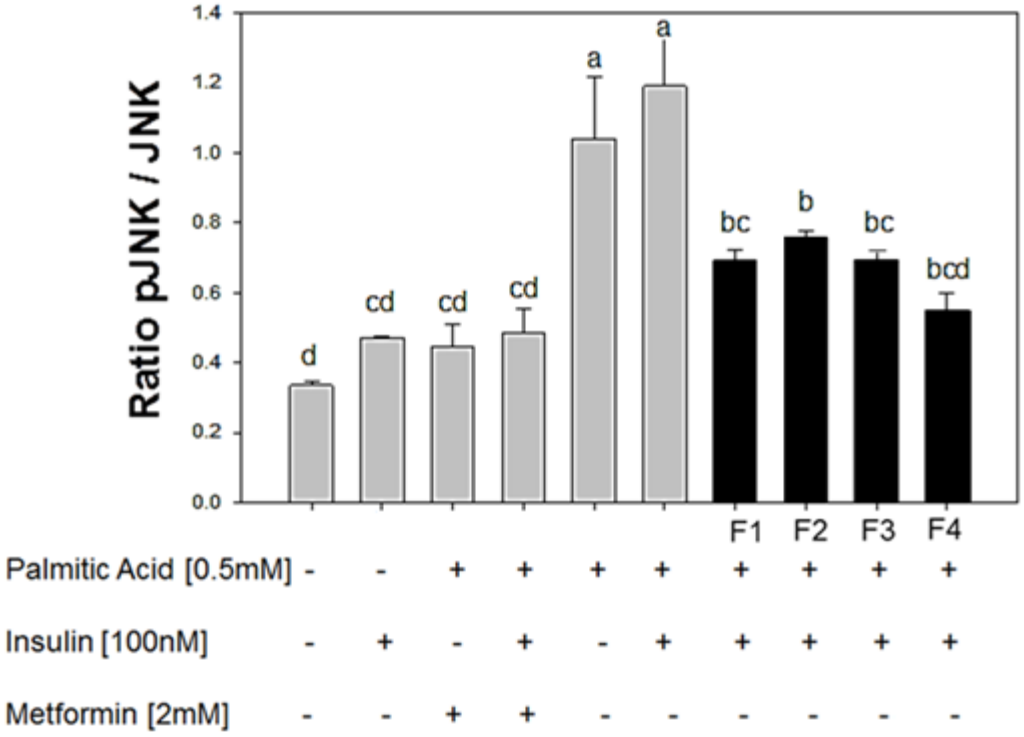

Figure 31. Effect of specific peach phenolic fractions on JNK activation in muscle cells (C2C12). Cells were treated with 0.5 mM of palmitic acid for 48 hours to induce insulin resistance and then treated with peach phenolics fractions co-incubated with palmitic acid for 16 hours, previous to be exposed to 100 nM of insulin for 10 minutes before assay. The western blot images are representative of three independent experiments. Graph is representative of both isomers of JNK. Bars are means ± S.E. (3= 5). Different letters denotes significant differences using one-way analysis of variance followed by tukey-kramer HSD.($P < 0.05$)

The results for JNK proteins show two bands in the western blot. This is due to the JNK protein having 2 isomers, one approximately 46 Kda and the other 54 Kda.[136] There is no differences between the two isoforms and both should be reported which is the case in many publications concerning JNK. From the above results of these sets of experiments, it shows that the positive control metformin works very well against this insulin resistant model, with positive effects on p-AKT activation, IRS1 ser307 reduction, and p-JNK reduction. All fractions of peach also showed beneficial effects on protein expression, at p-AKT activation and IRS1ser307 reduction, with fraction 3 being the most effective at activation p-AKT of all the fractions. In JNK activation, all fractions showed statistically the same level of reduction as metformin and the control cells level of JNK

From various concentrations of phenolic compounds tested, the concentration of phenolics that presented the highest increase in glucose uptake in the myotubes were used for further studies into ROS, G6P, and protein expression. From the compilation of these results, we can see possible areas where the peach phenolic fractions could be working to prevent IR skeletal muscle. As all fractions showed an increase in glucose uptake in myotubes, we can assume that each fraction is acting somewhere in the insulin signaling pathway, however, the fraction results from the G6P production and protein expression were not the same in levels, suggesting that the different categories of phenolic compounds are working in different areas of the insulin signaling pathway. The fractions could be exerting an effect on GK, AKT, or inhibiting JNK

Our positive control, metformin, is known to increase insulin sensitivity and reverse the effects of insulin resistance by working through AMPK activation. AMPK acts to change the AMP: ATP ratio in the cell, to stimulate catabolic pathways (glucose uptake, fatty acid oxidation) and inhibit anabolic pathways (synthesis). AMPK also works to up-regulate glucose transporters.[137] Metformin stimulates phosphorylation of the key regulatory site (Thr-172) on AMPK α2 catalytic subunits.[138]How metformin acts specifically on AMPK is still unknown. Recent proposed mechanism on how metformin phosphorylate AMPK includes proposing it acts by stimulating phosphorylation by upstream kinases of AMPK (AMPKK1 and AMPKK2) or by inhibiting de-phosphorylation by protein phosphatases.[139] Another theory is that metformin acts as an inhibitor of complex I of the respiratory chain complex.[140] However, recent findings using cell culture have proved these theories false as metformin did not stimulate activation of AMPK in cell-free assays by either of the two upstream kinases measured in their experiment (AMPKK1 or AMPKK2) and did not affect the cellular ADP to ATP ratio.[138] These researchers suggest metformin could be working through other upstream kinases other than AMPKK1 and AMPKK2 of the drug is modified inside the cell to an active form.[138]AMPK phosphorylation works at reversing insulin resistance by activating AKT, GLUT4, regulating PI3K and IRS-1, increasing hexokinase activity and inhibiting mTOR activation, all key parts of cellular mechanisms that affect insulin signaling and glucose uptake.[141-145] In regards to insulin signaling, AMPK activation increases glucose uptake in two major ways. AMPK can directly stimulate GLUT4 translocation to the cell membrane for glucose uptake into the cell. AMPK activation

leads to the inhibition of mTOR, a ser/thr protein kinase that activates IRS1 serine phosphorylation, negatively regulating insulin signaling. Metformin is also known to inhibit the production of intracellular ROS, a key player in the development of insulin resistance, most likely through AMPK induced PGC1a activity leading to SOD production.[146] Increasing ROS is a negative regulator of insulin signaling and a mediator of insulin resistance development. ROS generation occurs normally in the cell through an NADPH oxidase dependent mechanism, but over production can be triggered by several mechanisms including an overabundance of free fatty acids.[147] JNK activation for prolonged periods requires an overproduction of intracellular ROS which inactivate JNK phosphatases.[148] JNK activation by ROS promotes IRS1 ser307 phosphorylation which reduces the tyrosine phosphorylation of IRS1, leading to impairment in the insulin signaling pathway.[149-152] Studies show that GLUT4 mRNA and protein levels remain unchanged in muscle from normal as well as insulin resistant tissues indicating that the decreased glucose uptake is caused by impaired signaling promoting GLUT4 mobilization.[153-154] AMPK activation by metformin may also work though other mechanisms of action to increase glucose uptake in muscle, such as directly though GLUT4 mobilization, increasing hexokinase activity, and perhaps though preventing the inhibition of JNK phosphatases.[155-156] It has been proven difficult to identify one single signaling step mediating insulin resistance mediated solely by exposure to oxidants such as ROS as each signaling step functions as a complex network and not as a linear reaction chain as well as oxidant overproduction being only one of many negative stimuli of the insulin cascade,[157] therefore it is necessary to look at many different

aspects of insulin signaling to view possible mechanisms of treatments work. While ROS induced JNK production is a major player in insulin resistance development, studies show that treatment with JNK inhibitors only partially reduced insulin resistance, implying that there are also additional determinants to development of the syndrome.[158] Figure 32 shows how metformin works in reversing the insulin resistant effect on skeletal muscle.

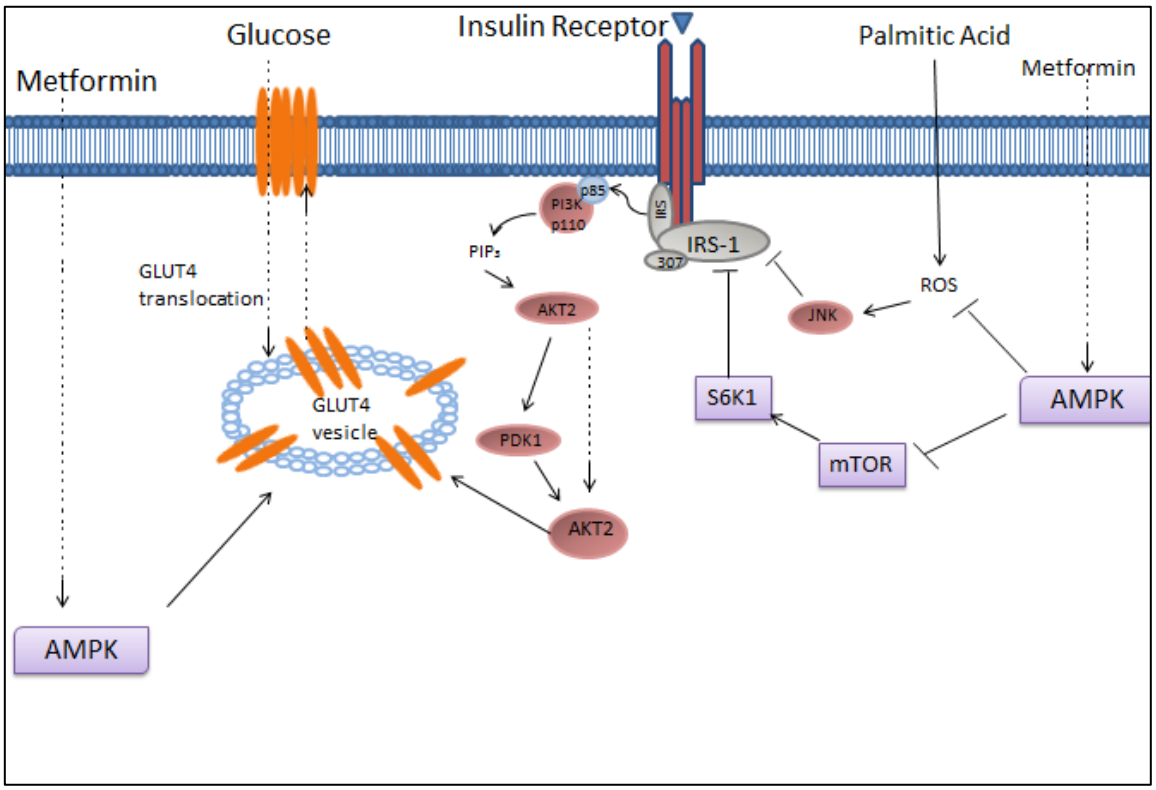

Figure 32. Diagram of metformin mechanism of action on insulin resistant muscle. Metformin activates AMPK in the cell which works directly on glucose transporter mobility as well as inhibiting mTOR activity in the cell, which works through the activation of S6K1 for IRS1 serine phosphorylation, inhibiting insulin receptor tyrosine phosphorylation, a key step in insulin signaling.[159-161]

In our results we show that while palmitic acid induced ROS overproduction occurs in the cell and is a major player in the development of insulin resistance, at our 48 hour testing period none of the peach fractions were able to decrease ROS levels. If the fractions are working as antioxidants on ROS production, then perhaps at this time frame of testing their antioxidant activity has been depleted and further ROS inhibition has stopped. As the phenolic fractions are able to reduce other aspects of ROS induced insulin resistance such as JNK activation they could be working through AMPK preventing the inactivation of JNK phosphatases.[156] Metformin also reduces the intracellular ROS accumulation in the cell by acting through AMPK.[162]The activation of JNK and IRS1 serine phosphorylation are directly linked, as JNK activates the IRS1 activation. This is seen true in our results for JNK and IRS1ser307 phosphorylation in muscle showing similar results in low activation for normal cells, high activation for palmitic acid induced cells, and slightly less activation for metformin and peach fraction cells, with all treatments showing similar reduction in activation levels similar to metformin. These results could indicate the phenolic compounds are working in a mechanism similar to metformin on JNK activation. The same trend is seen for AKT activation as well with the phenolic fractions all increasing the p-AKT to levels comparable with normal non-treated cells. Only fraction 3 showed slightly statistically significant difference from the other fractions for p-AKT activation, however not to the same levels as metformin. While research has shown that metformin can act directly on AKT through AMPK [141] this may or may not be the case for the phenolic fraction components. It has been reported that quercetin can up-regulate AMPK and inhibit JNK

phosphorylation and activation.[137,147,163] Others report studying chlorogenic acid affects in muscle cells found that chlorogenic acid can phosphorylate AMPK and activate AKT, but does not enhance IRS1 association to p85 protein, a step in the insulin signaling pathway.[109] Also, catechins and procyanidins have been shown to increase GLUT4 mobility to the cell membrane.[164] Having all of these individual compounds as well as showing similar effects on the biological assays, these fraction components may be working through many mechanisms to increase glucose uptake and insulin sensitivity. Like reported in the literature for quercetin and chlorogenic acid, the fractions could be regulating AMPK activity in the cell as well as inhibiting JNK phosphorylation, possibly through AMPK preventing the inactivation of JNK phosphatases, as well as behaving like catechin compounds and working directly on GLUT4 mobility to the cell membrane. While AMPK is a potential site for the compound's activity, the insulin signaling pathway is a complex signaling cascade where many proteins are involved in the uptake of glucose. The AKT activation from positive control metformin is very high, even more than the normal insulin stimulated cells and while the fractions increased AKT activation to the level of the normal cells they did not improve activation to the level of metformin. One reason for this is that the phenolics work on more than one point of the insulin signaling cascade simultaneously. Metformin's activation of AMPK aids in the activation of AKT[165] and as this is the main effect of metformin the AKT activation can be said to be the effect of AMPK activation via metformin. While the fractions may be working by this activation as well, the effect on the signaling of AKT is smaller possibly due to another mode of action on insulin signaling. Figure 33 depicts

possible mechanisms in which the peach fraction phenolics may be working on the insulin resistant model.

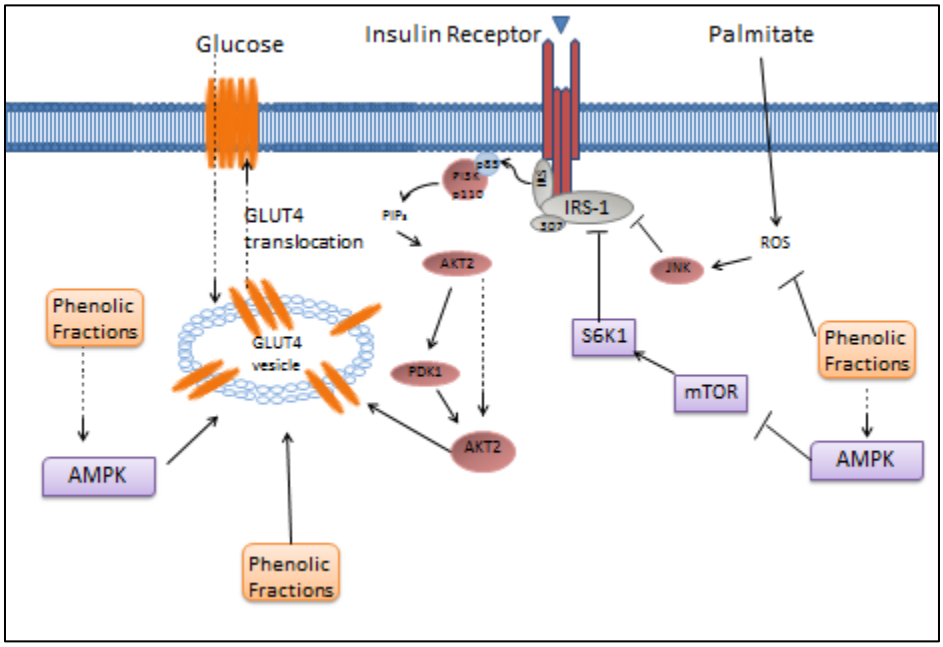

Figure 33. Diagram of possible mechanisms of action of peach phenolic fractions in insulin resistant myotubes. With increased AKT phosphorylation, reduced IRS1ser$_{307}$ and JNK phosphorylation, an increase in glucose uptake and G6P the phenolic fractions could be acting on multiple fronts to revert insulin resistance including acting directly on glucose transport mobility, reducing JNK activation and AKT activity. Phenolic fractions could be working like metformin in AMPK activation promoting glucose transporter mobility and inhibiting negative regulation of IRS1 serine phosphorylation.

In these biological cell assays we see that inducing the cells with 0.5 mM of palmitic acid causes inhibition of the insulin pathway, with reversal of this process with treated metformin and fruit phenolics. While this induction with palmitic acid has been

reported in the literature widely as a way to induce insulin resistance, However, palmitic acid has a low water solubility so for use in cell physiological systems must be in the form of a complex, usually with an albumin protein for its high affinity to fatty acids.[166] Palmitic acid complexes with albumin added to cell culture media must undergo absorption to the cell's membrane, trans-membrane diffusion, and desorption from the cell membrane once inside the cell.[166] While literature widely reports the amounts of palmitic acid: albumin complex treated into cell culture[92-95,104-108,167-173] it has been found that fatty acids bound to albumins directly contribute to fatty acid metabolism in the cell and the amount of bound: unbound palmitic acid to albumin plays a significant role in how much fatty acid is absorbed into the cells.[174-176] So in accordance to the literature we cannot say for certain the palmitic acid was taken up by the cells in its entirety, but we can see from our experiments palmitic acid was taken up at least in part by the pronounced effect on the inhibition of glucose uptake.[175]

4. SUMMARY AND CONCLUSIONS

4.1 Summary

In summary, we have used a common compound clean-up method as a mean to separate and recover phenolic compounds with high yield recovery and purity for testing in a developed model of skeletal muscle insulin resistance using mouse C2C12 myotubes. This determine the SPE fraction phenolics bioactive effects on cellular and macro-marker components in the cell with a key insight to how these compounds are mechanistically working in the cell. Fruit phenolics increase insulin stimulated glucose uptake, G6P, AKT phosphorylation, and decrease negative regulators of the insulin signaling pathway, IRS1 ser307 and JNK phosphorylation. In this study phenolic compounds had no effect on intracellular ROS accumulation.

4.2 Conclusions

Stone fruit phenolics from cultivars of Rich Lady peach, Black Splendor plum, and Spring Bright Nectarine were extracted and purified for their use as potential treatments in palmitic acid induced insulin resistant C2C12 myotubes. The fruit's effectiveness was analyzed by the results of insulin stimulated intracellular G6P production. Extracts were purified from sugars and organic acids by SPE reverse phase C18 silica gel and freeze-dried. Identification and validity of the phenolic extracts was done by HPLC-DAD and TLC chromatography methods. Extracts used in cells were dissolved in DMSO and expressed at μg freeze-dried powder/mL. Final DMSO concentration in the cells was 0.5%. Extracts were tested at levels of 0 – 300 μg/mL for

cytotoxicity testing, and were used at levels of 0, 10, and 100 µg/mL for glucose 6-phosphate assay. Myoblasts were fully differentiated into mature myotubes and exposed to palmitic acid (0.5mM) for 48hr to induce insulin resistance before and then treated with fruit extracts for 16hr and stimulated with insulin (100nM) for 30 minutes. Peach and nectarine showed to have a significant effect on glucose 6-phosphate accumulation in the cells at 1 and 10 µg/mL, respectively, while plum extracts showed no significant effects on the cells as compared to the positive control, metformin. Peach phenolic extract was chosen for further analysis into the significant effect on reverting insulin resistance. Peach extracts were purified from sugars and organic acids by SPE reverse phase C18 silica gel and separated into fractions via solvent and pH affinity. A yield study was performed to verify the effectiveness of the SPE methodology, resulting in an 85% yield recovery of phenolic compounds after purification. Identification of phenolic compounds in the peach fractions was done by HPLC-DAD and fractions quantified by wet based chemistry assay (Folin method) and expressed as µg Chlorogenic Acid Equivalent/mL. Myoblasts were fully differentiated into mature myotubes and exposed to palmitic acid (0.5mM) for 48hr to induce insulin resistance and then treated with fruit extracts for 16hr and stimulated with insulin (100nM) for 30 minutes prior to assays. Peach fractions showed no significant changes in ROS accumulation in the palmitic acid treated cells at any level from 0.1 to 5 µg CAE/ml but did show significant effects on glucose uptake, with the most pronounced effects at 1.0 µg CAE/ml for fraction 1, 0.1 µg CAE/ml for fraction 2 and 3, and 0.5 µg CAE/ml for fraction 4. These values were chosen for the remainder of the experimentation in the study. All fractions were able to

increase G6P intracellular concentration in palmitic acid treated cells to levels similar to metformin, with the exception of fraction 3 which showed no difference to palmitic acid. This could be due in part to large sample variation for this fraction. When running standard t-test at 95% CI, we see that there is a difference between F3 and palmitic acid treated cells. In addition, all fractions were able to decrease the JNK and IRS1 ser307 phosphorylation, both negative regulators of the insulin pathway. The fraction compounds could be working on multiple fronts, working similarly to metformin through AMPK activation, working directly on GLUT4, AKT, or IRS1 association with p85. As AMPK regulates JNK and IRS1 phosphorylation, which has a direct effect on AKT phosphorylation, the phenolic fractions could be regulating insulin signaling proteins through AMPK activation. Further research is needed to investigate the phenolic compounds effect on AMPK as it relates to protein phosphorylation in the insulin pathway.

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