AN ABSTRACT OF THE THESIS OF

Sarah M. Smith for the degree of Master of Science in Food Science and Technology presented on August 18, 2014.

Title: Metabolic Effects of Consumption of Quercetin, Ellagic Acid, Cherry, and Apple Phytochemical Extracts in High-Fat Fed Mice.

Abstract approved:

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Neil F. Shay

Metabolic improvements and changes in gene expression were measured in mice fed polyphenol-rich extracts derived from apples and sweet cherries, and the common phytochemicals, quercetin and ellagic acid. Polyphenol-rich extracts were produced by solid phase extraction and column chromatography. Extracts and purified compounds were mixed into a high-fat (HF, 60% of total kcal) obesigenic rodent diet and fed to C57BL/6J mice along with low-fat (LF, 10% of total kcal) and HF+treatment (HF+X) diets. Mice fed the HF control diet became obese and lost normal glucose control. Although no changes in weight gain or diet consumption were seen among the mice fed the high-fat diets, mice fed HF+X diets containing extracts and purified compounds exhibited improved metabolic parameters compared to mice fed the HF control diet. For example, six-hour fasted blood glucose levels measured at week six in mice fed HF+X diets, or HF diet supplemented with apple (HF+AE) or cherry extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA), showed lower baseline blood glucose levels when compared with

the HF-fed mice ($p=<0.05$). Glucose tolerance was also marginally improved in the HF+AE- and HF+QUE-fed mice ($p=<0.10$). A grip test, measuring strength, showed a trend toward improved strength in the HF+treatment groups when compared to HF-fed mice. Treadmill-like Rotarod testing evidenced a trend for improved endurance and balance in all HF+X groups, but with no significance. Serum cytokine levels measured at the end of the study demonstrated reduced levels of several inflammatory factors in HF+X-fed mice, indicating improved anti-inflammatory regulation compared to HF-fed mice. Finally, HF+X-fed mice showed increased relative expression for markers of fatty acid oxidation, such as CPT1-α, and ACOX-1, which was consistent with qualitative examination of lipid accumulation in liver tissue sections.

Quantitative measurements of cellular lipid accumulation were measured using a human HepG2 cell line model. HepG2 cells were treated with oleic acid (OA) to induce lipid accumulation along with the treatment compound of interest. Cells were then stained with Oil Red O and hepatic fat accumulation was measured using a spectrophotometer. Cells exposed to OA+QUE showed a significant decrease in lipid accumulation ($p=<0.05$) and other treatment groups showed a similar or slightly increased degree of lipid accumulation. Therefore, we conclude that compounds present in apples and cherries, quercetin and ellagic acid produce beneficial metabolic effects and that part of this improvement is mediated by phytochemical activation of nuclear hormone receptors.

Metabolic Effects of Consumption of Quercetin, Ellagic Acid, Cherry, and Apple Phytochemical Extracts in High-Fat Fed Mice

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Sarah M. Smith, Author

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ABBREVIATIONS

°C	Degrees centigrade
ABCA	ATP-binding cassette transporter, sub-family A
ACC	Acetyl coenzyme A carboxylase
ACO	Acyl-coenzyme A oxidase
AE	Apple Extract
ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
apoB	Apolipoprotein B
AST	Aspartate aminotransferase
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
CC	Cryptogenic cirrhosis
CDC	Centers for Disease Control and Prevention
CE	Cherry Extract
CHD	Coronary heart disease
CPT1α	Carnitine palmitoyl transferase-1α
CRP	C-reactive protein
CRP	C-reactive protein

CVD	Cardiovascular disease
CYP2A1	Cytochrome p450, family 1, subfamily a, polypeptide 2
CYP4A14	Cytochrome p450, family 4, subfamily a, polypeptide 14
CYP8B1	Cytochrome p450, family 8, subfamily b, polypeptide 1
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ellagic Acid
Eotaxin	Eotaxin
FAE	Fuji Apple Extract
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acids
FFQ	Food frequency questionnaire
FRAP	Ferric ion reducing antioxidant power
FW	Fresh weight
g	Grams
G-CSF	Granulocyte colony stimulating factor
GAE	Gallic Acid Equivalents
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSTT2	Glutathione S-transferase, theta-2

HDL	High-density lipoprotein
HF	High-fat
HF+X	High-fat plus treatment
HOMA-IR	Homeostatic model assessment-insulin resistance
HOX1	Heme oxygenase-1
HPLC	High-performance liquid chromatography
IAUC	Incremental area under the curve
IFN-g	Interferon gamma
IL-13	Interleukin 13
IL-1a	Interleukin 1 alpha
IL-1b	Interleukin 1 beta
IL-6	Interleukin 6
IL-9	Interleukin 9
Insulin	Insulin
IP-10	Interferon-gamma-inducible protein 10
KC	Keratinocyte chemoattractant
kcal	Kilocalorie
kg	Kilograms
L	Liters
LDL	Low-density lipoproteins
Leptin	Leptin
LF	Low-fat

LIX	Lipopolysaccharide-induced CXC chemokine
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LXR	Liver X receptor
M	Moles
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemotactic protein-1
mg	Milligrams
MIG	Monokine induced by gamma interferon
MIP-1a	Macrophage inflammatory protein-1 alpha
MIP-1b	Macrophage inflammatory protein-1 beta
MIP-2	Macrophage inflammatory protein-2
mL	Milliliters
mM	Millimoles
mm	Millimeter
mRNA	Messenger-RNA
N	Newton’s
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey

NHR	Nuclear hormone receptor
nM	Nanomole
NO	Nitric oxide
NQO1	NADPH dehydrogenase quinone-1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OA	Oleic Acid
Oatp1a4	Organic anion-transporting polypeptide 1a4
ORAC	Oxygen radical absorbance capacity
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PPAR	Peroxisome proliferator-activated receptors
PXR	Pregnane X receptor
QUE	Quercetin
RANTES	Regulated on activation, normal T cell expressed and secreted
RDAE	Red Delicious Apple Extract
Resistin	Resistin
RNA	Ribonucleic acid
RPL30	60S ribosomal protein L30
RPM	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SCD1	Stearoyl-coA desaturase-1

SEM	Standard error of the mean
SGLT	Sodium-dependent glucose transporters
SREBP-1	Sterol regulatory element binding protein 1
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
UT	Untreated
VLDL	Very low-density lipoproteins
w/w	Weight per weight
µg	Microgram
µL	Microliter
µM	Micromole

DEDICATION

I wish to dedicate this body of work to my family, my friends, and my God, because without them I could have never accomplished all that it took to get to this point. They have provided me with immeasurable amounts of support, love, and laughs and I can’t express my appreciation for them enough.

Thank you all.

CHAPTER I:
INTRODUCTION AND LITERATURE REVIEW

1. Introduction

As of 2010, more than one-third of adults in America are considered obese (Flegal et al.) and the prevalence of metabolic syndrome, a collection of clinical risk factors for cardiovascular disease, stroke, kidney disease, and type 2 diabetes mellitus, rose to 23% of U.S. adults (Beltrán-Sánchez et al.). Clinical risk factors for metabolic syndrome include hyperglycemia, hypertriglyceridemia, hyperlipidemia, waist circumference, blood pressure, and cholesterol levels. The International Diabetes Federation defines the metabolic syndrome as having central obesity (waist circumference ≥ 94cm for men and ≥ 80 cm for women) plus any two of the following four factors: 1) raised triglyceride level (≥ 150 mg/dL), 2) reduced HDL cholesterol (< 40 mg/dL in males and < 50 mg/dL in females), 3) raised blood pressure (systolic BP ≥ 130 mm Hg or diastolic BP ≥ 85 mm Hg), or 4) raised fasting plasma glucose (≥ 100 mg/dL) or previously diagnosed type 2 diabetes (Zimmet et al.). Beltrán-Sánchez et al. reported that the prevalence of metabolic syndrome decreased 2.6% in the last decade (2000-2010) along with hypertriglyceridemia (-9.2%) and elevated blood pressure (-8.3%). Despite this decrease in overall metabolic syndrome, the study found the prevalence of hyperglycemia and elevated waist circumference, or abdominal obesity, rose 7 and 10.7% respectively during the same time period. It can be postulated that the decrease in hypertriglyceridemia, elevated blood pressure, and low-density lipoprotein cholesterol levels is a result of an increased use of anti-hypertensive and lipid-modifying drugs to lower cardiovascular disease risk.

However, very little advancement aside from diet and lifestyle modifications has been made to reduce the prevalence of hyperglycemia or abdominal obesity.

It has been proposed that intake of certain fruit and vegetable phytochemical extracts have the ability to ameliorate some of the negative risk factors associated with metabolic disorders. Apples and cherries are two fruits popularly grown in the Pacific Northwest that also have a relatively high and diverse phytochemical composition. Previous studies have shown that apples and cherries and their phytochemical extracts are effective at decreasing the risk factors associated with metabolic diseases such as abdominal fat accumulation (obesity), diabetes, heart disease, and inflammation (Jayaprakasam et al., 2006; Kelley et al., 2006; Kelley et al., 2005; Seymour et al.). The Women's Health Study, a study of nearly 40,000 women, examined the association between cardiovascular disease and flavonoid intake. The results of the study showed that an increased intake of apples correlated with a decreased risk in developing type 2 diabetes and cardiovascular disease (Song et al, Sesso et al.). Other studies by Liu and Boyer and Johnston et al. demonstrated the ability of apples to inhibit lipid oxidation, reduce cholesterol, and improve glucose tolerance. Cherries are also able to help decrease the risk of developing metabolic diseases by reducing fat accumulation, body weight, cholesterol and triglyceride levels, improving glucose and insulin regulation in addition to acting as anti-inflammatory agents for various cytokines (Jayaprakasam et al., 2006; Seymour et al.; Kelley et al.; Zhou et al.).

Therefore, the aim of this study is to investigate the effectiveness of apple and cherry phytochemical extracts and the pure phytochemicals, quercetin and ellagic acid, to reduce the risk factors for prevalent metabolic disorders of abdominal fat accumulation, diabetes, heart disease, fatty liver disease, and overall inflammation. To determine the degree of effectiveness, both a rodent model and a human cell line model will be used to measure physiological and molecular parameters.

1.1 Metabolic Disease

Metabolic diseases encompass a variety of disorders such as obesity, fatty liver disease, heart disease, and type 2 diabetes. They are characterized by a lack of proper organ function as a result of abnormal chemical reactions in the metabolic pathway (MedLinePlus). A well-known example of this is Type 2 Diabetes, in which pancreatic beta-cell function is impaired as a result of hyperlipidemia or hyperglycemia. Recent prevalence estimates for the various metabolic diseases discussed are shown in **Table 1.1**.

Table 1.1. Prevalence estimates for a selection of metabolic diseases in the United States.

Metabolic Disease	Prevalence
Obesity	34.9% (2010)[1]
Type 2 Diabetes	9.3% (2012)[2]
Cardiovascular Disease	26.7% (2014)[3]
Hepatosteatosis	34.0% (2013)[4]

[1]Flegal et al.

[2]National Diabetes Statistics Report

[3]Go et al.

[4]Kim et al.

1.1.1 Obesity

According to an analysis of NHANES data compiled by Flegal et al., the prevalence of obesity in adults ages 20-74 has been gradually increasing between each NHANES assessment report. The publication looked at NHANES reports from 1999-2008. The NHANES reporting system classifies overweight as a BMI (weight in kilograms divided by height in meters squared) of 25.0 to 29.9 and obese as a BMI of 30.0 or higher. Obesity is further divided into grades: grade 1 (BMI, 30-<35), grade 2 (BMI, 35-<40) and grade 3 (BMI≥40). After

statistical analysis was completed, the authors found that age-adjusted obesity prevalence was 32.2% and 35.5% for men and women respectively. When overweight and obesity status were combined, there was an age-adjusted prevalence of 68.0%. The authors discuss the difference between the three reporting periods of 1976-1980, 1988-1994, and 1999-2000, stating that there was a 7.9 and 8.9 percent increase in obesity for men and women respectively from the first to second period and a 7.1 (men) and 8.1 (women) percent increase from the second to third period. Based on the trends observed by Flegal et al., the researchers propose a six to seven percentage point increase in obesity prevalence between 1999-2000 and 2008-2009 for both men and women. A CDC Morbidity and Mortality Weekly Report released in August of 2010 reported an obesity incidence of 26.7% for 2009, an increase of 6.9% from the 2000 estimate (Sherry et al.). The CDC report did not adjust for age so the overall percentage is lower; yet the predicted trend of a six to seven percent increase proposed by Flegal et al. is confirmed by the CDC reports.

With an increased prevalence in obesity comes an increased risk of physiological complications. Although the complications of obesity may not be as extreme as those of other diseases, complications associated with obesity will affect a larger population and require more long-term care, which will eventually contribute to an increase in competition for health-care resources in the coming years (Grundy). Obesity contributes to an increased risk of metabolic syndrome, cardiovascular disease, diabetes mellitus and other chronic diseases. As outlined by Grundy, the metabolic abnormalities that occur with metabolic syndrome, the

term used to describe the metabolic imbalances induced by obesity, are: dyslipidemia, hypertension, insulin resistance and glucose intolerance, and a pro-coagulant state.

Obesity is caused largely by excess energy intake, which results in excess fat accumulation in adipose and other tissues. It is normal for some nonesterified fatty acids to be seen in circulation in a fasting state, however, excessive circulating concentrations leads to an overload of lipids in tissues. This fat deposition in tissues other than adipose can result in a dysfunction in energy utilization in those tissues. The four main tissues targeted for fat nutrient overload in the body are skeletal muscle, pancreatic β-cells, liver, and the cardiovascular system (Grundy).

In muscle tissue, as non-esterified fatty acids (NEFA) are taken up into the muscle tissue, energy metabolism in the mitochondria is shifted to fatty acids, reducing glucose metabolism and leading to insulin resistance and hyperglycemia. In a study published by Roden et al., it was found that insulin resistance was induced by initially inhibiting glucose transport and phosphorylation followed by inhibition of glycogen synthesis and glucose oxidation. Grundy suggests that insulin resistance as a result of obesity is seen together with hyperinsulinemia. This results from the inability of the skeletal muscle to take up glucose, signaling the β-cells to secrete more insulin. The high concentration of nonesterified fatty acids also contributes to the continued increase in β-cell stimulation by training them to secrete insulin whenever

glucose is present in the serum. This overstimulation of β-cell secretion can impair function of the β-cells altogether and lead to type 2 diabetes as a result of reduced insulin secretion (Grundy).

The high concentrations of nonesterified fatty acids (NEFA) in the serum also promote hepatic uptake of the NEFAs. The increased hepatic uptake of fatty acids promotes the synthesis of triacylglycerol and cholesterol, increasing secretion of VLDL. An increase in circulating VLDL particles not only increases both triacylglycerol and cholesterol concentrations, but also decreases LDL particle size and concentration of HDL cholesterol. It has also been reported that obesity increases the activity of triacylglycerol lipase in the liver, also contributing to a decrease in LDL particle size and HDL cholesterol concentrations (Grundy). Obesity produces significant dysfunction of individual metabolic pathways, however, when multiple complications are produced in conjunction with each other, they can cause even more harm. It has been shown that elevated serum insulin, resulting from both skeletal muscle and β-cell dysfunction, can increase the amount of fatty acids, leading to an increase in VLDL-triacylglycerol synthesis, and an increase in coagulation proteins, inducing a procoagulant state (Grundy).

The cardiovascular system is also adversely affected by an obese state. With obesity frequently comes hypertension. It is suspected that hyperinsulinemia causes a raised arteriolar tone, which induces this hypertension (Grundy). The metabolic abnormalities that arise from an obese state also

contribute to an increased risk of coronary heart disease (CHD), which will be outlined below.

1.1.2. Diabetes

As outlined above, an excess intake of energy contributes to an increased risk of obesity and further, an increased risk of metabolic syndrome. In a study performed by Wilson et al, it was shown that in the presence of metabolic syndrome, the relative risk of developing Type 2 diabetes mellitus is increased nearly sevenfold. Hanson et al. performed a factor analysis on a population of 1,918 Pima Indians to determine which individual components of the metabolic syndrome were associated with an increased incidence of diabetes. The four factors measured were: insulinemia, body size, blood pressure, and lipid metabolism. The variables of metabolic syndrome that were measured were: body weight, waist circumference, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, fasting insulin x glucose, 2-h insulin x glucose, fasting insulin/glucose, and 2-h insulin/glucose. The researchers found that in both diabetic and non-diabetic participants the variables measured showed strong factor loadings, indicating that those variables could be considered major constituents of the factor. Among the group of nondiabetic participants, 16% developed diabetes after one to eight years (median 4.1 years). The strongest factors associated with diabetes incidence were insulinemia and body size (body weight and waist circumference). Lipids and blood pressure showed some relation, however, they were modest or weak in comparison (Hanson et al.).

This correlation between incidence of diabetes and the risk factors of insulinemia and body size further confirms the adverse effect that an obese state can have on the development of diabetes. It was mentioned above by Grundy that a high-fat, or obesigenic, diet increases both overall body size through increased fat absorption and storage in tissues, inhibits carbohydrate metabolism in skeletal muscle, and contributes to an increased prevalence of insulin dysfunction in the form of insulinemia or insulin resistance. These three factors observed in conjunction with the work done by Hanson et al. therefore supports an increased risk of developing diabetes with consumption of a high-fat diet or obese state.

1.1.3. Heart Disease

The risk of coronary heart disease (CHD) is also increased with metabolic syndrome, the consequences of which are exacerbated with obesity, as outlined above by Grundy. According to Chew et al., cardiovascular disease (CVD) risk is increased two-fold in people with metabolic syndrome. Wilson et al. reported similar results, determining that the relative risk for CVD and CHD to be more than or nearly doubled (CVD: men-2.88, women-2.25; CHD: men-2.54, women-1.54) in people with metabolic syndrome. The results from the Kuopio Ischemic Heart Disease Risk Factor Study, reported by Lakka et al., were in agreement with Chew and Wilson, reporting a 3.77- and 3.55-fold higher mortality from CHD and CVD respectively when metabolic syndrome was present. These statistics apply to both diabetics and non-diabetics with metabolic syndrome, since non-

diabetics with metabolic syndrome have a sevenfold increased risk of developing type 2 diabetes (Wilson et al.).

The factors of metabolic syndrome that are risk factors for CVD and CHD are blood glucose, serum insulin levels, dyslipidemia, blood pressure, BMI, and waist circumference. The factors with the greatest indicators of CHD and CVD risk have consistently been shown to be: body size, fat distribution, insulin and glucose (Hanson et al, Lakka et al.).

1.1.4. Hepatic steatosis

Hepatic steatosis arises from an imbalance between triglyceride (TG) uptake and excretion. Based on a rat model, 20% of TG consumed in the diet is delivered to the liver. Using a typical American diet of 100 grams of fat per day, ~20 grams of fat are being delivered to the liver each day (Cohen et al.). Adipocytes also secrete free fatty acids (FFA) to the liver as a result of TG hydrolysis, which accounts for approximately 20 grams/day. The combination of 20 grams of TG and 20 grams of FFA a day makes up the total TG content of the liver. In the obese state, the amount of fatty acids consumed in the diet and from adipose tissue is increased, which subsequently increases the amount of FFA in the liver. This flux of FFA to the liver is sufficient to cause steatosis (Cohen et al.).

The incidence of non-alcoholic fatty liver disease, or NAFLD, is closely related to obesity and its development risk is increased by the associated complications of metabolic syndrome, specifically diabetes and dyslipidemia.

Currently, NAFLD is considered the most prevalent liver disease in the developed world. The need for liver transplants as a result of NAFLD increased by 8.5% from 2001 to 2009 and is the third most prevalent indication for liver transplantation in the United States. A growing concern is the risk of recurring NAFLD after transplantation. Since NAFLD is caused primarily by diet and lifestyle, patients who have received a transplant are more likely to develop NAFLD in the allograft as well (Said).

In a study performed by El Atrache et al., 83 liver transplant patients with non-alcoholic steatohepatitis (NASH), a more severe form of NAFLD, or cryptogenic cirrhosis (CC) were monitored for pre- and post-transplant parameters. The study found that patients with metabolic syndrome, hypertension or on insulin had 34%, 32% and 37% recurrence of NASH compared to 13%, 12% and 6% without the complications.

1.1.5. Chronic Inflammation

Obesity has recently been shown to be associated with a greater abundance of macrophages, neutrophils, T-cells, B-cells, and mast cells in adipose tissue (Kanneganti and Dixit). The concentration of macrophages in adipose tissue from obese compared to lean individuals is three times greater and correlates with metabolic dysfunction. The primary macrophage present in adipose tissue of obese individuals is the M1 macrophage, which secretes high concentrations of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β. Conversely, M2 macrophages are associated with metabolic homeostasis and produce anti-

inflammatory molecules. In an obese state, there is a significant imbalance between quantity of M1 and M2 macrophages in favor of M1 macrophages, which plays an essential role in inducing obesity-induced inflammation. An increase in inflammatory T cells, B cells and mast cells and a decrease in regulatory T cells also correlate with chronic inflammation as a result of obesity by inducing M1 macrophage-inducing cytokines IL-6, TNF-α, IFN-γ (Kanneganti and Dixit; Rodríguez-Hernández et al.).

Weisberg et al. further supported these findings in their study of lean and obese mice and respective macrophage levels in adipose tissue. The researchers found that quantity of proteins characteristic of macrophages correlated significantly with body mass and body adipocyte size. They also found that macrophages are responsible for the majority of the adipose tissue expression of pro-inflammatory markers TNF-α and IL-6 and that these markers as well as MCP-1 and IL-1β positively correlate with the amount of white adipose tissue present.

Cytokines secreted by macrophages can induce adipocyte lipolysis, releasing free fatty acids and inducing toll-like receptor 4 (TLR4) in the macrophage. This activation in the macrophage stimulated the secretion of NF-κB and consequently the release of more pro-inflammatory mediators (COX-1, IL-1β, IL-6, and TNF-α) and further induces the chronic inflammatory response as a result of obesity (Johnson et al.).

Chronic inflammation is a significant health concern because it has a major effect on the development of chronic metabolic disorders such as metabolic syndrome, fatty liver disease, heart disease, and type 2 diabetes in addition to other health complications (**Figure 1.1**). Elevated CRP levels are a commonly used measurement for chronic inflammation and are associated with adiposity, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and low HDL cholesterol. Additionally, circulating levels of pro-inflammatory cytokines TNF-α and IL-6 are associated with an increased risk of developing cardiovascular disease and insulin resistance/diabetes (Rodríguez-Hernández et al.).

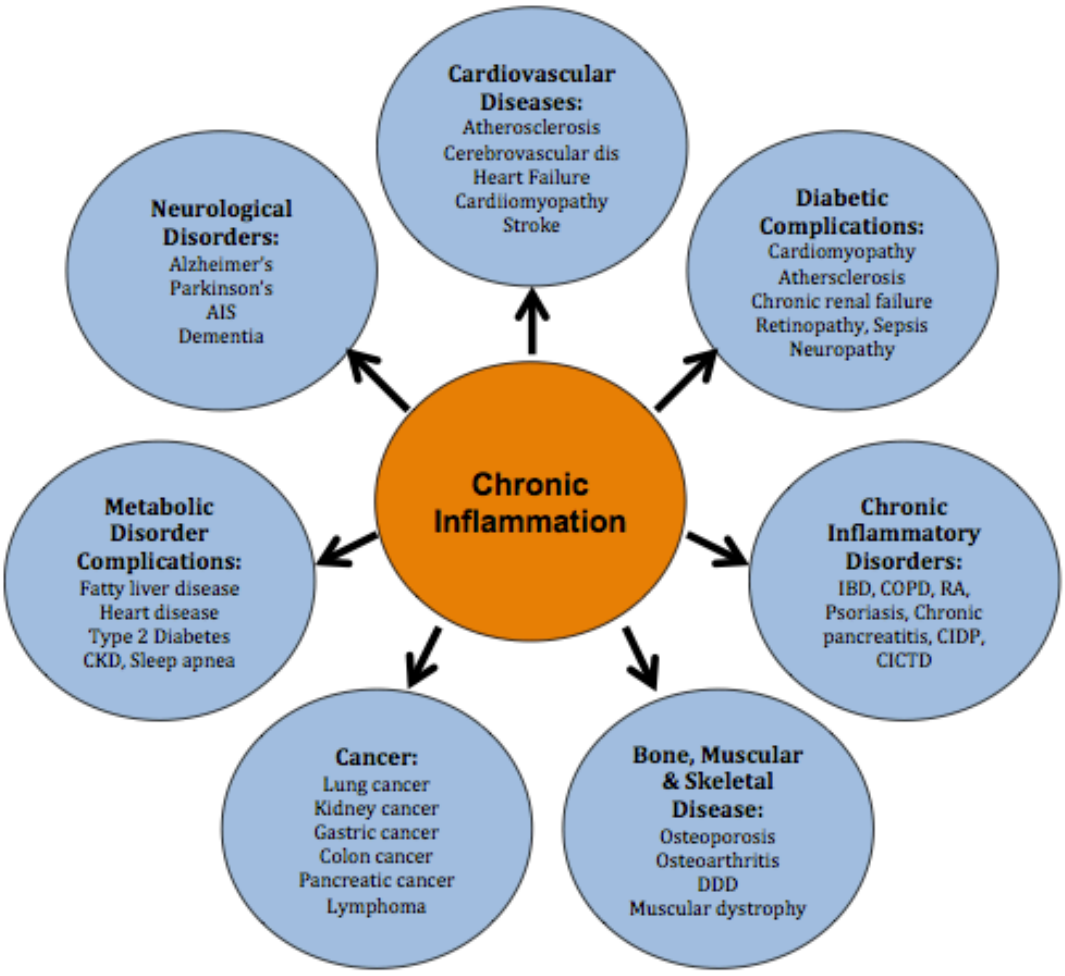


Figure 1.1. Diseases, disorders, and complications associated with chronic inflammation (adapted from Emma Olliff, CureJoy.com)

1.2. Therapies for Metabolic Diseases

1.2.1. Drug Therapies

The pharmaceutical industry has developed a number of drug therapies to treat the individual complications of metabolic disease as well as the overall disease. No one drug has been shown to effectively attenuate metabolic syndrome,

however there has been effective treatment for the individual complications of metabolic syndrome. Statins are a common therapy for lowering cardiovascular risk by reducing LDL- and VLDL-cholesterol accumulation, but unfortunately don't significantly elevate HDL-cholesterol levels. Fibrates, a known PPAR-α agonist, have been shown to regulate dyslipidemia associated with metabolic syndrome and reduce cardiovascular events. Metformin and thiazolidinediones (glitazones), a known PPAR-γ agonist, have been shown to reduce the incidence of diabetes or slow the progression of diabetes. Both metformin and thiazolidinediones have also been shown to reduce the risk of macrovascular events such as coronary heart disease and stroke, and even reduce the risk of death (Chew et al.). Still, to effectively treat metabolic syndrome, extensive management of multiple pharmacological therapies is needed to effectively remedy all risk factors.

1.2.2. Diet and Lifestyle Therapies

The most common therapy for metabolic syndrome is diet and lifestyle modification consisting of moderate weight loss, increased physical activity, and dietary modification. Exercise and weight reduction can attenuate insulin sensitivity and reduce the prevalence of diabetes and metabolic syndrome (Chew et al., González-Castejón et al.).

Diet therapies for metabolic syndrome typically consist of a reduced calorie diet low in fat and high in fruits and vegetables to promote moderate weight loss and better nutrient intake. Fruits and vegetables are promoted because of their nutrient-dense and relatively low-calorie qualities. These plant

foods contain a high concentration of phytochemicals, compounds that have the ability to regulate various biological pathways to promote anti-obesity, anti-inflammatory, and antioxidant effects. Dietary phytochemical consumption has been shown to reduce the risk of obesity by decreasing body fat mass, BMI and weight, as well as the complications that arise from it, such as blood pressure, triglyceride accumulation, inflammation, cholesterol levels, and blood lipids (González-Castejón et al, Rahman et al.).

The Women's Health Study and the Finnish study (men and women), both large scale population studies, looked specifically at flavonoids, a sub-class of phytochemicals, and their effect on metabolic and chronic diseases. The Finnish study showed an inverse relationship between flavonoid intake and total (men and women) and coronary mortality (women only), as well as a reduced risk for cerebrovascular disease, lung cancer, and asthma (Knekt et al, 2002; Knekt et al, 1996). The Women's Health Study did not support an association between total or individual flavonoid intake and cardiovascular disease risk or diabetes risk, however, both studies showed an inverse relationship between whole food product consumption and a decreased risk. Apples, tea and broccoli showed a non-significant inverse association with cardiovascular disease while apples and tea also showed a significant association with a reduced risk of Type 2 diabetes (Song et al, Sesso et al.). These findings support the theory that it is the sum of the food components as a whole that has a beneficial effect on metabolic diseases, not just their individual flavonoid components.

1.3. Apples

1.3.1. Phytochemical Composition and Concentration of Apples

In the United States, 22% of the phenolics consumed from fruits are from apples. Apples are ranked second for total antioxidant activity as well as total phenolic content. Apples also have the highest concentration of free phenolics, or phenolics not bound to other compounds, making them more available for uptake into tissues. It is important to note that the apple flesh or pulp contains a relatively low concentration of apple phenolics, with the majority of the phenolics found in the apple peel. Phenolic content of apples also widely varies among different varieties. The primary phenolic compounds found in apples are: quercetin derivatives, catechin, phloridzin, chlorogenic acid, cyanidins, and gallic acid (Boyer and Liu; Barbosa et al.) An important issue regarding phenolic compounds in apples is their bioavailability, or ability to be taken up by tissues. It has been found that the phenolics in apples are either enzymatically hydrolyzed then taken up by the intestinal cells (quercetin and phloridzin), glucuronidated in the liver (quercetin), metabolized by the gut microflora (chlorogenic acid), or simply absorbed by intestinal epithelial cells (catechin and epicatechin). Little is known of how these compounds are absorbed in a whole fruit model (Liu and Boyer).

1.3.2. Apples and Fat Accumulation

According to a review by Liu and Boyer, apples have the ability to inhibit lipid oxidation as well as reduce cholesterol levels. It is suspected that the antioxidant activity of apple phenolics successfully reduces the rate of LDL oxidation. In a study performed by Pearson et al., six commercial apple juices and red delicious whole apple, peel and flesh were compared for amount of LDL oxidation inhibition. When 5uM of gallic acid equivalents (GAE) of each sample was added to an LDL solution, it was found that the degree of inhibition varied among the apple juices, ranging between 9-34% while whole apple, peel and flesh had an LDL oxidation inhibition of 34, 38, and 21 percent, respectively.

Aprikian et al. studied 40 male Wistar rats fed diets with a 0.3% cholesterol and one diet supplemented with 15% lyophilized apples (apple diet) and the other (control diet) with 13% fructose/glucose/sucrose mixture. In the apple group, there was a significant drop in plasma and liver cholesterol concentrations as well as an increase in HDL. It was suggested that apples also reduced cholesterol absorption in the body due to an increased cholesterol concentration in the feces. Based on these findings it is shown that apples are able to reduce the amount of LDL oxidation as well as reduce serum and liver cholesterol, reduce cholesterol absorption, and improve the lipoprotein profile of serum.

Vidal et al. demonstrated similar results studying lipoprotein synthesis and secretion in human Caco-2/TC7 enterocytes supplemented with apple

polyphenol extracts. Cells were supplied with complex lipid micelles and both intracellular and secreted lipid levels were measured. When enterocytes were supplemented with apple polyphenols, a decrease in esterification of cholesterol and secretion of lipoproteins by the enterocytes was observed. Researchers found that the apple polyphenols inhibited apolipoprotein B (apoB) synthesis, but apoB mRNA levels were not modified. Further exploration was performed to observe the contrasting effects of whole apple extract, their flavonoid monomers and a mixed monomer solution, and the procyanidins fraction on lipid secretion and synthesis. Only whole apple extract showed a decrease in secreted phospholipids, triglycerides, and cholesteryl ester and intracellular cholesteryl ester concentrations when measured against the flavonoid monomers and monomer mix (chlorogenic acid, phloridzin, epicatechin, and catechin). However, apple procyanidins were able to replicate the effect of whole apple extract by inhibiting both cholesterol ester synthesis and lipoprotein secretion. This indicates that the procyanidins, not the individual flavonoid monomers or mixture of flavonoids that are the active compounds for lipid synthesis and secretion.

1.3.3. Apples and Diabetes

In the Women's Health Study, dietary flavonoid intake was measured by semi-quantitative FFQs to determine their association with Type II diabetes and the markers for insulin resistance and inflammation (Song et al.). Researchers found that apples were the only flavonoid-rich food that was associated with a reduced risk of Type II diabetes. With the consumption of one apple a day, the relative

risk of Type 2 diabetes was reduced 28%. Inflammatory markers and insulin resistance were not affected by apple consumption. The study also looked at total flavonoid intake and intake of subtypes of flavonoids (quercetin, kaempferol, myricetin, apigenin, and luteolin) and whether they were also associated with a reduced risk. None of the subtypes of flavonoids or total flavonoids, which were associated with apple products, showed an association with a reduced risk, leading the authors to speculate that other compounds were associated with the protective effect of apples and Type II diabetes. A major phytochemical compound of apple peels, quercetin, was also associated with a decreased risk of type II diabetes in the Finnish study of 10,000 male and female participants (Knekt et al., 2002).

Johnston et al. further investigated this association between apples and a reduced risk for Type II diabetes in their observation of apple juice consumption on plasma glucose concentrations and circulating hormones. Participants were given either a control water or apple juice solution (clear or cloudy juice) with 25g glucose load and glucose and insulin were measured. The results showed that consumption of apple juice versus water had a statistically significant effect on the parameters measured. There was a significant effect of apple consumption on the area under the curve (treatment x time) measurement for glucose over the three-hour time period. The authors stated that this was consistent with a delayed absorption of glucose. Some differences were seen between the cloudy and clear juices and their effect on glucose concentration and the incremental area under the curve (IAUC) values. For both juices, a lower glucose

concentration was observed early on at 15 minutes. During the first increment from time zero to 30 minutes, both juices showed a statistically significant decrease in IAUC when compared to the control, and the cloudy juice maintained this difference for the 30 to 90 minute increment as well. Apple juice also showed a lower treatment x time interaction for insulin concentrations in the first 90 minutes when compared to the control. The authors discussed several possibilities for this delayed glucose absorption following the consumption of apple juice. They proposed that phloridzin (or phloretin) inhibits the sodium-dependent glucose transporters (specifically SGLT1) in the intestinal lumen, preventing glucose uptake and improving overall glucose tolerance (Johnston et al.).

It was also reported by Barbosa et al. that various varieties of apple have an inhibitory effect on α-amylase and α-glucosidase activity *in vitro* using inhibitory assays relevant for managing hyperglycemia. A dose-dependent inhibitory response was seen for both α-amylase and α-glucosidase in the aqueous and ethanolic extracts. Peel extracts had a higher α-glucosidase inhibitory activity while pulp extracts had higher α-amylase inhibitory activity. The correlation between percentage inhibitory activity and total phenolic content of the apples was also measured. A correlation was found between percentage α-glucosidase inhibitory activity and total phenolic content, but no correlation was observed for α-amylase activity.

1.3.4. Apples and Cardiovascular Disease

The Women's Health Study showed that apple intake was associated with a reduction in risk for both cardiovascular disease and cardiovascular events (Sesso et al.). Apple consumption translated to a 13-22% decrease in risk of cardiovascular disease, depending on the amount of apples consumed. The study showed no association was seen between quercetin and a reduced risk of cardiovascular disease; however the women ingesting flavonoids at the 95th percentile level saw a 35% reduction in risk for cardiovascular events, but no significantly reduced risk in cardiovascular disease. These findings were consistent with the outcomes of a Finnish cohort study of over 5,000 men and women (Knekt et al.). The study reported that apple intake was associated with a reduced risk of coronary mortality. When the highest and lowest quartiles were compared, a risk reduction of 43 and 19% was seen for women and men respectively. The level of consumption at this level was greater than 71 grams/day for women and greater than 54 grams/day for men, which translate to about half of a medium-sized apple a day.

Much of apples effect on reducing cardiovascular disease is attributed to its cholesterol-lowering ability and antioxidant capacity. In a study performed by Décordé et al., forty hamsters were fed an atherogenic diet for twelve weeks supplemented with mashed apple or apple juice. Various cholesterol and antioxidant enzyme levels were measured to determine the effect of these compounds on the hypercholesterolemic hamsters. Both apple and apple juice

effectively reduced plasma cholesterol, non-HDL cholesterol, liver superoxide dismutase and glutathione peroxidase activities, and TBARS (thiobarbituric acid reactive substances) and increased plasma antioxidant capacity when compared to the controls. Aortic fatty acid streak area was also increased from 48 to 93%. The results show that prolonged consumption of apples or apple juice can prevent the development of atherosclerosis by improving both antioxidant status and the serum lipid profile.

The protective effect of apples on cardiovascular risk can be attributed to the apple's high phytochemical concentration as well as fiber and vitamin content. Serra et al. focused on determining the active ingredient(s) within the apple that were responsible for the bioactive response of lowering cholesterol. Correlations between the bioactive response (total cholesterol, LDL-C, triglycerides and ox-LDL) and chemical composition (polyphenols, antioxidant activity, fiber, and other components) of the apple varieties were measured. The correlations showed that catechin, epicatechin and procyanidins B1 were the major phytochemical compounds responsible for the cholesterol-lowering effect of apples. Soluble fiber, β-carotene, and antioxidant activity were also found to be contributing factors.

1.4. Cherries

1.4.1. Phytochemical Composition and Concentration of Cherries

Cherries are a nutrient dense food filled with nutrients and bioactive food components. Phytochemicals found in cherries are: anthocyanins, quercetin and carotenoids. The total phenolic content of sweet cherries can range from 44.3 to 87.9 mg gallic acid equivalents (GAE)/100g fresh weight (FW) according to a study of 13 sweet cherry cultivars performed by Usenik et al. Similar concentrations were seen in studies by Kim et al. and McCune et al., showing total phenolic concentrations ranging from 92.1 to 146.8 mg GAE/100g FW and 160 to 170 mg GAE/100g FW respectively. The majority of the phenolics in Bing sweet cherries are found in the skins and flesh, with lesser amounts found in the pits. The major phenolics found in cherries are: quercetin, hydroxycinnamic acid, beta-carotene, and anthocyanidins (cyanidin, pelargonidin, delphinidin, peonidin, and malvidin). Various flavones, flavanols, flavanones, and isoflavonoids are also found in cherries but to a lesser degree (McCune et al.).

Anthocyanins are the hallmark phenolic compound in cherries with concentrations up to 80.2 mg/100g FW in sweet cherries. In Bing sweet cherries, cyanidin makes up over 90% of the total anthocyanin content with minor amounts of peonidin (6%) and pelargonidin (<1%) (McCune et al.). Gao and Mazza identified cyanidin-3-rutinoside and -3-glucoside as the major anthocyanins in sweet cherries by method of HPLC. Peonidin-3-rutinoside and -3-glucoside were

identified as minor anthocyanins. Kim et al. confirmed these results by using HPLC and pH differential method to measure anthocyanin content.

Hydroxycinnamates are the primary type of phenol present in cherries, comprising around 40% of the total phenolic content. Hydroxycinnamates consist of neochlorogenic acid and *p*-coumaroylquinic acid (McCune et al, Gonçalves et al.). Flavonols and flavan-3-ols are also seen in noticeable concentrations in cherries. The most common flavonols found in cherries are quercetin, kaempferol, catechin and epicatechin. The quercetin derivative quercetin-3-rutinoside is seen in the highest concentration in sweet cherry varieties (Kim et al.) whereas the other three flavonols are seen at a relatively lower concentration (Gonçalves et al.).

1.4.2. Cherries and Fat Accumulation

In a study performed by Jayaprakasam et al. (2006), C57BL/6J mice were first fed a high-fat (60% kcals) diet to induce obesity and hyperglycemia and then switched to a high-fat diet supplemented with cherry anthocyanins (cyanidin, pelargonidin, and delphindin) to observe their ability to ameliorate obesity and insulin resistance. There was a 24% reduction in body weight gain for the anthocyanin-fed mice when compared to the high-fat control. The anthocyanin group also saw a reduction in liver lipid accumulation as well as liver triacylglycerol concentration.

In another study observing the effect of cherries *in vitro*, Dahl-ss rats were fed a diet enriched with tart cherries (Seymour et al.). After 90 days of feeding, a reduction in total cholesterol and triglycerides in both the liver and serum, as well as an increase in hepatic mRNA markers for PPAR-α and acyl-coenzyme A (CoA) oxidase (ACO) activity was observed. PPAR-α is involved in lipid metabolism and regulates ACO activity, which promotes fat oxidation and reduced hepatic fat storage. This increased activity of PPAR-α and ACO with whole tart cherry supplementation is likely responsible for the lower hepatic total cholesterol and triglyceride concentrations as well as reduced hyperlipidemia and hepatic steatosis (Seymour et al.).

In a feeding study of 18 healthy men and women, diets were supplemented with 280 grams/day of Bing sweet cherries for 28 days (Kelley et al., 2006). Blood samples were taken before, during, and after cherry supplementation. No effect on the plasma concentrations of total-, HDL-, LDL-, or VLDL-cholesterol, triglycerides, sub-fractions of HDL, LDL, VLDL, or their particle size and number was observed with cherry consumption. The authors attributed this lack of change in blood lipids to the fact that their study looked at healthy adults, while other studies showing a beneficial effect of cherries on blood lipids were seen in people with obesity-related metabolic changes or pre-existing blood lipid irregularities. Since participants showed no blood lipid irregularities at the start of the study, it is reasonable that supplementing their diet with cherries did not show a reduction since levels were not high to start off with, therefore no reduction was needed.

1.4.3. Cherries and Diabetes

Cherries have a relatively low glycemic index of 22 when compared to other fruits (Foster-Powell et al.). This suggests that cherries have a potential glucose-lowering effect. In the study by Jayaprakasam et al. (2006) mentioned above, anthocyanin-fed mice showed improved glucose tolerance when compared to the high-fat control. In addition, islet cell architecture and insulin staining were maintained despite elevated insulin levels when compared to the control. In a previous study by Jayaprakasam et al. (2005), anthocyanin and anthocyanidin fractions from the same *Cronus* fruits were isolated and purified to observe their ability to individually stimulate insulin secretion from rodent pancreatic β-cells. Cyanidin-3-glucoside and delphinidin-3-glucoside were found to the most effective at stimulating insulin secretion in the presence of 4mM and 10mM glucose. Similar results were seen in the study in Dahl-ss rats performed by Seymour et al. After 90 days of feeding, a reduction in insulin, hyperlipidemia, and fasting glucose were observed, indicating an improved insulin regulation and therefore a reduced risk of diabetes.

1.4.4. Cherries and Heart Disease

It has already been highlighted that cherries have been shown to reduce fat accumulation and body weight as well as lower cholesterol and triglyceride levels in both serum and liver (Jayaprakasam et al, 2006; Seymour et al.), some of the primary risk factors for cardiovascular disease. A study by Xia et al. further investigated the mechanistic pathways and specific anthocyanin compounds

responsible for cherries beneficial effect on cardiovascular risk using a cell culture study model in mouse peritoneal macrophages and macrophage-derived foam cells. They found that both cyanidin-3-glucoside and peonidin-3-glucoside effectively reduced total, free and esterified cholesterol in both cell models in a dose-dependent manner, indicating that these anthocyanin compounds are inducing apoAI-mediated cholesterol efflux from the cells. The authors further investigated the mechanisms by which cholesterol was reduced by measuring the relative expression of ABCA1, LXR-α and PPAR-γ in both peritoneal macrophages and foam cells. A dose-dependent increase in gene expression was seen for ABCA1, LXR-α, and PPAR-γ with the highest level of expression observed at 24h, 12h, and 12h respectively. For all three markers, cyanidin-3-glucoside had a greater effect on relative gene expression than peonidin-3-glucoside. When this PPAR-γ- LXR-α-ABCA1 pathway was inhibited or blocked, cholesterol efflux did not occur, confirming this as the active pathway for cholesterol efflux. Cyanidin-3-glucoside and peonidin-3-glucoside are two of the primary anthocyanins found in Bing sweet cherries, so it can be assumed from the results of this study that cherries will have also some level of beneficial effect on cholesterol efflux, thus reducing cholesterol accumulation in cells and overall cardiovascular risk.

1.4.5. Cherries and Inflammation

Inflammation and oxidative stress are a major cause of chronic inflammatory diseases such as diabetes and cardiovascular disease. Various fruits and

vegetables have been shown to reduce the risk for these chronic inflammatory diseases. In a study by Kelley et al. (2006), 18 adult men and women supplemented their diets with 280g/d of Bing sweet cherries. After 28 days of feeding, a decrease in CRP (25%), RANTES (21%), and nitric oxide (NO) (18%) was observed. Plasma levels for IL-6, intercellular adhesion molecule-1, and tissue inhibitor of metalloproteinases-2 were not affected by cherry consumption. Therefore, the authors proposed that there was some anti-inflammatory effect of sweet cherries through the selective modification of CRP, RANTES, and NO.

In a later study by Kelley et al. (2013), the same human feeding model was used; 28 days of supplementation with sweet cherries. In this second study, several biomarkers of inflammation and inflammatory diseases were reduced after 28 days of consumption such as CRP, ferritin, PAI-1, IL-18, endothelin-1, advanced glycation end products and epidermal growth factor. Most markedly decreased were CRP (20.1%), ferritin (20.3%) and advanced glycation end products (29.0%). An increase in the IL-1 receptor antagonist (27.9%) was also observed.

Although Kelley et al. did not observe an effect on concentration of interleukin-6 (IL-6) with cherry supplementation, Zhou et al. demonstrated that tart cherry extract, tart cherry anthocyanins and pure tart cherry cyanidin-3-*O*-glucoside added to adipose stem cells reduced the LPS-induced secretion of IL-6. The authors also showed a synergistic effect between atorvastatin (Lipitor) and tart cherry extract and atorvastatin and cyanidin-3-*O*-glucoside in reducing IL-6

secretion. These findings could reduce the need for use of pharmacological therapies to reduce adipose inflammation.

1.5. Beneficial Effects of Polyphenols

Polyphenols are found ubiquitously in plant foods and beverages such as fruits, vegetables, tea, wine, etc. (Bravo). In fruits, the predominant polyphenolic compounds are flavonols, which found primarily in the skins of the fruits. The total polyphenolic content of fruits can range anywhere from 2mg/100g FW (pears) up to 1200mg/100g FW (blackcurrants) and are highly variable depending on environmental conditions, ripeness, processing method, variety, and storage (Bravo).

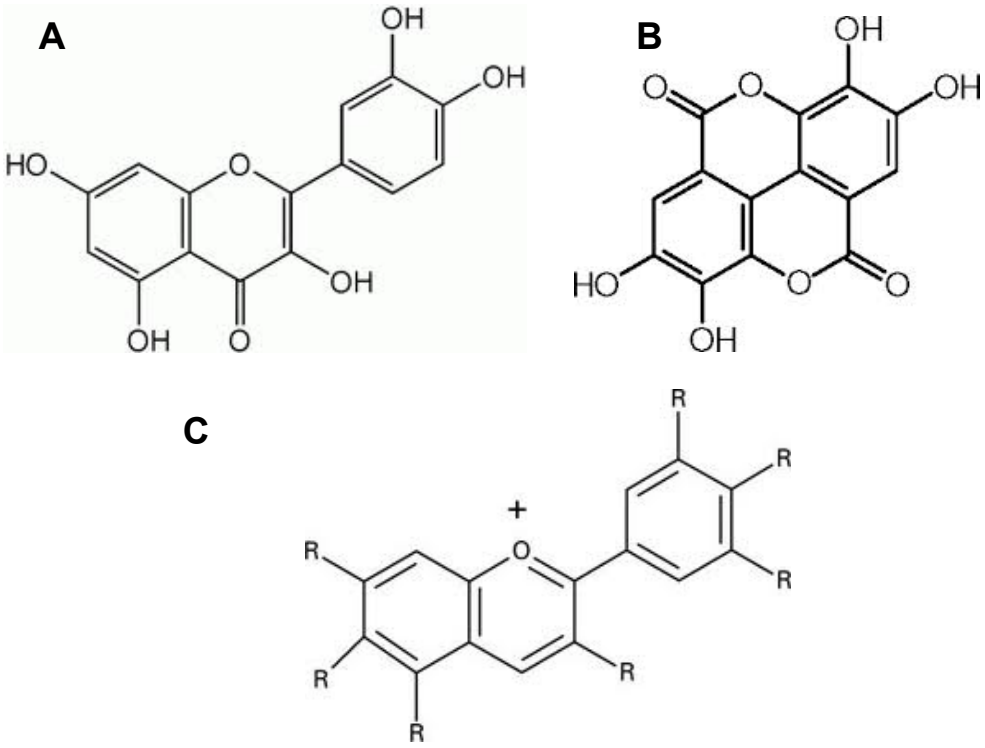


Figure 1.2. Structure of phytochemical compounds found in apples, cherries, and other fruits. Chemical structures represent those of quercetin (A), ellagic acid (B), and a general anthocyanin structure (C).

1.5.1. Bioavailability of Phytochemicals

The bioavailability of polyphenolic compounds is highly variable depending on the structure and solubility of the compound. Polyphenols can be divided into two categories of solubility: extractable and non-extractable. Extractable polyphenols are usually small or medium in size and can be extracted with different solvents such as water, menthol, ethanol, and acetone. Non-extractable polyphenols are large molecules or phenols that are bound to dietary fiber or protein and are not soluble in the solvents listed above (Bravo). Extractable polyphenols were minimally excreted after enzymatic exposure, indicating that they are thoroughly digested and/or absorbed in the gut. Non-extractable polyphenols, however, were seen in substantial quantities in the feces, indicating these compounds' resistance to absorption and/or digestion in the intestine. The absorption of polyphenolic compounds varies widely depending on the molecular structure. Some small and free polyphenols are absorbed directly into the gut mucosa, while other compounds like flavonols and flavonoids must undergo hydrolysis in either the small or large intestine where their glycoside groups are often hydrolyzed to their aglycone forms. Once absorbed they are quickly transformed into their methylated, glucuronidated or sulfated derivatives (Rahman et al.). The majority of polyphenolic metabolism, however, occurs in the liver (Bravo). Overall, the bioavailability of polyphenols is limited due to their low amount of absorption and rapid elimination.

1.5.2. Biological Activity of Polyphenols

Polyphenols have a wide range of biological activity including antioxidant activity, radical scavenging activity, anti-inflammatory response, lipid, cholesterol and insulin regulation and reduction, as well as ameliorating the risk factors and causes for chronic and metabolic diseases.

1.5.2.a. Quercetin

Quercetin (**Figure 1.2A**) has been shown to attenuate inflammatory genes (Chuang et al; Kleemann et al.), reduce insulin resistance (Chuang et al.), reduce TG, fat, and cholesterol accumulation (Moon et al, Panchal et al, 2012), inhibit adipogenesis (Moon et al.) decrease body weight (Moon et al.), decrease blood pressure (González-Castejón et al.) protect against radical scavenging activity (Kleemann et al.), reduce cancer risk, especially lung cancer (Knekt et al, 2002), as well as induce nuclear receptors such as PPAR-γ (Moon et al.), Nrf2 (Panchal et al, 2012) and inhibit others like NFκB (Panchal et al, 2012; Kleemann et al.). Quercetin was also shown to reduce total overall mortality (Knekt et al, 2002).

1.5.2.b. Ellagic Acid

Ellagic Acid (**Figure 1.2B**) exhibits free radical scavenging activity (Yoshimura et al.), inhibits resistin secretion (Yoshimura et al.), improves cardiovascular remodeling and ventricular function (Panchal et al, 2013), improves hepatic steatosis (Yoshimura et al, Panchal et al, 2013), improves glucose tolerance (Panchal et al, 2013), and improves serum lipid profile (Yoshimura et al.), and

induces nuclear receptors PPAR-α (Yoshimura et al, Panchal et al, 2013) and protein levels for Nrf2 (Panchal et al.) as well as reduces protein levels for NFκB (Panchal et al, 2013).

1.5.2.c. Cyanidin

Cyanidin, or specifically cyanidin-3-glucoside, is a common anthocyanin (**Figure 1.2C**) that is able to reduce lipid, triglyceride, and cholesterol accumulation (González-Castejón et al., Xia et al., Jayaprakasam et al., 2006), ameliorate hyperglycemia and insulin sensitivity (Sasaki et al.; Jayaprakasam et al., 2005), normalize adipocytokine secretion (González-Castejón et al.), reduce weight gain (Jayaprakasam et al., 2006) and decrease inflammation (González-Castejón et al; Zhou et al.).

1.5.2.d. Phloridzin

Phloridzin is shown to reduce body weight and food intake, improve glucose tolerance and insulin secretion, and attenuate the lipid profile (cholesterol, LDL, VLDL, triglycerides) in rats induced with diabetes (Najafian et al.).

1.5.2.e. *Resveratrol*

Resveratrol demonstrates the ability to increase insulin sensitivity (Baur et al, Chuang et al.), improve motor function (Baur et al.), reduce inflammation (Chuang et al.), reduce plasma fasted glucose (Baur et al.), improves liver

histology (Baur et al.) and increase overall survival (Baur et al.), as well as induce the activity of nuclear receptor PPAR-γ (Baur et al, Chuang et al.).

1.6. Nuclear Hormone Receptors

Nuclear receptors are transcription factors that typically require activation through the binding of a ligand. Ligands may include lipids, proteins, drugs, and phytochemicals. Once activated, these nuclear receptors regulate the expression of target genes that influence a multitude of biological processes from general metabolism to development and reproduction. Nuclear receptors all demonstrate a similar structure (**Figure 1.6**) with 3 main regions: an NH_2-terminal region, containing AF-1, a DNA-binding domain at the core, and a large COOH-terminal region, which includes the ligand-binding domain, dimerization interface, and AF-2. AF-1 is a ligand-independent transcriptional activation function while AF-2 is ligand-dependent. After ligand binding, the nuclear receptor undergoes a conformational change to promote recruitment of co-activator proteins to allow transcription to occur (Chawla et al.).

Two distinct nuclear receptor models exist: the classic nuclear steroid hormone receptors and the adopted orphan nuclear receptors. Steroid hormone receptors bind to DNA as homodimers and their ligands are typically steroid hormones synthesized in the endocrine system. The steroid hormones bind to the receptors at a high affinity and regulate metabolic and developmental processes critical for proper biological function. Adopted orphan nuclear receptors perform as heterodimers bound to retinoid X receptor (RXR) and some

are responsive to dietary lipids, which act as their ligands. Since some of the receptors respond to lipids and bind with lower affinities, their activity is highly affected by dietary intake. The main function of these adopted orphan nuclear receptors is to maintain lipid homeostasis within the body, activating a metabolic cascade upon ligand binding. There are many other receptors, called orphan nuclear receptors, meaning that they simply have not been shown to bind to a physiological ligand. Once a physiological ligand is discovered, they become "adopted" orphan nuclear receptors. (Chawla et al.) A table depicting the nuclear receptors most relevant to this thesis research and their known agonists is listed below (**Table 1.2**)

Figure 1.3. General nuclear hormone receptor structure (Chawla et al.)

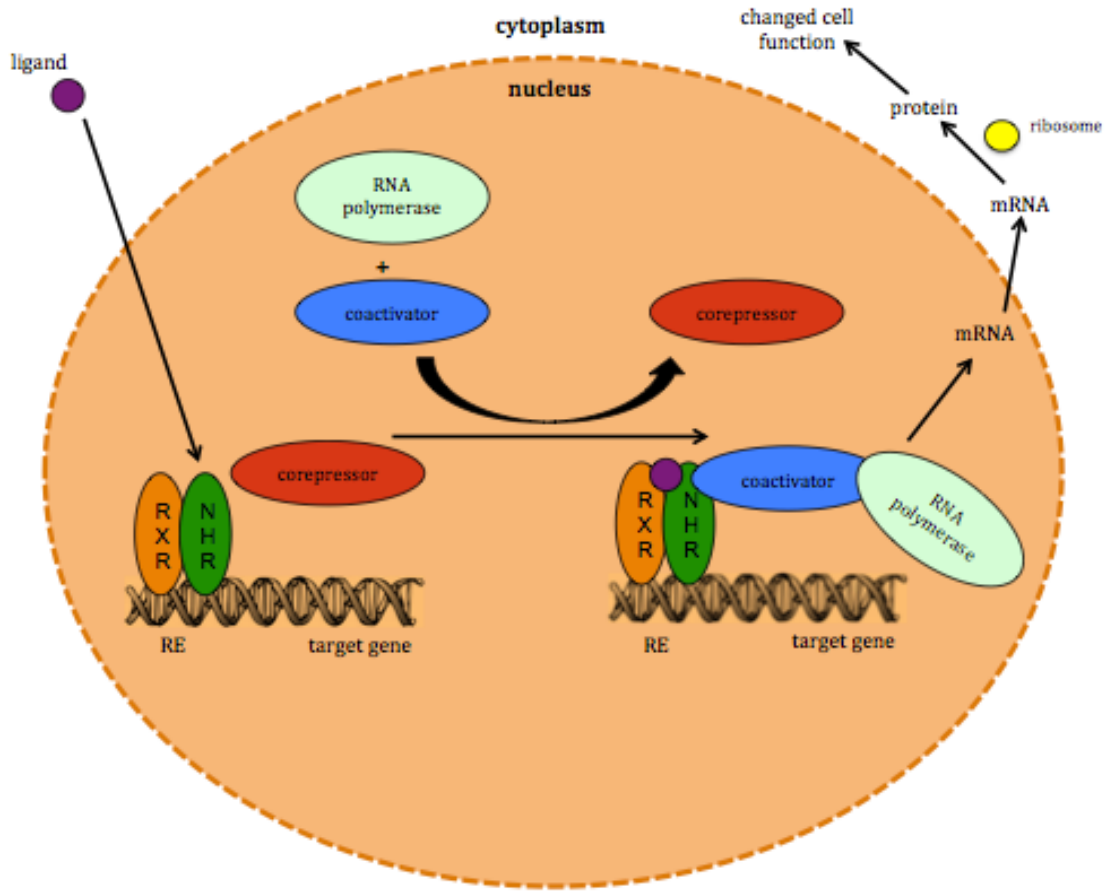


Figure 1.4. Mechanism of nuclear hormone receptor action*.

RXR=retinoid X receptor, NHR=Nuclear hormone receptor, RE=Receptor element
(Adapted from University of Oslo School of Pharmacy)

Table 1.2. Relevant adopted orphan nuclear receptors and their known agonists.

Nuclear Hormone Receptor	General Function	Known Agonists	Natural Product Agonists
PPAR-α	Fatty acid catabolism	Fibrates, Fatty acids	Soy isoflavones (Mezei et al., 2003; Mezei et al, 2006) Tart cherries (Seymour et al.) Onion peel extract (Moon et al.) Plum (Utsunomiya et al.)
PPAR-γ	Adipogenesis, cellular differentiation, insulin sensitization	Fatty acids, Eicosanoids, Glitazones, Triterpenoids	Tart cherries (Seymour et al.) Grape skin extract (Park et al.) Plum (Utsunomiya et al.) Cyanidin-3-glucoside and Peonidin-3-glucoside (Xia et al.)
PXR	Detoxification and elimination; xenobiotic metabolism	Xenobiotics, Steroids, Rifampicin	St. John's Wort (Watkins RE et al.) Soy isoflavones (Li et al., 2007; Li et al., 2009)
Nrf2	Antioxidant stress pathway, oxidative stress prevention	Quercetin	Grape polyphenols (Chuang and McIntosh) Curcumin, Resveratrol and Caffeic Acid (Rahman et al.)

Nrf2 is a transcription factor, but is not a member of the nuclear hormone receptor family (Chawla et al.).

CHAPTER II:

METABOLIC EFFECTS OF CONSUMPTION OF HIGH-FAT DIETS SUPPLEMENTED WITH APPLE AND CHERRY PHYTOCHEMICAL EXTRACTS, QUERCETIN, AND ELLAGIC ACID IN C57BL/6J MALE MICE

1. INTRODUCTION

Apples and cherries are phytochemical-rich foods with diverse phytochemical content, some of the more abundant compounds being quercetin, anthocyanins, and chlorogenic acid (Hyson, McCune et al.). Fruit was processed to isolate and extract polyphenol-rich extracts. These extracts, along with the purified phytochemicals quercetin and ellagic acid, were added to a high-fat rodent diet and fed to C57BL/6J mice. Previous studies have shown that these phytochemical-rich fruit extracts can serve to ameliorate the risk factors associated with the development of metabolic diseases (Hyson, McCune et al., Gourineni et al., Johnston et al., Boyer and Liu).

The C57BL/6J mouse strain was chosen based on its ability to mimic the negative effects of consumption of a high-calorie, high-fat diet by humans. When mice are fed a normal diet, they remain normal weighted and healthy; however, when fed a high-fat diet, these mice overeat and become obese. As mentioned in Chapter 1, obesity results in an increased incidence of metabolic abnormalities and chronic inflammation, leading to the development of metabolic disorders such as diabetes, hepatosteatosis and cardiovascular disease. For the purpose of our study, we sought to observe the effect of phytochemicals specifically on the metabolic factors of body weight, food intake, glucose tolerance, insulin regulation, mobility, balance and endurance, lipid metabolism, and inflammatory response.

We hypothesize that feeding high-fat fed mice a diet enriched with apple and cherry phytochemical extracts and selected pure phytochemicals will improve

their metabolism by ameliorating one or more metabolic abnormalities accompanying obesity.

2. MATERIALS AND METHODS

2.1. Preparation of Apple and Cherry Extracts

The fruit extraction protocol was adapted from He and Liu, Sun et al. and Gourineni et al. Ninety kilograms of Bing sweet cherries were donated by Blue Mountain Horticultural Society (Walla Walla, WA) and were pitted, destemmed, and frozen. Cherries were kept frozen at -20°C and defrosted for 20 hours before pureeing, using a KitchenAid blender (model #: KSB560CU1) in batches, yielding 40 L of puree. Thirteen liters of the puree were filtered using a bladder press lined with a fine bladder bag; 7.5 L liquid was collected and the solids were discarded. The remaining 27 L of cherry puree were treated with 10 mL pectinase and refrigerated for 18 hours to reduce the clogging of an overly thick puree encountered during the bladder press filtration. Treated cherry juice was centrifuged at 3000 RPM for 30 minutes at ambient temperature using a Sorvall RC-5C and an Eppendorf 5810-R centrifuge (Rotors: PTI 096-062032, A-4-81) The supernatant was collected and combined with the previously obtained filtrate. Solid phase extraction was performed using a custom-built separatory column packed with 4 L adsorbent polymer beads (Amberlite FPX-66, Rohm and Haas, Philadelphia, Pennsylvania) fed by a peristaltic pump (Manostat Model72-640-000, The Barnant Company, Barrington, IL). Briefly, cherry juice was loaded onto the column until beads were saturated. After loading, the column was

rinsed of residual juice components by passing deionized water until five times the initial juice volume had been passed. Adsorbed compounds were then recovered by passing USP grade ethanol (95% w/w) through the column until the beads became colorless and the eluent ran clear. After extraction the recovered solution was evaporated under vacuum at 50°C to remove excess ethanol and freeze-dried for approximately 30 hours. Dried extract was 18 g and was stored in sealed containers at 4°C.

For the apple extraction procedure, 49 kg of Red Delicious and 58.5 kg of Fuji apples were donated from Blue Mountain Horticultural Society (Walla Walla, WA) and stored under refrigeration at 4°C. Apples were peeled and peels were soaked in 0.1 M citric acid to prevent oxidation; apple flesh was discarded. Peels were squeezed dry and blended for five minutes in a KitchenAid blender (model #: KSB560CU1) at a 2:1 ratio of 80% ethanol (mL) to apple peels (grams). Blended puree was then filtered with a Büchner funnel under vacuum through Whatman #2 filter paper. Solids were collected and re-homogenized a second time with 80% ethanol and filtered again. Juice was evaporated under vacuum at 50°C to remove excess ethanol, then freeze-dried for 30 hours using a Virtis Consol 4.5 (Warminister, PA; Serial # 200554) freeze-drier. Juice was frozen in trays overnight until solid and then freeze-dried at -40°C and 100milliTorr vacuum. The freeze-dried product demonstrated evidence of residual sugars and/or pectin due to a viscous quality. Solids were re-dissolved in water and treated with 0.1% pectinase and kept at 4°C overnight. The supernatant was collected and solids were discarded. This liquid was run through a separatory column filled with

approximately 500mL of Amberlite beads as described above and eluted with ethanol. The eluent was then evaporated under vacuum at 50°C and then freeze-dried for 30 hours. Dried extract yields were 10 g for Red Delicious and 12 g for Fuji and extracts were stored in sealed containers at 4°C.

2.2. Quantification of Phytochemicals by HPLC

Anthocyanins were analyzed by HPLC. Total anthocyanins were measured by AOAC method 2005.02 (pH differential method). Polymeric color and browning were measured by bisulfite bleaching. Total phenolics were measured by the Folin-Ciocalteu spectrophotometric method. Phenolics were analyzed by HPLC using the method that has been proposed as an AOAC method for phenolic analysis. Antioxidants were measured by the Ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. (Waterhouse).

2.3. Mouse diet studies

Forty-eight, 6-week old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and randomly divided into six experimental groups (n=8): Low-fat group (LF) (10% calories from fat), High-fat group (HF) (60% calories from fat) and five “High-fat treatment” groups (HF+X): high-fat plus 0.2% Quercetin (MP Biomedicals, LLC, Solon, OH; Cat #: 152003) (HF+QUE), 0.2% Ellagic Acid (Alfa Aesar, Ward Hill, MA; A15722) (HF+EA), 0.2% Bing Cherry phytochemical extract (HF+CE) and 0.2% Fuji and Red Delicious Apple

phytochemical extract (HF+AE). Mice were housed four to a cage under standard conditions of paper bedding, controlled climate, good hygiene, and access to nesting materials. Mice were acclimated for two weeks with access to standard chow diet and water ad libitum and a 12-hour light/dark cycle and then divided into their respective treatment groups and provided their experimental diets ad libitum for 10 weeks. Experimental diets were prepared by Research Diets Inc. (New Brunswick, NJ, USA) (**Table 1**). Mouse body weights and food intake were measured weekly. At the end of the study, food was withheld from mice for six hours prior to anesthetization via inhalation of 5% isofluorane. Blood was collected by cardiac puncture and glucose level was measured using a ReliOn® Ultima Blood Glucose Monitoring System (ReliOn® Ultima, Abbott, Alameda, CA). Plasma samples were collected after centrifuging at 2000 RPM with an Eppendorf 5417R microfuge for 15 minutes at 4 C, then stored at -80 C. Liver and epididymal adipose were collected and weighed; tissue was stored in RNAlater® (Ambion®, PN: AM7021), remaining tissue was frozen at -80C. Mouse liver cell RNA were isolated from tissue samples by homogenizing tissue in Trizol® (Ambion®, #: 15596-026) following the suggested product protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee at Oregon State University.

Table 2.1 Composition of experimental diets

Diets	LF		HF		HF+X*	
	gm	*kcal*	gm	*kcal*	gm	*kcal*
Casein, 80 mesh	200	800	200	800	200	800
L-Cysteine	3	12	3	12	3	12
Corn Starch	506.2	2025	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	68.8	275
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205
Mineral Mix S10026	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
X**	0	0	0	0	1.56	0
%						
Protein	19	20	26	20	26	20
Carbohydrate	67	70	26	20	26	20
Fat	4	10	35	60	35	60
Total	--	100	--	100	--	100
Kcals/gram	3.8	--	5.2	--	5.2	--

*HF+X: HF+QUE, HF+EA, HF+AE and HF+CE

**X: Quercetin, Ellagic Acid, Cherry (Bing) or Apple (Fuji and Red Delicious) phytochemicals

Table 2.2. Mouse study schedule

	Week of Study									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Weight Measurements	X	X	X	X	X	X	X	X	X	X
Diet Consumption	X	X	X	X	X	X	X	X	X	X
Baseline Glucose						X				X
Glucose Testing						X				
5-Day Diet Measurements							X			
Grip Strength Test								X	X	
Rotarod Endurance Test								X	X	
Metabolic Cages								X	X	
Necropsy										X

2.5. Five Day Diet Measurements

Mouse body weight and food intake were measured daily over the course of five consecutive days in week 4. The amount of food consumed and weight gained between each day was measured and recorded. Spillage was collected and accounted. The average daily food intake for each diet group (n=8, 2 cages) was analyzed for differences in consumption.

2.6. Intraperitoneal glucose tolerance test

A glucose tolerance test was performed at week six of the study. Glucose levels were measured using a ReliOn® Ultima Blood Glucose Monitoring System (ReliOn® Ultima, Abbott, Alameda, CA) by collecting a sample of tail blood every 30 minutes over a two hour period. Food was withheld from mice for six hours prior to initial baseline glucose measurement. Mice were anesthetized using isofluorane inhalation at 5% concentration, a tail cut, 3-4mm wide and 2mm deep, was made under anesthesia and an initial, baseline glucose measurement was taken. After baseline glucose level was measured at time 0, 10 µL of 20% glucose in 0.9% saline solution per 1 g body weight was injected into the abdomen of the mouse. Mice were then removed from anesthesia and placed into clear plastic restraining tubes at the time of each remaining blood glucose measurement (Andrikopoulos et al., Ayala et al., Heikkinen et al., Christensen et al.).

2.7. Rotarod Endurance Test

The Rotarod 3375-4R with 60Hz/115V Process Control RotaRod 337500 (TSE Systems, Bad Homburg, Germany, Serial No: 050620-3 and 050620-04) was used. Two mice at a time were placed in separate sections of the horizontal cylinder, which rotated and increased in speed as the trial progressed. The rod is suspended above a metal floor high enough to incentivize the mice to avoid falling. The average of 3 test trials was used. Speed protocol for rotarod was as follows: acceleration from 0 rpm to 3 rpm followed by acceleration from 3 rpm to 15 rpm. Latency time was measured when mice fell off the cylinder; a longer latency time indicates better balance and/or endurance. Mice were trained on the rotarod in 3 trials, 24 hours prior to testing. Testing followed consisted of three timed trials with a 60 second break between each trial. Times were averaged for each mouse and then reported as an average time for all mice in treatment group (n=8).

2.8. Grip Strength Test

Chatillon DFE-010 Series Digital Force Gauge (C.S.C. Force Measurement, Inc. Agawam, MA) was used to measure grip force. Mice were allowed to clasp their front paws to a metal grate hooked up to a force meter. The mouse was then gently pulled backward by the base of its tail and the maximum grip force (in Newton's) was measured. The grip measurement was repeated three times for each mouse with a 60 second break in between each trial.

2.9. Metabolic Cages

One mouse per treatment group was placed into a metabolic cage equipped with food and water ad libitum for 24 hours. Small plastic cylinders under a metal grate floor collected all urine and feces excreted by mouse. Mouse and food weights were measured before and after mice were placed in cage. This procedure was repeated until all eight mice in the treatment group had spent 24 hours in the metabolic cages.

2.10. Hepatic tissue fixation and staining

Hepatic tissue fixation and staining was done according to protocol outlined by Carson in *Histotechnology: A Self-Instructional Text* (2009).

2.11. Statistical analysis

All data are expressed as the mean ±standard error of the mean (SEM). Multi-way analysis of variation (ANOVA) was performed on Prism 6 (GraphPad Software, La Jolla, CA). Post-hoc testing was performed using Tukey's multiple comparison test.

2.12. Automated Serum Analysis

Serum was collected at time of necropsy by heart puncture. Serum was analyzed for triglycerides, total cholesterol, HDL (LDL was calculated), liver cytosolic enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase

(AST)). Serum chemistry parameters were analyzed utilizing a Vitros 250 (Ortho-Clinical Diagnostics, Rochester, NY).

2.13. Cytokine Method

Cytokines were measured in 96-well plates by three different MILLIPLEX® MAP kits from Millipore (Millipore, Billerica, MA). The Mouse Acute Phase Magnetic Bead Panel 2 (Cat. #MAP2MAG-76K) was used to measure C-Reacitve Protein (CRP) levels. The Mouse Cytokine/Chemokine Magnetic Bead Panel (Cat. #MCYTMAG-70K-PX32) was used to measure 32 cytokines simultaneously (Eotaxin, G-CSF, GM-CSF, IFNg, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1a, MIP-1b, MIP-2, RANTES, TNFa, VEGF; Abbreviations and functions of cytokines can be found in **Appendix Table 2.2**). The Mouse Adipokine Magnetic Bead Panel (Cat. #MADKMAG-71K) was used to simultaneously measure 7 adipokines (IL-6, Insulin, Leptin, MCP-1, PAI-1, Total Resistin, TNFa). A total of 10-25 uL of serum and an equal amount of antibody-immobilized beads were added to each well following the manufacturer's instructions. Plates were sealed and incubated overnight at 4C in the dark on a plate shaker. After incubation, the plates were washed twice and detection antibodies were added to each well. Plates were sealed and incubated at room temperature, in the dark, on a plate shaker for 30 minutes to one hour according to the manufacturer's instructions. Plates were washed 2-3 times according to manufacturer's instructions and 100 uL of Wash Buffer were added to all wells.

Plates were read on a Luminex 200 instrument using xPONENT software. Data was analyzed by ANOVA using Graph Pad software.

3. RESULTS

Total phenolics and total anthocyanins in apple and cherry phytochemical extracts were measured (**Table 2.3**). Red Delicious apple extract (RDAE) and Fuji apple extract (FAE) contained similar total phenolic concentrations, only differing by approximately 19 g GAE/100g extract, with RDAE being the greater of the two. AE used in the study was a 50/50 w/w mix of RDAE and FAE extracts, having a total phenolic content of 60.6 g GAE/100g. Cherry extract (CE) contained noticeably less phenolics overall, about one-fifth the concentration of AE. AE also had more than three times greater concentration for total anthocyanins than CE, with the RDAE having greater than three times that of FAE. Assays for ORAC and FRAP were also performed. For ORAC, RDAE and FAE had relatively similar capacities, however, CE had nearly 70% greater values than AE. However, for the FRAP assay, CE had a markedly lower value than AE; nearly eight times lower. RDAE had a FRAP value noticeably greater than FAE.

Body weight of mice was measured weekly and analyzed by repeated-measures one-way ANOVA (**Figure 2.1A)**. No significant difference was observed among high-fat treatment groups; however all high-fat groups were different from the low-fat control group ($p=<0.0001$). On average, HF mice were approximately 14 grams heavier than the LF mice. Weekly diet consumption was

also measured and no difference was observed in regards to energy consumed among high-fat fed mice (**Figure 2.1B**). A 24-hour diet consumption test was run over the course of 5 days, where weight of diet consumed was measured daily (**Figure 2.1C**). No significant difference in energy consumed was observed among high-fat treatment groups, only between the LF and HF groups ($p=<0.05$).

Baseline glucose measurements were taken at weeks 6 and 10. Mice were fasted for six hours prior to blood draw and glucose concentrations are expressed as mg/dL (**Figure 2.2**). At week 6, all high-fat treatment groups with extracts or phytochemicals had significantly lower baseline glucose measurements when compared to the HF group (HF+AE: $p=<0.0001$, HF+CE and HF+QUE: $p=<0.05$, HF+EA: $p=<0.001$) and were statistically similar to the LF mice. At week ten, HF+CE, HF+QUE, and HF+EA groups had an intermediate baseline glucose concentrations when compared to HF and LF control groups, and HF+AE treatment group had baseline glucose levels that were similar to those HF control mice.

A glucose tolerance test was performed at week six and is expressed as the difference in area under the curve (AUC) relative to the average AUC for the HF control group (**Figure 2.3**). When ANOVA was performed on HF-fed mice only, a significant difference was seen ($p=0.0214$), and marginally significant improvement for HF+AE and HF+QUE-fed mice was observed ($p=<0.10$). The LF control group had significantly improved glucose tolerance when compared to the high-fat fed groups ($p=<0.001$).

At eight and nine weeks, grip strength was measured. The test recorded the maximum grip force exerted in three trials and is expressed as an average grip force relative to the mouse's body weight (N/g body weight). It was observed that despite a similar body weight, mice from all high-fat treatment groups showed a trend toward improved grip strength when compared to the HF group, and had values intermediate to the LF and HF controls (**Figure 2.4**). The LF control mice had greater grip force per gram body weight compared to the HF control group ($p=<0.001$).

A Rotarod test was performed during weeks eight and nine to measure endurance and coordination of mice among treatment groups (**Figure 2.5**). Mice from HF+AE, HF+CE, and HF+QUE treatment groups showed a trend toward improved endurance and mobility when compared to the HF group, and were intermediate between the two control groups. HF+EA showed no difference when compared to the HF control group. LF mice showed significant improvement in endurance and mobility when compared to HF control mice ($p=<0.05$)

At week ten, serum was collected from mice at necropsy and a serum glucose and blood lipid panel was performed (**Table 2.4)**. No difference in serum glucose levels was observed among high-fat treatment groups, and all high-fat diet groups were statistically higher than the LF control ($p=<0.05$). LDL, VLDL, HDL, and triglycerides levels showed no difference among all high-fat treatment groups, but HDL levels were higher in HF-fed mice compared to the LF-fed mice ($p=<0.001$). Serum cholesterol levels were improved in HF+AE-fed mice and were intermediate in value to the two LF- and HF-fed control groups. The

remaining high-fat treatment groups showed no differences compared to the HF control group for serum cholesterol. Creatinine levels were decreased in HF+EA- and HF+QUE-fed mice when compared to HF- and LF-fed mice ($p=<0.0001$). Levels for ALT and AST showed no difference among diet groups.

Serum hormone and cytokine levels were also measured (**Table 2.5)**. No significant difference was observed between HF-fed mice for any of the hormone or cytokines measured. Despite the absence of statistical significance, qualitative trends were observed. CRP levels were decreased in HF+QUE-fed mice relative to HF-fed mice. GM-CSF levels were elevated in HF+AE-fed mice while they were depressed in HF+CE-fed mice. Mice fed HF+AE and HF+QUE diets had noticeably reduced IL-1a, IL-6 and insulin levels when compared to HF-fed mice, respectively, with the two groups being intermediate between the two controls for serum insulin levels, not different from either LF- or HF-fed mice. IP-10 levels were reduced in HF+CE-fed mice to levels below that of both LF and HF control mice. Serum KC levels were reduced in HF+CE- and HF+QUE-fed mice compared to HF-fed mice. Leptin levels were marginally ($p=<0.10$) ameliorated in mice fed HF+CE and HF+EA diets. HF+CE-fed mice had reduced LIX levels when compared to both control diet groups. Mice fed HF+AE and HF+EA diets had lower M-CSF levels when compared to HF-fed mice. HF+EA-fed mice also showed reduced MIP-1a and MIP-1b levels along with HF+QUE-fed mice for MIP-1a. HF+AE-fed mice had noticeably reduced MIP-2 and RANTES levels compared to HF-fed mice. Serum PAI-1 levels were markedly reduced in mice fed HF+AE and HF+CE diets compared to mice fed LF and HF diets. Finally, only

HF+EA mice had reduced resistin levels when compared to the HF control mice. No qualitative trends were observed among all diet groups for serum GM-CSF, IL-1a, IL-6, IP-10, KC, LIX, M-CSF, MIP-1a, MIP-1B, MIP-2, PAI-1, and RANTES levels.

At the end of week ten, mice were sacrificed and organ tissues were collected and weighed. Body weights in grams and in grams per body weight are shown in **Table 2.6**. Liver weights of mice fed HF+AE, HF+CE, and HF+QUE diets were intermediate to both control groups, however, when expressed relative to overall body weight, no significant difference was observed. All HF-fed groups showed reduced kidney weight when compared to the HF-fed control and were intermediate between the two LF- and HF-fed mice with HF+QUE-fed mice being statistically similar to the LF-fed control. When kidney weight was measured as a percentage of total body weight, all HF-fed groups had a lower organ weight per body weight when compared to the LF- and HF-fed mice, however, all groups were similar to the HF control. Lastly, inguinal adipose tissue was collected and no difference was observed among all HF-fed groups when compared to the HF-fed mice for both organ tissue weight and the weight relative to overall body weight.

Cross-sections of liver tissue were collected and stained with trichrome stain to identify areas of fat accumulation and fibrosis and a representative image for each group is shown in **Figure 2.6**. White globules represent fat accumulation within the cells, dark red circles are cell nuclei, and red staining indicates cytosol. When comparing the two control samples, liver from HF-fed mice has a

noticeably greater amount of fat accumulation than the LF-fed mice. Cell nuclei are no longer located in the center of the cells of the HF-fed mice as the fat globules have shifted them toward the cell periphery. Hepatocytes from HF+AE- and HF+CE-fed mice appear to have an accumulation of fat more similar to that of the LF-fed mice than the HF-fed mice. The mice fed HF+QUE and HF+EA diets also showed noticeably reduced fat accumulation when compared to the HF-fed mice, however, to a lesser degree than the HF+AE- and HF+CE-fed mice.

Table 2.3. Quantification of phenolics in purified apple and cherry phytochemical extracts.

	RDAE[1]	FAE	Total AE[2]	CE
Total Phenolics (g GAE/100g)	70	51	61	12
Total Anthocyanins (mg/100g)	1086	348	717	230
ORAC (mmol Trolox/100g)	395	483	439	630
FRAP (mmol Trolox/100g)	847	595	721	90

[1]RDAE=Red Delicious Apple Extract; FAE=Fuji Apple Extract; AE=Apple Extract, CE=Cherry Extract; GAE=Gallic Acid Equivalents; ORAC=Oxygen Radical Absorbance Capacity; FRAP=Ferric Reducing Ability of Plasma
[2]Total AE is the average of RDAE and FAE extract value

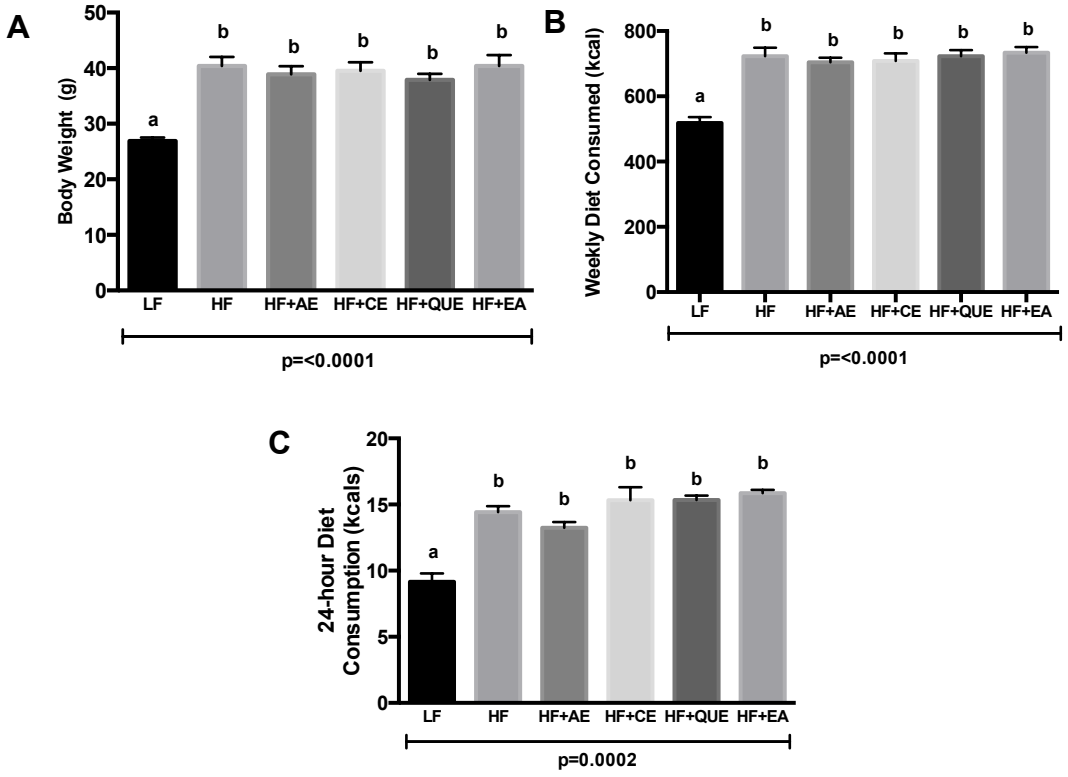


Figure 2.1. **Body weight (A), Weekly diet consumption (B), and 24-hour diet consumption (C) in C57BL/6J mice fed various phytochemical containing diets for 6 weeks**. Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Weekly diet consumption was measured as total for feeding group (n=8) and 24-hour diet consumption for individual mice. Values are expressed as means, n=8. Where letters differ, values are statistically different as measured by repeated-measures one-way ANOVA ($p<0.05$).

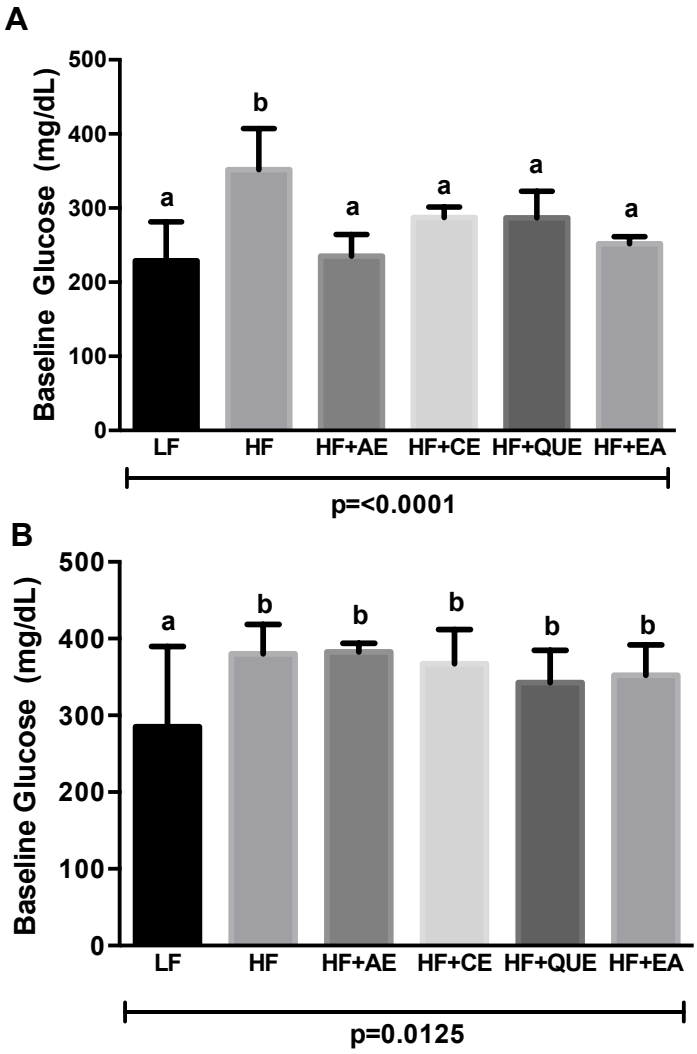


Figure 2.2. Glucose concentration of male C57BL/6J mice fed various phytochemical containing diets for 6 (A) and 10 (B) weeks. Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are expressed as the mean ± SEM, n=8. Values that do not share a letter differ ($p<0.05$).

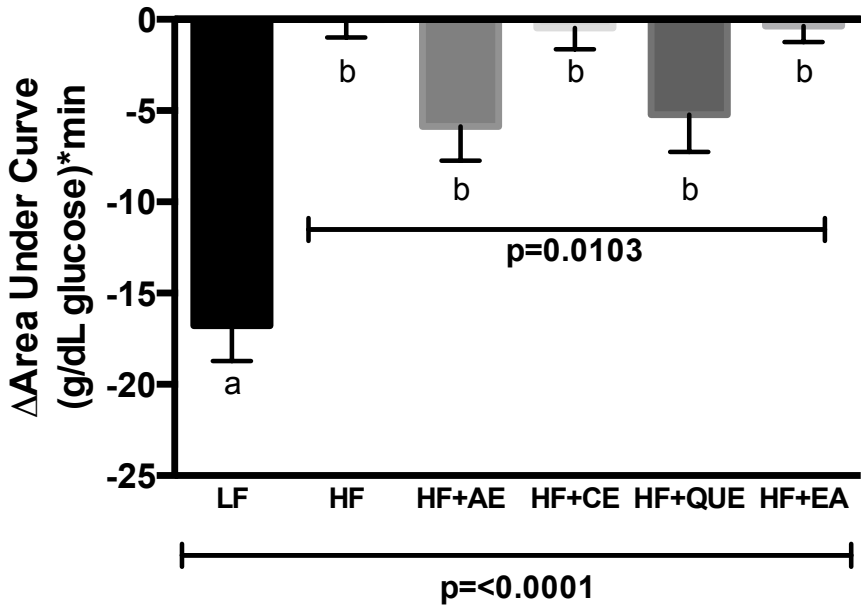


Figure 2.3. **Glucose tolerance test measured as area under the curve relative to HF group (ΔAUC) in male C57BL/6J mice fed various phytochemical containing HF-diets for 6 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). AUC measurements were subtracted from the average AUC for HF mice. Values are expressed as the mean ± SEM, n=8. Values that do not share a letter differ ($p<0.05$).

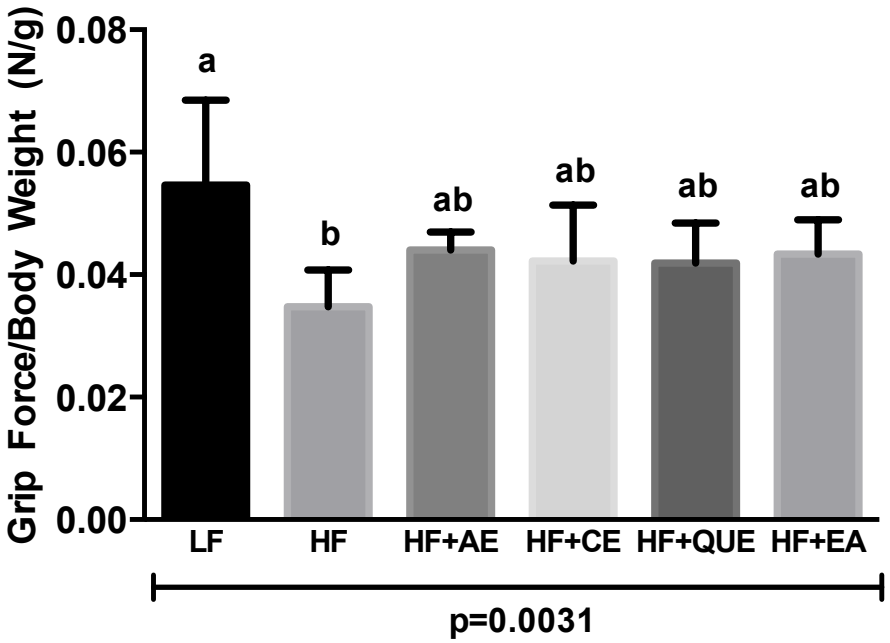


Figure 2.4. **Grip force relative total body weight in male C57BL/6J mice fed various phytochemical containing diets for 9 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are expressed as the mean ± SEM, n=8. Values that do not share a letter differ ($p<0.05$).

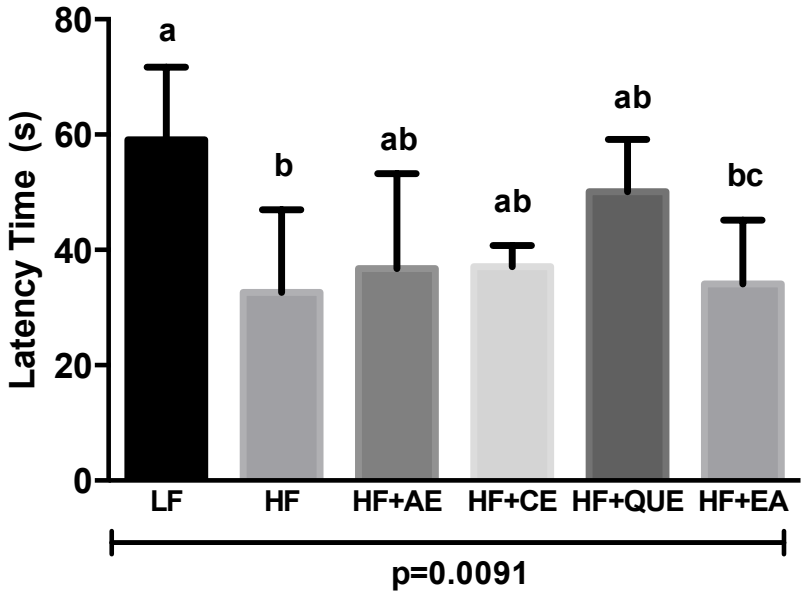


Figure 2.5. **Rotarod latency time of male C57BL/6J mice fed various phytochemical containing diets for 9 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Time is expressed as the average of 3 test trials. Values are expressed as the mean ± SEM, n=8. Values that do not share a letter differ ($p<0.05$).

Table 2.4. Serum glucose and lipid levels in male C57/BL6J mice fed various phytochemical containing diets for 10 weeks[1]

	LF[2]	HF	HF+AE	HF+CE	HF+QUE	HF+EA
Glucose mmol/L	13.3±2.8[a]	21.4±0.6[b]	24.4±0.6[b]	22.7±1.4[b]	20.8±0.9[b]	19.2±1.0[b]
LDL- mmol/L	ND[3]	1.09±0.22[a]	0.60±0.32[a]	1.02±0.19[a]	0.61±0.17[a]	0.99±0.22[a]
VLDL- mmol/L	0.41±0.06[a]	0.43±0.01[a]	0.44±0.09[a]	0.51±0.04[a]	0.42±0.01[a]	0.46±0.02[a]
HDLC- mmol/L	1.5±0.4[a]	3.0±0.1[b]	2.9±0.2[b]	3.0±0.1[b]	3.1±0.2[b]	3.3±0.1[b]
Cholesterol- mmol/L	2.04±0.35[a]	4.51±0.28[b]	3.69±0.80[ab]	4.55±0.23[b]	4.13±0.31[b]	4.62±0.30[b]
Triglycerides- mmol/L	0.90±0.14[a]	0.94±0.01[a]	0.95±0.20[a]	1.10±0.08[a]	0.93±0.02[a]	1.00±0.05[a]
Creatinine- umol/L	14±2[a]	15±1[a]	14±1[a]	15±1[a]	5±1[b]	4±0[b]
ALT- U/L	375±246[a]	152±36[a]	69±6[a]	83±14[a]	156±63[a]	209±154[a]
AST- U/L	568±337[a]	212±61[a]	111±28[a]	156±47[a]	250±74[a]	218±124[a]

[1]Values are expressed as means ± SEM of each group, n=3-7
Values that do not share a letter differ ($p<0.05$).
[2]Diet groups: Low-fat (LF), high-fat (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA).
[3]ND: Value not detected

Table 2.5. Serum hormone and inflammatory cytokine levels in male C57/BL6J mice fed various phytochemical containing diets for 10 weeks[1]

	LF[2]	HF	HF+AE	HF+CE	HF+QUE	HF+EA
CRP- ng/mL	0.40±0.06[a]	0.70±0.04[b]	0.71±0.06[b]	0.60±0.03[b]	0.50±0.04[ab]	0.63±0.06[b]
Eotaxin (pg/mL)	476±102[a]	648±88[a]	774±182[a]	578±157[a]	856±118[a]	746±50[a]
G-CSF (pg/mL)	132±18[a]	224±37[a]	180±26[a]	255±47[a]	222±15[a]	179±18[a]
GM-CSF (pg/mL)	52.7±13.4[a]	48.2±11.0[a]	65.3±40.2[a]	13.1±4.3[a]	40.1±13.9[a]	36.4±17.5[a]
IFN-g (pg/mL)	5.06±0.56[a]	4.01±0.45[a]	5.61±2.44[a]	4.49±1.56[a]	2.92±0.60[a]	4.42±0.38[a]
IL-1a (pg/mL)	121±50.4[a]	121±50.4[a]	102±43.9[a]	180±102[a]	144±57.6[a]	262±46.5[a]
IL-1b (pg/mL)	10.3±2.06[a]	9.08±0[a]	28.4±10.3[a]	11.7±5.41[a]	13.2±5.68[a]	24.8±10.2[a]
IL-6 (pg/mL)	4.45±0.88[a]	7.22±0.60[a]	4.62±1.94[a]	7.37±2.77[a]	3.02±1.20[a]	7.40±3.54[a]
IL-9 (pg/mL)	111±56.6[a]	82.0±36.0[a]	136±79.5[a]	185±74.1[a]	273±74.6[a]	150±52.1[a]
IL-13 (pg/mL)	108±26.8[a]	161±24.6[a]	140±28.8[a]	138±13.1[a]	125±21.8[a]	200±45.6[a]
IP-10 (pg/mL)	197±35[a]	174±13[a]	188±25[a]	126±5[a]	166±10[a]	162±13[a]
Insulin (pg/mL)	67.2±20.6[a]	615±126[b]	364±137[ab]	634±109[b]	475±112[ab]	868±224[b]
KC (pg/mL)	77±21[a]	138±23[a]	194±53[a]	109±42[a]	102±15[a]	120±22[a]
Leptin (ng/mL)	2.01±0.27[a]	21.9±1.25[b]	19.5±2.69[b]	15.5±2.25[b]	19.3±2.55[b]	17.8±3.24[b]
LIX (ng/mL)	11.5±2.35[a]	13.3±2.74[a]	16.7±4.10[a]	6.82±0.69[a]	22.8±5.94[a]	18.5±3.37[a]
MCP-1 (pg/mL)	29.2±6.59[a]	44.1±9.60[a]	37.9±16.3[a]	41.9±17.8[a]	31.3±9.57[a]	68.6±18.9[a]

Table 2.5 Continued. Serum hormone and inflammatory cytokine levels in male C57/BL6J mice fed various phytochemical containing diets for 10 weeks[1]

	LF[2]	HF	HF+AE	HF+CE	HF+QUE	HF+EA
M-CSF (pg/mL)	9.96±1.72[a]	14.6±2.47[a]	9.99±1.85[a]	12.5±2.69[a]	14.8±1.24[a]	6.94±2.02[a]
MIG (pg/mL)	120±30.3[a]	47.7±7.21[a]	56.7±7.89[a]	73.0±25.2[a]	77.2±16.5[a]	101±23.3[a]
MIP-1a (pg/mL)	12.9±2.31[a]	31.8±4.66[a]	31.9±6.62[a]	31.7±9.57[a]	18.3±2.89[a]	24.6±6.04[a]
MIP-1b (pg/mL)	53.8±10.1[a]	46.0±9.58[a]	50.0±11.4[a]	56.8±9.53[a]	40.6±6.93[a]	24.8±9.93[a]
MIP-2 (pg/mL)	113±22.3[a]	110±29.4[a]	72.8±33.3[a]	97.5±43.8[a]	144±20.7[a]	118±34.4[a]
PAI-1 (pg/mL)	1460±426[a]	2160±141[a]	784±304[a]	794±282[a]	1620±301[a]	1780±498[a]
RANTES (pg/mL)	9.97±2.05[a]	13.4±2.42[a]	9.82±2.50[a]	18.4±4.89[a]	12.4±2.46[a]	20.1±6.99[a]
Resistin (ng/mL)	0.23±0.22[a]	5.95±0.46[b]	5.02±0.78[b]	5.27±0.35[b]	5.20±0.77[b]	4.66±0.70[b]

CRP: C-reactive protein, G-CSF: Granulocyte colony stimulating factor, GM-CSF: Granulocyte-macrophage colony stimulating factor, IFN-g: Interferon-g, IL: interleukin, IP-10: Interferon gamma-induced protein 10, KC: Keratinocyte chemoattractant, LIX: Lipopolysaccharide-induced CXC chemokine, MCP-1: monocyte chemotactic protein-1, M-CSF: Macrophage colony stimulating factor, MIG: Monokine induced by gamma interferon, MIP: Macrophage inflammatory protein, PAI: Plasminogen activator inhibitor, RANTES: Regulated on activation, normal T cell expressed and secreted.

[1]Values are expressed as means ± SEM of each group, n=3-7. Values that do not share a letter differ ($p<0.05$).

[2]Diet groups: Low-fat (LF), high-fat (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA).

Table 2.6. Organ tissue weights, weights as a percentage of average final body weight, and liver triglycerides of male C57BL/6J mice fed various phytochemical containing diets for 10 weeks [1]

		LF[2]	HF	HF+AE	HF+CE	HF+QUE	HF+EA
Liver	grams	0.92±0.09[a]	1.48±0.14[b]	1.24±0.06[ab]	1.30±0.10[ab]	1.22±0.08[ab]	1.52±0.13[b]
	g/g BW[3]	3.43±0.32[a]	3.61±0.19[a]	3.19±0.06[a]	3.25±0.14[a]	3.20±0.14[a]	3.71±0.17[a]
Kidney	grams	0.13±0.01[a]	0.17±0.01[b]	0.15±0.01[ab]	0.15±0.01[ab]	0.14±0.01[a]	0.16±0.01[ab]
	g/g BW	0.49±0.02[a]	0.44±0.03[ab]	0.38±0.02[b]	0.37±0.02[b]	0.36±0.02[b]	0.40±0.02[ab]
Inguinal Adipose	grams	0.31±0.02[a]	1.24±0.07[b]	1.35±0.07[b]	1.25±0.08[b]	1.21±0.10[b]	1.18±0.06[b]
	g/g BW	1.16±0.09[a]	3.07±0.16[b]	3.46±0.09[b]	3.17±0.19[b]	3.16±0.22[b]	2.94±0.15[b]

[1]Values are expressed as means ± SEM of each group, n=8
[2]Diet groups: Low-fat (LF), high-fat (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA).
[3]g/g BW: grams organ weight per gram overall body weight; TG: Triglycerides
[4]Values that do not share a letter differ ($p<0.05$)

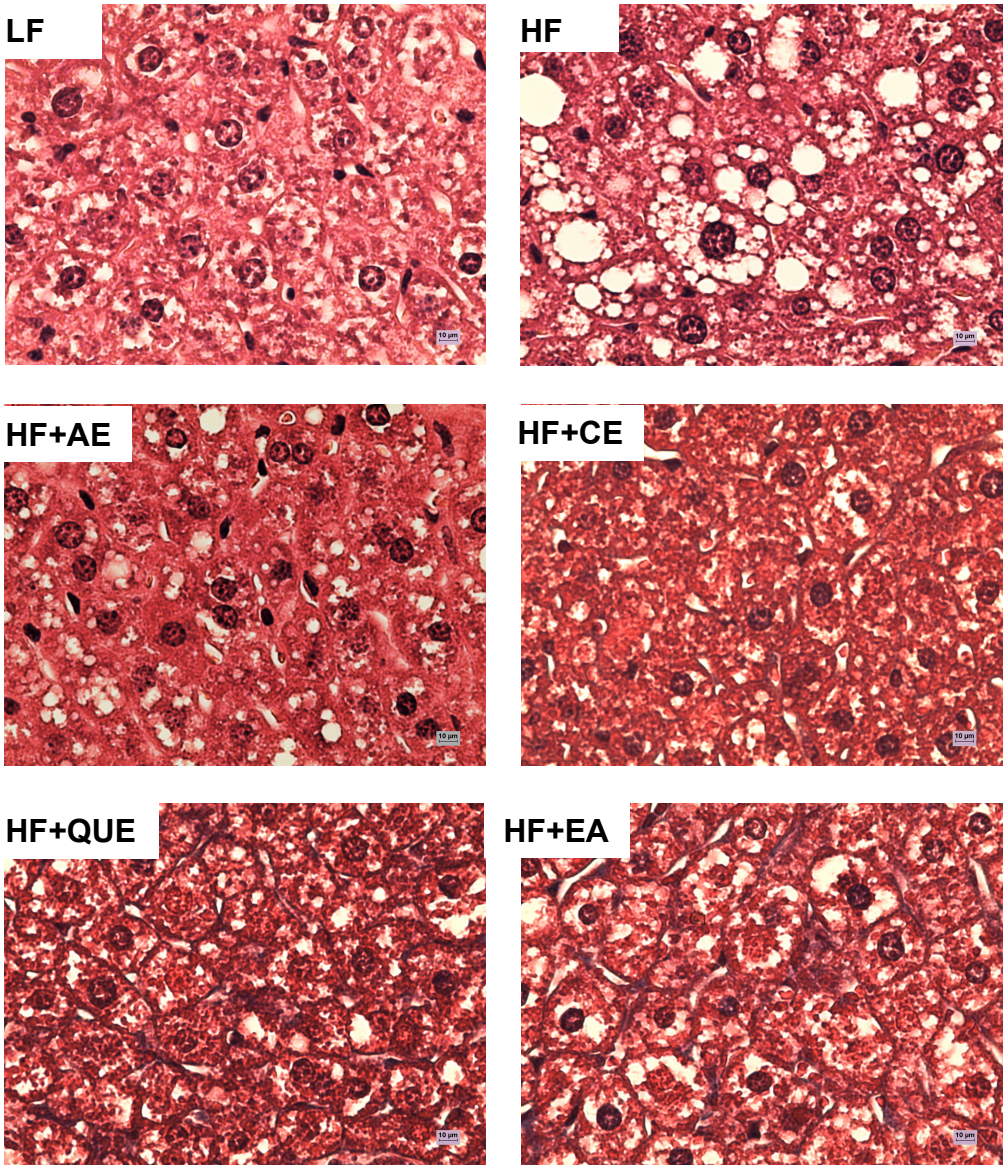


Figure 2.6. Cross-section of C57BL/6J mouse livers stained with trichrome stain. Slides were observed under 40x magnification using a Nikon Eclipse 50i microscope (Nikon Corporation, Japan; Serial #: 211880) fitted with an Infinity1-3C camera (Lumenera Corporation, Atlanta, GA; Serial #: 0186795). Scale: 0.5”=50µm.

4. DISCUSSION

Consumption of a high-fat diet that leads to obesity is a major health issue in the United States and around the world. This obese state is associated with an increased risk of developing diabetes, cardiovascular disease, hepatosteatosis, and increased inflammation. In animal models, phenolic- and anthocyanin-rich extracts have been shown to ameliorate some of the metabolic dysfunction that results from obesity (Jayaprakasam et al., 2006; Prior et al.; Gourineni et al.; Moon et al.; Baur et al.). Although research has been done to observe the effects of both whole apple and cherry fruits and their phytochemical extracts, there is an opportunity to observe the effects of these extracts in the C57BL/6J mouse model of obesity.

This animal model allows us to easily induce obesity by providing a high-fat diet and then monitors the degree of metabolic dysfunction over a reasonably low span of time. A diet of 60% kcals from fat was used to ensure that obesity occurred over the course of our 10-week study. However, similar studies using 45% kcals from fat found similar degrees of weight gain over the same amount of time (Prior et al.). We sought to mimic the high-fat, high calorie diet consumed by many Americans. In our study, percent kcals from protein was kept constant at 20% and carbohydrate levels were normalized relative to fat content (70% for LF and 20% for HF). For future studies, it may be beneficial to use a 45% fat diet with added sucrose to more realistically mimic the Western diet, which is high in both fat and sugar consumption.

No significant difference in final body weight was observed among the HF-fed mice. This result is consistent with the findings in previous studies of similar design (Baur et al., Gourineni et al.) On average, the HF mice were approximately 14 grams heavier than the LF-fed mice, and based on our observations, this additional weight was almost entirely adipose. When weekly diet consumption in kilocalories was observed, no difference was observed in energy consumed among the HF-fed mice. Kilocalories consumed by LF-fed mice was significantly different from all HF-fed mice, suggesting that the reduced weight gain in the LF-fed mice can be attributed to the lesser kcals consumed since no difference in grams of food consumed was observed.

Both Gourineni et al. and Jayaprakasam et al. (2006) showed that phytochemical extracts were effective at improving glucose metabolism in obese subjects by reducing baseline glucose, plasma insulin, HOMA-IR, and improving glucose tolerance. Similar results were observed in our study. At week 6, baseline glucose measurements were reduced for all high-fat treatment groups relative to the HF-fed mice and were similar to the LF mice. A glucose tolerance test was also performed at week 6. A significant difference was seen among high-fat-fed mice ($p=0.01$), however, despite an improved area under the curve (AUC) measurement for HF+AE- and HF+QUE-fed mice (-5.86 and -5.23 respectively), no difference between individual treatment groups was observed (**Figure 2.3**). At week 10, the difference in baseline glucose was less notable with HF+QUE, HF+EA, and HF+CE having an intermediate concentration relative to HF and LF-fed mice. HF+AE had baseline glucose measurements similar to

HF-fed mice. Interestingly, however, serum insulin levels at week 10 were reduced in HF+AE- and HF+QUE-fed mice and intermediate between the LF- and HF-fed mice. Contrary to the results observed by Gourineni et al., no differences were seen in week 10 serum glucose measurements for any of the high-fat-fed mice despite reduced serum insulin levels in HF+AE- and HF+QUE-fed mice. We suspect that the 60% fat diet induced a state of obesity that was too far progressed to detect a distinctive difference between treatment groups due to advanced dysfunctional metabolism of nutrients (Grundy) and potentially damaged islet cell function (Jayaprakasam et al., 2006).

Motor function, balance and strength were measured using a grip strength test and a rotarod endurance test. In a study by Baur et al. it was reported that the livers of resveratrol-treated mice had a significantly greater number of hepatic mitochondria than the high-calorie control diet and a trend toward increased citrate synthase activity, an indicator of mitochondrial content. It is well understood that exercise and reduced caloric intake can increase the number of mitochondria in the liver, but this study showed that resveratrol supplementation produced a similar effect in the presence of a high-calorie diet. Congruently, Baur et al. observed that motor function of HF-fed mice was also improved with resveratrol without a significant reduction in body weight. In our study, HF+AE-, HF+CE-, and HF+QUE-fed mice showed a trend toward improved balance and mobility when compared to the HF group and had latency times intermediate between the LF- and HF-fed mice. No difference was observed for HF+EA mice. Grip strength was also improved in all high-fat treatment groups relative to the

HF-fed mice and was intermediate between the LF- and HF-fed mice. The mechanism by which this improvement occurs is uncertain, however, it can be postulated that increases in mitochondrial number or mitochondrial activity may play a role. Measurements of mitochondrial number in hepatic cells would confirm this effect. Location of fat deposits could also be a potential cause of this improved performance if the high-fat treatment diets up-regulated pathways for fat deposition in adipose tissue rather than in vital organs, however, a more thorough body scan would need to be performed to confirm. If this was the case, we should have seen a difference in inguinal adipose measurements among the high-fat-fed mice, however, no difference was observed.

Serum was collected at week 10 and analyzed for blood lipids, hormone and cytokine levels. No difference was observed in high-fat-fed mice for LDL, VLDL, HDL, and triglycerides. Again, it is suspected that no change was detected as a result of progressed obesity in all high-fat-fed groups by the end of the study. It has also been proposed that the alternative Western diet described previously of 45% fat and added sucrose may be able to show a greater distinction in triglyceride levels compared to the 60% fat diet. Serum cholesterol was improved in HF+AE-fed mice when compared to the HF-fed mice, indicating that cholesterol metabolism or efflux pathways were affected by the AE treatment. Creatinine levels were decreased in HF+EA- and HF+QUE-fed mice when compared to HF- and LF-fed mice. Elevated serum creatinine is indicative of renal failure and is associated with high blood pressure and diabetes mellitus (Coresh et al.). The distinctly lower creatinine levels for HF+QUE- and HF+EA-

fed mice indicates that these treatments may favorably impact renal function potentially during the development of diabetes or hypertension. Leptin levels were decreased for HF+EA and HF+CE mice. Leptin is associated with regulating feeding behavior and body weight and is generally elevated in obese people (DePaoli). Therefore, a decrease in leptin levels may indicate an improved feeding behaviors and metabolism in these treatment groups. Resistin levels were also decreased in HF+EA-fed mice. In rodents, resistin is produced in adipose tissue and plays a role in the regulation of glucose metabolism and insulin sensitivity. There is some controversy surrounding the association of resistin and obesity, however, there is growing evidence that high levels of resistin may be a biomarker of metabolic disease (Steppan and Lazar).

Serum cytokine levels indicate that the inflammatory response was affected by the high-fat treatment diets. HF+AE-fed mice saw a decrease in multiple cytokine levels, indicating a lessened inflammatory response by decreasing cell differentiation into macrophages and secretion of immune and inflammatory response signals (i.e. leukocytes, fever, sepsis, blood clotting, etc.) by macrophages. A similar response was seen in HF+CE-, HF+QUE-, and HF+EA-fed mice, where macrophage, granulocyte, and neutrophil production was inhibited and secretion of inflammatory factors that induce the immune response was also lessened.

Mouse kidneys, inguinal adipose, and liver tissues were taken and weighed at the time of necropsy. Kidney weight per gram body weight was reduced in all high-fat-fed treatment mice to values lower than both the LF- and

HF-fed mice. Cui et al. found that magnolia extract was effective at attenuating obesity-associated renal inflammation and oxidative stress and had a dose-dependent response on decreasing kidney weight. Therefore, the decreased kidney weight observed in the high-fat treatment diets may be a result of the ability of apple and cherry extract, quercetin, and ellagic acid to attenuate renal inflammation that can develop from consumption of a high-fat diet. No difference in inguinal adipose weight was observed among high-fat-fed mice, which was not surprising due to the absence of a reduction in body weight in high-fat treatment mice. Liver weight was not significantly reduced in any of the diet groups (including LF-fed mice) when normalized to overall body weight.

However, cross sections of liver tissue revealed less fat accumulation in high-fat treatment mice relative to the HF-fed mice. No significant difference in hepatic triglyceride content was observed when an enzymatic assay was used to measure cellular triglyceride levels (data not shown). However, qualitative histological observations clearly illustrate a reduction in white fat globules, especially in the HF+AE- and HF+CE-fed mice, which were more similar to the LF-fed mice than the HF-fed mice. HF+QUE- and HF+EA-fed mice also demonstrated lessened fat accumulation relative to the HF-fed mice, but to a lesser degree. This reduction in liver fat accumulation or distribution suggests that the polyphenol-rich extract and pure polyphenols are impacting the metabolism of fat in the liver, but the precise mechanism is uncertain. Baur et al. saw a reduction in size and weight of livers with resveratrol treatment, however, with no alteration of plasma lipid levels. Our study saw no reduction in size and

weight of livers or plasma lipid levels, however, it is apparent from histological examination of liver tissue that fat accumulation in the liver was reduced with high-fat treatment diets.

Taken together, the results of this study demonstrate that polyphenol-rich apple and cherry extracts as well as quercetin and ellagic acid are able to attenuate some of the metabolic dysfunctions of mice consuming a high-calorie/high-fat diet. High-fat treatment diets were able to improve glucose tolerance and insulin sensitivity, balance, motor function, and strength, as well as reduce serum levels of inflammatory cytokines, hormones, cholesterol, and hepatic fat accumulation. Remarkably, these physiological changes occurred without a significant reduction in body weight or diet consumption. Further investigation is needed to determine the precise mechanism of action behind these physiological changes, but it is noteworthy that a small concentration of polyphenols is able to reduce some of the negative metabolic consequences that ensue from consumption of an obesigenic diet. The low concentration in the animal diets translates to a human equivalent consumption of only 3.5 medium apples, 2 cups of cherries, and 1.25 g or quercetin or ellagic acid per day, which are very attainable amounts with intentional consumption.

CHAPTER III:
HEPATIC GENE EXPRESSION IN C57BL/6J MICE FED DIETS CONTAINING APPLE AND CHERRY PHYTOCHEMICAL EXTRACTS, QUERCETIN AND ELLAGIC ACID FOR 10 WEEKS

1. INTRODUCTION

As mentioned previously, consumption of phytochemicals have been shown to ameliorate some of the negative metabolic effects of an obese diet state. Chapter 2 illustrates the specific physiological metabolic effects that were ameliorated with the consumption of apple and cherry phytochemical extracts, quercetin and ellagic acid. Chapter 3 aims to understand these effects on a molecular level by observing the related transcriptional pathways, specifically nuclear hormone receptors and their target genes.

Nuclear hormone receptors are transcription factors that activate and/or repress sets of genes relating to specific metabolic pathways. These receptors are often promiscuous and can bind many different molecules as ligands. Three receptors of particular interest to this study are: PPAR-α, which is involved in lipid oxidation, PPAR-γ, which regulates adipogenesis and glucose control, and PXR, which is involved in xenobiotic, bile acid, and cholesterol metabolism and excretion (Chawla et al).

We hypothesize that phytochemicals are able to enter the cell and act as a ligand for the nuclear hormone receptor and activate or repress gene transcription. Therefore, we will be able to observe activation or repression of mRNA in the livers of mice fed phytochemical-containing diets by means of RT-PCR measurement.

2. MATERIALS AND METHODS

2.1. Tissue Extraction

Hepatic RNA was extracted from all mice samples using Trizol® reagent (Life Technologies, Pleasanton, CA) following the product protocol for tissue homogenizations. Concentrations of RNA were determined by spectrophotometry and integrity of isolated RNA was confirmed by agarose gel electrophoresis. Extracted hepatic RNA was suspended in DEPC treated water and stored in -20°F freezer for future use.

2.2. Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR protocol was adapted from methods described by Nam and Knutson and Real-time PCR Handbook (Life Technologies). Isolated RNA was reversely transcribed using Applied Biosystems® High Capacity cDNA Reverse Transcription Kits (Life Technologies). RNA was added at a volume equivalent to 1µg RNA and DEPC water was added to RNA sample to reach 10µL. Ten microliters of 2X RT master mix was added to each RNA/DEPC water sample for a total volume of 20µL. Master mix was prepared by mixing 2.0µL10X RT Buffer, 0.8µL 25X dNTP mix (100mM), 2.0µL 10X RT Random Primers, 1.0µL Multiscribe™ Reverse Transcriptase, and 4.2µL Nuclease-free water. RT thermal cycle was run under the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, and then held at 4°C until removed and placed into the freezer for storage.

Pathway-specific genes were quantified by real-time PCR performed on an Applied Biosystem®. 7900HT Fast thermal cycler using SensiMix SYBR Master Mix (Origene, Rockville, MD) to detect PCR products. PCR samples were prepared in a 96-well plate. Samples were prepared using the following mixture: 12.5 µL 2X SensiMix SYBR Master Mix, 1.0µL of 10µM Primer Mix (Life Technologies, prepared from 50µM stock), 9.0µL DEPC treated water, and 2.5µL RT template (diluted to 1/50 original concentration). Standard curve samples were prepared at concentrations of 1/10, 1/100, 1/1000, and 1/1000 original RT concentration and plated in triplicate for all primers tested. ΔΔct method for PCR and housekeeping gene RPL30 were used. PCR reaction was run under standard thermal cycle: 10 min at 95°C followed by 40 cycles of: 5 sec at 95°C and 20 sec at 60°C. Relative mRNA levels for samples were calculated using standard curve data and one-way ANOVA was performed to determine significant differences. Melt curves were observed to confirm purity of primers.

2.3. Statistical analysis

All data are expressed as the mean mRNA level ± standard error of the mean (SEM). Multi-way analysis of variation (ANOVA) was performed on Prism 6 (GraphPad Software, La Jolla, CA). Post-hoc testing was performed using Tukey's multiple comparison test.

3. RESULTS

RT-PCR analysis demonstrated a significant difference in relative expression in the hepatic mRNAs carnitine palmitoyltransferase 1-alpha (CPT1α), acyl-CoA oxidase-1 (ACOX1), stearoyl-coA desaturase-1 (SCD1) and C-Reactive Protein (CRP).

For CPT1α, the HF+AE and HF+EA groups had a significantly increased expression compared to the HF-fed mice, 165% and 154% greater expression respectively ($p=<0.05$) (**Figure 3.1**). The cherry (HF+CE) and quercetin (HF+QUE) treatment groups also showed increased relative expression, 122% and 129% but were statistically similar to HF-fed mice.

ACOX1 mRNA expression was also increased in some of the high-fat treatment groups (**Figure 3.2**). HF+AE-, HF+QUE- and HF+EA-fed mice showed increased ACOX1 expression by 333, 257 and 259% respectively when compared to the HF-fed mice ($p=<0.05$). HF+CE-fed mice also showed increased ACOX1 expression, 242% greater than the HF-fed mice.

HF+EA-fed mice had an increased SCD-1 expression of 369% compared to HF-fed mice ($p=<0.05$) (**Figure 3.3**). HF+AE-, HF+CE-, and HF+QUE-fed mice also increased relative SCD-1 expression by 269, 163 and 141% greater than the HF group respectively.

C-reactive Protein (CRP) levels were increased by 297 and 138% in HF+CE- and HF+QUE-fed mice compared to the HF-fed mice (**Figure 3.4)**. HF+AE- and HF+EA-fed mice decreased CRP expression by 67% and 97%

relative to the HF-fed mice. All groups, however, were statistically similar to HF-fed mice.

Other mRNA tested include: HOX1 (**Figure 3.5**), Oatp1a4 (**Figure 3.6**), CYP4A14 (**Figure 3.7**), NQO1 (**Figure 3.8**), GSTT2 (**Figure 3.9**), CYP8B1 (**Figure 3.10**), CYP1A2 (**Figure 3.11**), and LPL (**Figure 3.12**), however, no significant differences were observed in relative expression of these mRNAs.

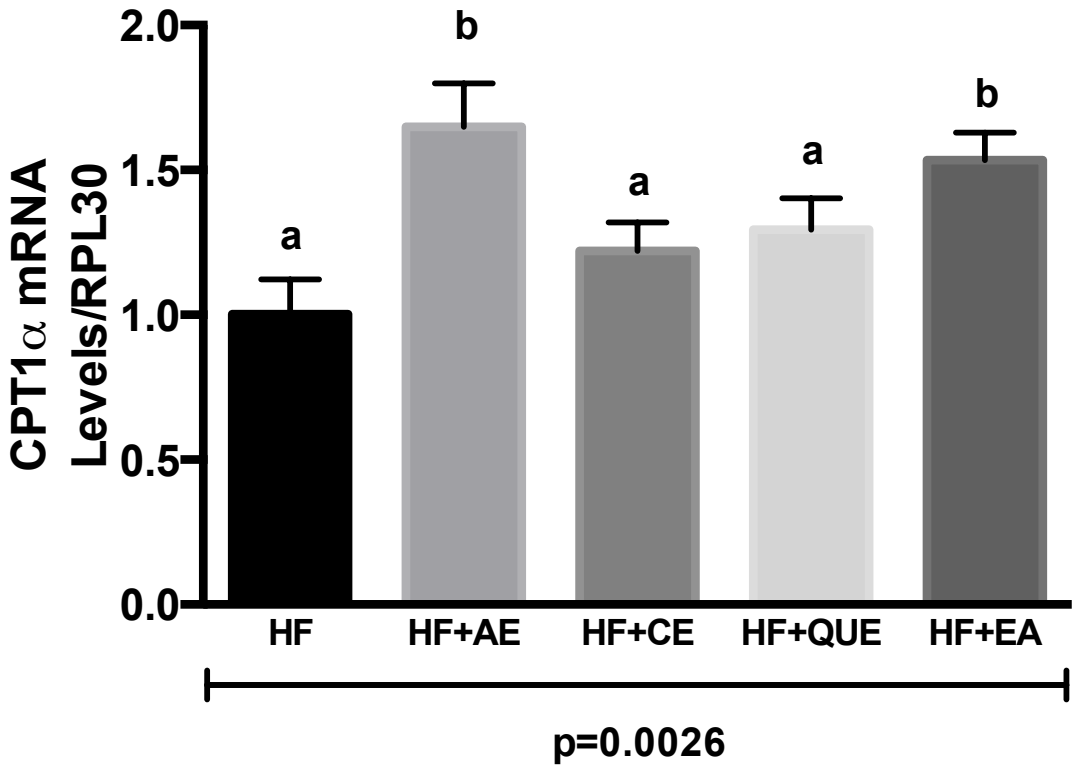


Figure 3.1. **Hepatic carnitine palmitoyl transferase 1-alpha (CPT-1α) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=7-8. Values that do not share a letter differ ($p<0.05$).

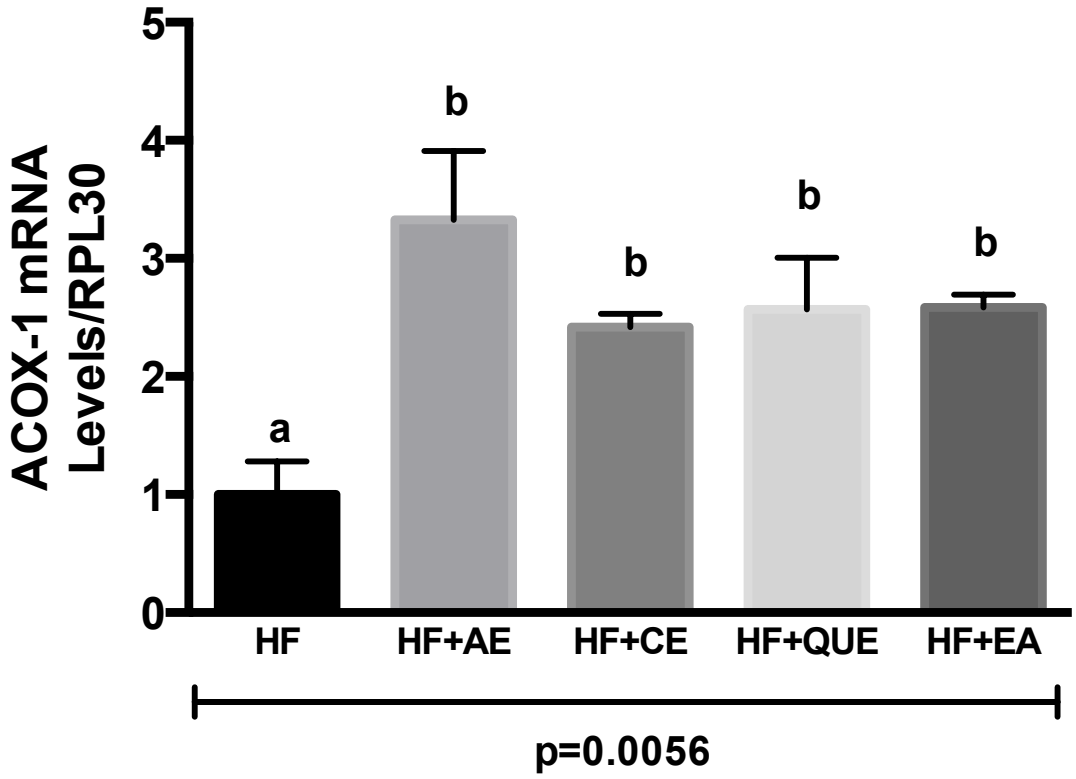


Figure 3.2. **Hepatic Acyl-CoA oxidase-1 (ACOX-1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=6-8. Values that do not share a letter differ ($p<0.05$).

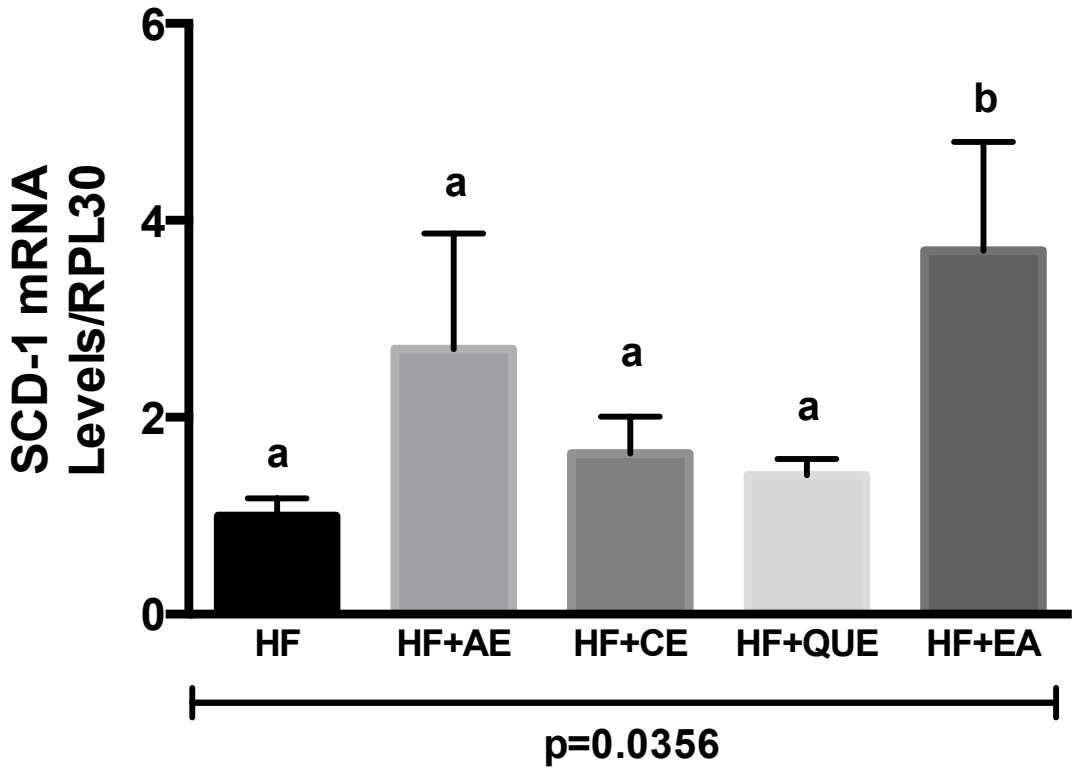


Figure 3.3. **Hepatic stearoyl-CoA desaturase-1 (SCD-1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=4-8. Values that do not share a letter differ ($p<0.05$).

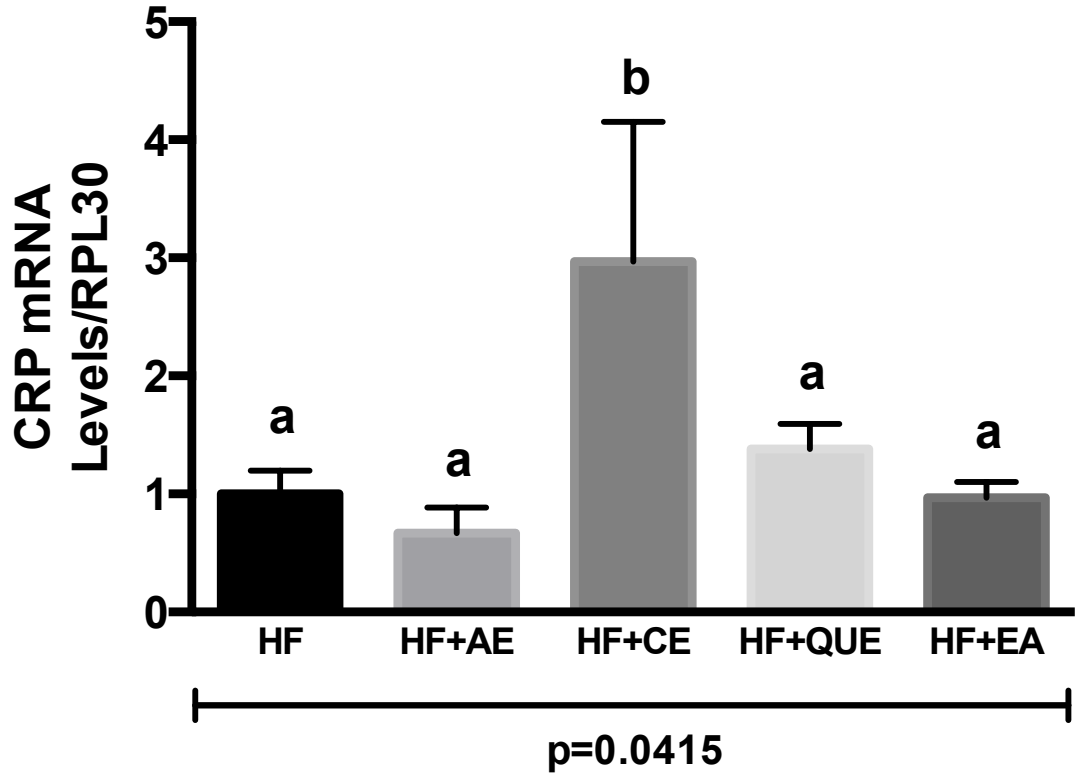


Figure 3.4. **Hepatic C-reactive protein (CRP) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=3-4. Values that do not share a letter differ ($p<0.05$).

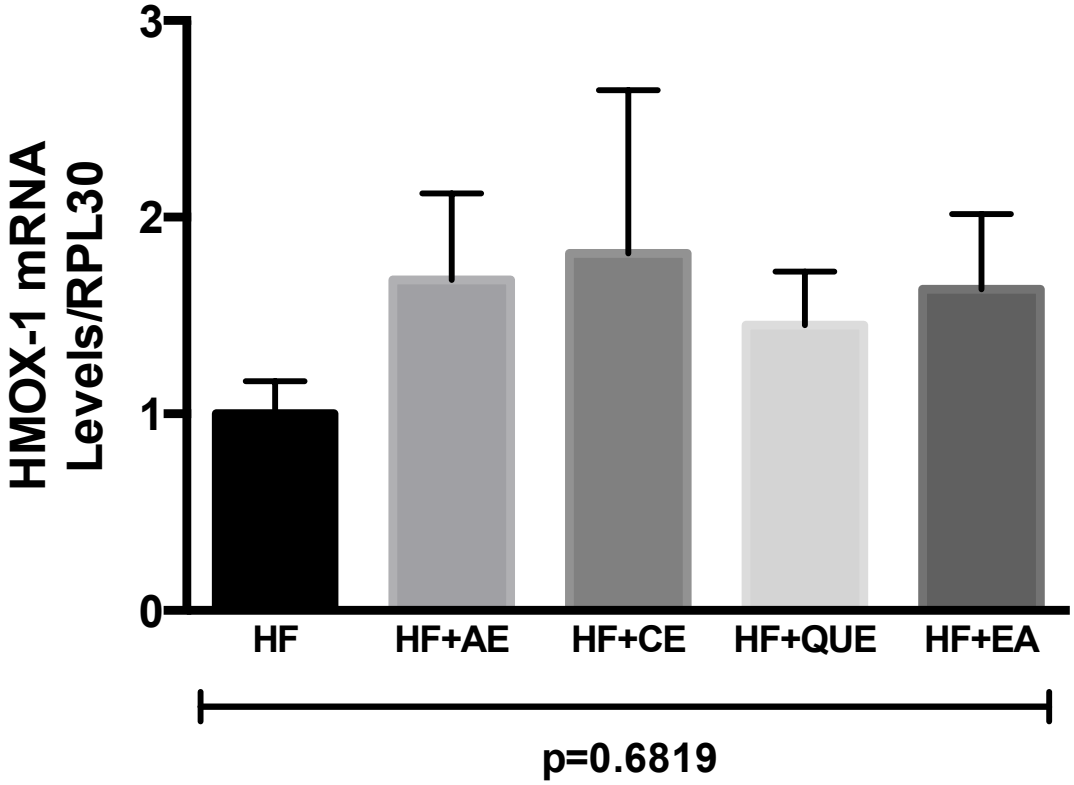


Figure 3.5. **Hepatic heme oxygenase-1 (HOX-1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=3-4.

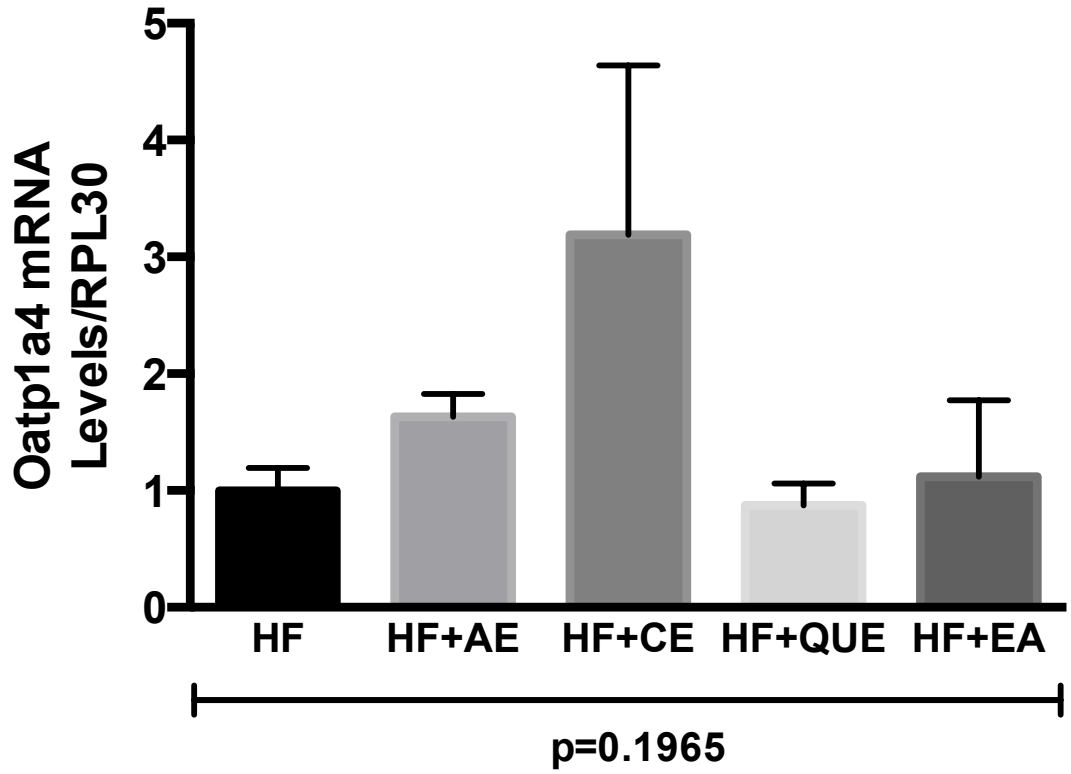


Figure 3.6. **Hepatic organic anion-transporting polypeptide 1a4 (Oatp1a4) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=4.

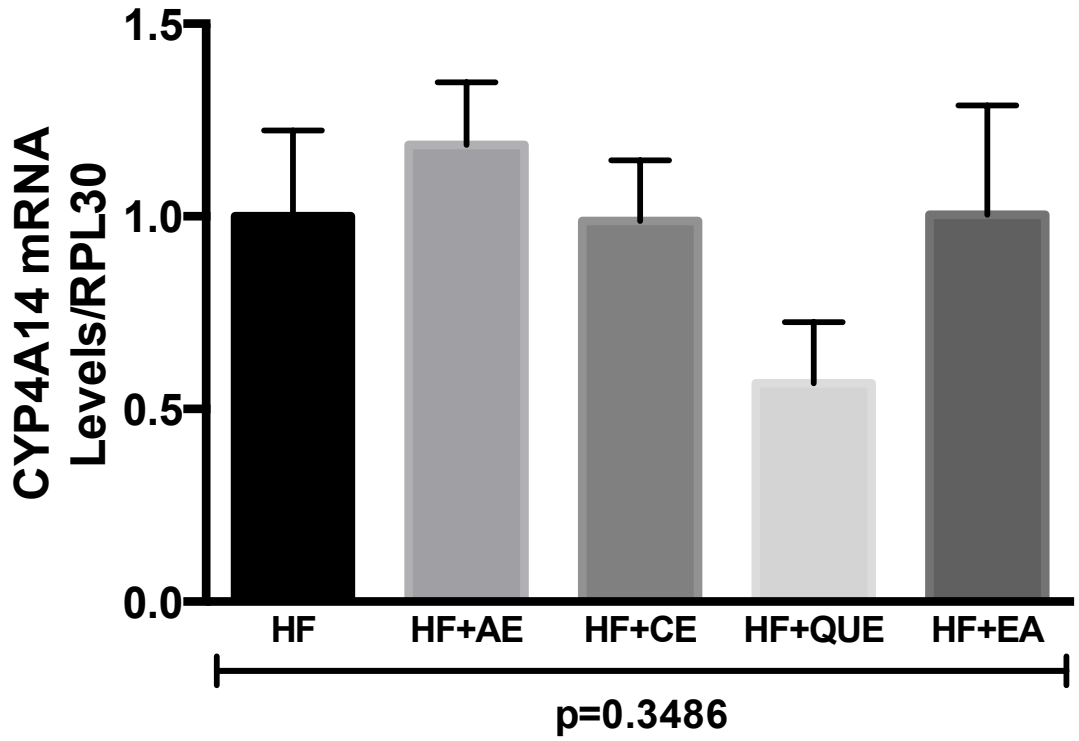


Figure 3.7. **Hepatic cytochrome p450, family 4, subfamily a, polypeptide 14 (CYP4A14) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=7-8.

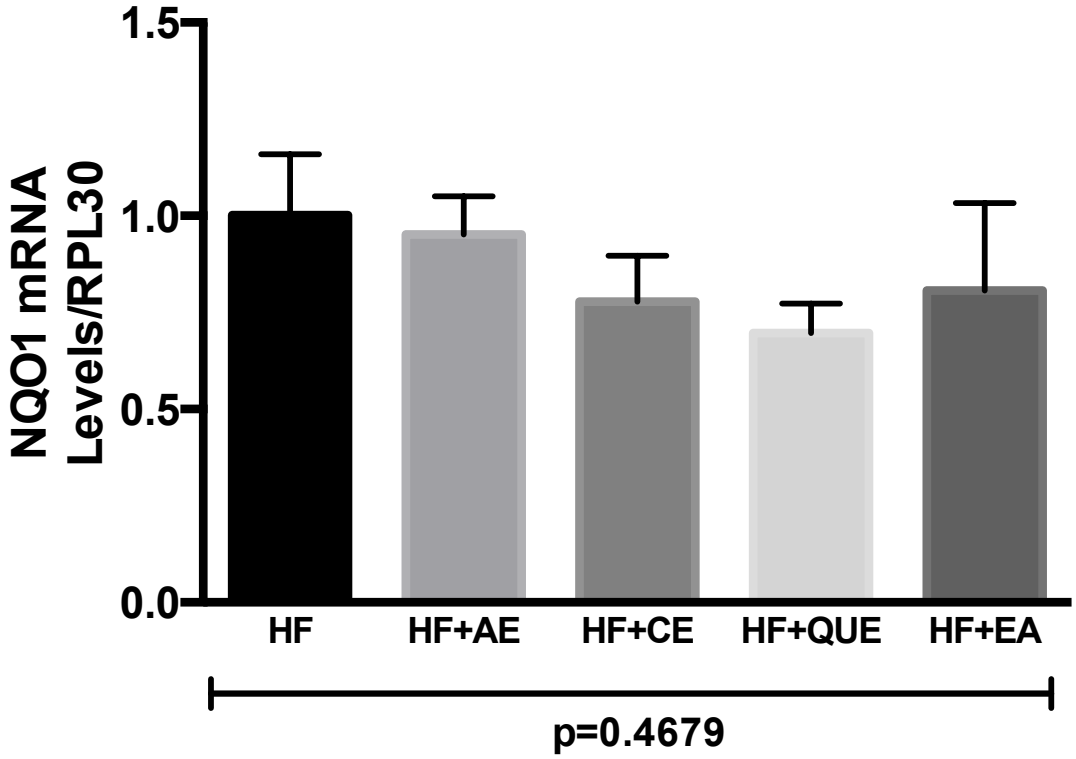


Figure 3.8. **Hepatic NADPH dehydrogenase, quinone-1 (NQO1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=6-8.

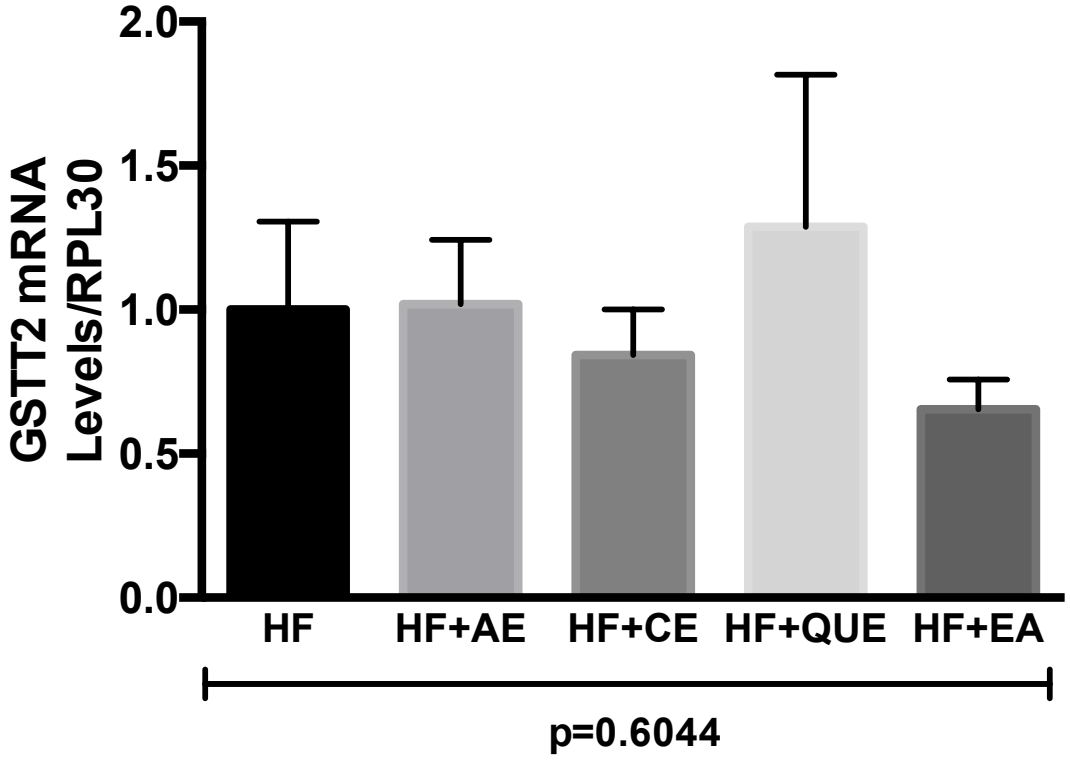


Figure 3.9. **Hepatic glutathione S-transferase, theta-2 (GSTT2) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=3-4.

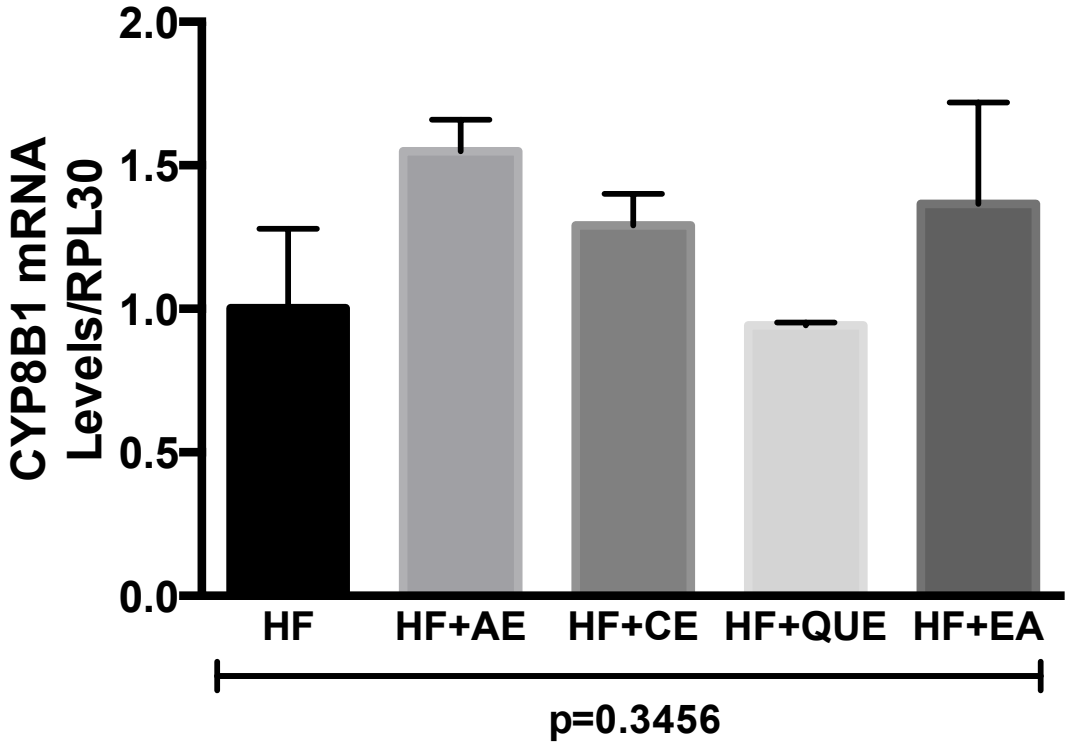


Figure 3.10. **Hepatic cytochrome p450, family 8, subfamily b, polypeptide 1 (CYP8B1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=3-4.

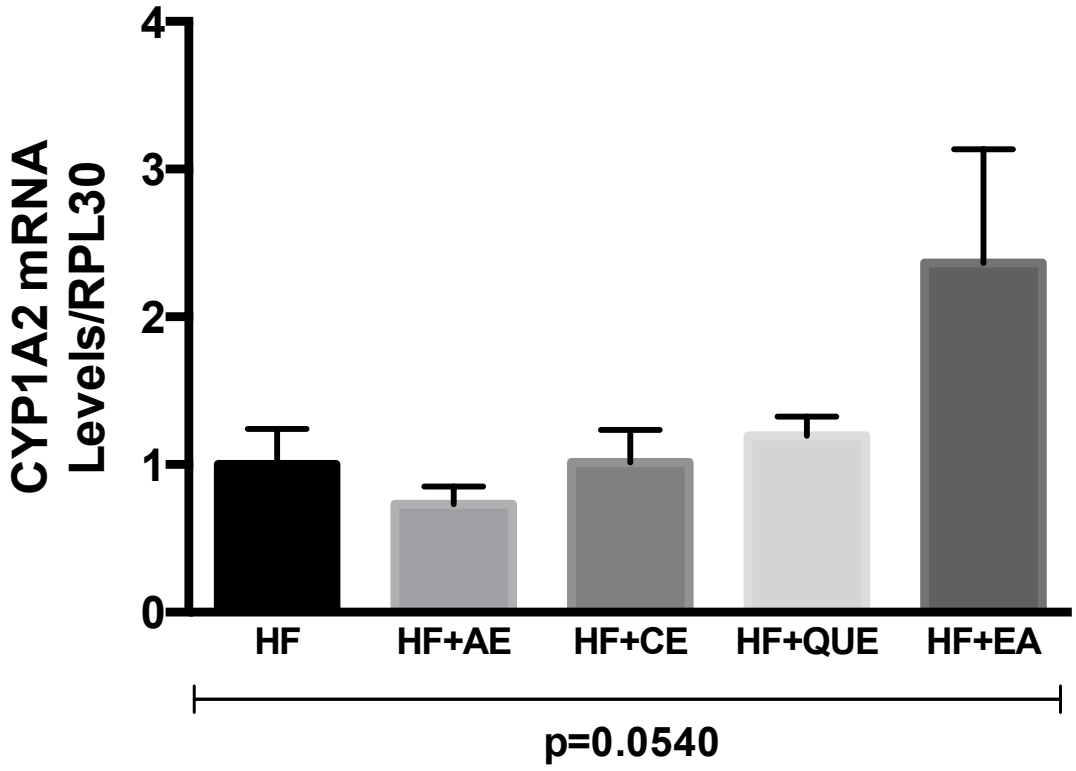


Figure 3.11. **Hepatic cytochrome p450, family 1, subfamily a, polypeptide 2 (CYP2A1) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the high-fat (HF) diet. Values are expressed as the mean ± SEM, n=5-8.

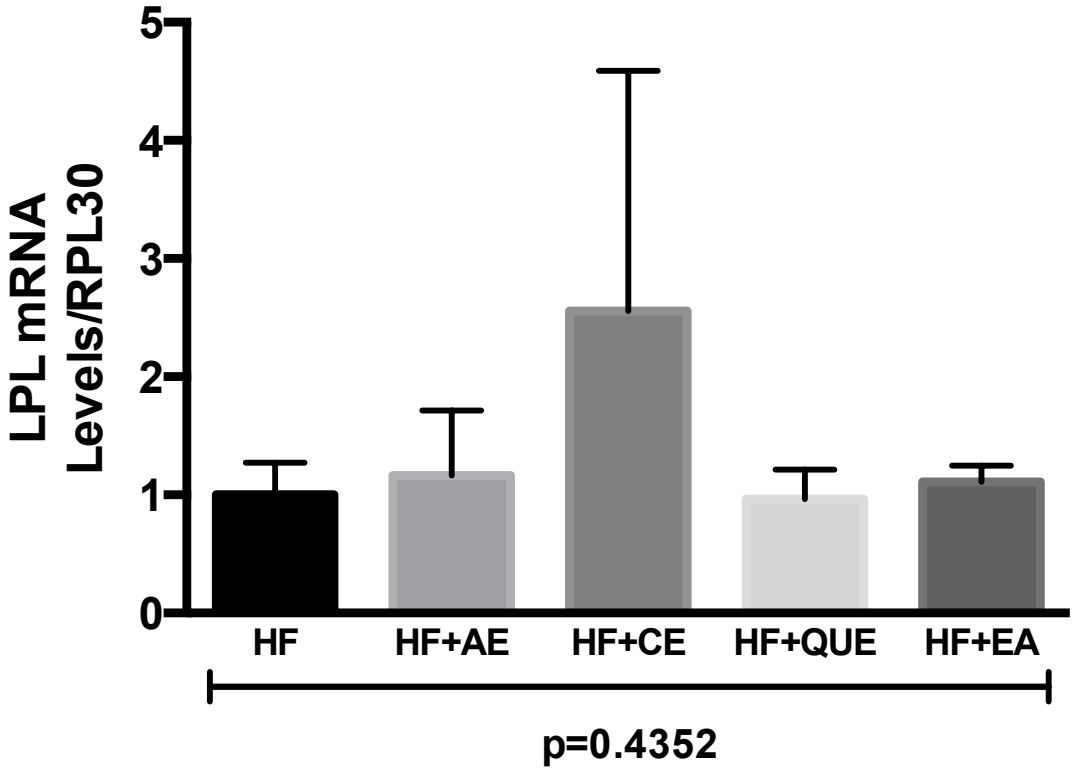


Figure 3.12. **Hepatic lipoprotein lipase (LPL) mRNA levels in male C57BL/6J mice fed various phytochemical containing diets for 10 weeks.** Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are normalized to RPL30 gene expression and are expressed as a fold difference versus the HF diet. Values are expressed as the mean ± SEM, n=2-4.

4. DISCUSSION

Chapter 2 illustrated the physiological benefits that phytochemical-rich extracts and pure phytochemicals had on ameliorating metabolic dysfunction as a result of obesity. This chapter reports the molecular interactions and specific metabolic pathways impacted by the phytochemical-rich diets that may have brought about the physiological changes observed. It has been observed by many researchers that phytochemicals are able to impact the pathways of nuclear hormone receptors, particularly PPARα (Seymour et al.; Mezei et al, 2003, 2006; Moon et al.), PPARγ (Mezei et al., 2003; Park et al.; Moon et al.), PXR (Li et al., 2007, 2009), and the transcription factor, Nrf2 (Chuang and McIntosh).

As stated in Chapter 1, the PPAR receptors are considered "promiscuous", as they are activated by many different ligands. Fatty acids and fibrates have been found to serve as ligands for PPARα, along with other natural compounds such as soy isoflavones (Mezei et al., 2003, 2006) and tart cherry powder (Seymour et al.). In 2003, Mezei et al. observed an antidiabetic and hypolipidemic effect of soy isoflavones and attributed the effects to an increase in PPAR pathway activation. Luciferase activity of PPARα confirmed that the soy isoflavones were in fact activating PPARα. In 2006, Mezei et al. went on to further confirm the role of soy isoflavones in activating PPARα by using a PPARα-knockout model. What the researchers observed was a lessened reduction of serum triglyceride levels in the PPARα knockout mice, indicating that PPARα was required for the full effect of soy isoflavones and fenofibrate to be observed. The expression of carnitine palmitoyl transferase-1α (CPT1α) was also

measured by Mezei et al. (2006), to confirm PPARα activity. CPT1α is responsible for mitochondrial uptake of fatty acids, designating them for β-oxidation. The CPT1α gene is strongly regulated by PPARα and serves as a good marker for measuring PPARα activity (Hsu et al.). Other genes that are regulated by PPARα are Acyl-CoA oxidase-1 (ACOX1) and Stearoyl-CoA desaturase-1 (SCD1) (Hsu et al., Seymour et al.).

In our mice, we saw an increase in CPT1α activity in all HF treatment groups, with significant increases in HF+AE- and HF+EA-fed mice. Similar results were observed for ACOX1 activity, in which all treatment groups had increased mRNA expression relative to HF-fed mice, and HF+AE-, HF+QUE- and HF+EA-fed mice had significantly increased expression. SCD1 expression paralleled the results seen for CPT1α and ACOX1 with increased expression in all HF-treatment groups and significant increase for HF+EA-fed mice relative to HF-fed mice. The increased relative expression for these three mRNAs in all HF-treatment groups suggests a PPARα agonist-like effect of AE, CE, QUE and EA, with greatest potency in HF+AE- and HF+EA-fed mice.

C-reactive protein (CRP) levels were also increased in HF+CE-fed mice when compared to HF-fed mice. C-reactive protein is produced in the liver and levels rise when there is inflammation throughout the body. It is considered an important acute-phase protein that plays a role in leptin and insulin resistance. Elevated levels of CRP have been linked to diabetes mellitus, metabolic syndrome, decreased adiponectin, and obesity (Timar et al., Fernández-Bergés et al.). The mRNA levels of CRP observed here are not in agreement with the

serum CRP levels reported in Chapter 2 where HF+CE mice exhibited CRP levels that were slightly less than both HF- and HF+AE-fed mice with HF+QUE-fed mice having the lowest CRP levels of HF+treatment-fed mice. The significantly elevated CRP mRNA in the livers of the HF+CE-fed mice could indicate an elevated overall inflammation in the body that was not reflected in the serum. It is important to note that RT-PCR was run on a sample size of n=4 and may not be representative of complete group. Trials of whole group (n=8) should be run to confirm results. RT-PCR of tissues other than the liver should also be performed to confirm inflammatory state.

Although other studies have shown that food powders or phytochemical extracts alter mRNA expression for PPARγ (Seymour et al., Park et al., Moon et al.), PXR (Li et al., 2007, 2009), and Nrf2 (Chuang and McIntosh), no significant change in hallmark mRNA expression was observed for these nuclear receptors in our study.

Some quantitative trends were observed for the HOX-1, CYP1A2, Oatp1a4, and LPL mRNAs. HOX-1 was increased 168, 187, 145, and 164% for HF+AE, HF+CE, HF+QUE, and HF+EA respectively. HOX-1 is positively regulated by Nrf2 expression (Chuang and McIntosh), suggesting that the phytochemicals and phytochemical-rich extracts may act be activating Nrf2. CYP1A2 also showed a 237% increased expression for HF+EA-fed mice. CYP1A2 is associated with multiple nuclear hormone receptors; however, of interest to this study is its role in Nrf2 expression (Aleksunes and Klaassen). The distinctive increase in CYP1A2 mRNA expression for HF+EA-fed mice indicate

that EA might play a role in Nrf2 activation. Oatp1a4 mRNA expression was increased by 320% in HF+CE-fed mice. Oatp1a4 is associated with PXR activity (Aleksunes and Klaassen), suggesting CE's potential role in drug, xenobiotic, and cholesterol metabolism and excretion. Finally, LPL mRNA relative expression was increased by 256% in HF+CE-fed mice. LPL is an upstream adipogenesis gene that is regulated by PPARγ activation. LPL also plays an important role in regulating fatty acid metabolism and triglyceride concentration (Zhang et al.). Additional trials need to be completed to confirm these results, however, initial trends suggest potential pathways of nuclear hormone activation.

CHAPTER IV:

THE EFFECTS OF APPLE AND CHERRY POLYPHENOL EXTRACTS AND QUERCETIN AND ELLAGIC ACID ON FAT ACCUMULATION AND METABOLISM IN HEPG2 CELL LINE

1. INTRODUCTION

The results of Chapters 2 and 3 demonstrated both the physiological and molecular effects that phytochemicals can have on ameliorating the metabolic dysfunctions that occur as a result of obesity. One of the most notable distinctions observed was the reduced fat accumulation in the mouse livers as well as increased hepatic gene expression of PPARα-regulated genes. To confirm the results observed in the animal study model, a human hepatoma cell (HepG2) model stimulated with oleic acid (OA) was used. HepG2 cells were used because they serve as a reliable model for observing lipoprotein metabolism and fatty acid oxidation (Hsu et al.). Oleic acid was chosen because it is commonly used to stimulate an increased free fatty acid (FFA) uptake and concentration in cells (Pang et al., Chang et al.). This human cell line model mimicked that used in our animal study where OA-treatment serves as the HF-treatment group.

2. MATERIALS AND METHODS

2.1. Cell Culture

Cell culture design was adapted from protocol developed by Chang et al. HepG2 is a hepatocellular carcinoma cell line from American Type Culture Collection (ATCC, Manassas, VA, ATCC #HB-8065). HepG2 cells were maintained in 10% Fetal Bovine Serum (FBS) (Gibco® by Life Technologies™, Grand Island, NY, Ref #10437-028) Dulbecco's Modified Eagle Medium (DMEM) (Gibco® by Life Technologies™, Grand Island, NY, Ref #11965-092) supplemented with 0.2% gentamicin (Gibco® by Life Technologies™, Grand Island, NY, Ref #15710-064)

and 0.2% fungizone (Gibco® by Life Technologies™, Grand Island, NY, Ref #15290-018). Cells were cultured in a humidified condition at 37°C and 5% CO_2. To evaluate the effect of the extracts and purified compounds on these two cell lines, HepG2 cells were seeded with 1.4 x 10^6 cells/mL or 5 x 10^6 cells/well (cell count was allowed a count of ± 10%) in 6-well plates under the previously stated culture conditions for 24 hours. Cell medium was then replaced with 1% FBS DMEM and incubated for another 24 hours. Cells were then treated with control (DMSO or Ethanol) or treatment compounds at suitable concentrations, (quercetin: 100umol/L, ellagic acid: 6.5umol/L and cherry, and Red Delicious and Fuji apple extracts: 50ug/mL) as determined by MTT Assay. Quercetin was dissolved in DMSO at maximum solubility of 30mg/mL (100mM). Ellagic acid was dissolved in ethanol (9.8mg in 50mL ethanol) to achieve maximum concentration (650uM). Extracts (preparation for extracts is described in Chapter 2) were dissolved in DMSO at a concentration of 50mg/mL. After 24 hours of incubation with the treatment medium, medium was removed and Oil Red O staining (see Section 2.4) was performed on cells.

2.2. Oil Red O Staining and Quantification

Medium is removed from cells and each well is washed with 2 mL PBS and removed completely. Two mL of 10% room temperature formalin (mixture of 27% of 37% formalin- Merck, Cat # K36658003, 63% distilled water and 10% 10X PBS) is added to each well and incubated at room temperature for 10 minutes. After incubation, formalin is discarded and another 2 mL of fresh formalin is

added to each well and incubated for at least 1 hour. Next, formalin is removed and discarded. Cells are washed with 2 mL of distilled water. Water is removed and cells are washed with 2 mL of 60% isopropanol (Merck, Cat # K36543834) for 5 minutes at room temperature. Isopropanol is removed and cells are dried completely at room temperature. Once dry, 1 mL of Oil Red O working solution (60% of stock solution, Sigma Cat # 0-0625) is added to each well and incubated at room temperature for 10 minutes. Oil Red O solution is removed and distilled water is immediately added to the well. Cells are washed 4 times with distilled water. Water is then discarded and wells can be observed under photomicroscope for analysis.

To quantify degree of staining, all the water is removed from wells and cells are allowed to dry. To elute the Oil Red O dye, 1 mL of 100% isopropanol is added and incubates at room temperature for 10 minutes while gently shaking. The isopropanol with Oil Red O is pipetted up and down several times to ensure that all Oil Red O is in solution. The solution is transferred to a 1.5 mL Eppendorf tube and optimal density (absorbance) is measured at 500 nm using 100% isopropanol as a blank.

2.3. MTT Assay

HepG2 cells were seeded at 50,000 cells/mL in 96-well plates with 10% FBS DMEM medium for 24 hours. Medium was removed and replaced with 1% FBS medium for another 24 hours. Next, medium was removed and replaced with treatment medium at varying degrees of concentration (quercetin: 0-200umol,

ellagic acid: 0-6.5umol, apple and cherry extract: 0-100mg/mL) in addition to a blank (1% FBS medium) and control (DMSO or ethanol) sample. DMSO and ethanol concentrations were normalized for treatment concentrations at tolerable levels of 0.2% and 1.0% respectively as determined by previous MTT assay results (figures not included). Cells were incubated for 24 hours in treatment medium. Next, medium was replaced with MTT (Molecular Probes® by Life Technologies™, Eugene, OR, Ref #M6494) treated medium (10% MTT reagent in 1% FBS medium) and incubated for 4 hours at 37°C and 5% CO_2. After incubation, MTT treated medium was removed and 200 uL DMSO was added to each well and incubated at room temperature for 10 minutes. Plate was then read for absorbance at 490 nm using a plate reader. Cell survival was calculated as a percentage of the control. Tolerable maximum concentration was determined as the maximum concentration at which no distinguishable cell death occurred as measured by one-way ANOVA statistical analysis.

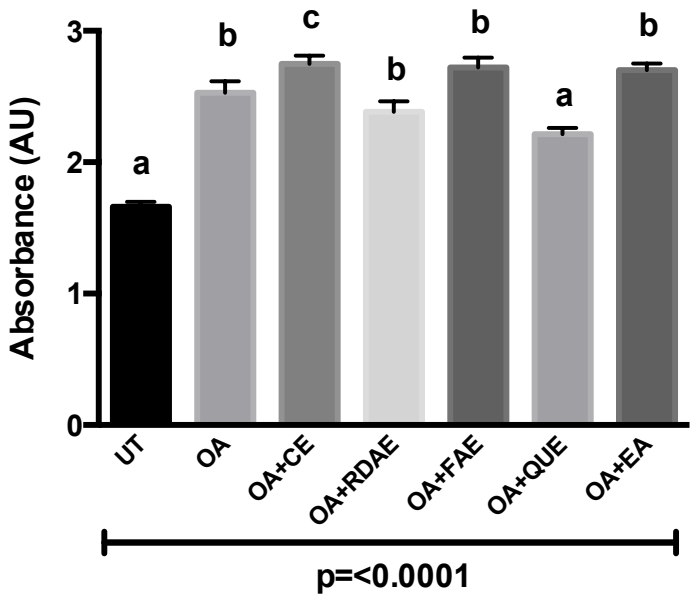
2.4. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Multi-way analysis of variation (ANOVA) was performed using Prism 6 (GraphPad Software, La Jolla, CA). Post-hoc testing was performed using Tukey's multiple comparison test.

3. RESULTS

MTT assay showed safe levels of QUE and EA up to 100uM and 6.5uM respectively (Figures not shown). Based on these values, a safe concentration of 50ug/mL for RDAE, FAE, and CE was chosen.

After cells were incubated for 24 hours with treatment media, cells were stained with Oil Red O and absorbance at 500 nm was measured to quantify degree of lipid accumulation. A significant difference was observed between UT and all OA-treated cells ($p=<0.0001$). OA+QUE-treated cells showed a 12.6% decrease in fat accumulation ($p=<0.05$) and OA+RDAE showed a reduction of 5.4% compared to OA-treated cells. OA+CE-, OA+FAE-, and OA+EA-treated cells showed a slight, but not significant, increase in lipid accumulation of 8.7, 7.5, and 6.7% respectively, compared to the OA-treated mice.



Treatment	Absorbance (AU)
UT	1.66 ± 0.04[a]
OA	2.53 ± 0.09[b]
CE	2.75 ± 0.06[c]
RDAE	2.39 ± 0.08[b]
FAE	2.72 ± 0.07[b]
QUE	2.21 ± 0.05[a]
EA	2.70 ± 0.05[b]

Figure 4.1. Fat accumulation of HepG2 cells as measured by Oil Red O staining. HepG2 cells were seeded in 6-well plates at 1.4 x 10^6 concentration and treated with various treatment compounds: UT= Untreated (1% FBS), OA= 1000uM Oleic Acid, CE= 1000uM Oleic Acid and 50ug/mL Cherry Extract, RDAE= 1000uM Oleic Acid and 50ug/mL Red Delicious Apple Extract, FAE= 1000uM Oleic Acid and 50ug/mL Fuji Apple Extract, QUE= 1000uM Oleic Acid and 100uM Quercetin, EA= 1000uM Oleic Acid and 6.5uM Ellagic Acid. After 24-hours with treatment media, Oil Red O staining protocol was followed and degree of staining was quantified by reading absorbance at 500nm using a spectrophotometer. Values are expressed as the mean ± SEM, n=6. Values that do not share a letter differ ($p<0.05$).

4. DISCUSSION

A reduction in hepatic lipid accumulation was observed in QUE and RDAE treatment groups, demonstrating the beneficial effects of these compounds on ameliorating fatty acid uptake in a high-fat-fed state. It is interesting to note that of the three phytochemical-rich fruit extracts observed, that RDAE was the most effective at reducing lipid accumulation. HPLC analysis of extracts showed that RDAE had a higher total phenolics and a significantly greater anthocyanin concentration than both those of FAE and CE. It should also be noted that quercetin and quercetin metabolites were among the most prominent phenolics present in the RDAE (Figure not shown). Therefore, it could be hypothesized that quercetin is the active ingredient in RDAE responsible for its ability to reduce hepatic lipid accumulation.

Chang et al. demonstrated similar hepatic lipid-reducing effects with their mulberry (MWE) and mulberry anthocyanin (MAE) extracts. The study design used a mulberry anthocyanin concentration about 2-10-fold greater than the dose used in our study (0.1-0.5mg/mL). A dose-dependent response was observed with MAE treatment, showing a reduction in lipid accumulation of up to 37.2% at the highest concentration (0.5mg/mL). They also explored the mechanism behind the lipid-lowering effect and found that MWE and MAE activated adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, which led to an inhibited expression of acetyl coenzyme A carboxylase (ACC), increased expression of carnitine palmitoyl transferase-1 (CPT1), and stimulated fatty acid

oxidation. The researchers also found that MAE suppressed sterol regulatory element binding protein 1 (SREBP-1) and fatty acid synthase (FAS).

Therefore, the results from Chang et al. as well as the results obtained during our animal study allows us to conclude that QUE and RDAE have the ability to reduce lipid accumulation in the liver and that this is caused, at least in part, by the activation of PPARα and its downstream products. However, further experimentation in Western blot analysis and/or RT-PCR is needed to confirm this mechanistic effect in our HepG2 cell line model.

SUMMARY AND FUTURE WORK

A two-part, animal and human cell line study was conducted to observe the metabolic improvements and changes in gene expression that were impacted by intake of polyphenol-rich extracts derived from apples and sweet cherries, and the common phytochemicals, quercetin and ellagic acid. Part one was a rodent study where mice were fed high-fat obesigenic diets supplemented with extracts or purified compounds. Throughout the course of the study, mice were monitored for changes in metabolic function and performance. Mice fed the HF control diet became obese and lost normal glucose control. In the absence of changes in weight gain or diet consumption, the HF+treatment-fed mice demonstrated improved metabolic parameters compared to the HF-fed mice. At week 6, fasted blood glucose levels were reduced in all HF+treatment-fed mice relative to the HF-fed mice ($p=<0.05$). Glucose tolerance was also improved in the HF+AE- and HF+QUE-fed mice ($p=<0.05$). A grip test indicated improved strength, in HF+AE-, HF+QUE- and HF+EA-fed mice ($p < 0.05$) and a moderate improvement in HF+CE-fed mice compared to HF-fed mice ($p=<0.10$). A Rotarod test appeared to increase in endurance and balance in HF+treatment-fed mice, but this improvement was not statistically significant. There was a noticeably reduced inflammatory response in HF+treatment-fed mice, indicating an anti-inflammatory effect, however, significance was not determined for individual values. Lastly, CPT1-α, and ACOX-1 mRNA levels, were increased in HF+treatment-fed mice relative to the HF-fed mice, indicating increased gene expression of PPAR-α-

related genes and fatty acid oxidation. This quantitative data was consistent with qualitative examination of liver tissue sections stained.

Part two of the study utilized a human cell line model of fatty acid uptake where quantitative measurements of cellular lipid accumulation were performed. HepG2 cells were treated with oleic acid (OA) and a compound of interest. Oleic acid was used to stimulate cellular lipid accumulation and treatment compounds were observed for their ability to affect the degree of lipid accumulation. After incubation with treatment media, cells were stained with Oil Red O dye to quantify hepatic fat accumulation and absorbance was measured using a spectrophotometer. Cells exposed to OA+QUE showed a significant decrease in lipid accumulation ($p=<0.05$). Other treatment groups showed a similar or slightly increased degree of lipid accumulation. Based on the results observed from both parts of the study, we conclude that the compounds present in apples and cherries, quercetin and ellagic acid produce beneficial metabolic effects and that part of this improvement may be mediated by phytochemical activation of nuclear hormone receptors.

Future directions for this line of research include observing different fruit phytochemical and whole food compounds and gene expression and metabolic profiling. It would be beneficial to look at the other phytochemical compounds present in apples and cherries, such as chlorogenic acid or catechin, to observe how they impact metabolism. This observation of individual compounds alongside the phytochemical-rich extract would allow us to pinpoint the active ingredients in each extract. Observing different varieties of apples and cherries

would also provide valuable insight into how different quantities and composition of phytochemicals impact metabolic pathways. Both individual phytochemicals and different varieties of fruit extracts should be monitored at both the physiological and molecular/genetic level. In addition to the physiological and molecular testing performed in our study, additional studies into gene expression and metabolic profiling should be performed. Using a PPAR-α knockout mouse model would confirm whether the extracts were in fact inducing PPAR-α or another nuclear hormone receptor. Microarray analysis would also allow us to measure a large number of genes simultaneously and pinpoint specifically which genes or molecular pathways are being altered with phytochemical treatment. Finally, a metabolomics study would let us see how the phytochemicals or extracts are metabolized, where they are metabolized, and what the phytochemical's active elements or biological endpoints are.

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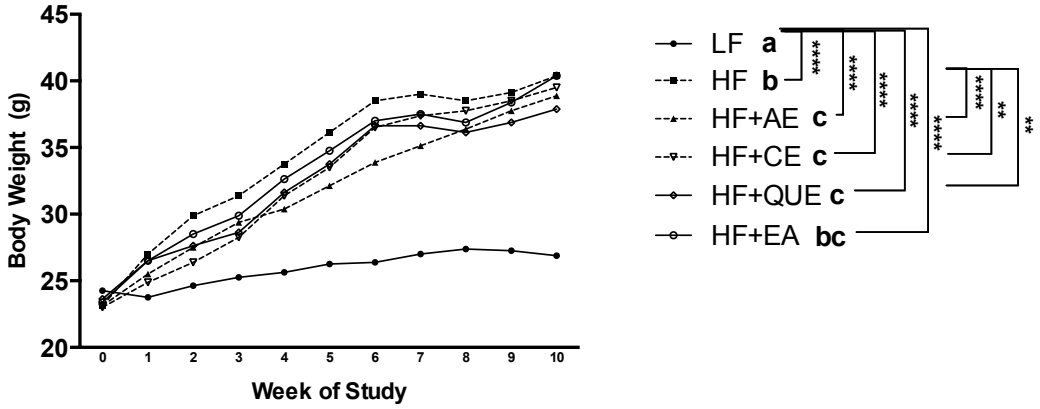
APPENDIX



Appendix Figure 1.1. **Body weight of C57BL/6J mice fed various phytochemical containing diets for 10 weeks**. Mice were fed either a low-fat diet (LF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% w/w of either apple phytochemical extract (HF+AE), cherry phytochemical extract (HF+CE), quercetin (HF+QUE), or ellagic acid (HF+EA). Values are expressed as means, n=8. Where letters differ, values are statistically different as measured by repeated-measures two-way ANOVA ($p<0.05$). Asterisks denote degree of significance (**: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

Appendix Table 1.1. Weekly body weights of male C57BL/6J mice fed various phytochemical containing diets for 10 weeks[1].

	LF[2]	HF	HF+AE	HF+CE	HF+QUE	HF+EA
Week 0	24±0.3	23±0.7	23±0.7	23±0.5	24±0.4	23±0.5
Week 1	24±0.8	27±1.1	26±0.8	25±0.6	27±0.5	27±0.6
Week 2	25±0.7	30±1.3	28±0.9	26±0.7	28±0.6	29±0.8
Week 3	25±0.6	31±1.6	29±1.1	28±0.6	29±0.9	30±1.2
Week 4	26±0.6	34±1.9	30±1.1	31±1.0	32±0.8	33±1.3
Week 5	26±0.7	36±2.0	32±1.3	34±1.3	34±1.0	35±1.4
Week 6	26±0.8	39±2.0	34±1.3	37±1.5	37±1.0	37±1.6
Week 7	27±0.5	39±1.9	35±1.2	37±1.6	37±1.2	38±1.8
Week 8	27±0.7	39±1.5	36±1.1	38±1.7	36±1.2	37±1.8
Week 9	27±0.5	39±1.4	38±1.4	39±2.0	37±1.1	38±1.8
Week 10	27±0.7	40±1.6	39±1.5	40±1.5	38±1.1	40±2.0

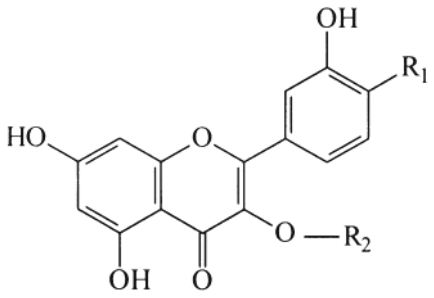
Appendix Table 1.2. Cytokine and hormone abbreviations and definitions

Abbreviation	Full Name	Function
CRP	C-reactive protein	C-reactive protein is a protein found in the blood, the levels of which rise in response to inflammation
Eotaxin	Eotaxin	Selectively recruits eosinophils by inducing their chemotaxis, and therefore, is implicated in allergic responses
G-CSF	Granulocyte colony stimulating factor	Glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells and release them into the bloodstream
GM-CSF	Granulocyte-macrophage colony stimulating factor	Functions as a white blood cell growth factor; stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes
IFN-g	Interferon gamma	Critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control; activator of macrophages
IL-13	Interleukin 13	Secreted by many cell types, but especially T helper type 2 (Th2) cells, that is a mediator of allergic inflammation and disease
IL-1a	Interleukin 1 alpha	Responsible for the production of inflammation, as well as the promotion of fever and sepsis
IL-1b	Interleukin 1 beta	Mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis
IL-6	Interleukin 6	Secreted by T cells and macrophages to stimulate immune response

IL-9	Interleukin 9	Produced by T-cells and specifically by CD4+ helper cells that acts as a regulator of a variety of hematopoietic cells; stimulates cell proliferation and prevents apoptosis
Insulin	Insulin	A peptide hormone produced by beta cells of the pancreas and is central to regulating carbohydrate and fat metabolism in the body. It causes the cells in the liver, skeletal muscles, and fat tissue to absorb glucose from the blood
IP-10	Interferon-gamma-inducible protein 10	Secreted by several cell types in response to IFN-γ. Attributed to several roles, such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis.
KC	Keratinocyte chemoattractant	Involved in chemotaxis and cell activation of neutrophils; overexpression in murine keratinocytes, monocytes and macrophages follows in response to PDGF and M-CSF
Leptin	Leptin	A 16-kDa adipokine that plays a key role in regulating energy intake and expenditure, including appetite and hunger, metabolism, and behavior. It is one of the most important adipose-derived hormones
LIX	Lipopolysaccharide-induced CXC chemokine	Associated with cell migration and activation in neutrophils
M-CSF	Macrophage colony stimulating factor	Influences hematopoietic stem cells to differentiate into macrophages or other related cell types

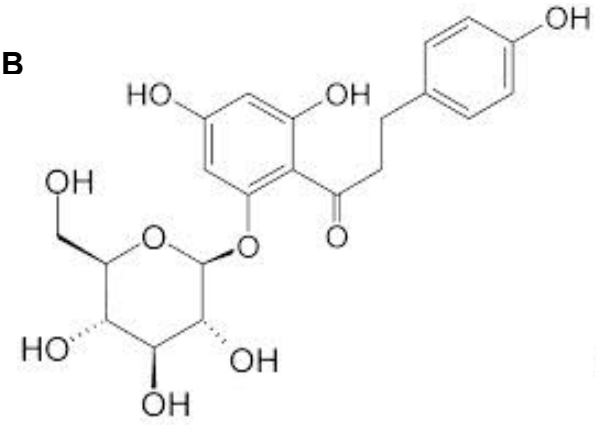
MCP-1	Monocyte chemotactic protein-1	Recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection
MIG	Monokine induced by gamma interferon	T-cell chemoattractant, which is induced by IFN-γ
MIP-1a	Macrophage inflammatory protein-1 alpha	Involved in the acute inflammatory state in the recruitment and activation of poly-morphonuclear leukocytes
MIP-1b	Macrophage inflammatory protein-1 beta	Chemoattractant for natural killer cells, monocytes and a variety of other immune cells
MIP-2	Macrophage inflammatory protein-2	Secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells
PAI-1	Plasminogen activator inhibitor-1	Inhibitor of fibrinolysis, the physiological process that degrades blood clots, and is thus a marker for fibrosis
RANTES	Regulated on activation, normal T cell expressed and secreted	Chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites
Resistin	Resistin	In primates, pigs, and dogs, resistin is secreted by immune and epithelial cells, while, in rodents, it is secreted by adipose tissue; increases the production of LDL in human liver cells and also degrades LDL receptors in the liver

A



Flavonol	R_1	R_2
Rutin	OH	Rutinose
Quercitrin	OH	Rhamnose
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H

B



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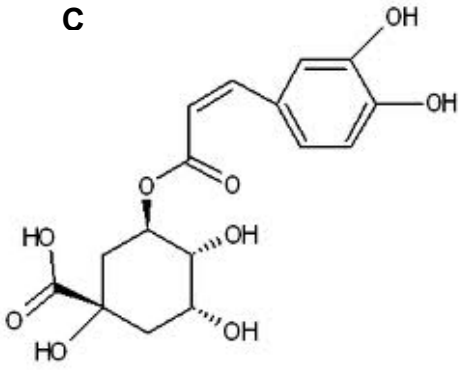

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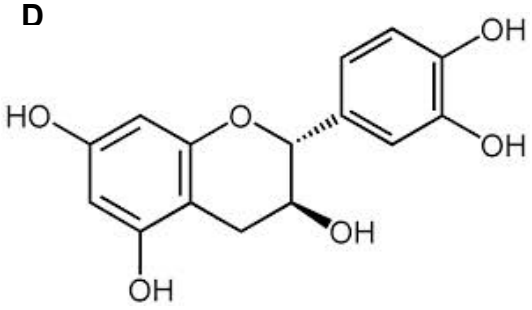

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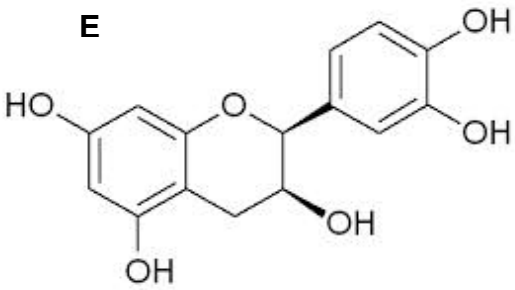

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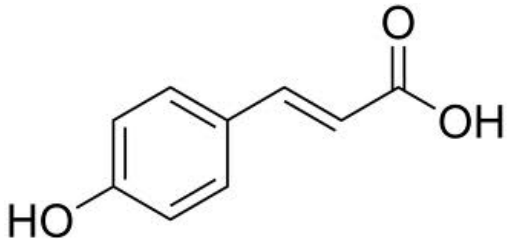
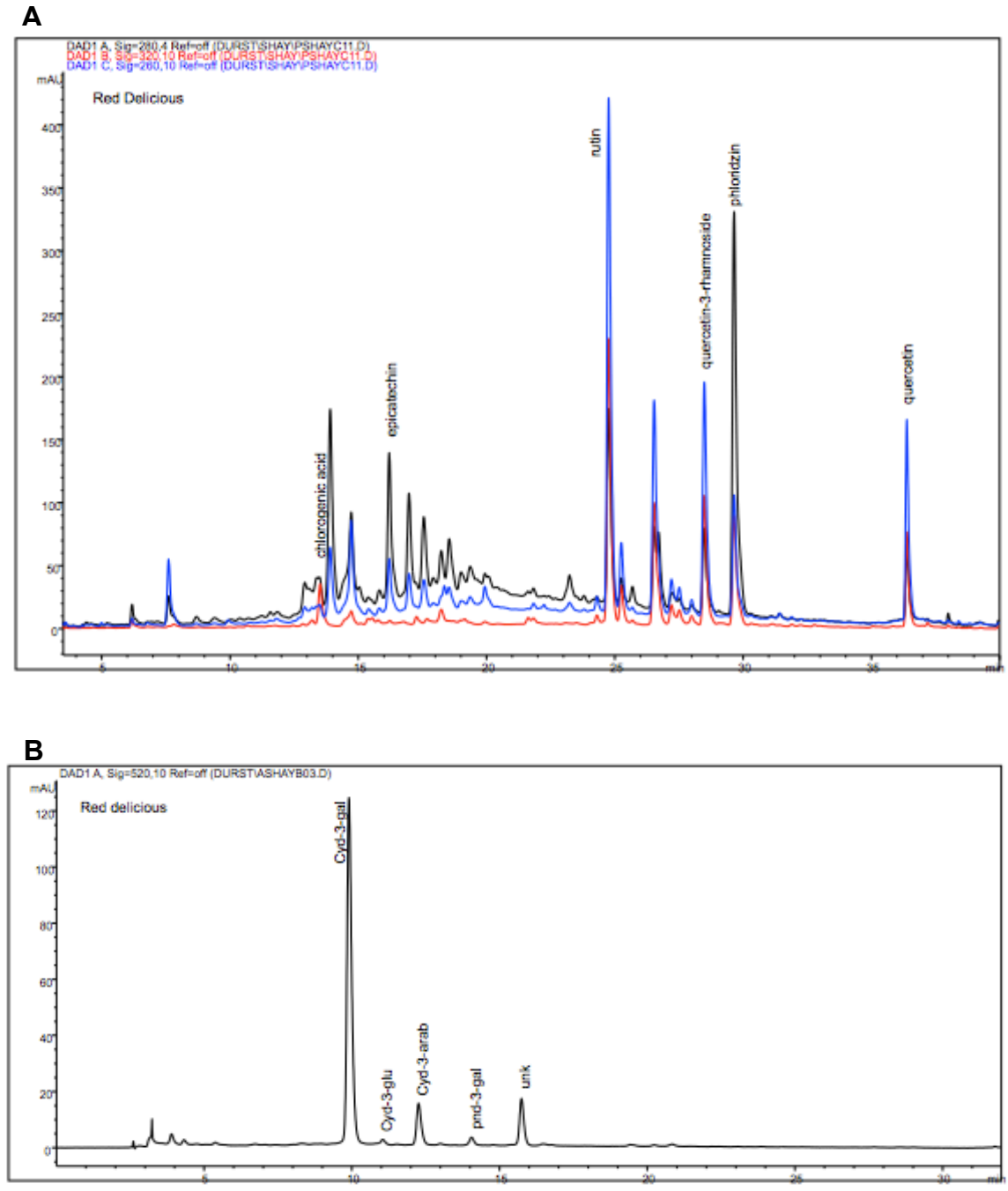
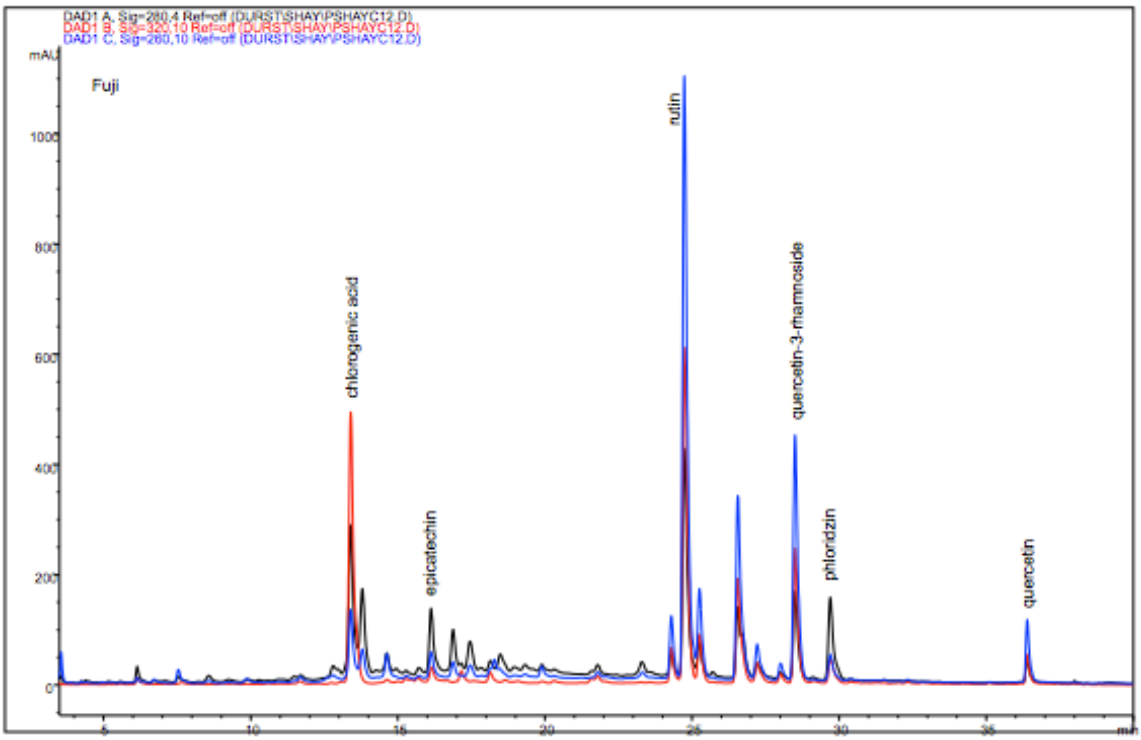


Appendix Figure 1.2. Phytochemical structure of most abundant phytochemicals in apple and cherry phytochemical extracts as determined by HPLC. A: quercetin and quercetin conjugates and metabolites, B: phloridzin, C: chlorogenic acid, D: catechin, E: epicatechin, F: coumaric acid.

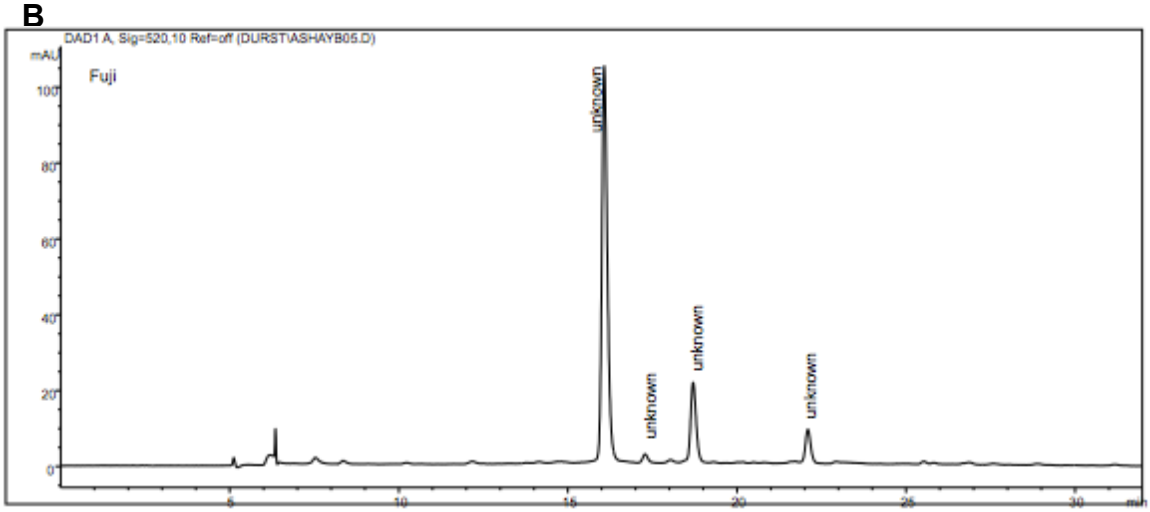


Appendix Figure 1.3. HPLC graph of Red Delicious apple extract phenolics (A) and anthocyanins (B).

A



B



Appendix Figure 1.4. HPLC graph of Fuji apple extract phenolics (A) and anthocyanins (B).