GRAPE EXTRACTS FOR TYPE 2 DIABETES TREATMENT THROUGH SPECIFIC INHIBITION OF α-GLUCOSIDASE AND ANTIOXIDANT PROTECTION

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ABSTRACT

Research was conducted to investigate the effect of phenolic compounds derived from inherently rich antioxidant grape extracts (GE) on \( \alpha \)-glucosidase inhibitory activity \textit{in vitro} and \textit{in vivo} blood glucose control, oxidative stress, and inflammation associated with obesity-induced type 2 diabetes. Because intestinal \( \alpha \)-glucosidase plays a key role in the digestion and absorption of complex carbohydrates, the inhibition of this enzyme is a metabolic target for managing diabetes by improving post-prandial blood glucose control. Initially, red Norton wine grape (\textit{Vitis aestivalis}) and pomace extracts were evaluated and determined to have notable phenolic content and antioxidant properties. Next, grape skin (GSE) and pomace extract (GPE) were tested and both had \textit{in vitro} yeast and mammalian \( \alpha \)-glucosidase inhibitory activity. The GSE was 32-times more potent at inhibiting yeast \( \alpha \)-glucosidase than acarbose, a commercial oral hypoglycemic agent. From HPLC and LC-MS analysis, three phenolics from the GSE (resveratrol, ellagic acid, and catechin) were identified as active inhibitory compounds. The acute administration of GPE (400 mg/kg bw) to mice reduced postprandial blood glucose level by 35% following an oral glucose tolerance test compared to the control. The daily supplementation (250 mg/kg bw) of GSE and GPE for 12-weeks to mice affected fasting blood glucose levels, oxidative stress, and inflammatory biomarkers associated with obesity and type 2 diabetes. At the end of the study, the GSE group gained significantly (P < 0.05) more weight (24.6 g) than the control, high fat, or GPE groups (11.2, 20.2, 19.6 g, respectively). Both GSE and GPE groups had lower fasting blood glucose levels (119.3 and 134.2 mg/dL, respectively) compared to the high fat group (144.6 mg/dL). The 12-week supplementation of GSE was associated with a higher plasma oxygen radical absorbance capacity (ORAC), lower liver lipid peroxidation as measure by TBARS, and lower levels of inflammation as measured by plasma C-reactive protein compared to the high fat group. In conclusion, our collective observations from these studies provide insight into the potential effects of antioxidant rich grape extracts on diabetes-related biomarkers through a dual mechanism of antioxidant protection and specific inhibition of intestinal \( \alpha \)-glucosidases.
In memory of my grandparents,

Patrick and Stella Hogan
and
Donna Griffith Richman

They were always very supportive and interested in my education since kindergarten and I know that they would be very proud of me.
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<tr>
<td>$\alpha$</td>
<td>alpha</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>emission wavelength</td>
</tr>
<tr>
<td>$\lambda_{ex}$</td>
<td>excitation wavelength</td>
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<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
</tr>
<tr>
<td>HLB</td>
<td>hydrophilic-lipophilic balance</td>
</tr>
<tr>
<td>HPLC</td>
<td>reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>concentration of inhibitor that inhibits enzyme rate by 50%</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
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<tr>
<td>LC-MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>$M$</td>
<td>molecular weight</td>
</tr>
<tr>
<td>$m$</td>
<td>mass</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>ORAC</td>
<td>oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>$p$NPG</td>
<td>$p$-nitrophenyl $\alpha$-D-glucoside</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
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<td>UV</td>
<td>ultraviolet</td>
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CHAPTER 1: INTRODUCTION

Type 2 Diabetes

Type 2 diabetes is a serious chronic health condition with increasing prevalence worldwide. In the United States, this epidemic is spreading rapidly and affects 24 million children and adults (or 7% of the population) (1). Even more alarming is that these numbers continue to be on an up surge. It is estimated that in approximately two decades more than 390 million individuals globally will have diabetes (2). In addition to serious health complications associated with diabetes, there is an enormous economic cost, in 2002 alone more than $132 billion in healthcare expenses in the United States were attributed to diabetes (3, 4). If untreated, this metabolic condition can lead to a suite of devastating conditions including blindness, kidney failure, and nerve damage (5). Furthermore diabetes is associated with an increased risk of developing cardiovascular diseases (5). Considering the chronic nature of this disease and the limitations of current therapies such as high secondary failure rates and adverse side effects, there remains a consensus for the need to develop and explore new therapeutic agents that have the potential to combat this epidemic.

Oral $\alpha$-Glucosidase Inhibitors

The pathogenesis of type 2 diabetes has been attributed to two key metabolic anomalies: peripheral insulin resistance and pancreatic $\beta$-cell dysfunction (i.e. impaired insulin synthesis and/or release) (6). The lack of insulin or resistance to insulin results in an environment of excess glucose or hyperglycemia. The chronic elevation of blood glucose is the principal cause of the well-known complications associated with diabetes including retinopathy, nephropathy, neuropathy, and coronary heart disease (5). Clinical and epidemiological data indicate that decreasing glycemia is an effective strategy of reducing the vascular associated complications (7, 8). Thus, improving blood glucose homeostasis is a target area for potential therapeutic agents. For example, a strategy in the treatment and management of type 2 diabetes is the use of oral $\alpha$-glucosidase inhibitors, which represent a large sector of the lucrative anti-diabetes drug market (more than $300 million in sales) (4). Oral hypoglycemic agents delay the absorption of complex carbohydrates by inhibiting $\alpha$-glucosidase, an enzyme present in the brush border of the
small intestine (9). Individuals with diabetes or those with an elevated blood glucose levels (also known as having impaired fasting glucose or “pre-diabetes”) may use these drugs in order to treat or prevent the induction of type 2 diabetes (5, 10, 11).

Commercially available oral $\alpha$-glucosidase inhibitors, such as acarbose (Precose®) have been widely marketed and prescribed to treat type 2 diabetes (10, 12). These expensive medications, however, are often associated with a variety of undesirable symptoms as a result of undigested starch into the colon. For instance, bloating, abdominal discomfort, diarrhea, and flatulence were seen in nearly 20% of patients treated with acarbose (13). Hence, great interest is currently being devoted to alternative $\alpha$-glucosidase inhibitors derived from natural sources and foods that may be effective, safe, tolerable, and less expensive.

**Oxidative Stress**

When there is an imbalance between anti-oxidants and pro-oxidants in biological systems oxidative stress can ensue (14). During the past decade much attention has been focused on the role of oxidative stress in diseases. There is growing recognition that oxidative stress may contribute to the pathogenesis of complications associated with diabetes (15). Individuals diagnosed with diabetes and experimentally induced animal models have exhibited oxidative stress as a result of persistent and chronic hyperglycemia, resulting in diminished activity of antioxidant enzymes and the promotion of free radical generation (16-18). Furthermore, it is suggested that oxidative stress related to hyperglycemia induces the generation of free radicals which contributes to the development and progression of diabetes and its complications (17-22). For instance, abnormally high levels of free radicals cause membrane damage due to peroxidation of lipid membranes, protein glycation, and depletion of antioxidant defense enzyme systems all of which can lead to cell and tissue damage. Antioxidant enzymes that can be affected include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) all of which function as part of the cellular endogenous antioxidant defense system by scavenging highly reactive and promiscuous radical oxygen species (ROS) (18, 23). Research continues to identify potential triggers of oxidative stress associated with type 2 diabetes, as well as to establish a link between hyperglycemia and
oxidative stress. Thus, complementary strategies that achieve both antihyperglycemic and antioxidant status have been suggested and investigated in diabetes research in an effort to better understand the link between diabetes and oxidative stress.

**Inflammation**

Data are accumulating that suggests oxidative and inflammatory stresses are implicated in the alterations of insulin response, and therefore increase the propensity of developing diabetes associated with obesity (24-26). For example, there is indication that both obesity and type 2 diabetes biochemically exhibit properties of a persistent state of low-grade inflammation (27-29). For instance, these metabolic conditions have been associated with inflammatory processes such as increased levels of proinflammatory molecules (30). Over time, chronic low-level inflammation may have a direct role in the development of diseases and associates with disease risk. In particular, pro-inflammatory cytokines and acute phase proteins such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interleukin-1(IL-1), and C-reactive protein (CRP) may contribute to the development of insulin resistance and further worsening of blood glucose control (31). It is speculated that with lower glucose concentrations over time along with sustained antioxidant protection may result in a lower systemic inflammatory response as reflected by lower levels of biomarkers such as CRP (32, 33). The current scientific evidence indicates that there may be a multi-faceted link between oxidative stress, inflammation, and hyperglycemia associated with type 2 diabetes, and obesity (30, 34).

**Natural Product Research**

Alternative antidiabetes strategies have been conducted using natural products with potential dual bioactive properties for addressing hyperglycemia and the parallel oxidative and inflammatory stress (35). For example, grapes have been well characterized as a natural source of notable bioactive compounds with antioxidant, health promoting, and disease protective qualities (36-38). Over 1600 phytochemicals have been identified in grapes including resveratrol, anthocyanins, catechins, ellagic acid, lutein, melatonin, lycopene, quercetin, and many other potent antioxidants (39). The polyphenolic compounds which are widely distributed in grape seeds, skins, and stems have been reported to contribute to the beneficial properties (36-38, 40). Grape extract
supplementation improved insulin resistance and suppressed oxidative stress in rats (38). Research continues to determine if grape-derived phenolic compounds have a corollary benefit not only for a role in blood glucose control but also as an antioxidant that may reduce oxidative stress (41).

**Justification**

The overall goal of this study was to identify and develop bioactive food components that have an antidiabetic effect through the mechanisms involving \( \alpha \)-glucosidase inhibition, antioxidant protection, and anti-inflammatory effect. In particular, this project explored whether antioxidant rich grape extracts (GE) as a potent \( \alpha \)-glucosidase inhibitor, have the potential to be developed as an appropriate antidiabetic agent. Intestinal \( \alpha \)-glucosidase was the macromolecular structure of primary focus because of its direct activity in hydrolyzing oligosaccharides derived from starch to glucose in the small intestine and thereby metabolic carbohydrate digestion and absorption (42, 43). Various \( \alpha \)-glucosidase inhibitors have been reported to lower postprandial blood glucose and to provide beneficial effects for diabetes management (12, 44-48). From preliminary investigations in our laboratory, specific GE were discovered to exhibit potent *in vitro* \( \alpha \)-glucosidase inhibition. This newly identified natural food-derived phenolic extract had a 32-fold stronger *in vitro* yeast \( \alpha \)-glucosidase inhibition compared to acarbose, the commercially available \( \alpha \)-glucosidase inhibitor. Further investigation of this \( \alpha \)-glucosidase inhibitor revealed that GE strongly inhibits mammalian \( \alpha \)-glucosidases but had no inhibition on pancreatic \( \alpha \)-amylase. These preliminary results suggested that GE specifically inhibits \( \alpha \)-glucosidases. After conducting fundamental enzyme kinetic assays, GE was determined to function as a competitive inhibitor of \( \alpha \)-glucosidases, indicating the need for GE to be available shortly before or during starch consumption. Based on these *in vitro* findings, the following study hypothesis was that GE would ameliorate hyperglycemia, oxidative stress, and improve inflammatory status in mice with diabetes or at an increased risk of developing type 2 diabetes as a result of a high fat diet. Thus, the GE may be a nutraceutical facilitator in the prevention and treatment of diabetes, through a mechanism initially
stemming from the inhibition of intestinal $\alpha$-glucosidases and subsequent lower rise in blood glucose levels. To test this hypothesis the following objectives were investigated:

**Objectives**

**Objective 1. Determine and characterize the phenolic active constituents in GE that exert $\alpha$-glucosidase inhibition**

A potent $\alpha$-glucosidase inhibitory activity in grape phenolic extracts (GE) prepared from red grapes was identified. For example, anthocyanins found in high concentrations of red grape skins were initially speculated to be contributing to the inhibitory effect. However, a white wine GE also revealed strong inhibition, suggesting that the active inhibiting constituents are likely from other polyphenolic molecular groups in addition to the deep burgundy pigmenting anthocyanins. The identification of active constituents in GE, combinations of GE phenolic extracts fractions, and a number of phenolic standards normally contained in grape were attempted using HPLC and LC-MS. Additionally, enzyme kinetic parameters were determined using Michaelis-Menten kinetics (i.e. Lineweaver-Burke and Dixon plot extrapolations) with the active GE fractions with potent enzyme inhibitory activity.

**Objective 2. Determine the *in vitro* antioxidant profile of GE**

The antioxidant property of GE was further characterized using established antioxidant capacity methods. For example the aim of this part of the project was to measure the antioxidant potential that GE possesses by employing routine freeze drying methods, extraction techniques for phenols, and testing for antioxidant properties. The antioxidant protective qualities were measured by determining the total phenolic content, total anthocyanin content, total flavonoid content, oxygen radical absorbance capacity (ORAC), and the scavenging activity of the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH).
Objective 3 and Objective 4. Acute and long-term in vivo effect of GE on hyperglycemia in mice at risk for diabetes

The anti-hyperglycemic effects of commercially available α-glucosidase inhibitors such as acarbose and miglitol are well established in both animal studies and human clinical trials (11, 46-50). From in vitro studies it was determined that GE potently inhibits rat α-glucosidases with an inhibition constant ($K_i$) of 0.296 mg/mL. The in vivo effects of GE administration on postprandial response and glucose homeostasis in streptozotocin (STZ) injected mice with diabetes were investigated from a 2-hour oral glucose tolerance test (OGTT). The long-term animal study entailed a 12-week supplementation of GE along with a high fat “Western” diet study to assess pertinent biomarkers of type 2 diabetes (i.e. glucose and insulin).

Objective 5. Determine the influence of GE on oxidative stress and inflammatory state in vivo

Blood samples were collected at the termination of the 12-week high fat study in order to determine the effect of GE supplementation on oxidative stress status in mice. In particular, assays were conducted to determine the ORAC of blood samples, activity of antioxidant enzymes and liver lipid peroxidation. Enzyme activities were measured for cytosolic glutathione peroxidase (GPx) and liver lipid peroxidation was evaluated by thiobarbituric reactive acid substances (TBARS). Furthermore, inflammatory response to the GE supplementations was determined by measuring the acute phase inflammatory C-reactive protein, CRP. The working hypothesis for the objectives addressing the 12-week feeding study was that supplementation of grape phenolics would lead to improved concentrations of fasting blood glucose through a mechanism involving chronic attenuation of postprandial hyperglycemia by α–glucosidase inhibition and a corollary reduction in the formation and generation of biological radical species associated with oxidative stress and inflammation. As such supplementation of an oral hypoglycemic agent such as grape extracts (GE) may provide evidence in regards to an effective strategy for addressing type 2 diabetes.
Summary

The incidence of diabetes increased by approximately 80 percent in the United States between 1995 and 2005 despite the increased number and availability of anti-diabetic drugs (51). This trend in diabetes diagnosis emphasizes the existing opportunity to identify effective, safe, and natural therapeutic agents for managing diabetes in the 21st century especially in light of the fact that most individuals chose not to heed the advice of health professionals advocating diet and exercise. In this research, GE was proposed to be a natural product for the treatment and prevention of type 2 diabetes as a result of potent GE inhibition on mammalian α-glucosidase activity, a key enzymes relevant to blood glucose control. Results from the preliminary animal studies have provided the foundation for future endeavors, including conducting additional animal studies to test the effectiveness and safety of the administration of GE.
CHAPTER 2: REVIEW OF LITERATURE

Type 2 Diabetes

Type 2 diabetes is a serious disease which can affect almost every organ in the body. This condition can produce various dysfunctions in the body, including a variety of circulatory system disorders, retinopathy, altered immune and intestinal functions, nephropathy, and peripheral neuropathy (52, 53). There are 20.8 million children and adults in the United States who suffer from diabetes and this number is projected to double by 2025 (4). Not only is this rapidly evolving disease causing an enormous health burden to individuals often resulting in mortality, it comes with profound financial burden (54). The consensus exists for a need to discover and develop alternative and adjuvant therapeutic compounds in addition to the conventional interventions to address the unmet health crisis associated with diabetes (5).

Pathologically, diabetes mellitus is characterized by two key metabolic anomalies. One is described as a reduced efficiency of glucose metabolism resulting from insulin resistance or insensitivity (55). The second factor related to the pathogenesis of diabetes is the dysfunction or deterioration of insulin producing pancreatic β-cells (56). These endocrine malfunctions are inter-linked in that the reduction in insulin secretion results from dysfunction in pancreatic β-cell and concomitant hyperglycemia. Although the underlying causes are not clearly known, oxidative stress has been suggested as a mechanism that contributes to the deleterious modification of pancreatic β-cells in type 2 diabetes (56).

A hallmark feature of type 2 diabetes is hyperglycemia or presence of high blood glucose levels (5). The epidemic of type 2 diabetes and the recognition that maintenance of healthy blood glucose levels can reduce morbidity has given precedence for strategies targeting hyperglycemia (5). Research has indicated that hyperglycemia worsens oxidative status as a consequence of the generations of free radicals which can damage macromolecules ranging from DNA to lipids (57). Furthermore, chronic and persistent hyperglycemia is believed to be contributing to the suite of micro and macro vasculature complications associated with diabetes including retinopathy, nephropathy, neuropathy, and circulatory system disorders (58-62). The encouraging news is that clinical and
epidemiological data indicate that decreasing glycemia is an effective strategy of reducing the vascular complications associate with diabetes (7, 8).

Regarding dietary nutrients, type 2 diabetes is related, in part, to the carbohydrates (i.e. starch) in the diet and the absorption of glucose derived from these food macromolecules into the blood stream. Starch is made of amylose, which is a linear polymer of glucose residues linked by 1,4-glycosidic bonds, and of amylopectin (63). In metabolic pathways, mammalian starch digestion primarily occurs in the lumen of the small intestine by \( \alpha \)-amylase to yield both linear maltose (a disaccharide formed from two units of glucose joined with an \( \alpha \) (1-4) linkage) and branched isomaltose oligosaccharides (46). Neither of these sugars (i.e. maltose or isomaltose) can be absorbed into the bloodstream without further hydrolysis by \( \alpha \)-glucosidases (46). Similarly, sucrose, a disaccharide comprised of a glucose and a fructose molecule, can be hydrolyzed by \( \alpha \)-glucosidase into monosaccharides in the small intestine. In vivo these saccharides are the substrates for hydrolase enzymes which contribute to the rise of blood glucose levels.

**\( \alpha \)-Glucosidase**

Blood glucose control is influenced in part by \( \alpha \)-glucosidase as a result of its key metabolic role in carbohydrate digestion and absorption in the proximal small intestine (46). This starch-catalyzing enzyme is classified in a sub-family under the glycoside hydrolase family 13, also known as the \( \alpha \)-amylase family (63). The glycoside hydrolase family is the largest sequence-based family of glycoside hydrolases (63). Numerically, \( \alpha \)-glucosidase is referred to by its enzyme classification number: EC 3.2.1.20 and is found in the brush border of the small intestine where it hydrolyzes either sucrose or maltose (i.e. terminal, non-reducing 1,4-linked \( \alpha \)-D-glucose residues and releases \( \alpha \)-D-glucose) (63). The general enzymatic mechanism has all of the characteristics of hydrolase enzymes (Figure 2.1).

![Figure 2.1. Structure and general glycoside hydrolase reaction.](image)
For example, the hydrolysis of complex carbohydrate macromolecules into smaller absorbable units such as disaccharides and monosaccharides is the result of glycoside hydrolase activity. Mechanistically, hydrolysis of intermediates in the reaction release the non-reducing end product (glucose monosaccharide) which becomes available for rapid absorption in the small intestine and release into the circulatory system (63).

**α-Glucosidase Inhibitors**

In terms of pharmaceutical drug development, α-glucosidase inhibitor candidates have been designed with the purpose of reducing the rate of digestion of carbohydrates in the small intestine. Most α-glucosidase inhibitors have been reported to be competitive, reversible inhibitors of membrane-bound intestinal α-glucosidase enzymes (64). These oral “starch blockers” are effective at lowering postprandial glucose levels without causing hypoglycemia because carbohydrates can be absorbed more distally in the small intestine or be available for colonic metabolism (65). This sector of the pharmaceutical industry is lucrative; oral anti-diabetes agents account for more than $10 billion in the global diabetes prescription market (66).

Consequently, a variety of anti-diabetes agents have been developed and prescribed in an effort to delay intestinal carbohydrate absorption (67). Two approved oral α-glucosidase inhibitors in the United States designed to reduce postprandial hyperglycemia are acarbose (Precose®) and miglitol (Glycet®) (12, 46, 47). Other anti-diabetes agents are continuously being identified and investigated for their appropriateness as additional interventions with the ability to achieve postprandial glycemic control (67, 68). Unfortunately, these drugs have gastrointestinal side effects that often limit their acceptance and tolerability by the general public (6). For example, the use of acarbose, which non-specifically inhibits α-amylase and α-glucosidase, has been associated with adverse gastrointestinal side effects such as bloating, abdominal discomfort, diarrhea, and flatulence (11, 69). It was reported that these gastrointestinal annoyances occurred in about 20% of patients taking this drug (10). The problem arises due to the increase in colonic bacterial fermentation which results in gas or flatulence. For example, carbohydrates resistant to digestion and those that escape absorption in the small intestine are available for colonic bacterial fermentation resulting in the production
of short chain fatty acids (acetic, butyric, and propionic acids) and gases (CO$_2$, CH$_4$, and H$_2$) (65, 70).

As such, a major advantage and therapeutic opportunity exists for developing natural compounds that have antidiabetes properties and may result in fewer side effects. A number of naturally derived anti-diabetes candidates may already be determined as generally recognized as safe (GRAS) under the FDA food ingredient rules (71). The GRAS status of newly identified anti-hyperglycemic agents facilitates their acceptance as a safe nutraceutical anti-diabetic agent, which would be an obvious advantage for future application in human clinical trials and FDA health claim approval (71). It is recognized that in order to address the public’s concern and to demonstrate that complementary medicines are effective and safe, all anti-diabetes agent candidates need to be subjected to the same standards demanded in traditional or conventional medical therapies used to address diabetes.

To address this concern, studies have demonstrated the effectiveness of $\alpha$-glucosidase inhibitors for diabetes treatment in animal and human clinical studies. For instance, acarbose has been shown to reduce postprandial serum glucose and insulin concentrations, improve metabolic control, and produce improvements in nephropathy associated with diabetes in mice (49, 72). Additionally, acarbose has been shown to exert beneficial effects on ischemia/reperfusion damage and pancreatic β-cell damage by attenuating hyperglycemic episodes (73, 74). Furthermore, this $\alpha$-glucosidase inhibitor was reported to prevent age related glucose intolerance and improve insulin sensitivity in fructose fed rats (47, 49). Collectively, these and other studies provide evidence for the efficacious potential of $\alpha$-glucosidase inhibitors as countermeasures against diabetes and associated complications.

Human clinical studies also suggest an effective and safe role of $\alpha$-glucosidase inhibitors on glycemic control and insulin response, though there is no evidence that the inhibitors contribute to a reduction in mortality or morbidity (72). Consequently, developing optimal treatment strategies to achieve long-term glycemic control continues to be under the scrutiny of organization such as the American Diabetes Association (ADA). The ADA recommends the following targets for glycemic control: hemoglobin A1c less than 7%, fasting blood glucose of 80-120 mg/dL, and post-prandial blood
glucose of 100-140 mg/dL (75-77). A recent meta-analysis on α-glucosidase inhibitors for type 2 diabetes revealed that α-glucosidase inhibitors significantly decreased the serum level of glycosylated hemoglobin by 0.68-0.77% (11). This decrease may not seem biologically significant, but it is a reflection of successful long-termed improvement in blood glucose with regards and reference to the target levels determined by the ADA. For example, hemoglobin A1c (glycosylated hemoglobin) is recommended to be less than 6% for people without diabetes; for people with diabetes HbA1c levels are recommended to be 7% or less (78).

In another study involving a human randomized controlled prospective trial, acarbose was highlighted as a potent α-glucosidase inhibitor that could prevent or delay the manifestation of “pre-diabetes” or impaired glucose tolerance (IGT) to becoming diagnosed type 2 diabetes (12). This finding gained the attention of many in light of the fact that the CDC has estimated that 57 million people have pre-diabetes (1). Thus, the importance of this finding is related to the consensus that IGT following a 2-h plasma glucose challenge between 140 mg/dl to 199 mg/dl is an indicative characteristic of peripheral insulin resistance (75). Consequently there exists a need to address this population that is likely to be diagnosed with type 2 diabetes. Furthermore, the presence of IGT is associated with an increased risk of diabetes and the development of complications such as cardiovascular disease and retinopathy (79, 80). Overall, the consensus continues to grow which advocates the use of oral hypoglycemic agents in combination with conventional therapies such as diet and exercise to achieve healthy blood glucose levels (5).

**Oxidative Stress**

Reactive oxygen species (ROS) are formed in the body as a result of ubiquitously occurring biological reactions involving electron transfer and hydrogen abstraction. Although there are benefits of oxidative species produced by cells, destruction of the cell’s structure, functions, and integrity can result, which has been associated with the propagation of various diseases (81). At the cellular level, when ROS surpass the reduction-oxidation balance threshold they cause oxidative stress. For example, highly reactive and promiscuous ROS can wreak havoc to macromolecules such as lipids,
proteins, and DNA which can lead to cellular dysfunction and disease (82). In particular, oxidative damage to DNA, lipids, and proteins, is associated with the pathogenesis of numerous diseases such as diabetes, cancer, inflammation, infection, neurodegenerative, and cardiovascular diseases (57, 81). The damage imposed on biological systems as a result of the imbalance of oxidative species is known as oxidative stress.

During states of oxidative stress the function and the role of oxidative processes in biological and macromolecular systems can be protected by antioxidants (57). Cellular defenses against oxidative stress involve utilizing both dietary and endogenous antioxidants to scavenge and quench ROS in the cell. These dietary, enzymatic and non-enzymatic antioxidant defenses are necessary to maintain antioxidant–prooxidant homeostasis (34). For example, antioxidants can be in the form of exogenous dietary antioxidant such as vitamins A, C, and E or in the form of endogenous cellular enzymatic and non-enzymatic antioxidants such superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione (GSH) (14). Abnormally high levels of free radicals cause membrane damage in part due to peroxidation of lipid membranes, protein glycation, and depletion of antioxidant defense enzymes all of which can lead to cell and tissue damage (57, 81). For example, enzymatic antioxidants, such as glutathione peroxidase, are reported to be lower in diabetes animals (83). Thus, when the balance is tipped in the direction of pro-oxidants then excessive amount of ROS are generated which can cause damage and/or dysfunction to multiple biological sites such as DNA, cells, membranes, as well metabolic pathways (81, 84).

A variety of techniques that measure biomarkers or end products of free radical oxidative processes are used to estimate oxidative stress (34). The endogenous enzymes such as SOD, GPx, and CAT are biomarkers of oxidative stress and can be measured to determine their in vivo activity against ROS (18, 23, 57). Additionally, parameters such as the oxygen radical absorbance capacity (ORAC) in plasma, serum, urine, and/or saliva can be measured (34). Other biomarkers associated with lipid peroxidation such as thiobarbituric reactive acid substances (TBARS) and peroxidation end products like malondialdehyde (MDA) can be measured as indicators of oxidative stress in vivo and have been found to be elevated in individuals with diabetes (17, 34).
Oxidative stress has been suggested to play a role in the pathogenesis of complications associated with obesity and diabetes (14, 57, 85). For example, individuals diagnosed with diabetes and experimentally induced animal models have exhibited oxidative stress due to persistent and chronic hyperglycemia, resulting in diminished activity of antioxidant enzymes and the promotion of free radical generation (15-18). It is suggested that oxidative stress can be triggered by hyperglycemia which induces the generation of free radicals that contribute to the development and progression of diabetes and its associated complications (17-22). For example, type 2 diabetes is often characterized by pancreatic β cell dysfunction and insulin resistance, both of which may be a result of oxidative stress (15, 57, 85-88). The progression of type 2 diabetes is not only accelerated by oxidative stress but also plays a role in generating ROS as a result of chronic and persistent elevation of glucose concentrations. Another form of oxidative stress associated with type 2 diabetes involves the formation and accumulation of glycated proteins (referred to as advanced glycated end products, AGEs) due to the oxidation of sugars (i.e. glucose, sucrose, and maltose) (89). More specifically, AGEs constitute a mass of compounds formed by non-enzymatic reactions of reducing sugars with amino acids, peptides, proteins and lipids (90). It has been determined that glycated proteins can produce almost 50 times more radicals than non-glycated proteins (91). Free radicals can keep this pathway of destruction cycling as they have been shown to participate in AGEs formation (81). Additionally, the binding of AGEs to their specific receptors results in the generation of ROS and thus more oxidative stress (92). The good news is that antioxidants have been shown to inhibit AGE formation and ROS generation (93, 94). These are beneficial qualities in light of the findings showing that increased AGE formation are implicated as one of the key mechanisms that is involved in complications attributed to hyperglycemia (89).

In diabetes, a reduction in antioxidant defense reservoirs may begin with a low intake of protective antioxidants and phyto-compounds in the diet (34). Phyto-phenolic intake has been found to be inversely correlated with higher plasma lipid peroxidation (34). Additionally, antioxidant enzymes may be depleted in obese individuals who are at an increase risk of developing type 2 diabetes (95). Consequently, these population would be susceptible to oxidative stress and this has been demonstrated in a rat model using a
In the early stages of obesity there may be an initial elevation in antioxidant enzymes to counteract oxidative stress, whereas chronic obesity associated ROS persistently deplete the reservoirs of antioxidant enzymes (96, 97). Overall, there is a growing recognition that long term oxidative stress is associated with diabetes in part because of the increase in ROS generated from hyperglycemia, production of glycation endproduct, and depletion of both exogenous and endogenous antioxidant reservoirs (14, 98). To this end, antioxidant rich phenolic compounds found in nature may not only lower hyperglycemic states in vivo but also play a corollary role in lowering oxidative stress induced by type 2 diabetes.

**Inflammation**

Research indicates that low-level inflammation has a direct role in the development of diseases and associates with disease risk. In particular, data suggest that oxidative and inflammatory stresses are implicated in the alterations of insulin response, and therefore promote diabetes (24-26). Insulin has been shown to reduce proinflammatory transcription factors and thus have an anti-inflammatory effect (30). For example, inflammatory cytokines and acute phase proteins such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 (IL-1), and C-reactive protein (CRP) play active roles in the development of insulin resistance associated with type 2 diabetes (31). As a result of the associated oxidative stress, type 2 diabetes may be an inflammatory condition (29). In addition to insulin resistance, oxidative stress can cause inflammation by stimulating an increase in proinflammatory transcription nuclear factor (NFκB)(99). NFκB is considered a proinflammatory gene regulator because it is a transcription factor that is known to induce the gene transcription of more than 200 proinflammatory genes (100). For example, NFκB will cause a proinflammatory immune response by inducing the transcription of the proinflammatory cytokines such as TNF-α, IL-1, IL-6, and CRP (99). Evidence suggests that obesity and type 2 diabetes are associated with these proinflammatory molecules as a result of the ROS associated with these conditions (30, 99). Furthermore, studies are beginning to establish a multi-faceted
association between oxidative stress, inflammation, hyperglycemia, type 2 diabetes, and obesity \((30, 34)\). For example, during states of hyperglycemia there is an increased generation of ROS and activation of proinflammatory transcription factors \((99)\). In particular, hyperglycemia in a post-prandial state has been shown to increase the production of plasma cytokines that are associated with inflammation such as CRP \((32, 33)\). In another study, dietary restriction in an obese population for 4 weeks resulted in a significant reduction in oxidative stress and inflammatory mediators \((101)\). The authors suggested that overconsumption of macronutrients which results in an influx of circulating blood glucose may be associated with the generation of oxidative and inflammatory stress \((101)\). Collectively, there is growing evidence which supports the hypothesis that there may be a link between obesity, type 2 diabetes, hyperglycemia and inflammation.

A working hypothesis in this area of research is that delaying the establishment of type 2 diabetes through achieving and maintaining blood glucose control may reduce the probability of initiating persistent low-level inflammation. A therapeutic intervention that has “extra glycemic” effects may reduce long-term complications associated with the disease associated with oxidative stress and corollary inflammation. Furthermore, a goal of health professionals is to address diabetes in the early or developing stages, when the metabolic abnormalities such as hyperglycemia or insulin resistance are less severe \((102, 103)\). Unmanaged hyperglycemia associated with type 2 diabetes worsens not only oxidative status but also inflammatory status. The link between oxidative stress, proinflammatory states, and hyperglycemia associated with type 2 diabetes continues to be intensely investigated. Naturally derived compounds may have antidiabetes bioactive properties.

**Nutraceutical Potential of Grape Extracts**

With the surge in the incidence of type 2 diabetes worldwide there is an expanding interest in the area of naturally derived antioxidants with bioactive properties as a new strategy for addressing hyperglycemia and the parallel oxidative and inflammatory stress associated with diabetes \((27)\). For example, in the past three decades considerable research efforts have been devoted to the discovery and development of
natural or plant-derived α-glucosidase inhibitors that may be promising oral anti-diabetes agents for the treatment of diabetes (104-109). Mechanistically, phenolic compounds found in plants (i.e. tea, raspberries, strawberries, blueberries, and blackcurrants) have been found to be involved in the inhibition of α-glucosidase (109-111). Green tea extract, for instance, has been shown to inhibit rat intestinal α-glucosidases with a 50% inhibition concentration (IC$_{50}$) of 0.735mg/ml (109). The oral administration of green tea extract (300 mg/kg bw for 4 weeks) was found to remarkably reduce the fasting blood glucose (by 54%) in STZ-induced diabetic rats (112). In another investigation, a procyanidin extract derived from pine bark extract (Pycnogenol®) was found to potently inhibit the α-glucosidases associated with type 2 diabetes (104, 113). Other naturally derived phenolics such as anthocyanins have been identified as having α-glucosidase inhibitory activity (110). The area of natural product research is growing exponentially worldwide and more bioactive compounds are anticipated to be identified and further investigated for their medicinal utilities.

**Grapes**

The global production and consumption of grapes remains high with growing consensus of potential health promoting properties in the matrix of compounds found in this fruit (39). Grapes have been well characterized as a natural source of notable bioactive phyto-compounds with antioxidant, health promoting, and disease protective qualities (28-30). For example, over 1600 phytochemicals have been identified in grapes including resveratrol, anthocyanins, catechins, ellagic acid, lutein, melatonin, lycopene, quercetin, and many other potent antioxidants (39). As such, grapes constitute one of the major sources of phenolic compounds among different fruits (114). The rich polyphenolic compounds found in grape seeds, skins, and stems have been reported to account for their health promoting and disease protective qualities (28-31). For example, antioxidant phenolic compounds like phenolic acids, polyphenols, and flavonoids are believed to scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyyl and thus inhibit the oxidative mechanisms that lead to degenerative diseased conditions (i.e. diabetes, Alzheimer’s, atherosclerosis, and certain cancers) (115, 116).
The antioxidant activity of grapes has been positively correlated with their phenolic composition such as anthocyanins, flavonols, flavan-3-ols, procyanidins and phenolic acids (117-121). Additionally, these compounds with antioxidant potential have been shown to reduce hydroperoxide formation and inhibit both lipid and protein oxidation (122). Furthermore, a variety of research has provided promising evidence that grapes may reduce the risk of cardiovascular disease by decreasing platelet aggregation, improving endothelial function, and reducing blood pressure (39). The grape phenolic extract has also demonstrated in vitro protection of low density lipoprotein from oxidation and prevention of oxidative injury by stimulating endogenous antioxidant enzyme systems (121, 123). The antioxidant potential of grapes has been studied for the prevention of certain cancers such as colon and skin cancers (39). Although the medicinal uses on grape research are expanding, studies investigating the biological effects of grape extracts on diabetes and their implication on oxidative stress are limited.

An anti-diabetes effect was demonstrated when grape-derived extracts with high amounts of procyanidins were supplemented to high fructose fed rats which resulted in improved insulin resistance and suppressed oxidative stress (30). It is possible that grape derived phenolic compounds may have dual or multiple benefits not only for their role in blood glucose control but also as a naturally rich antioxidant which can reduce the generation of ROS (32). Thus, grapes may be a cost-effective, alternative anti-hyperglycemic source for treating diabetes. The use of grape extracts (GE) as a nutraceutical with respect to diabetes treatment may hold tremendous advantages compared to currently available and often expensive α-glucosidase inhibitor drugs (4, 13, 66). In contrast to currently available α-glucosidase inhibitors, grape products are consumed worldwide everyday in the form of whole grapes, raisins, wine, and grape juice and are already considered GRAS. To this end, developing GE into a safe and efficacious nutraceutical would be rationale and advantageous. Recent animal studies have shown that the oral ingestion of grape skin extract at a dose of 2000 mg/kg had no acute toxicity or mutagenicity in mice, and no adverse effects were observed in rats after administration of grape skin extract at 2150 mg/kg bw/day for 3 months (124, 125). Additionally, very few people have allergy problems associated with grapes, which is an additional benefit for achieving a GRAS status (39). Overall, these data support the safety
of grape phenolic extract as a dietary component for human consumption and more grape research is needed to add to the body of literature regarding its potential anti-diabetes properties.

Taking advantage of the bioactive and antioxidant potential that grapes contain is an added niche for the lucrative grape industry (126). In the state of Virginia the wine grape industry has grown to more than 100 commercial wineries. The further elucidation and characterization of the overall antioxidant profiles and the potential of health protection found in Virginia grown grapes would enhance this industry in the Commonwealth and at the same time provide consumers additional awareness of the benefits of consuming foods comprised of phenolic compounds, such grapes, raisins, juices, and wines. In particular, the Virginia native Norton red wine grape (*Vitis aestivalis*) has grown in popularity, especially in Southern and Midwestern states in addition to Virginia such as, Missouri, and Arkansas (127). This particular grape variety has good wine characteristics and is highly adaptable to local growing conditions, primarily humid regions with comparatively long growing seasons (128). The Norton grape is generally resistant to the primary fungal diseases, which has enhanced its attractiveness to wine growers due to increasing concerns on environmental protection and pesticide avoidance (128). However, information about the beneficial components and bioactive health promoting qualities of the Norton grape is very limited, especially in regards to its phenolic profile and antioxidant properties (129).

In the state of Virginia, grapes are a major crop grown primarily for winemaking. Virginia wine grape production has been steadily increasing from 2004 (3,400 tons; $4.42 million) to 2006 (6,500 tons; $9.36 million) (126). The grape remains an important agricultural crop in the United States where in 2007, almost 7 million tons of grapes were produced with more than half grown for wine production (136). Winemaking generates the accumulation of grape pomace at an estimated amount of approximately 20% of the harvested grapes. As such, grape pomace continues to accumulate and may be an exceptional bioresource of value-added compounds with therapeutic properties (130, 137).
Grape Pomace

Because grapes are readily accessible in virtually unlimited supply, the cost for using this agent for diabetes prevention and management is of interest to scientists and the grape agriculture sector. Furthermore, grape pomace is an accumulating biomass in the wine industry and is attracting more interest as a potential useful medicinal byproduct (130, 131). Although grape pomace is generally regarded as a waste product a potential opportunity exists due to the phenolic compounds comprised in the skin and seeds of grape pomace. Potential uses of grape pomace as a source for novel therapeutics for addressing diseases is limited (130, 132-135). No research has addressed the utility of grape pomace for blood glucose control. Recently our lab discovered for the first time that grape pomace extract (GPE) potently inhibits intestinal $\alpha$-glucosidases, key enzymes in the digestive system responsible for starch digestion and absorption. Furthermore, we demonstrated that oral administration of GPE significantly improved postprandial glycemic control in mice induced with diabetes. It is speculated that glucose-lowering properties of grape pomace has the potential to greatly increase the value of grape crops and financially benefit grape cultivators.

Similar to its parent source, grape pomace is a biomass known to contain a variety of phenolic compounds which provide for its potential antioxidant property. Phenolic components such as flavonols and resveratrol remain in pomace after wine production (138). Some research indicates that pomace may be a potential rich bioresource of antioxidant and active polyphenolic compounds, such as anthocyanins and flavonols (139). For example, the antioxidant protective capacity of phenols was shown in rats pretreated with a grape pomace extract containing gallic acid, catechin, epicatechin, and procyanidins followed by free radical exposure (CCl$_4$) in which there was a reduction in liver lipid peroxidation (130). The authors of this study suggested that the antioxidants from the grape pomace administration was due in part to the phenols scavenging the free radicals (130). Further evidence of grape phenolic antioxidant protection was observed when a mixture of polyphenols from grape seed extract (300 mg) in a meal reduced postprandial plasma lipid peroxidation and enhanced plasma antioxidant capacity (140).

Another example of antioxidant utility of a byproduct similar to grape pomace was shown with a pomegranate juice byproduct was found to have antioxidant properties
(141). In particular, after eight weeks of pomegranate peel supplementation cattle gained more weight than the animals on standard feed. Additionally the animals supplemented with the pomegranate byproduct had higher blood levels of alpha-tocopherol. Overall, the authors explained that the pomegranate peels enhanced bodyweight similar to that of antibiotics and hormones without unwanted side effects and the peels may yield meat with higher levels of beneficial antioxidants (141). Similar to pomegranate pomace, grape pomace may have a corollary benefit not only for its role in blood glucose control but also as an antioxidant which can reduce the generation of reactive oxygen species (ROS). For example, compounds with antioxidant and antiglycation properties have been found to be effective in addressing diabetes (142). To this end, grape pomace, a rich antioxidants source, may be beneficial in attenuating the formation and accumulation ROS which would promote health. Additional research is needed to determine if pomace can be leveraged as a having therapeutic potential in addressing health conditions such as diabetes and promoting health due to its inherent polyphenolic composition.

In summary, with an unprecedented surge in obesity globally and incidence of diabetes rising, there is an undisputable need to discover new treatments (1). Grape phenolic compounds from sources like the skin and pomace of this fruit may be useful for improving blood glucose levels. Because grape pomace is currently treated as a low-value remnant byproduct, providing evidence of these bioactive properties may significantly enhance the overall value of grape production in the state of Virginia and other grape producing regions, in the US and across the globe. In terms of improving blood glucose control, the general biological mechanism that reflects this potential bioactive property through specific inhibition on α-glucosidase by grape phenolic extracts is presented in Figure 2.2.
Figure 2.2. Alpha-glucosidase inhibition by grape extract (GE) in the small intestine.

The schematic above illustrates the enzymatic digestion of carbohydrate (starch and sucrose) in biological systems and the proposed inhibition of intestinal $\alpha$-glucosidase by grape extracts (GE) and subsequent decrease in blood glucose levels that are often exacerbated in populations at risk for or diagnosed with type 2 diabetes.

Conclusion

It is well acknowledged that diet and physical activity strategies are an effective means of reducing the risk of developing type 2 diabetes for individuals, but may not be a realistic prescription, as evidenced by the steady rise in obesity rates and the low proportions of individuals who meet physical activity recommendations. The search for natural $\alpha$-glucosidase inhibitors for treating hyperglycemic conditions in combination with conventional therapies is of importance in order to reduce the risk of developing more debilitating diabetes-associated complications (i.e. neurological, renal, and cardiovascular). Furthermore, oxidative stress arising from the production of ROS has been implicated in type 2 diabetes as a result of hyperglycemia (15, 17, 19, 20, 87, 93, 143-146). Collectively, biomarkers of oxidative stress can be measured from techniques
using plasma ORAC and specific AOX enzymes. The results from these oxidative parameters help to clarify the health status of oxidative stress associated conditions like diabetes in vivo. Preliminary grape phenolic extract findings have provided the bases for the continuation of this research interest; in particular, specific grape extracts displayed an extremely potent inhibitory activity on α-glucosidase. These naturally grape-derived α-glucosidase inhibitors not only could be developed into antidiabetes agents but also be formulated into functional foods or dietary supplements for the prevention and management of type 2 diabetes. In particular, grape extracts may suppress the activity of α-glucosidase, thereby delaying the appearance of glucose in the blood after a meal. To this end, the grape and grape pomace phenolic compounds may be potent inhibitors of mammalian intestinal α-glucosidases as well as a potent antioxidant. Naturally derived oral hypoglycemic agent may have the potential to be developed as a novel, tolerable, and cost-effective anti-diabetes agents. Type 2 diabetes is a complicated metabolic condition which provides many interesting avenues of investigation and merits additional research to clarify our understanding of the role that natural compounds such as grape extracts play in blood glucose control, oxidative stress, and inflammation associated with type 2 diabetes.
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CHAPTER 3: ANTIOXIDANT PROPERTIES AND BIOACTIVE COMPONENTS OF NORTON (*Vitis aestivalis*) AND CABERNET FRANC (*Vitis vinifera*) WINE GRAPES

**Abstract**

Three wine grapes, Norton (*Vitis aestivalis*), Cabernet Franc clone1, and Cabernet Franc clone313 (*Vitis vinifera*), collected from a Virginia vineyard, were evaluated and compared for their antioxidant properties and phenolic profiles. All grape extracts exerted notable antioxidant activities. Their ORAC values for these particular varieties were not significantly different from one another, ranging from 22.9 to 26.7 µmol TE/g of fresh grape. The Cabernet Franc clone1 grape was determined to have the most DPPH• radical scavenging activity (8.8 µmol TE/g) compared to the Norton or Cabernet Franc clone313 extracts (7.9 µmol TE/g and 5.4 µmol TE/g, respectively). The Norton grape variety contained significantly higher total phenolic, anthocyanin, and flavonoid content than either of the Cabernet Franc grapes (P < 0.05). The hydroxybenzoic acids were the major phenolic acids in all the grape extracts with gallic acid dominating and representing 55%, 60%, and 65% of total phenolic acid content in the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 grapes, respectively. The Norton grape variety was found to be rich in malvidin-diglucoside and malvidin-glucoside, while the malvidin-diglucoside was negligible in the Cabernet Franc grape variety. The results suggest a varietal difference of grapes on antioxidant properties and phenolic compound composition in Virginia-grown grapes typically used for vinification. This study adds to the body of evidence of the health promoting potential of grapes, especially in regards to the less known Virginia native Norton grape.

**Keywords**

Red wine grapes, Norton, *Vitis aestivalis*, phenolic composition, antioxidant properties
Introduction

A growing body of epidemiological studies has associated the consumption of grapes, wine, and grape juice with a wide variety of health-promoting effects particularly the reduced risk of cardiovascular diseases (1-12). The beneficial effects of grape and relevant grape derived food products are believed to be related to the phenolic compounds (13, 14). Grapes constitute one of the major sources of phenolic compounds among fruits (15). Antioxidative phenolic compounds like phenolic acids, polyphenols and flavonoids have been shown to scavenge free radicals such as superoxide, peroxyl, and hydroxyl radicals, and thus impact on the reduction/oxidation (redox) mechanisms that may lead to degenerative diseased conditions (i.e. Alzheimer’s, atherosclerosis, diabetes, and certain cancers).

The antioxidant activity of grapes has been positively associated with their phenolic composition such as anthocyanins, flavonols, flavan-3-ols, procyanidins, and phenolic acids (16-20). Additionally, these compounds have been shown to reduce hydroperoxide formation and inhibit both lipid and protein oxidation (21). The grape phenolic extract has also demonstrated, in vitro, the potential to protect low density lipoprotein (LDL) from oxidation (20) and prevent oxidative injury by modulating the expression of antioxidant enzyme systems (22). In addition to antioxidant protection, the grape phenolics have been reported to inhibit platelet aggregation (23), and exert antimicrobial (24), anti-inflammatory (25), and anti-aging activities (26). Concord grape seeds rich in procyanidins have been reported to have potential vasodilatation properties (27, 28). Overall, there exists scientific evidence of the health promoting qualities of consuming grapes and grape derived products.

The Norton grape (Vitis aestivalis) has grown in popularity, especially in Southern and Midwestern states such as Virginia, Missouri, and Arkansas (29). This particular grape variety has good wine characteristics and is highly adaptable to local growing conditions, primarily humid regions with comparatively long growing seasons (30). Moreover, the Norton grape is generally resistant to the primary fungal diseases, which has enhanced its attractiveness to wine growers due to increasing concerns on environmental protection and pesticide avoidance (30). However, information about the
beneficial components and bioactive health promoting qualities of the Norton grape is very limited, especially with regard to its phenolic profile and antioxidant properties. To this end, the purpose of this study was to characterize the phenolic composition and antioxidant properties of the Virginia native Norton grape compared to the Cabernet Franc (*Vitis vinifera*) wine grape from extracts that were analyzed prior to any vinification processing.

**Materials and Methods**

**Sample preparation**

Three Virginia wine grapes: Norton, Cabernet Franc clone 1, and Cabernet Franc clone 313 were evaluated in this study. The Norton grape is a North America native variety widely cultivated in Virginia vineyards for the principle purpose of making red wine. The two Cabernet Franc red wine grapes share the same genetic background but with slight mutational differences (as clones 1 and 313). Each red grape variety was collected from three vineyard blocks from the Blackstone vineyard (Blackstone, VA) using a randomized complete block (RCB) design. Each block, consisting of three vine panels, was analyzed for antioxidant properties and component composition. The obtained results represent the mean measurement taken from 3 blocks. After removing the seeds, 45.0 g of pulp and skin were homogenized in 100 mL of methanol, and then placed in a water bath (Thermo Scientific, San Jose, CA) under shaking for 30 minutes. The extracts were filtered through Whatman No. 4 filter paper. The residues were extracted in 100 mL of 70% aqueous methanol (v/v) in a water bath under shaking for 30 minutes. Following filtration, both filtrates were combined. Extracts were concentrated in a rotary evaporator at 45°C to a final volume of 45 mL. The sample grape extracts were then lyophilized and stored at -20°C until further investigation.

**Total phenolic content (TPC)**

The total phenolic content (TPC) of grape extracts was determined using Folin-Ciocalteu reagent (Sigma, St. Louis, MO) with gallic acid as the phenolic standard (31). In brief, appropriate dilutions of extracts were mixed with Folin-Ciocalteu reagent and
saturated sodium carbonate solution at ambient temperature. After incubation for 2 hours, the absorbance of blue color developed in each assay mixture was recorded at 760 nm using a Genesys 10-UV scanning (Thermo Electron Corporation, Madison, WI). The TPC value of the grape extracts was expressed as milligram of gallic acid equivalent per gram of fresh grape tissue (mg/g).

**Total anthocyanin content (TAC)**

Total anthocyanin content (TAC) of the grape extracts was quantified using a pH differential method (32). This assay is based on the premise that the anthocyanin pigments undergo reversible structural modifications as a result of a change in pH. The colored oxonium or flavylium form predominates at pH 1.0 and the colorless hemiketal form or carbinol form is detected at pH 4.5 (32). As a result, the difference in absorbance is proportional to the anthocyanin content. To conduct the TAC assay, 100 mL of 0.025 mol/L potassium chloride (pH=1.0) and 0.4 mol/L sodium acetate (pH=4.5) buffer solutions were prepared. Each sample and standard (cyanidin 3-glucose) (Sigma, St. Louis, MA) was diluted with 0.025 mol/L potassium chloride buffer (pH 1.0) and the absorbance was measured at 520 nm and 700 nm with a UV spectrophotometer (Thermo Electron Corporation, Genesys 10-UV scanning, Madison, WI, USA) against a reagent blank (distilled water). A second aliquot of each sample was diluted to the same value with 0.4 M sodium acetate buffer (pH 4.5) and measured at 520 and 700 nm. The absorbance (A) was calculated using the equation: $A = (A_{520nm, pH=1.0} - A_{700nm, pH=1.0}) - (A_{520nm, pH=4.5} - A_{700nm, pH=4.5})$. The TAC was calculated and expressed as mg of cyanidin 3-glucoside equivalent per gram of fresh grape tissue (mg/g).

**Total flavonoid content (TFC)**

Each grape extract was analyzed for total flavonoid content (TFC) according to a previously reported colorimetric method with modification (33). Specifically, a volume of 1 mL of lyophilized grape extract or 1 ml of rutin standard (Sigma, St. Louis, MO) was mixed with 0.3 mL of 0.7 mol/L sodium nitrite (NaNO₂), 0.3 mL of 0.8 mol/L aluminum chloride (AlCl₃), and 2 mL of 1 mol/L sodium hydroxide (NaOH). All samples were analyzed in duplicate and compared against a blank at an absorbance of 510 nm (Thermo
Electron Corporation, Genesys 10-UV scanning, Madison, WI, USA). Results were expressed as milligram rutin equivalent per gram of fresh grape tissue (mg/g).

**Oxygen radical absorbance capacity (ORAC)**

The oxygen radical absorbance capacity (ORAC) assay was conducted to measure the peroxyl radical scavenging activity of each grape by following a method reported previously (34). A water soluble vitamin E analog was used as the standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™). A fluorescein stock solution (100 µmol/L) in phosphate buffer (75 mM, pH 7.4) was prepared and kept at 4°C in the dark. Fresh working fluorescein solution (100 nmol/L) was prepared by diluting the stock solution with the phosphate buffer. The fluorescein solution was added to each grape extract sample or Trolox standards prepared in phosphate buffer in a black 96-well plate and incubated for 20 minutes at 37°C. The reaction was initiated by adding the peroxyl radical generator, 0.36 mol/L 2,2′-Azobis-2-amidinopropane (AAPH), prepared in the phosphate buffer. The fluorescence was measured ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 535$ nm) every minute using a Victor³ multi-label plate reader (Perkin-Elmer, Turku, Finland) maintained at 37°C until the reading had declined to less than 5% of the initial reading (e.g. 20 minutes). Standards and samples were run in triplicate. Results for ORAC were determined by regression relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value of each grape extract was expressed as micromoles of Trolox equivalents per gram sample (µmol TE/g).

**DPPH radical scavenging activity**

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging antioxidant capacity assay was determined by using a previously reported method (35). This antioxidant capacity assay, based on the reduction of the free radical DPPH, was carried out spectrophotometrically (Victor³ multi-label plate reader, PerkinElmer, USA). The reaction mixture contained 100 µL of sample extract and 100 µL of 0.208 mM DPPH’ solution. The absorption at 515 nm was determined immediately after the reaction was initiated. Each plate was read once every minute for 1.5 hours. The relative DPPH’
scavenging capacity for each grape extract sample was expressed as micromoles of Trolox equivalents per gram of sample (µmol TE/g).

**HPLC analysis of phenolic acid composition**

Grape phenolic acid profiling was performed on an Agilent 1200 quaternary high performance liquid chromatography system (Agilent Technologies, Palo Alto, CA) equipped with a photodiode array detector. Phenolic acids were separated on a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size 5µm) (Phenomenex, Torrance, CA) using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H$_2$O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H$_2$O, 2:30:68, v/v/v) (36). The solvent gradient was linear programmed from 10 to 100% solvent B in 42 minutes with a flow rate of 1.0 mL/min. Identification of phenolic acids was accomplished by comparing the retention time and absorption spectra of peaks to the standard compounds. Quantification of individual phenolic acids was conducted using the total area under each peak with external standards.

**HPLC-MS determination of anthocyanin composition**

Chromatographic separation was carried out on a reverse-phase C18 Alltech Prevail column (150mm x 4.6mm, particle size 5 µm) (Alltech Associates, Deerfield, IL) using 5% formic acid in water (v/v) (A) and methanol (B) as the mobile phases. The gradient condition was 0-2 min, 15% B; 2-30 min, 15-45% B; and 30-45 min, 45-15% B (v/v). Other chromatographic conditions were as follows: flow rate, 0.8 mL/min; dual detection, 380 nm and 525 nm. Anthocyanins in grape extracts were analyzed on a Hewlett-Packard 1100 coupled with a DAD and a Finnigan LCQDUO mass spectrometer (Thermo Scientific, Waltham, MA). The mass spectrometer was operated in positive electrospray ionization at a temperature of 350º C and a voltage of 4 kV. Mass scan spectra were measured from m/z 200 up to m/z 700 at 1000 amu/second.

**Statistical Analysis**

Data were reported as mean ± SD (Standard Deviation) for triplicate determinations. The mean values within each test were compared by a two-sample *t*-test.
Significance of variety differences was determined by analysis of variance. Difference was considered statistically significant when the P-value was < 0.05. A two-tailed Pearson's correlation test was conducted to determine the correlations among means.

**Results and Discussion**

**Total phenolic content**

The concentrations of total phenolic content (TPC) in three Virginia-grown grape varieties are presented in Table 3.1. The Norton grape revealed the highest TPC at 1.8 mg gallic acid equivalents/g fresh grape weight, followed by Cabernet Franc clone1 (1.5 mg GAE/g) and Cabernet Franc clone313 (0.6 mg GAE/g). Cabernet Franc clone313 had significantly lower TPC compared to the Norton or Cabernet Franc clone1 (P < 0.05), although both Cabernet Franc grapes shared similar genetic backgrounds. This distinct difference in TPC values suggests that Virginia-grown Norton grapes may be a better source for phenolic compounds than Virginia-grown Cabernet Franc grapes and even a slight genetic alternation can significantly affect grape phenolic content. However, the TPC of Cabernet Franc clone313 grape is comparable to previous findings which reported that three seedless red grape varieties (Thomson, Flame, and Black) contained TPC of 0.26, 0.85 and 0.92 mg GAE/g, respectively (37). Others have reported a slightly higher phenolic content in red wine grapes ranging from 1.4 to 3.1 mg GAE/g fresh grape weight (38). The TPC values determined for the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 red wine grapes had higher TPC values than seven red table grapes, which varied from 0.13 to 0.36 mg/g (39). This difference may be a consequence of the influence of geographical location in addition to varietal differences. In particular, grape phenolics may be significantly affected by growing conditions related to the geographical region of the cultivar such as soil composition, irrigation, light intensity, etc (39, 40). For example, the TPC in Flame seedless grapes was more than two times higher when grown in the USA (0.86 mg/g) compared to being grown in Spain (0.36 mg/g) (37, 39). To minimize the interfering influence of these agronomic factors, the grape samples investigated for this study were collected from one harvest season (fall 2007) and from three different blocks of each variety.
Total anthocyanin content

Red grapes and wines are a major source of dietary anthocyanins which can be absorbed as intact or hydrolyzed molecules after ingestion ([41]). The total anthocyanin content (TAC) of the grape extracts are presented in Table 3.1. The Norton grape was detected to have the highest TAC (0.93 mg/g), followed by the Cabernet Franc clone1 (0.64 mg/g) and the Cabernet Franc clone313 (0.17 mg/g). These differences suggest a potential impact of grape variety on anthocyanin content. The TAC of the Norton and Cabernet Franc grapes was lower than previous reported ranges from 1.0 to 2.8 mg C3GE/g measured from 7 different red wine grapes ([42]). However, the Virginia-grown Norton and Cabernet Franc wine grape varieties had higher anthocyanin content compared to previously reported table grapes with 0.07 to 0.15 mg anthocyanins per gram ([39]). The TAC in Norton grape skins grown at the Purdue University Research Farms in the state of Indiana was determined to be 8.8 mg/g ([43]). On average, the skin accounts for approximately one tenth of the weight of a whole fresh grape. Hence, the estimated anthocyanin content reported in the Indiana-grown grapes would be about 0.9 mg/g, which is comparable to the values determined in the Virginia-grown grapes for the present study. In the Norton grape variety, anthocyanins were the major phenolic compounds accounting for over half of the TPC. In comparison, the contribution of anthocyanin content in the Cabernet Franc clone313 was determined to be less than one third of the TPC. The TAC for both the Virginia-grown Norton and Cabernet Franc variety correlated significantly with the TPC (P < 0.05).

Total flavonoid content

The three wine grape extracts varied significantly in total flavonoid content (TFC) ranging from 0.48 mg RE/g for the Cabernet Franc clone313 to 1.19 mg RE/g for the Norton (P < 0.05), Table 3.1. The flavonoid range of these grapes was slightly higher than that of seven table grape varieties previously reported, which ranged from 0.13 mg/g to 0.31 mg/g ([39]). Furthermore, it was determined that the flavonoids of the wine grapes analyzed in this study substantially contributed TPC (66-76mg flavonoids/100mg TPC). These results agree with previous findings that flavonoids constitute the majority of
phenolic compounds in grapes. In the Norton grape variety, 78% of flavonoids were determined to be anthocyanins. In comparison, the Cabernet Franc clone313 grape was calculated to be comprised of 65% of non-anthocyanin flavonoid compounds. The TFC of all three Virginia-grown wine grapes investigated in this study was significantly correlated with TPC and TAC (P < 0.05).

**Oxygen radical absorbance capacity (ORAC)**

All three Virginia-grown wine grape extracts displayed strong ORAC values ranging from 22.9 to 26.7 µmol TE/g of fresh grape (Figure 3.1). The variation in ORAC values among the three different grapes was much smaller than the differences determined for their TPC, TAC, and TFC. The ORAC values for the Virginia-grown wine grape extracts were lower than methanolic extracts of 11 grape varieties grown in India where ORAC values were reported to range from 43.7 to 46.8 µmol TE/g (16). However, our detected ORAC range was significantly higher than that of a red grape acetone extract which was reported to be 7.4 µmol TE/g (44). The differences of these ORAC results compared to our values may be explained in part by the varietal differences of grapes for each study, vineyard/geographical location, as well as the differences in extraction preparations for antioxidant estimation. Interestingly, the Cabernet Franc clone313 was found to have a higher ORAC value than the Norton variety, despite the latter having twice the amount of TPC. There was no statistically significant correlation detected between the TPC and ORAC value for either of the Norton or Cabernet Franc varieties. The ORAC assay was utilized in this study to measure the activity of the grape extract against peroxyl radicals. This suggests the ability of the grape extract to scavenge peroxyl radicals is not solely dependent on their phenolic compound content. It is likely that there are other components in the extracts capable of interacting with peroxyl radicals as well as other reactive species.

**DPPH radical scavenging activity**

Both of the Virginia-grown grape varieties exerted DPPH’ scavenging activity. The highest DPPH’ scavenging activity was detected in the Cabernet Franc clone1 (8.8 µmol TE/g) followed by the Norton (7.9 µmol TE/g) and Cabernet Franc clone313 (5.4
µmol TE/g) (Figure 3.2). No correlation was detected between the DPPH• scavenging activities of the grape extracts and their ORAC values. This may be due to the possibility that the grape extracts were involved in additional and/or separate redox scavenging mechanisms while interacting with the specific radicals associated with the DPPH assay. For example, the ORAC assay was conducted with the purpose of assessing the antioxidant activity against peroxyl radicals. As such, a report of higher ORAC activity does not necessarily suggest a stronger DPPH• scavenging ability.

**Phenolic acid composition**

Phenolic acids are one of the major phenolic compounds found in grapes. Grape phenolic acids have been shown to exert strong antioxidant and anti-cancer activities (45, 46). The consumption and ingestion of red wine significantly increases plasma levels of phenolic acids such as caffeic acid and 4-O-methylgallic acid (45, 47). The phenolic acids in grapes are divided into two groups based on their chemical structures: hydroxybenzoic and hydroxycinnamic derivatives. The hydroxybenzoics includes gallic, syringic, vanillic, and ferulic acids, while the hydroxycinnamates consists of caffeic, caftaric, and coumaric acids. Gallic acid is reportedly the main phenolic acid in both grape skin and seeds (48). In red wine, gallic acid also is the predominant phenolic acid and accounts for 46% of total phenolic acid content in wine (49). These findings are similar to the gallic acid values detected in the Virginia-grown Norton and Cabernet Franc varieties of this study. In particular, the gallic acid components accounted for 55%, 60%, and 65% of total phenolic acid content in the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 grapes, respectively (Table 3.2). Other phenolic acids detected in these selected Virginia-grown grapes include chlorogenic, vanillic, caffeic, coumaric, and ferulic acids. Gentisic, 4-OH benzoic, and syringic acids were not present in detectable amounts in the extracts. Total phenolic acids of 131.8 µg/g in the Norton grape were more than 4 times higher than that of the Cabernet Franc clone1 (28.1µg/g) and Cabernet Franc clone313 grapes (25.7 µg/g). As a comparison, these ranges are lower than that of an acetone-methanol-water extract of three major Champagne grape varieties (330-480 µg/g fresh weight)(50). The hydroxycinnamic acid levels of 2.9-9.2 µg/g in the Norton and Cabernet Franc grapes were comparable to the reported range of
2-26 µg/g measured from nine red grapes (38). Furthermore, the hydroxycinnamic acid content of the Norton (9.2 µg/g) and Cabernet Franc clone1 (6.8 µg/g) grapes were similar to that of the Red Globe grape (8.4 µg/g) (39). The phenolic acid profiles of the Norton and Cabernet Franc grapes were different. The Norton grape was dominated by gallic and vanillic acids, which accounted for 92.6% of total phenolic acid content in the grape. No chlorogenic or coumaric acids were detected in the Norton grape. The Cabernet Franc clone1 and Cabernet Franc clone313 grapes shared a similar phenolic acid profile except that Cabernet Franc clone1 was found to contain a notably higher caffeic acid content than the Cabernet Franc clone313.

**Percent anthocyanin composition**

Five anthocyanin glucosides were detected in the three Virginia-grown wine grapes including malvidin-diglucoside, petunidin-glucoside, peonidin-glucoside, malvidin-glucoside, and cyanidin-diglucoside (Table 3.3). The anthocyanin profiles were distinctly different between the three grape varieties. In particular, the major anthocyanins in the Norton grape variety were malvidin-diglucoside and malvidin-glucoside, which accounted for 45% and 31% of the total anthocyanins, respectively. These findings were in agreement with previous studies conducted on the Norton grape which reported that malvidin glucosides are the major anthocyanins in Norton grape skin and wine (51). In contrast to the Norton, the Cabernet Franc grapes contained minor or non-detectable levels of malvidin-diglucoside. The most abundant anthocyanin in the Cabernet Franc grapes was malvidin-glucoside, which represented 78% and 46% of total anthocyanins in the Cabernet Franc clone1 and Cabernet Franc clone313, respectively. These results agree with previous reports suggesting that malvidin 3-glucoside was the major anthocyanin in the red wine grapes (52, 53). Interestingly, the anthocyanin compositions within the two Cabernet Franc grapes were found to be significantly different (P < 0.05). The Cabernet Franc clone313 grape contained substantially more cyanidin-diglucoside. This clone was determined to have cyanindin-diglucosides which accounted for 42% of the total anthocyanin content. However, this anthocyanin component was not detected in the Cabernet Franc clone1 grape samples. This distinct difference in anthocyanin compositions among the different Cabernet Franc clones could
be largely associated with their genotypes, which has been reported as an important
determinant of the phenolic composition in grapes (50, 54, 55).

**Conclusion**

In summary, all three Virginia wine grapes tested exhibited remarkable
antioxidant properties. However, their antioxidant activities and compositions were
considerably different although all the samples were collected from the same growing
season and location. Flavonoids were the major phenolic compounds in the wine grapes
tested. The Norton grape variety contained significantly higher total phenolic,
anthocyanin, and flavonoid content than the Cabernet Franc grapes (P < 0.05), indicating
an important impact of grape genotypes on their antioxidant content. This study adds to
the body of evidence of the health promoting potential of grapes including the less known
Norton (*Vitis aestivalis*) grape. Future studies are needed to address the effect of vintage,
seasonal variation, maturity of grape, and the influence of vinification on the antioxidant
properties and phenolic profile of wine grapes.

**Acknowledgments**

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### Table 3.1. Total phenolic, total anthocyanin, and total flavonoid contents of Norton and Cabernet Franc grape varieties

<table>
<thead>
<tr>
<th></th>
<th>Norton</th>
<th>Cabernet Franc clone 1</th>
<th>Cabernet Franc clone 313</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total phenolic content (GAE mg/g)</strong></td>
<td>1.82 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.47 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total anthocyanin content (C3GE mg/g)</strong></td>
<td>0.93 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.17 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total flavonoid content (RE mg/g)</strong></td>
<td>1.19 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

GAE mg/g, C3GE mg/g, and RE mg/g represent mg of gallic acid equivalents, mg of cyanidin 3-glucoside equivalent, and mg of rutin equivalent per gram of fresh grape sample, respectively. For each measurement, the data marked by different letters in a row indicate significant difference (P < 0.05).
Table 3.2. Phenolic acid composition of the Virginia grown wine grapes

<table>
<thead>
<tr>
<th></th>
<th>Norton (µg/g)</th>
<th>Cabernet Franc clone1 (µg/g)</th>
<th>Cabernet Franc clone313 (µg/g)</th>
</tr>
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<tbody>
<tr>
<td>Gallic acid</td>
<td>72.6 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Chlorogenic acid</td>
<td>ND</td>
<td>2.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Vanillic acid</td>
<td>49.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>9.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Coumaric acid</td>
<td>ND</td>
<td>4.2 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Ferulic acid</td>
<td>0.6 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total hydroxybenzoic acids</td>
<td>122.6</td>
<td>21.3</td>
<td>22.8</td>
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<td>Total hydroxycinnamic acids</td>
<td>9.2</td>
<td>6.8</td>
<td>2.9</td>
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<tr>
<td>Total phenolic acids</td>
<td>131.8</td>
<td>28.1</td>
<td>25.7</td>
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</tbody>
</table>

The phenolic composition of the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 grape extracts were determined by HPLC and expressed as µg/g fresh grape sample (mean ± SD, n=3). For each measurement, the data marked by different letters in a row are significantly different (P < 0.05).
Table 3.1. Anthocyanin composition of Norton and Cabernet Franc grape varieties

<table>
<thead>
<tr>
<th></th>
<th>Molecular ion (m/z)</th>
<th>Norton (% of total)</th>
<th>Cabernet Franc clone1 (% of total)</th>
<th>Cabernet Franc clone313 (% of total)</th>
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</thead>
<tbody>
<tr>
<td>Malvidin-diglucoside</td>
<td>655</td>
<td>45.5</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Petunidin-glucoside</td>
<td>479</td>
<td>5.5</td>
<td>2.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Peonidin-glucoside</td>
<td>463</td>
<td>3.3</td>
<td>10.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Malvidin-glucoside</td>
<td>493</td>
<td>31.2</td>
<td>77.9</td>
<td>46.1</td>
</tr>
<tr>
<td>Cyanidin-diglucoside</td>
<td>611</td>
<td>4.5</td>
<td>ND</td>
<td>42.3</td>
</tr>
<tr>
<td>Other</td>
<td>10.1</td>
<td>8.1</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Total area of peaks</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1. Oxygen radical absorbance capacity (ORAC) values of Norton and Cabernet Franc grape varieties. The ORAC values of the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 grape extracts were expressed as Trolox equivalents per gram of fresh grape sample (µmol TE/g) mean ± SD (n=3). Bars with different letters are statistically significant (P < 0.05).
Figure 3.1. DPPH radical scavenging capacity of Norton and Cabernet Franc grape varieties. The results of the Norton, Cabernet Franc clone1, and Cabernet Franc clone313 grape extracts were expressed as Trolox equivalents per gram of fresh grape sample (µmol TE/g) mean ± SD (n=3). Bars with different letters are statistically significant (P < 0.05).
CHAPTER 4: IN VITRO YEAST AND MAMMALIAN $\alpha$-GLUCOSIDASE INHIBITORY ACTIVITY OF NORTON VITIS AESTIVALIS GRAPE SKIN PHENOLIC EXTRACTS

Abstract

Because intestinal $\alpha$-glucosidase plays a key role in carbohydrate digestion and subsequent blood glucose absorption, the inhibition of $\alpha$-glucosidase provides a metabolic target for managing diabetes and improving post-prandial glucose control. Preliminary screenings of over 300 naturally derived extracts revealed that a grape skin extract (GSE) exhibited $\alpha$-glucosidase inhibitory properties. The enzyme inhibitory activity of a Norton ($Vitis aestivalis$) GSE was investigated and compared with other varieties ($Vitis vinifera$) of GSE. In yeast the $\alpha$-glucosidase inhibitory activity of Norton GSE was 32-times stronger ($IC_{50}=10.5\mu g/ml$) than acarbose ($IC_{50}=341.8 \mu g/ml$), a commercial oral hypoglycemic agent. When compared to the yeast enzyme, the Norton GSE was more effective at inhibiting yeast $\alpha$-glucosidase as compared to the rat intestinal $\alpha$-glucosidases. The GSE was determined to have an $IC_{50}$ value of 0.384 mg/mL on rat $\alpha$-glucosidases. Enzyme kinetic experiments demonstrated that GSE was a competitive inhibitor of both yeast and mammalian $\alpha$-glucosidases but had no inhibition on pancreatic $\alpha$-amylase or lipase, suggesting that GSE is likely a specific $\alpha$-glucosidase inhibitor. Three Norton GSE phenolic compounds, including resveratrol, ellagic acid, and catechin, were detected to strongly inhibit yeast $\alpha$-glucosidase activity. Additional studies are needed to identify the active $\alpha$-glucosidase inhibiting constituents in GSE and to clarify the inhibiting mechanism and possible synergistic effect of GSE constituent(s) on specific intestinal $\alpha$-glucosidases including sucrase, maltase, and isomaltase.

Keywords

Norton, $Vitis aestivalis$, grape skin extract, phenolic phytocompounds, $\alpha$-glucosidase, enzyme inhibition and kinetics
Introduction

An unprecedented 33 percent of U.S. adults over the age of 20 are obese, according to the U. S. Centers for Disease Control and Prevention (1). These numbers are especially alarming due to the fact that numerous deleterious health consequences manifest as a result of obesity such as coronary heart disease and type 2 diabetes. Furthermore, it is estimated that 57 million US citizens have impaired glucose tolerance, which if left uncontrolled the condition will eventually progress into type 2 diabetes, currently affecting 24 million Americans (2). One strategy to combat the escalating risk of developing diabetes is to reduce an individual’s blood glucose levels by addressing diet, exercise, and the use of oral hypoglycemic agents. Use of hypoglycemic agents reduce postprandial blood glucose levels, which is considered a key contributing factor in the development of type 2 diabetes (3).

Postprandial hyperglycemia is, in part, related to the amount and digestion rate of consumed starch, which is the primary source of blood glucose. A growing body of evidence suggests that inhibition of specific carbohydrate digestive enzymes (i.e. α-amylase and α-glucosidase) provides a beneficial effect for both preventing and treating type 2 diabetes by attenuating postprandial blood glucose levels (3, 4). To this end, synthetic compounds like acarbose have been developed and are widely used with the purpose of inhibiting these starch metabolism enzymes. However, acarbose and other commercially available inhibitors have their drawbacks due to availability, cost, and negative side effects (4, 5). As a result, naturally derived phyto-compounds are continuously being screened for their bioactivity regarding anti-hyperglycemia.

In terms of improving hyperglycemia, a variety of natural compounds have been identified as being beneficial in the treatment of diabetes (6-8). In particular, phenolic compounds found in plants (i.e. tea, raspberries, strawberries, blueberries, and blackcurrants) have been found to be involved in the inhibition of α-glucosidase (8-10). We recently identified grape skin phenolic extracts from the Virginia-grown Norton grape to potently inhibit α-glucosidase during our initial screening on more than 300 food extracts and compounds. As a popular natural antioxidant supplement, grape skin is
known to be comprised of a wide variety of bioactive phytochemicals especially phenolic compounds such as anthocyanins, flavonols, proanthocyanidins, and phenolic acids (11-13). Furthermore, a growing body of epidemiological and experimental studies has associated the consumption of grape derived products with health promoting qualities (14-17). To our knowledge, the preliminary screening revealed for the first time that the Norton grape skin phytopenolics have a strong inhibitory effect against α-glucosidase, a rational target for preventing and treating diabetes. The red Norton grape (Vitis aestivalis) is a wine grape grown in Southern and Midwestern states such as Virginia, Missouri, and Arkansas (18). This particular grape variety is highly adaptable to local growing conditions, primarily humid regions with comparatively long growing seasons and is generally resistant to the primary fungal diseases (19).

The purpose of this study was to investigate the effectiveness of a Norton grape skin extract and its phenolic constituents on yeast and rat intestinal α-glucosidases as well as other grape skin varieties and digestive enzymes for comparison. The initial experiments utilized yeast α-glucosidase, which is readily available in a pure form and has been widely used for preliminary nutraceutical and medicinal investigations as a model for screening potential inhibitors (7, 10, 20-23). Acarbose, a commercially available oral hypoglycemic agent and often used as a positive control, has been reported to have a more pronounced inhibitory effect on rat intestine α-glucosidase as compared to yeast α-glucosidase (24). This indicates that the difference in inhibitory activity of specific compounds depends on the origin of α-glucosidase species. As a result, in an effort to compare the inhibitory influence of grape derived extracts on α-glucosidase, both yeast and mammalian forms of α-glucosidase were used to further clarify the enzyme inhibitory effect of the GSE. Enzyme kinetic assays were conducted in order to establish the presence of a dose-dependent response, mode of enzyme inhibition (i.e. competitive, non-competitive, un-competitive or mixed), the inhibitor concentration which inhibits the enzyme activity by 50% (IC$_{50}$), and the inhibitor constant ($K_i$) which reflects the dissociation constant for inhibitor binding. Furthermore, open column fractionation and HPLC analysis were performed in an effort to identify specific phytochemicals in GSE with α-glucosidase inhibitory activity.
Materials and Methods

Materials

Yeast type I α-glucosidase (EC 3.2.1.20), rat intestinal acetone powder (N1377-5G), porcine pancreatic type VI-B α-amylase (EC 3.2.1.1), porcine pancreatic type II lipase (EC 3.1.1.3), p-nitrophenyl α-D-glucoside (pNPG), oleic acid 4-methylumbelliferyl ester, acarbose, orlistat, resveratrol, and phenolic acids standards were purchased from the Sigma Chemical Co. (St. Louis, MO). The solvents acetone, methanol, acetonitrile, trifluoroacetic acid, and acetic acid were HPLC grade (Fisher Scientific Co. Atlanta, GA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Grape skin extraction

Red wine Norton grapes (Vitis aestivalis) grown at the Blackstone vineyard (Blackstone, VA) were obtained in the fall of 2007 and delivered to the Enology Laboratory at Virginia Tech. The Norton grape variety is a native Virginia grape; its bioactive components and antioxidant profile have been previously investigated in our laboratory for antioxidant properties (25). The skins from grapes were hand-peeled and then lyophilized. The dried grape skins were milled to a fine powder with a Thomas Wiley mini-mill (Swedesboro, NJ) and extracted by four different solvents including water, 50% acetone, 80% acetone, and methanol at 1:10 ratio (m/v). The extracts were then filtered through Whatman No. 4 filter paper to remove unwanted residues. After removing the organic solvent, the filtrates were frozen and lyophilized (prepared in dry form by rapid freezing and dehydration, while in the frozen state under high vacuum) to obtain the GSE. For comparison, additional skin extracts were obtained from a white Chardonnay grape (Vitis vinifera), a red Cabernet Franc (Vitis vinifera), and a commercial grape skin extract. The commercial extract was an aqueous ethanol extract derived from a red grape skin (variety unknown) (RFI ingredients, Blauvelt, NY).
Measurement of yeast and mammalian $\alpha$-glucosidase inhibition

Both the yeast and mammalian $\alpha$-glucosidase activity was assayed using the substrate p-nitrophenyl-$\alpha$-D-glucopyranoside (pNPG), which is hydrolyzed by $\alpha$-glucosidase to release the product p-nitrophenol, a color agent that can be monitored at 405 nm (20). The assay was conducted by mixing 80 µL of the sample solution (1 mg/mL) with 20 µL of the enzyme solution (1 U/mL) and incubated in a 96-well plate at 37 ºC for 3 minutes under constant shaking. After incubation, 100 µL of 4 mM pNPG solution in 0.1 M phosphate buffer (pH 6.8) was added and the reaction was conducted at 37 ºC. The release of p-nitrophenol from pNPG was monitored at 405 nm every minute for 75 minutes spectrophotometrically with a plate reader (Victor 3, PerkinElmer, Turku, Finland). The $\alpha$-glucosidase activity was determined by measuring area under the curve (0-75 minutes) for each sample and compared with that of the control. The results were expressed as the percent of $\alpha$-glucosidase inhibition.

Measurement of $\alpha$-amylase inhibition

The $\alpha$-amylase inhibitory activity was determined by incubating a total of 500 µL of each GSE with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing $\alpha$-amylase solution (0.5 mg/mL) at 25 ºC for 10 minutes. After preincubation, 500 µL of a 0.5% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were then incubated at 90 ºC in a water bath for 10 minutes and cooled to room temperature. The reaction mixture was then diluted after the addition of 15 mL of distilled water, and absorbance was measured at 540 nm with the plate reader. The readings were compared with the controls containing buffer in place of the sample extract.

Measurement of lipase inhibition

Lipase inhibition was determined by measuring the amount of 4-methylumbelliferone product released by lipase spectrofluorometrically following a previously reported method, with modification (26). The reaction mixture was prepared
with 25 μL of the GSE sample or lipase inhibitor (Orlistat, O4139, SIGMA) and 25 μL of 16.7 U/mL of lipase in Tris-HCl, pH 8.0 buffer solution. The reaction was initiated by adding 50 μL of 0.1M 4-MU oleate in Tris-HCl, pH 8.0 buffer solution. After incubation at 37 °C for 30 minutes, the rate of release of the 4-methylumbelliferone product was measured with a plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The readings were compared with the controls containing buffer in place of sample extract.

**Kinetics of α-glucosidase inhibition by GSE**

The enzyme reaction was performed according to the above reaction conditions with GSE and pNPG at various concentrations. Inhibition type (i.e. competitive, non-competitive, mixed, etc.) was determined by Lineweaver-Burk plot analysis of the data, which were calculated from the data according to Michaelis-Menten kinetics. Additional enzyme kinetic parameters for the inhibitors were determined by constructing appropriate reciprocal plots. In particular, from these plots the inhibitor concentration which inhibits the enzyme activity by 50% (IC$_{50}$), and the inhibitor constant ($K_i$) indicating the concentration required to produce half maximum inhibition of the enzyme were determined.

**Fractionate GSE and identify active fractions**

The GSE were fractionated with open column chromatography and by solid-phase extraction (SPE), the active fractions were identified through an activity-driven approach. In brief, the GSE was first separated into tannins and non-tannin fractions with an open column using SPE with Sephadex LH-20. Five grams of Sephadex LH-20 was hydrated in 20 ml of 100% methanol for 5 hours and then loaded into a glass column (13 mm x 600 mm). The column was continually washed with methanol until the matrix was stable. The GSE (20 mg) was reconstituted in 1 ml of 20% methanol and then loaded to the column. The non-tannin fraction in the GSE was first eluted with methanol. The tannins in the GSE bound with the LH-20 were then eluted with 50% acetone. Our α-glucosidase assays revealed that the non-tannin fraction contained inhibitor candidates. Therefore, the non-tannin fraction was further separated using SPE with Oasis HLB.
(hydrophilic-lipophilic balance) reversed-phase sorbents (Waters Corp., Milford, MA. Briefly, the fraction was dried with a rotary evaporator and reconstituted in 20% methanol. The solution was loaded to the SPE cartridge and eluted with 30% methanol (fraction 1), 40% methanol (fraction 2), 60% methanol (fraction 3), 80% methanol (fraction 4), and 100% methanol (fraction 5) to afford five non-tannin fractions.

Identification of the active α-glucosidase inhibitors in GSE by HPLC and LC-MS

After testing fractions for α-glucosidase inhibitory activity, the combined fractions with the strongest inhibition were further separated by reverse phase high-performance liquid chromatography (HPLC, Waters Corp., Milford, MA) using a C18 column (Porasil, 4.6 mm × 250 mm). In regards to the strongest yeast α-glucosidase inhibitor, fractions 1 and 2 were separated by the binary mobile phase consisting of 0.04% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B). A gradient program was applied as follows: 0-20 minutes with 30% solvent B; 21-35 minutes with 30-60% solvent B; 35-42 minutes with 60-100% solvent B; 43-45 minutes with 100% solvent B; at a flow rate of 1 mL/min. The eluent was monitored at 280 nm and 360 nm. Fractions of the HPLC eluent was collected at 3 minute intervals after injection (a total of 15 fractions were collected). The chromatograph process was repeated 3 times to obtain sufficient amount of each fraction. All the fractions were dried in a SpeedVac (Savant AES 1010, GMI Inc., Ramsey, Minnesota) and assayed for α-glucosidase inhibitory activity. In addition, the combined fraction 1 and 2 was analyzed with HPLC-MS to identify the active compounds. This was performed with an Agilent (Wilmington, DE) 1050 quaternary HPLC pump and DAD detector. The HPLC was interfaced to a MicroMass (Waters Corp., Milford, MA) Platform mass spectrometer equipped with an APCI-ES ionization chamber. The HPLC column output was split 1:10 with 1 part going into the MS and 9 parts going into the DAD detector. The HPLC separation was performed on a Waters C18 column (100 × 2.1 mm, 5 μm). The binary mobile phase consisted of (A) 1% aqueous formic acid and (B) acetonitrile (CH₃CN) containing 1% formic acid. A gradient program was applied as follows: 0-5 minutes with 5% solvent B; 6-20 minutes with 5-75% solvent B; 21-25 minutes with 75-100% solvent B; 26-30 minutes with 0% solvent B; at a flow rate of 0.2 mL/min. Data were collected
with the UV detector at 280 nm. The MS conditions for analysis in the negative ion mode included a cone voltage of 31 V, a capillary voltage of 3.15 kV, and a source temperature of 120 °C. A scan time of 1.4 seconds and interscan delay of 0.05 seconds was used (m/z 200–700 and 500–1200 amu).

**Statistical Analysis**

Data were reported as mean ± SD (Standard Deviation) for triplicate determinations. Statistical analysis was conducted by repeated measures ANOVA and Tukey’s post hoc comparison tests were performed to identify differences among means when differences were identified (SPSS for Windows, version 10.0.5., 1999). Statistical significance was declared at P-value < 0.05.

**Results**

**GSE inhibition on yeast α-glucosidase**

The GSE (from Norton) was identified as an inhibitor of yeast α-glucosidase during our initial screening of hundreds of food extracts and compounds (data not shown). To optimize the extraction efficiency for GSE, we prepared GSE with different solvents and compared their effects on yeast α-glucosidase. The 50% acetone GSE had the strongest inhibitory activity, followed by water and 80% acetone extracts (Figure 4.1). The methanol extract had the lowest activity. Therefore, for all subsequent investigations the GSE was prepared and analyzed from the 50% acetone extract. The inhibitory activity of GSE against α-glucosidase was further analyzed with acarbose as a positive control (Figure 4.2). The GSE was found to inhibit 92% of yeast α-glucosidase activity. The detected degree of inhibition was more potent than that of acarbose, which reduced the enzyme activity by 48% under the same enzyme reaction concentration. Interestingly, the extract of the whole Norton grape showed remarkably lower inhibitory activity on α-glucosidase. In a subsequent experiment, an extract from Norton grape flesh was prepared and found to have no inhibition on yeast α-glucosidase (data not shown). These observations suggest that the active components are particularly localized in the grape skin as opposed to the flesh. To test whether the inhibition is specific to the glucosidase enzyme family, we examined the effect of GSE on other digestive enzymes.
including pancreatic α-amylase and lipase and found no inhibition from the GSE when
dosed up to 2 mg/ml (data not shown). Collectively, these findings suggest the GSE may
be a specific yeast α-glucosidase inhibitor.

**Yeast α-glucosidase inhibitory effect of the GSE from different sources**

To evaluate whether the skin extracts prepared from other grapes also possess
similar inhibitory activity against yeast α-glucosidase, we collected and acquired 50%
acetone extracts from different grape sources for comparison. Four different grape skin
extracts were compared for their yeast α-glucosidase inhibition (*Figure 4.3*). To
represent a white grape, a Chardonnay extract was used and the other GSE were red
grapes including the red Norton extract, a red Cabernet Franc, and a commercial red
grape skin extract. The commercial extract was an aqueous ethanol extract from a red
grape skin (variety unknown). All the samples were assayed under the same
concentration (14.3 µg/ml). The results demonstrated that all the grape skin extracts
possessed strong inhibitory activity on yeast α-glucosidase. The GSE prepared from
Norton grape inhibited 61% of α-glucosidase activity, followed by the Cabernet Franc
(47%), Chardonnay (35%), and commercial GSE (26%). As a result of these findings, all
subsequent yeast α-glucosidase inhibitory activity investigations were conducted by
using the 50% acetone GSE prepared from the Norton grape variety.

**Kinetics and dose-dependent inhibition of yeast α-glucosidase by GSE**

To further elucidate the inhibitory action of GSE, the enzyme kinetics and dose-
response of GSE against yeast α-glucosidase was investigated. The enzymatic reactions
were conducted under the same conditions as previously described. Results revealed that
the GSE had noticeable inhibition on α-glucosidase activity at a concentration as low as
7.1 µg/mL in the reaction (*Figure 4.4A*). A dose-dependent inhibition of α-glucosidase
by GSE was also observed in the reaction with concentrations ranging from 1.4 to 285.7
µg/mL (*Figure 4.4B*). At a concentration of 14.3 µg/mL the GSE exerted a stronger
inhibition than 285.7 µg/mL of acarbose. From these results, it was determined that GSE
inhibits over 90% of yeast α-glucosidase activity at a concentration of 28.6 µg/mL or
higher. In regards to the 50% inhibition concentration, GSE was found to be 32-times
more effective (IC$_{50}$=10.5µg/mL) than acarbose (IC$_{50}$=341.8µg/mL) in inhibiting yeast α-glucosidase. Research studies investigating the enzymes that fall under the glycoside hydrolase family 13, such as α-amylase and α-glucosidase (EC 3.2.1.20) have shown that this family of enzymes shares a common reaction mechanism and several short conserved sequences (27). Hence, the combination of these findings and our observations indicate that the inhibiting mechanism of GSE on yeast α-glucosidase differs from that of acarbose, which inhibits both α-amylase and α-glucosidase. Collectively, the data suggest that the Norton GSE is likely an inhibitor specifically targeting α-glucosidases.

**Inhibition constant (K$_{i}$) and IC$_{50}$ of GSE for yeast α-glucosidase**

To determine the mode of inhibition of GSE on α-glucosidases, the kinetic reactions with GSE and pNPG were conducted at different concentrations and Lineweaver-Burk plots were constructed (Figure 4.5). The Lineweaver-Burk double reciprocal plot for this set of data resulted in a set of lines crossing the y (1/v) axis at the same point. This intersecting site indicates that the $V_{max}$ was unchanged in the presence of the GSE inhibitor. Accordingly, the GSE enzyme kinetic data revealed the intersecting point for the set of GSE concentrations occurred on the 1/v axis ($1/V_{max}$), suggesting that GSE is a competitive inhibitor on α-glucosidases. The inhibition constant (K$_{i}$) and IC$_{50}$ of GSE on α-glucosidase was determined to be 9.1 and 10.5 µg/mL, respectively. The low dissociation constant for binding of inhibitor to enzyme, (K$_{i}$), is indicative of the GSE having a high affinity for α-glucosidase and was associated with a low IC$_{50}$. Furthermore, the extrapolation of the enzyme kinetics suggest that the GSE was an inhibitor that inhibited the α-glucosidase by binding to half the enzyme active sites at equilibrium in the absence of substrate or other competitors.

**Purification and identification of active compounds in GSE with yeast α-glucosidase inhibitory activity**

GSE was first fractionated into tannins and non-tannin fractions. As shown in Figure 4.6, non-tannin fraction showed noticeably stronger inhibitory activity against α-glucosidase. Therefore, the non-tannin fraction was further separated with a SPE-HLB cartridge to afford 5 fractions. Fractions 1 and 2 had noticeably higher enzyme inhibitory
activity as compared to fractions 3, 4, or 5. As a result, fractions 1 and 2 were combined and then separated by HPLC to yield 15 fractions and each of these fractions was further tested for yeast α-glucosidase enzyme inhibitory activity. The results showed that several of these fractions inhibited α-glucosidase activity, suggesting that GSE contains multiple active compounds with enzyme inhibitory activity. Because it is challenging to analyze each fraction for composition, we used HPLC-MS to determine the individual compounds in fraction 1 and 2 collected from the SPE-HLB fractionation. The MS data suggested the combined fractions contained quercetin, catechin, resveratrol, and gallic, cinnamic, caffeic, ellagic, vanillic, syringic, and ferulic acids. These 10 identified compounds were further analyzed for their inhibition against yeast α-glucosidase. Three phenolic compounds, including resveratrol, ellagic acid, and catechin, were detected to strongly inhibit yeast α-glucosidase activity and their IC₅₀ were determined to be 20.4, 28.6, and 40.3 µg/mL, respectively. Interestingly, the IC₅₀ values were higher for purer compound fractions compared to extractions which were comprised of multiple compounds. These results suggest that the GSE is more potent at inhibiting yeast α-glucosidase when multiple compounds are present possibly acting in a synergistic manner as opposed to single compound presence (i.e. resveratrol, ellagic acid, and catechin).

**Inhibition of mammalian α-glucosidase by GSE**

Rat intestinal acetone powder was used as a model to better mimic the mammalian digestive system as compared to the purer yeast α-glucosidase. The mammalian α-glucosidases are categorized as α-glucosidase type II (28). This type of α-glucosidase was assayed under the same methods as described for the yeast α-glucosidase. The mammalian enzymes prepared from rat intestine that were investigated included the α-glucosidase complexes consisting of sucrase, maltase, and isomaltase. Results revealed that the 50% acetone Norton GSE (1 mg/mL) inhibited 86% of rat α-glucosidase activity, while acarbose (0.4mg/mL) showed 74% inhibition (Figure 4.7). The Norton GSE was identified to have strong dose-dependent inhibition on rat α-glucosidases at concentrations of 0.2, 0.4, and 1 mg/mL. When compared to the yeast enzyme, the Norton GSE was more effective at inhibiting yeast α-glucosidase as compared to the rat intestinal α-glucosidases. This may be the consequence of the GSE
selectively inhibiting the purer form of α-glucosidase in the yeast as opposed to when the GSE is attempting to inhibit the mammalian α-glucosidase complex enzymes present in mammalian species.

**Red and white GSE inhibit mammalian α-glucosidases**

The red Norton GSE, a white Chardonnay GSE, and a commercial GSE were compared for their inhibitory activity on the mammalian α-glucosidase. Similar to the yeast α-glucosidase findings, the white GSE also exhibited strong inhibition on rat α-glucosidases (59% inhibition), though weaker than that of the Norton GSE (86% inhibition, Figure 4.8). The commercial GSE inhibited the mammalian α-glucosidase activity by 45% at a dose of 1 mg/mL. Anthocyanins are known to be contained in high amounts in red grapes and it has been previously reported that acylated anthocyanins inhibited α-glucosidases (9). In an effort to identify the active inhibiting constituents in the Norton GSE, a group of standard compounds likely to be contained in grape skin including resveratrol, catechin, epicatechin, quercetin, rutin, and several phenolic acids were examined. However, none of these standards exerted notable inhibitory activity on rat α-glucosidases at 1mg/mL (data not shown). Collectively, the findings suggest that GSE constituents in addition to anthocyanins are participating in the inhibition of mammalian α-glucosidases. The 2-fold mammalian α-glucosidase inhibition difference between the red Norton grape skin and the commercial grape skin may be attributed to the varietal difference of the grape source or to the difference in the method of solvent extraction.

**Enzyme kinetic profile: Inhibition constant (Kᵢ) and IC₅₀ of GSE for rat α-glucosidase**

To clarify the type of inhibition of the GSE on rat α-glucosidases, the kinetic reactions with GSE and pNPG were conducted at different concentrations and Lineweaver-Burk plots were constructed (Figure 4.9). The Lineweaver-Burk double reciprocal plot for this set of data resulted in a set of lines crossing the y (1/v) axis at the same point. This intersecting site indicates that the V_max was unchanged in the presence of the GSE inhibitor. The GSE enzyme kinetic data revealed the intersecting point for the
set of GSE concentrations occurred on the $1/v$ axis ($1/V_{\text{max}}$), suggesting that GSE is a competitive inhibitor on rat $\alpha$-glucosidases and thus competes with the substrate for the active site of the enzyme. These findings indicate that increasing the concentration of the substrate will decrease the chance of the GSE binding to the active site of the $\alpha$-glucosidase. Thus, if the substrate concentration is high enough the $\alpha$-glucosidase will reach the same $V_{\text{max}}$ as it would without the GSE inhibitor. However, the $\alpha$-glucosidase would require a higher concentration of substrate to achieve this and so the $K_m$ of the enzyme will also be higher. The Dixon plots revealed that the inhibition constant ($K_i$) of GSE was 0.296 mg/mL. For a comparison of the potency of GSE, acarbose has been reported to have a $K_i$ of 0.068mg/mL. As such, the GSE was detected to be a less potent rat $\alpha$-glucosidase inhibitor as compared to acarbose, which had a 4-fold stronger inhibiting affect. It is postulated that because GSE was analyzed as an extract mixture, that once identified and collected in a purer concentration the active constituents in this GSE may have a comparable or stronger inhibitory effect than that of acarbose. Finally, the GSE was determined to have an $IC_{50}$ value of 0.384 mg/mL on rat $\alpha$-glucosidases which is relatively strong in comparison to other natural $\alpha$-glucosidase inhibitors such as oolong tea extract ($IC_{50} = 1.34$ mg/mL) and green tea extract ($IC_{50} = 0.735$ mg/mL) (29).

**Discussion**

The effectiveness of $\alpha$-glucosidase inhibitors for diabetes treatment is well documented in numerous animal and human clinical studies. For instance, acarbose has been shown to reduce postprandial serum glucose and insulin concentrations, improve metabolic control, and produce a dose-dependent amelioration of diabetic nephropathy in the diabetic ($db/db$) mouse (30, 31). A recent meta-analysis on $\alpha$-glucosidase inhibitors for type 2 diabetes revealed that $\alpha$-glucosidase inhibitors significantly decreased the serum level of glycosylated hemoglobin by 0.68-0.77% (4). Recently, $\alpha$-glucosidase inhibitors have gained more attention due to a randomized controlled prospective trial (STOP-NIDDM), showing that acarbose could prevent or delay the progression of impaired glucose tolerance from becoming established type 2 diabetes (32).

In the past three decades, considerable research effort has been devoted to the discovery and development of novel $\alpha$-glucosidase inhibitors as potential therapeutic
agents for diabetes. A variety of natural compounds and extracts have already been identified as being beneficial in the treatment of diabetes (6, 22, 29, 33-35). In particular, phenolic compounds found in plants have been found to be involved in the inhibition of α-glucosidase (8, 9). The oral administration of a green tea extract (300 mg/kg bw for 4 weeks) remarkably reduced fasting blood glucose (by 54%) in STZ-induced diabetic rats (36). Additionally, a procyanidins-rich pine bark extract (Pycnogenol®) was found to potently inhibit α-glucosidases, which has been associated with its antidiabetic effect in patients with type 2 diabetes (22, 37). Furthermore, a group of natural acylated anthocyanins have been shown to exert α-glucosidase inhibitory activity (9). It is well established that the grape is rich in phenolic compounds including flavonols, anthocyanins, and procyanidins which may account for its antioxidant bioactive property. Studies on other grapes and grape derived bioactive properties and components related to hyperglycemia and diabetes are limited (14, 17, 38-40). This study demonstrated for the first time that Norton (V. aestivalis) grape skin extract (GSE) potently inhibits both yeast and mammalian α-glucosidases.

The yeast α-glucosidase used in these studies is readily available in pure form and has been widely been used for nutraceutical and medicinal investigations (20, 21, 29, 41). In an effort to further explore the effectiveness of GSE on α-glucosidase, mammalian enzyme studies were also conducted to better mimic the human digestive system. The GSE was not found to inhibit the activity of pancreatic α-amylase or lipase, suggesting that the GSE is likely a specific α-glucosidase inhibitor. The GSE was found to inhibit yeast and mammalian α-glucosidase in a dose-dependent manner. In the yeast studies, the inhibition potency was determined to be even stronger than that of the commercially available oral hypo-glycemic pharmaceutical, acarbose. However, in the mammalian studies, acarbose was determined to be almost 4 times more potent that the GSE. This may be the consequence of the GSE selectively inhibiting specific α-glucosidases instead of inhibiting all the starch digesting enzymes associated with the mammalian α-glucosidase complex of enzymes (i.e. sucrase, maltase, and isomaltase). As a result, the uninhibited α-glucosidases in the complex are able to hydrolyze the substrate even in the presence of the GSE inhibitor. Additionally, in regards to our assay conditions, pNPG has a strong affinity for α-glucosidases, thus, it is a specific substrate for the intestinal α-
glucosidase complex including sucrase, maltase, and isomaltase. The results from mammalian enzyme assays revealed that GSE inhibited rat α-glucosidases. However, we did not determine whether GSE inhibits one specific enzyme or affects the whole intestinal glucosidase complex (i.e. sucrase, maltase, and/or iso-maltase). To this end, it will be important to clarify which enzyme(s) in this complex is the target for the GSE as well as to identify the active GSE phenolic compounds.

The presence of anthocyanins in the GSE were speculated to be contributing to the inhibitory effect in agreement with previous findings that revealed acylated anthocyanins have α-glucosidase inhibitory activity (9). However, a white wine Chardonnay GSE was also found to have strong inhibitory qualities, suggesting that the active inhibiting constituents were likely from other phenolic groups in addition to the anthocyanins on the basis that the white grape is deficient in these pigmenting phenolics. The kinetic reactions revealed that GSE inhibited the yeast and mammalian α-glucosidase through a competitive mechanism. Collectively, the inhibition studies provide useful information for the design of new inhibitor candidates for α-glucosidase.

Conclusion

From the activity-driven approach, active compounds from the GSE were fractionated and identified to exhibit potent yeast α-glucosidase inhibition. It is well known that tannin compounds have a unique ability to combine and form complexes with proteins. As such, tannins have been shown to nonspecifically inhibit a variety of enzymes. Grape skins are rich in tannin compounds. Therefore, the GSE was first fractionated into tannin and non-tannin fractions with the LH-20 column, which is a traditional technique used for the separation of tannin compounds. After conducting the yeast α-glucosidase enzyme assays, it was clear that the non-tannin fraction was a remarkably stronger inhibitor of α-glucosidase as compared to the tannin fraction. As a result of this discovery, the non-tannin fraction was further separated using SPE and several fractions were identified to have strong inhibition on yeast α-glucosidase activity. The fractions with the strongest inhibitory activity were analyzed by HPLC and LC-MS. Three compounds in the Norton GSE, resveratrol, ellagic acid, and catechin were identified to strongly inhibit yeast α-glucosidase activity. Individually the inhibitory
activity of these compounds were noticeably lower than that of GSE, suggesting that they may act synergistically against $\alpha$-glucosidase or there may be other constituents in GSE which are also inhibiting or facilitating the inhibition of the yeast $\alpha$-glucosidase enzyme. Overall, the yeast $\alpha$-glucosidase study findings revealed that the Norton GSE and 3 of its phenolic components (resveratrol, ellagic acid, and catechin) strongly inhibit yeast $\alpha$-glucosidase activity. These phenolics may be ideal candidates for utility in future mechanism studies regarding hyperglycemia and glycosylation which is present in diabetes (42, 43).

To better understand the inhibition of GSE on mammalian $\alpha$-glucosidases, the inhibiting action of GSE on specific rat $\alpha$-glucosidases (i.e. sucrase, maltase, and isomaltase) needs to be clarified in future studies. Furthermore, in vivo studies are needed to determine if GSE can suppress the absorption of glucose by way of an $\alpha$-glucosidase inhibitory mechanism in the small intestine.

**Acknowledgments**

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Figure 4.1. Yeast α-glucosidase inhibitory activity of GSE extracted by different solvents. From the red wine Norton grape (*Vitis aestivalis*), water, 50% acetone (50 ACE), 80% acetone (80 ACE), and 100% Methanol grape skin extract (GSE) we tested for inhibitory activity, respectively. All samples were tested at the same concentration by weight (285.7 µg/mL in the reaction). The α-glucosidase activity was determined by measuring *p*-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 ºC for 75 minutes.
Figure 4.2. Yeast α-glucosidase inhibitory activity of GSE, whole grape extract, and acarbose. From the Norton red wine grape (*Vitis aestivalis*) 50% acetone grape skin extracts (GSE) was tested and compared to whole grape 50% acetone extract (pulp and skin of Norton grape) and to a positive control (Acarbose) at the same concentration (285.7 µg/mL in the reaction). The α-glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes.
**Figure 4.3.** Comparison of different grape skin extracts for their inhibitory activities on yeast α-glucosidases. 50% acetone grape skin extracts from Norton (NR) and Cabernet Franc (CV) are from red grape varieties. 50% acetone extract from Chardonnay (CH) is a white grape skin extract. Commercial skin extract (CM) was donated by RFI ingredients (Blauvelt, NY) which is an aqueous ethanol extract of red grape skin (unknown variety). All the samples were assayed under the same concentration (14.3 µg/ml). Bars with the different letters are significantly different ($P < 0.05$).
Figure 4.4. Kinetics and dose-dependent inhibition of yeast α-glucosidase activity by Norton GSE.  

A. Enzyme kinetics of α-glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 ºC for 75 minutes in the absence or presence of 50% acetone Norton GSE at different concentrations ranging from 0 to 285.7 µg/mL.  

B. Dose-dependent inhibition of Norton GSE was tested with concentrations ranging from 0 to 285.7 µg/mL. Acarbose is the positive control and denoted as AC (285.7 µg/ml). Results are expressed as means ± SD; bars with different letters are statistically significant (P < 0.05).
Figure 4.5. Lineweaver-Burke plots of yeast α-glucosidase inhibition by 50% acetone Norton GSE. The enzyme reaction was performed with each designated concentration of pNPG (0.4-2 mM) in the absence or presence of GSE at different concentrations (0, 4, 8, 16, and 32 µg/ml). The reaction was conducted at 37 ºC for 75 minutes. Inhibition mode (i.e. competitive) was determined by Lineweaver-Burk plot analysis of the data, which were calculated from the results according to Michaelis-Menten kinetics.
Figure 4.6. Yeast α-glucosidase inhibitory activity of Norton GSE fractions. The GSE was first fractionated to tannin and non-tanin fractions by SPE with LH-20. The non-tanin fraction (20% methanol) was then fractionated into five fractions (F1-F5) with SPE-HLB (eluted with 30, 40, 60, 80, and 100% methanol, respectively). The enzyme assays were conducted at the concentration of 8 µg/mL for each fraction.
Figure 4.7. Dose-dependent Inhibition of Norton GSE on rat α-glucosidases. Grape skin extract (GSE) derived from red Norton *V. aestivalis* grapes was analyzed at concentrations of 0.2, 0.4, and 1 mg/mL. Acarbose is the positive control and denoted as AC (0.4 mg/ml). The α-glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes. Results are expressed as means ± standard deviation of the mean; bars with different letters are statistically significant (P < 0.05).
Figure 4.8. Comparison of different varieties of GSE for their inhibitory activities on rat α-glucosidases. 50% acetone GSE from Norton (red wine grape), Chardonnay (white wine grape), and a commercial grape skin extract (RFI Ingredients, Blauvelt, NY) which was an aqueous ethanol extract from an unknown variety were measured for enzyme inhibition activity at a concentration of 1 mg/ml. Rat α-glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes. Results are expressed as means ± standard deviation of the mean; bars with different letters are statistically significant (P < 0.05).
Figure 4.9. Enzyme kinetic inhibition plots of rat α-glucosidase inhibition by the Norton GSE. The enzyme reaction was performed with different concentrations of substrate, pNPG (0.4-2 mM), in the absence or presence of Norton GSE at different concentrations: 0 (○), 0.2 mg/mL (▲), 0.4 mg/mL(■), and 1mg/mL(♦). The reaction was conducted at 37 °C for 75 minutes. Inhibition type (i.e. competitive) was determined by Lineweaver-Burk plot analysis of the data, which were calculated from the result according to Michaelis-Menten kinetics.
CHAPTER 5: VALUE-ADDED POTENTIAL OF GRAPE POMACE: A BIORESOURCE WITH ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY PROPERTIES IN VITRO AND IN VIVO

Abstract

The purpose of this study was to investigate the therapeutic potential of grape pomace, a byproduct in the wine industry. Eighty percent acetone extracts from different pomace samples were evaluated for in vitro antioxidant and α-glucosidase inhibitory activity as well as in vivo postprandial anti-hyperglycemic effects in STZ-induced diabetes mice. Phenolic composition and antioxidant properties of each pomace were measured and their enzyme inhibitory activity was evaluated with α-glucosidase, α-amylase, and lipase. Red grape (Norton) pomace and white grape (Chardonnay) pomace contained notable amounts of phenolic and flavonoid compounds with pronounced free radical scavenging activities. The red grape pomace extract (GPE) had the highest oxygen radical absorbance capacity (ORAC), 245.3 μmol TE/g of dry pomace weight followed by the white GPE (198 μmol TE/g). All the extracts had comparable DPPH radical scavenging activity by quenching at least 66% of the radicals per mg of pomace. The red and white GPE inhibited both yeast and mammalian α-glucosidase activity. At 20 mg/mL the red GPE inhibited more mammalian α-glucosidase activity (47%) compared to the white GPE (39%). The oral administration of the red GPE (400 mg/kg bw) to STZ-induced diabetic mice significantly suppressed postprandial blood glucose levels 35%. This study suggests that the red GPE may provide novel anti-diabetes effects as a result of dual antioxidant and postprandial antihyperglycemic bioactivities.

Keywords

Grape pomace phenolics, antioxidant, α-glucosidase, enzyme inhibition, postprandial hyperglycemia, type 2 diabetes, oral glucose tolerance test, STZ-injected mice
Introduction

Diabetes affects nearly 24 million people in the United States, surging more than 3 million in the last two years (1). This alarming increase in the incidence of diabetes emphasizes an urgent need for novel health-promotion and therapeutic strategies. One approach to managing diabetes has been investigated using phytocompounds in the diet that improve glycemic control by specifically inhibiting α-glucosidases (2-4). Research in this area adds to the existing tools and resources used to facilitate individuals at risk or with diabetes to properly control postprandial blood glucose levels (5, 6). Both synthetic and naturally derived compounds are known to reduce postprandial hyperglycemia by inhibiting a key carbohydrate metabolizing enzyme in the small intestine such as α-glucosidase (7, 8). The phenolic molecular structure of inhibitors may be a key element that contributes to this bioactive property. To this end, a variety of phenolic compounds comprised in plants have been found to be potent inhibitors of carbohydrate hydrolyzing enzymes (4, 9-11). From a series of previous experiments in our laboratory, we have identified that a phenolic rich grape skin extract from both red and white wine grape varieties can inhibit both yeast and mammalian starch metabolizing enzymes in vitro (12). Our laboratory continued to investigate alternative grape derived extracts such as pomace as a novel and cost effective source of nutraceutical agents with health promoting properties.

In addition to improving glucose control, grape pomace extract supplementation may play an additional role by providing potential antioxidant benefits for managing oxidative stress associated with chronic conditions such as diabetes. In particular, studies have indicated that hyperglycemia is associated with increased oxidative stress (13, 14). For example, antioxidant activity of grape pomace may be a consequence of the phenols comprised in grapes and grape products contributing to reduce oxidative stress associated with diabetes in vivo (15-17). Furthermore, the antioxidant protective capacity of phenols was shown in rats pretreated with a grape pomace extract containing gallic acid, catechin, epicatechin, and procyanidins followed by free radical exposure (CCl₄) in which there was a reduction in liver lipid peroxidation (18). The authors of this study suggested that the antioxidants from the grape pomace administration was due in part to the phenols scavenging the free radicals (18). Further evidence of grape phenolic antioxidant
protection was observed when a mixture of polyphenols from grape seed extract (300 mg) in a meal reduced postprandial plasma lipid peroxidation and enhanced plasma antioxidant capacity (19).

A number of studies are in agreement that grapes are a natural source of notable bioactive phenolic compounds with health promoting and disease protective qualities (20-22). For example, wine, grapes, and grape seed extracts are a major source of polyphenolic components such as anthocyanins, flavanols, catechins, and proanthocyanidins (23-27). Because grape skins and seeds are the predominant constituents in the pomace, this biomass is speculated to be a rich source of natural antioxidants and other bioactive compounds (18, 28). Grape pomace is a waste product generated in the wine industry (29). Large amounts of this byproduct accumulate annually which leads to a waste-management issue (30). In 2005, wine grape production in the US was approximately 3.7 million tons (31, 32). Twenty percent of this amount results in the mass of pomace (32). Although the uses of pomace are limited it has been recycled as organic fertilizers, manure, and animal feed. There exists a continued interest in the development of additional value to this byproduct. Limited research has been conducted with grape pomace as an alternative bioresource for medical utility or novel therapeutics for addressing diseases (18, 29, 33-37). To our knowledge this is the first report of an α-glucosidase inhibitory property of pomace.

In this study, we analyzed two varieties of grape pomace for their antioxidant activity and enzyme inhibitory effects on digestive enzymes namely glucosidase, amylase, and lipase. In addition, the phenolic composition of each pomace was determined. Our results suggest that the red GPE possess significant antioxidant activity and anti-postprandial hyperglycemic effect through specific inhibition of glucosidase. The data suggests a great potential of developing the red GPE as a novel dietary antidiabetic agent, which, in turn, could result in the development of a more cost-effect nutraceutical source for promoting and improving health.
Materials and Methods

Materials

Yeast type I α-glucosidase (EC 3.2.1.20), rat intestinal acetone powder (N1377-5G), p-nitrophenyl α-D-glucoside (pNPG), acarbose, Folin-Ciocalteu reagent, gallic acid, rutin, Trolox™, fluorescein, 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and streptozotocin (STZ) were purchased from the Sigma Chemical Co. (St. Louis, MO). The acetone solvent was HPLC grade (Fisher Scientific Co., Atlanta, GA).

Pomace extract preparation

Red wine grape pomace (Norton), white wine grape pomace (Chardonnay), and red apple pomace was collected in the fall of 2007 from a local Virginia vineyard (Blackstone, VA, USA). Each variety of pomace was extracted with 80% acetone at 1:10 ratio (m/v) and then placed in a stir bath overnight. The extracts were filtered through Whatman No. 4 filter paper to remove unwanted residues. After evaporating off the organic solvent, the filtrates were frozen and lyophilized to obtain the pomace extracts. All extracts were stored at -20°C for further investigation (i.e. for in vitro antioxidant, enzyme inhibition/kinetic assays, and in vivo acute animal studies).

Total phenolic content (TPC)

The TPC of each pomace extract was determined using Folin-Ciocalteu reagent with gallic acid as the phenolic standard (38). In brief, appropriate dilutions of extracts were mixed with Folin-Ciocalteu reagent and 20% sodium carbonate (Na₂CO₃) at ambient temperature. After incubation for 2 hours, the absorbance of the blue color that developed in each assay mixture was recorded at 760 nm (Thermo Electron Corporation, Genesys 10-UV scanning, Madison, WI, USA). The TPC value of each pomace extract was expressed as micrograms of gallic acid equivalent per gram of pomace (μg GAE/g).
**Total flavonoid content (TFC)**

The pomace extracts were analyzed for TFC according to a previously reported colormetric method (39). In brief, 1 mL of each pomace extract or rutin standard was mixed with 0.3 mL of 5% sodium nitrite (NaNO₂), 0.3 mL of 10% aluminum chloride (AlCl₃), and 2 mL of 1M sodium hydroxide (NaOH). All samples were measured in duplicate and compared against a blank at an absorbance of 510 nm (Thermo Electron Corporation, Genesys 10-UV scanning, Madison, WI). Results were expressed as micrograms of rutin equivalent per gram of pomace (µg RE/g).

**Oxygen radical absorbance capacity (ORAC)**

The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity of each pomace extract with Trolox™ as the antioxidant standard according to a method reported previously (25). Fluorescein was used as the fluorescent probe and the peroxyl radicals were generated from AAPH in 75 mM phosphate buffer (pH 7.4). Standards and samples were measured in duplicate on a black 96-well flat bottom plate. The fluorescence of the reaction mixture was monitored and recorded every minute (λ<sub>ex</sub> = 485 nm and λ<sub>em</sub> = 535 nm) maintained at 37°C until the reading had declined to less than 5% of the initial reading with a Victor<sup>3</sup> multi-label plate reader (Perkin-Elmer, Turku, Finland). Results for ORAC were determined by using a regression equation relating Trolox™ concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value was expressed in micromoles of Trolox™ equivalents per gram of pomace (µmol TE/g).

**DPPH radical scavenging activity**

To obtain the antioxidant activity of pomace extracts the DPPH radical scavenging antioxidant activity assay was determined by using a previously reported method (40). This antioxidant capacity assay, based on the reduction of the free radical DPPH, was carried out spectrophotometrically on the platereader. The reaction mixture contained 100 µL of sample extract and 100 µL of 0.208 mM DPPH radical solution. The absorption at 515 nm was determined immediately after the reaction was initiated.
Each plate was read once every minute for 1.5 hours. The DPPH data was expressed as the percent of DPPH radical quenching activity per milligram of pomace extract (% DPPH inhibition/mg).

**Yeast and mammalian α-glucosidase activity**

Both the yeast and mammalian α-glucosidase activity was assayed using the substrate p-nitrophenyl-α-D-glucopyranoside (pNPG), which is hydrolyzed by α-glucosidase to release the product p-nitrophenol, a color agent that can be monitored at 405 nm (41). The assay was conducted by mixing 80 µL of the sample solution (1 mg/mL) with 20 µL of the enzyme solution (1 U/mL) and incubated in a 96-well plate at 37ºC for 3 minutes under constant shaking. After incubation, 100 µL of 4 mM pNPG solution in 0.1 M phosphate buffer (pH 6.8) was added and the reaction was conducted at 37ºC. The release of p-nitrophenol from pNPG was monitored at 405 nm every minute for 75 minutes spectrophotometrically with the platereader. The α-glucosidase activity was determined by measuring area under the curve (0-75 minutes) for each sample and compared with that of the control. The results were expressed as the percent of α-glucosidase inhibition. On the basis of the *in vitro* results, only the 80% acetone red wine grape pomace extract (GPE) was selected for further *in vivo* studies.

**In vivo Suppression of Blood Glucose Levels in STZ-injected Mice**

**Experimental animals**

Male 6-week old mice (C57BLKS/6Ncr, National Cancer Institute, Frederick, MD, USA) were housed in groups of four mice per cage and maintained on a 12-hour light-dark cycle at 20ºC to 22ºC. The animals were acclimatized for a 2-week period before starting the experiment and had *ad libitum* access to food and water. The mice were maintained on rodent feed (Harlan Tekland Global Diets 2018 rodent diet containing 60% of calories from carbohydrate, 23% of calories from protein, and 17% of calories from fat; digestible energy of 3.4 Kcal/g, Madison WI, USA) for the duration of the experiment. Animal husbandry, care, and experimental procedures were conducted in compliance with the “Principles of Laboratory Animal Care” NIH guidelines, as
approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech (approved protocol #07-115-FST).

**STZ induction of experimental diabetes in mice**

Diabetes was induced in 14-hour fasted 8-week old mice (25-27 g) by intraperitoneal injection of STZ dissolved in 10 mM sodium citrate buffer (pH 4.5) at a dose of 50 mg/kg bodyweight (bw). The STZ was dissolved in ice-cold citrate buffer protected from light and injected immediately to avoid STZ degradation. Five to seven days after STZ injection, mice with a fasting blood glucose (FBG) level higher than 126 mg/dL were considered to have diabetes and were randomly assigned to one of two groups (n=8 mice per group) (42).

**Oral glucose tolerance test (OGTT)**

The starch (i.e. maltose) challenge experiment was designed to determine the effect of grape pomace extract (GPE) in STZ-induced mice following OGTT. All mice were fasted for 14-hour with free access to water before the OGTT was conducted. The control group of mice (n=8) were given 0.2 mL of water by gavage. The treatment group (n=8) were administered 0.2 mL of GPE (400 mg/kg bw) by gavage. After 30 minutes post water or GPE administration, 0.2 mL of a sterile maltose dissolved in water (2 g/kg bw) was administered to each mouse by gavage. Approximately 5 µL of whole blood samples were collected from the tail vein of each mouse. The blood samples were acquired at 0 (just before maltose administration), 30, 60 and 120 minutes after the oral maltose challenge for whole blood glucose analysis. Blood glucose levels were measured with a blood glucometer and accompanying test strips (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI). This data was used to derive the percent induced glycemia (%IG) following oral glucose load at the time intervals for the control and GPE group. From the %IG versus time plot, the area under the glucose tolerance curve (AUC$_{0–120 \text{ min}}$) was calculated using a trapezoidal method (43). The total antihyperglycemic response (AUC$_{0–120 \text{ min}}$) was expressed as mean ± standard deviation.
Statistical Analysis

All data were presented as mean ± SD (Standard Deviation). Student’s paired t-test was used for analysis of paired data. The statistical significance comparing data between groups was assessed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range post-hoc tests. Statistical analysis was performed using SPSS (Windows, Version Rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was declared at a P value less than 0.05.

Results and Discussion

Phenolic profile: total phenolic content (TPC) and total flavonoid content (TFC)

The pomace extracts (i.e. red grape, white grape, and red apple) were measured and compared for TPC and TFC (Table 5.1). The apple pomace extract served as an additional pomace source for comparing the grape pomace results. All pomace extracts contained measurable amounts of phenolic and flavonoid compounds. The red grape pomace contained the highest TPC (30.4 μg GAE/g) followed by the white grape pomace (24.5 μg GAE/g). Both the grape pomace extracts contained more TPC when compared to the red apple pomace which contained the least amount of TPC (11.2 μg GAE/g). In a similar trend, the red grape pomace contained the highest amount of TFC (22.1 μg RE/g) followed by the white grape pomace (16.2 μg RE/g). The red apple pomace contained the least amount of TFC (5.7 μg RE/g). The red grape pomace extract contained statistically more TPC and TFC as compared to the apple pomace extracts (P < 0.05). The red and the white grape pomace extracts did not have statistically different TPC or TFC (P < 0.05).

Previous studies have also reported that apple and wine pomace contain substantial phenolic compounds. For example, apple pomace has been reported to contain measureable amount of TPC (0.3g/kg apple fresh weight contain 0.5 - 1.1 g/kg) and red wine pomace was reported to contain 2 g/L of TPC (34, 44). The TFC in pomace will vary depending on the grape variety (45). For instance, TFC was measured in a variety of Spanish wine pomace and it was reported that a red Merlot had higher TFC (199 mg/100g of dry weight) as compared to a white Viura variety (29 mg/100g of dry weight) (45). Other red and white wine grape sources (Reunite Lambrusco and Soave Doc
Classico, respectively) have been reported to contain a wide variety of phyto-phenolic constituents (46). In particular, red wine had notable amounts of resveratrol and gallic acid (2.8 and 10.1mg/L, respectively) and white wine had notable amounts of tyrosol and hydroxytyrosol (17.1 and 2.7 mg/kg, respectively) (46). Our findings and other reports demonstrate that varietal differences of the grape source will contribute to the differences in phenolic content.

**Antioxidant property: ORAC and DPPH radical scavenging activity**

Grapes have been shown to be a good source of phenolic compounds with antioxidant properties (47). Similar to other pomace antioxidant findings, our pomace extracts had comparable antioxidant activity (45). The ORAC assay measured the scavenging capacity of pomace extracts against peroxyl radicals and is reported in Table 5.1. The red grape pomace had the highest ORAC value (246.0 µmol TE/g) followed by the white pomace and apple pomace (198.3 and 168.8 µmol TE/g, respectively, P < 0.05). The ORAC values of grape extracts will vary depending on factors such as extraction, fractionation, and drying techniques. Additionally, the ORAC values of grape derived compounds will vary depending on the composition of the grape phenolic source. In particular, extracts from grape skins and pomace were reported to have ORAC values ranging from 1.38 to 21.4 µmol Trolox equivalents/mg whereas extracts from grape leaves had ORAC values ranging from 1.52 to 2.55 µmol Trolox equivalents/mg (48).

Other grape derived sources such as raisins have been found to have ORAC properties. For instance, golden raisins from a Californian grape were reported to have an ORAC value of 104.5 µmol TE/g (49). Additionally, the DPPH radical scavenging assay was conducted to attain a clearer antioxidant profile of the pomace samples. All of the pomace extracts were determined to possess DPPH radical scavenging activity (Table 5.1). The red grape, white grape, and apple pomace quenched the DPPH radicals by 66%, 67%, and 55%, respectively. No statistical differences were detected between these groups (P < 0.05). Similar percent inhibition of DPPH by naturally derived extracts has been reported. For example, methanol extracts from Bangalore blue grape pomace scavenged DPPH radicals by 67.3% at 50 mg/L (18). Overall, the phenolic and antioxidant results of our 80% acetone pomace extracts indicate that they contain
measurable phenolic compounds as evidence from the TPC, TFC data and have significant peroxyl radical and DPPH radical scavenging ability.

**In vitro α-glucosidase inhibition by grape pomace extract (GPE)**

The GPE had notable yeast and mammalian α-glucosidase inhibitory activity *in vitro*. The red GPE (285.7 µg/mL) inhibited yeast α-glucosidase activity (99% inhibition) followed by a pharmaceutical hypoglycemic agent, acarbose (48% inhibition) (*Figure 5.1*). The GPE was tested for inhibitory activity on pancreatic α-amylase and lipase and was found to have no effect on either of these enzymes (data not shown), suggesting that it may be a specific α-glucosidase inhibitor. The yeast α-glucosidase enzyme used in our experiments is readily available in pure form and has been commonly used for nutraceutical and medicinal investigations (41, 50-52). For example, yeast α-glucosidase was found to be inhibited by a maritime pine bark extract and a green tea extract (4).

Previously, our laboratory determined that 50% acetone grape skin extracts (14.3 µg/ml) possessed strong inhibitory activity on yeast α-glucosidase (12). In particular, we found that the extract prepared from a red Norton grape inhibited more yeast α-glucosidase activity (61%) as compared to a red Cabernet Franc and a white Chardonnay grape skin extracts (47% and 35% enzyme inhibition respectively) (12).

In an effort to further establish the effectiveness of GPE on α-glucosidase, the more biological relevant rat intestinal acetone powder was used. The mammalian α-glucosidases are categorized as α-glucosidase type II consisting of sucrase, maltase, and isomaltase and have been used to identify inhibitor candidates (53). Green tea extract, for example, has been reported to inhibit rat intestine α-glucosidase (IC$_{50}$ 735 µg/mL) (52).

In our study, both the red and the white GPE (20 mg/mL) exerted inhibition on mammalian α-glucosidase activity (*Figure 5.2*). The red GPE had significantly higher (P < 0.05) inhibitory activity (47%) than the white GPE (39%). A dose-dependent inhibition of rat α-glucosidase by the red GPE was observed in the reaction with concentrations ranging from 0.25 to 2.5 mg/mL (*Figure 5.3*). The red GPE inhibited over 64% of the rat α-glucosidase activity at a concentration of 2.5 mg/mL. Other naturally derived compounds are reported to have inhibitory activity. For example, we determined that a
red Norton grape skin extract to have an IC$_{50}$ value of 0.384 mg/mL which is relatively strong in comparison to other natural α-glucosidase inhibitors such as oolong tea extract (IC$_{50}$ =1.34 mg/mL) and green tea extract (IC$_{50}$ =0.735 mg/mL) (52). Additional experiments are planned in an effort to identify the active components in GPE and establish their inhibiting mechanisms against specific α-glucosidases namely sucrase, maltase, and/or iso-maltase. On the basis of the in vitro findings, only the 80% acetone red wine grape pomace extract (GPE) was selected for further in vivo studies.

In vivo effect of red GPE on postprandial blood glucose levels following OGTT

The red GPE (400 mg/kg bw) suppressed postprandial hyperglycemia in STZ-induced diabetes mice after a maltose challenge (2 g/kg bw) (Figure 5.4A). This inhibiting effect of GPE on postprandial glucose was similar, though less effective, to that of the commercially available drug to treat type 2 diabetes, acarbose (25 mg/kg, bw) (7). Although the inhibiting effect of GPE did not achieve statistical significance, there was a noticeable trend indicating the potential blood glucose lowering effect of GPE. Extrapolation of the data was conducted using the area under the curve to determine the overall percent glucose suppression between the control and GPE administered groups. The GPE group had a significant (P < 0.05) 35% reduction in the glucose AUC$_{0-120min}$ compared to the control group (Figure 5.4B). From our in vitro results, the biochemical mechanistic explanation for this blood glucose response is proposed to be the inhibitory effect of GPE on α-glucosidase activity in the small intestine. The GPE administration was found to result in the most consistent results as compared to OGTT conducted with other potential α-glucosidase inhibitor candidates identified previously in our laboratory (54). For example, grape skin and grape seed extracts (gavaged at a dose of 400 mg/kg bw) were used in OGTT experiments and the measured postprandial glucose results were variable and less consistent as compared to the GPE (data not shown). The in vivo results suggest that the GPE may lower the rise in postprandial blood glucose levels in populations with impaired glycemic responses therefore, exert an antidiabetes effect by limiting or delaying the absorption of dietary carbohydrates through specific inhibition of α-glucosidase.
The α-glucosidase inhibitory effect is important in regards to the bioactive function of pomace extracts pertaining to the attenuation of postprandial blood glucose. The role of postprandial hyperglycemia in the pathogenesis of type 2 diabetes has received increased attention as a promising therapeutic target (55). Postprandial hyperglycemia is, in part, related to the amount and digestion rate of consumed starch, which is the primary source of blood glucose (56). A growing body of evidence suggests that inhibition of key starch digestive enzymes provides a beneficial effect for both preventing and treating type 2 diabetes by attenuating postprandial blood glucose levels (55-58). We have revealed a potential GPE therapeutic effect on postprandial blood glucose levels after a maltose load. The mechanism by which these results evolved are proposed to be a consequence of phenolic grape extracts and their ability for delaying the metabolism of carbohydrates by the competitive inhibition of brush border α-glucosidase in the small intestine, similar to that of acarbose (12, 59). Similar potential antidiabetic effects of grape products have been reported. For instance, phenolic extracts from red grape wine administered for 6 weeks to rats with diabetes were associated with anti-diabetes activity (60). Additionally, grape seed extracts rich in flavonoids were demonstrated to have an antihyperglycemic effect in STZ-injected rats (i.e. 250 mg/kg bw of procyanidin extract) (17). The authors of these studies suggested that this antidiabetes response was a consequence of a delay in intestinal glucose absorption and/or through an insulin signaling pathway (17). Acute hypoglycemic effects have also been observed from other grape derived compounds. For example, a reduction of blood glucose levels was observed in STZ-injected rats administered extracts from the leaves of grape vines (250 and 500 mg/kg bw doses) following an OGTT (61). Furthermore, GSE supplementation at approximately 880 mg/kg bw/d (quantified to account for approximately 280 mg/kg bw of polyphenols) for 15-weeks has been reported to delay the development of atherosclerosis in hyperlipidemic rabbits (62). Collectively the evidence from our study and others is building in support of hypoglycemic agents derived from natural phenolic-plant sources such as grapes and their potential to improve glycemic control associated with diabetes.
Conclusion

This study provided evidence supporting the potential of the nutraceutical value-added development of grape pomace. To the best of our knowledge, this was the first report on the antihyperglycemic effect of antioxidant rich grape phenolics derived from pomace. The phenolic profile and antioxidant bioactive assessment was determined in which grape pomace extracts had measurable phenolic and antioxidant profiles in regards to TPC, TFC, ORAC, and DPPH values. The GPE exhibited notable in vitro yeast and mammalian α-glucosidase inhibitory activity. GPE (400 mg/kg bw) administration prior to an oral maltose challenge reduced the hyperglycemic response in STZ-injected mice. The possible mechanism of action of grape derived phenols to delay glucose absorption and improve blood glucose levels in populations at risk for type 2 diabetes may be a result α-glucosidase inhibitory activity in the small intestine. Our in vitro and in vivo results suggest that grape pomace may be a useful bioresource that contains natural antioxidants and anti-hyperglycemic compounds. These dual bioactive attributes derived from one phenolic bioresource could play a complementary and alternative role in managing the poorly regulated blood glucose levels associated with type 2 diabetes and corollary oxidative stress. Further research is merited to address the impact of the antioxidant rich GPE supplementation on improving blood glucose regulation, antioxidant status, and corollary oxidative stress associated with type 2 diabetes.

Acknowledgments

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References


47. Hogan, S.; Zhang, L.; Chung, H.; Huang, Z.; Zoecklein, B.; Li, J.; Zhou, K., Antioxidant properties and bioactive components of Norton (Vitis aestivalis) and Cabernet Franc (Vitis vinifera) wine grapes *LWT-Food Science and Technology* **2009**, *In review*.


Tables

**Table 5.1.** TPC, TFC, ORAC and DPPH of 80% acetone pomace extracts

<table>
<thead>
<tr>
<th></th>
<th>Red grape</th>
<th>White grape</th>
<th>Red Apple</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Phenolic Content</strong></td>
<td>30.4 ± 11.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.5 ± 6.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>11.2 ± 5.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(µg GAE /g)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Total Flavonoid Content</strong></td>
<td>22.1 ± 8.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.2 ± 5.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.7 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(µg RE /g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ORAC (µmol TE/g)</strong></td>
<td>245.3 ± 21.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>197.7 ± 25.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>168.8 ± 22.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>%DPPH Inhibition/mg</strong></td>
<td>66.1 ± 0.6</td>
<td>67.4 ± 4.1</td>
<td>54.6 ± 13.2</td>
</tr>
</tbody>
</table>

Total phenolic content (TPC) is expressed as µg of gallic acid equivalents (GAE) per gram of pomace; Total flavonoid content (TFC) is expressed as µg of rutin equivalents (RE) per gram of pomace. Oxygen radical absorbance capacity (ORAC) was measured in triplicate, values expressed as micromoles Trolox equivalents per gram of pomace (µmol TE/g). DPPH, 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable radical and reactive hydrogen acceptor for determining the DPPH radical scavenging activity of extracts is expressed as the percent quenching of the DPPH free radical per mg of pomace. For each measurement, the data marked by different letters for each assay are significantly different (P < 0.05).
Figure 5.1. *In vitro* screening of yeast α-glucosidase inhibitory activity. Phenolic sample extracts were tested at the same concentration (285.7 µg/mL in the reaction). FP-1 to FP-7 represent peptide fractions hydrolyzed and separated from milk proteins. Norton grape (*Vitis aestivalis*) pomace exerted the strongest inhibition on α-glucosidase (99% inhibition) followed by acarbose (48% inhibition). Acarbose, is a commercially available oral alpha-glucosidase inhibitor which served as the positive control. The α-glucosidase activity was determined by measuring *p*-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes. Results are expressed as mean ± standard deviation.
Figure 5.2. *In vitro* effect of 80% acetone grape pomace extracts on rat α-glucosidase activity. Comparison of the inhibitory effects of 80% acetone grape pomace extracts (20 mg/ml) on α-glucosidase activity were measured from red and white wine pomace samples acquired from the fall of 2007 in Virginia. The α-glucosidase activity was determined by measuring *p*-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes. Results are expressed as mean ± standard deviation. Bars with different letters are significantly different (P < 0.05).
Figure 5.3. Dose-dependent inhibition of rat α-glucosidase inhibition by Norton GPE at different concentrations. The α-glucosidase inhibitory activity of 80% acetone red wine grape pomace extract (GPE) was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37 °C for 75 minutes. Dose-dependent inhibition of GPE was tested with concentrations ranging from 0 to 2.5 mg/mL. Results are expressed as mean ± standard deviation.
Figure 5.4. *In vivo* postprandial blood glucose suppression by Norton GPE following an OGTT. **A.** Attenuating effects of the red wine Norton (*Vitis aestivalis*) grape pomace extract (GPE, 400 mg/kg bw) on postprandial blood glucose levels measured at 0, 30, 60, and 120 minutes post maltose challenge (2g/kg bw) in mice (n=8) induced with diabetes by STZ intraperitoneal injection. Data are mean ± SD. **B.** Percent of glucose suppression. Incremental calculation of area under the curve (AUC$_{0-120\text{min}}$) was calculated from whole blood glucose data acquired at timepoints from 0 to 120 minutes. * Indicates statistical significance (P < 0.05, n=8 mice per group) where GPE suppressed glucose level by 35% compared to control.
CHAPTER 6: 12-WEEKS OF GRAPE PHENOLIC EXTRACT SUPPLEMENTATION EFFECTS GLYCEMIC, OXIDATIVE, AND INFLAMMATORY BIOMARKERS IN MICE FED A HIGH FAT DIET

Abstract

The purpose of this study was to investigate the effect of red grape phenolic extracts on fasting blood glucose levels, oxidative stress, and inflammatory biomarkers associated with obesity and the development of type 2 diabetes. Mice (8-week old male C57BLK) were supplemented (250 mg/kg bw) with either grape skin extract (GSE) or grape pomace extract (GPE) for 12-weeks while on a high fat “Western” diet. After 12-weeks of treatment, mice in the GSE group gained significantly (P < 0.05) more weight (24.6 g) than the control, high fat, or GPE groups (11.2, 20.2, 19.6 g, respectively). At week-12, the GSE and GPE groups had lower fasting blood glucose levels (119.3 and 134.2 mg/dL, respectively) compared to the high fat group (144.6 mg/dL). The 12-week supplementation of GSE was associated with a higher plasma oxygen radical absorbance capacity (ORAC), lower liver lipid peroxidation as measure by TBARS, and lower levels of inflammation as measured by plasma C-reactive protein compared to the high fat fed group. The GPE supplemented group had a significantly lower CRP value compared to the high fat group. The data indicated a potential therapeutic role of grape phenolic supplementation for improving blood glucose levels and as a countermeasure against oxidative stress and corollary inflammation associated with populations habitually consuming a high fat diet who are at risk for developing type 2 diabetes.

Keywords

Grape skin and pomace phenolic extract, mice, type 2 diabetes, obesity, hyperglycemia, oxidative stress, inflammation, high fat diet
Introduction

Diet and physical activity strategies are an effective means of reducing the risk of obesity and developing type 2 diabetes for individuals, but may not be a realistic prescription, as evidenced by the steady rise in obesity and type 2 diabetes rates and the low proportions of individuals who meet physical activity recommendations (1). This chronic metabolic condition currently affects approximately 24 million adults and children in the United States, and this number is expected to double by 2025 (2). Furthermore, it is estimated that 57 million North Americans have impaired glucose tolerance or “pre-diabetes”; if left uncontrolled this condition can eventually progress into type 2 diabetes (2, 3). With an unprecedented 33% of United States citizens obese and the incidence of diabetes rising, there is an undisputable need to identify and develop effective and safe alternative strategies (4).

Improving blood glucose homeostasis is a target of potential therapeutic agents such as oral α-glucosidase inhibitors (5). These inhibitors delay absorption of complex carbohydrates by inhibiting α-glucosidase, an enzyme present in the brush border of the small intestine. A growing body of evidence suggests that inhibition of this enzyme provides a beneficial effect for both preventing and treating type 2 diabetes by attenuating postprandial blood glucose levels (6-9). Oral α-glucosidase inhibitor medications are often associated with a variety of intolerable side effects such as bloating, abdominal discomfort, and flatulence (10). Thus, great interest is being devoted to developing alternative α-glucosidase inhibitors derived from natural sources and foods that may exert fewer adverse side effects and be cost-effective.

Controlling blood glucose levels is especially important in obese and type 2 diabetes prone populations. High levels of glucose or hyperglycemia has been associated
with the promotion of oxidative stress and inflammation (11-15). For example, oxidative stress due to persistent and chronic hyperglycemia may result in diminished activity of antioxidant enzymes and increased reactive oxygen species (ROS) generation which may be linked to the development and progression of complications associated with diabetes (14-19). Mechanistically, postprandial hyperglycemia induces oxidative stress by various biochemical pathways (20). Once oxidative stress is established, ROS may damage macromolecules such as lipids and proteins caused by peroxidation of lipid membranes and glycation of proteins (15, 21). The research strongly suggests that the elevated chronic blood glucose is a key contributing factor for the generation of ROS and subsequent decrease in antioxidant defenses associated with both obesity and type 2 diabetes (17, 19). Another deleterious consequence of obesity and hyperglycemia associated with type 2 diabetes is the presence of persistent low-grade inflammation (12, 22, 23). Reports have shown that plasma C-reactive protein (CRP), an acute phase biomarker of an innate immune pro-inflammatory response, is distinctly higher in obese and hyperglycemic populations (24, 25). To this end, research continues to determine the potential multiple-interfacing link between hyperglycemia, oxidative stress, and low-grade inflammation in obese populations at risk for developing type 2 diabetes.

Alternative strategies have been proposed to use naturally occurring agents with potential dual bioactive properties for addressing hyperglycemia and the parallel oxidative and inflammatory stress associated with diabetes (26). Grapes have been well characterized as a natural source of notable bioactive compounds with antioxidant, health promoting, and disease protective qualities (27-29). The rich phenolic compounds found in grape seeds, skins, and stems have been reported to account for the beneficial
properties (27-30). For example, rats supplemented with grape derived extracts rich in procyanidins had improved insulin resistance and suppressed oxidative stress in rats (29). Research continues to determine if grape derived phenolic compounds have a corollary benefit not only for their role in blood glucose control but also as an antioxidant that may reduce oxidative stress (31).

Reducing blood glucose may be an effective strategy for reducing the risk of developing diabetes, oxidative stress, and inflammation in obese populations. Our laboratory has previously detected a potential dual antioxidant and α-glucosidase inhibitory activity of grape derived bioactive constituents in vitro and on the improved postprandial blood glucose control in vivo in STZ-injected mice with diabetes (31, 32). It has been shown that C57BL/6J mice develop obesity, insulin resistant diabetes, hypercholesterolemia, and hypertriglyceridemia when fed a high fat diet formulated to approximate a typical human diet in Western cultures (33). The purpose of this study was to assess the effect of grape extract supplementation (250 mg/kg bodyweight per day) on hyperglycemia, oxidative stress, and inflammation status in C57BLK/NCr mice fed a high fat diet.

**Materials and Methods**

**Materials**

The following kits and material were purchased and used to conduct this study:

Mouse/Rat Insulin enzyme linked immunoassay (ELISA) kit (Millipore Corporation, St.Charles, Missouri, USA, Cat.EZRMI-13K); Mouse CRP ELISA (Immunology Consultants Laboratory, Inc. Newberg, OR, USA, Cat.E-90CRP); cytosolic glutathione peroxidase (GPx) (Cayman Chemical Company, Ann Arbor, MI, USA, Cat.703102);
bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA, Cat.23227). Trolox™, fluorescein, 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The acetone solvent for grape skin and grape pomace extraction was HPLC grade (Fisher Scientific Co. Atlanta, GA).

**Grape skin and pomace extract preparation**

Red wine Norton grapes (*Vitis aestivalis*) grown at the Blackstone vineyard (Blackstone, VA, USA) were harvested in the fall of 2007 and transported to the Enology Laboratory at Virginia Tech. The Norton wine grape variety is a native Virginia grape with bioactive properties that have been investigated in our laboratory (31, 32). The skins from grapes were hand peeled and then lyophilized (prepared in dry form by rapid freezing and dehydration, while in the frozen state under high vacuum). The dried grape skins were milled to a fine powder with a Thomas Wiley mini-mill (Swedesboro, NJ) and extracted with 50% acetone at 1:10 ratio (m/v). The extracts were then filtered to remove unwanted residues with Whatman No. filter paper. After removing the organic solvent, the filtrates were frozen and lyophilized to obtain the grape skin extract (GSE). The Norton grape pomace was extracted with 80% acetone at 1:10 ratio (m/v) and then placed in a stir bath overnight. The extracts were filtered through Whatman No. 4 filter paper to remove unwanted residues. After evaporating off the organic solvent, the filtrates were frozen and lyophilized to obtain the grape pomace extract (GPE). The GSE and GPE were shipped to Research Diets Inc. (New Brunswick NJ, USA) for incorporation into a pelleted high fat diet.
Supplementation of GSE and GPE (250mg/kg bw) to High Fat Fed Mice

Male 6 week-old mice (C57BLK/6NCr, National Cancer Institute, Frederick, MD, USA) were used to conduct this 12-week supplementation study. Animal husbandry, care, and experimental procedures were conducted in compliance with the “Principles of Laboratory Animal Care” NIH guidelines, as approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech (approved protocol #07-115-FST). Throughout the duration of this study, the mice were housed in cages (4 mice/cage) at a regulated temperature (20–23 °C) and alternating 12-h light/dark cycles. The rodents had access to mouse chow and water ad libitum. Mice were fed a standard rodent chow (Harlan Tekland Global Diets 2018 rodent diet, Madison, WI, USA) for 2 weeks during the initial quarantine and acclimatization phase. This diet contains 17% of calories from fat (derived from soybean oil), 60% calories from carbohydrate, 23% of calories from protein, and with a vitamin mixture containing α-tocopherol (100 mg/kg feed) (Figure 6.1). After the acclimatization period, mice were randomly placed into one of four groups (n=12 per group), and fed a healthy control diet (C), high fat diet (HF), high fat diet + grape skin extract (HFSE), high fat diet + grape pomace extract (HFPE). The pelleted high fat diet (D12079B; Research Diets Inc., New Brunswick, NJ) contained 41% of calories from fat, 43% calories from carbohydrate, 17% of calories from protein, and had a vitamin mixture containing α-tocopherol (3350 mg/kg feed) (Figure 6.1). This diet was selected to leave the mice highly susceptible to diet-induced obesity and at an elevated risk of developing type 2 diabetes (34). The caloric value in a total of 1000 grams of feed comprised in this high fat feed matrix included: milk fat constituting 1800 kcal (equivalent to approximately 65% saturated fat, 30% monounsaturated fat, and 5% polyunsaturated fat) and corn oil constituting 90 kcal (equivalent to approximately 13%
saturated fat, 25% monounsaturated fat, and 62% polyunsaturated fat). The dosage of the GSE and GPE in the feed was incorporated into pellet form at a mixture of 2.4 g/kg of feed in order to dose each mouse at approximately 250 mg/kg bodyweight (bw) per day. Fresh feed, stored at 4°C in light and air protected containers, was weighed and dispensed every three days. Daily feed intake was determined by estimating the average daily consumption (g) intake and dose of grape extract. Bodyweight and fasting blood samples were determined at weeks 0, 2, 4, 6, 8, 10, and 12. For the termination timepoint (week-12), blood samples were collected into ice chilled heparinized tubes from the retro orbital venous plexus while under anesthesia to obtain plasma for insulin, oxidative, and inflammatory biomarkers. All mice were sacrificed with cervical dislocation under anesthesia. Liver organs were immediately excised, washed/chilled in ice-cold 0.9% NaCl, weighed, and then snap frozen with liquid nitrogen. The plasma and liver samples were stored for two-weeks at −20°C and −80°C, respectively (35).

**Fasting blood glucose**

At baseline and every 2-weeks (i.e. 0, 2, 4, 6, 8, 10, and 12) approximately 5 µL of whole blood was collected from the tail vein of each mouse after a 14-hour fast to measure blood glucose concentrations (mg/dL) with a glucometer and accompanying blood glucose test strip (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI).

**Plasma insulin**

Fasting plasma insulin levels were measured at the final timepoint (week-12) in duplicate, using a mouse enzymatic ELISA kit according to the manufacturer's recommendations. The assay was measured spectrophotometrically (Victor³ multi-label
platereader, PerkinElmer, Turku, Finland) by the increased absorbency at 450nm, corrected from the absorbency at 590nm and expressed as ng/mL.

**Plasma CRP**

During states of inflammation the acute phase C-reactive protein (CRP) can be detected in plasma. An immunoperoxidase assay for determination of CRP at the termination timepoint (week-12) was used. The plasma CRP level was measured by ELISA using a platereader at an absorbance of 450nm and expressed as ng/mL.

**Cytosolic glutathione peroxidase**

Blood samples were collected at the 12-week termination timepoint and analyzed for biomarkers associated with oxidative stress. The plasma antioxidant enzyme activity cytosolic glutathione peroxidase (GPx) was measured with an assay kit following the manufacturer’s instructions. The GPx assay was measured spectrophotometrically with the platereader at an absorbance of 340 nm (nmol/min/mL). The bicinchoninic acid (BCA) protein assay was used for the determination of the total protein (µg/mL) in each plasma sample following manufacturer’s instructions. The GPx values were expressed as mmol/min/g.

**Plasma ORAC**

Plasma oxygen radical absorbance capacity (ORAC) was measured to determine the influence of grape phenolic supplementation on oxidative stress status following the 12-week study. The ORAC assay measures the peroxyl radical scavenging activity of blood samples with Trolox as the antioxidant standard (36). A water soluble vitamin E analog was used as the standard, Trolox™. A fluorescein stock solution (100 µmol/L) in
phosphate buffer (75 mM, pH 7.4) was prepared and kept at 4°C in the dark. The fluorescein solution (final concentration of 100 nmol/L) was added to each plasma sample or standards prepared in phosphate buffer in a black 96-well plate and incubated for 20 minutes at 37°C. The reaction was initiated by adding the peroxyl radical generator, 0.36 mol/L AAPH, and the fluorescence was measured ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 535$ nm) using the platereader maintained at 37°C until the reading had declined to less than 10% of the initial reading (e.g. 20 minutes). Standards and samples were run in duplicate. The ORAC value of each plasma sample was expressed as micromoles of Trolox equivalents per milligram of protein concentration for each sample ($\mu$mol TE/mg) as determined from the BCA assay.

**Liver lipid peroxidation**

Liver lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) and was expressed as micrograms of malondialdehyde (MDA) per gram of liver specimen using a previously protocol with slight modification (37). In brief, equal amounts of liver tissue from each mouse was weighed (0.8 g) and homogenized in 10 mL distilled de-ionized water with 0.002% propyl gallate and 0.004% ethylenediaminetetraacetic acid (EDTA) (w:v), 0.1ml of 2.0% sodium dodecyl sulfate (SDS) using a homogenizer (Virtishear 225318, The Virtis Company Inc., Gardiner, NY). EDTA was used to prevent the liver samples from in vitro lipid peroxidation during sample preparation (38, 39). Next, 9 ml of distilled water was used to rinse the homogenizer head and then mixed into the homogenate with vortexing. For each sample, 1.0 ml of the homogenate was transferred into 4.0 ml of 0.38% TBA solution (0.51% SDS and 9.38% acetic acid, (w: v)). After vortexing, each sample mixture was incubated
in a 95°C waterbath (Model 10l, Fisher Scientific, Pittsburgh, PA) for 60 minutes and cooled to room temperature. After cooling, 5.0 ml of 1-butanol and pyridine solution at a ratio of 15:1 was added to each sample mixture, thoroughly vortexed, and then centrifuged at 10°C at 3000 rpm for 15 minutes. The absorbance of the upper organic layer of the centrifuged solution was measured at a wavelength of 532 nm with a spectrophotometer (Model 21D Spectronic, Milton Roy Co., Rochester, NY). The final unit of TBARS value was calculated and expressed as mg of malondialdehyde (MDA) equivalent per kg of liver (mg MDA equivalent/kg).

**Statistical Analysis**

All data are expressed as the mean ± standard deviation (SD). First the data was analyzed for the statistical assumptions for equal variance or the homogeneity of variance and normality or the normal distribution using the Levenes and Shapiro-Wilk tests. The data was then analyzed using one-way analysis of variance followed by post hoc Duncan’s multiple range tests for comparison of differences among all groups (SPSS for Windows, Version 13.0, SPSS Inc., Chicago, IL, USA). A significant difference was defined as a P-value < 0.05.

**Results**

**Estimated extract dosage and caloric intake and effect of diet on growth**

Both the HFSE and HFPE diets were well tolerated by the mice; all animals survived the 12-week feeding study. The HFSE and HFPE groups had similar average daily extract dosage, 234.72 ± 26.1 and 246.91 ± 26.0 mg/kg of bodyweight, respectively. Feed intake was comparable in all groups (approximately 3.5 g/d) which equates to approximately 0.875 mg/day of phenolic extracts in GSE or GPE. The control and high
fat diets were not iso-caloric; as such the control group habitually consumed fewer calories per day as compare to any of the high fat fed groups. The mice maintained their palatability for both the GSE and GPE dosage of (250 mg/kg bw per day) incorporated extracts into the high fat diet.

Bodyweight differences were detected (Table 6.1). The incorporation of the GSE had a notable impact on bodyweight; by week-12 the HFSE group gained significantly (P < 0.05) more weight (24.6 g) compared to the C, HF, or HFPE groups (11.2, 20.2, 19.6 g, respectively). At week-12 the HFSE group weighed 20.3% more than the HFPE group even though both groups were estimated to consume approximately the same amount of kilocalories per day (16.45 kcal/d) (Figure 6.2). The control group weighed significantly less than all groups throughout the study and gained the least amount of overall weight (11.2 ± 2.8 g) (P < 0.05). At week-12 the HF and HFPE groups gained statistically similar amounts of weight (20.2 ± 3.4 and 19.6 ± 3.6 g, respectively). At termination, the weights of the liver organs were determined for each of the C, HF, HFSE, and HFPE groups (1.0, 1.5, 2.4, 1.9 g, respectively). The liver weight of the HFSE group was 37.5% and 20.8% heavier than the HF and HFPE groups, respectively. The liver to body weight ratio of the HFSE group was statistically larger compared to the C, HF, or HFPE groups (P < 0.05) (Figure 6.3).

**Effect of GSE and GPE supplementation on fasting blood glucose levels**

The fasting blood glucose (mg/dL) response to each diet was variable but detected to be suppressed at week-12 in the C, HFSE, and HFPE groups compared to the HF group (Table 6.2). In particular, after all the mice had been acclimatized to diet treatments for 2 weeks, no significant differences were identified for fasting blood
glucose levels between the C, HF, HFSE, or HFGE groups (90, 85, 95, and 98 mg/dL, respectively). By week-6 the fasting blood glucose levels were significantly influenced by the high fat diets independent of GSE or GPE supplementation. In particular, the C group had significantly lower (P < 0.05) fasting blood glucose levels (84 mg/dL) compared to the HF, HFSE, or HFPE groups (111, 103, 102 mg/dL, respectively). By week-12 the fasting blood glucose levels for the C, HFSE, and HFPE groups were all lower than the HF group (Table 6.2). The HFSE group had fasting blood glucose levels (119.3 mg/dL) that were statistically similar to that of the C group (110.3 mg/dL). The HFPE group had lower fasting blood glucose levels (134.2 ± 20.2 mg/dL) compared to the HF group (144.6 ± 17.3 mg/dL) and this decrease did not achieve statistical significance (P > 0.05). The long term supplementation of GSE with a high fat diet resulted in fasting blood glucose levels similar to that of the healthy control group. This improved fasting blood glucose trend observed in the HFSE group which became more regularized to the healthy control levels was not observed in either the HF or HFPE groups.

The lower concentration of fasting blood glucose may be linked to an improvement of insulin sensitivity or insulin resistance in mice in response to the treatment with grape extracts. However, there was no significant difference in the insulin concentrations detected between any of the groups when measured at the 12-week timepoint (Table 6.3). The plasma insulin variability especially within the HF group was such that clear conclusions could not be drawn from this particular study.
Effect of GSE and GPE supplementation on oxidative stress and inflammation

Following 12-weeks of diet treatments, a variety of antioxidant biomarkers were measured (Table 6.3). The HFSE group had a significantly higher ORAC value (52.6 umol TE/mg) compared to the C or HFPE groups (43.1, 43.4 umol TE/mg, respectively). The HF group had similar ORAC values when compared to the HFSE group (49.8 vs. 52.6 umol TE/mg, respectively). Supplementation of an antioxidant and phyto-phenolic rich red GSE (250 mg/kg bw per day) was associated with a trend of improved antioxidant capacity in the plasma and this antioxidant protective potential was not detected in the HFPE group from the ORAC values. The plasma GPx was not significantly influenced by any of the feeding treatments (Table 6.3, P > 0.05). Although not significant, the HF group was determined to have the highest GPx activity as well as the most variability. The HFPE group was found to have the least plasma GPx activity indicating less lipid peroxidation detoxification occurring in this group of mice compared to the HF group. The effects of grape extracts on liver lipid peroxidation in mice are shown in Table 6.3. The control group had the highest levels of liver lipid peroxidation (8.2 mg MDA/kg liver) (P < 0.05). The HFSE group had a non-significant 19.5% reduction in liver malondialdehyde (MDA) level compared to the HF group (4.05 vs. 5.03 mg MDA/kg liver, respectively P = 0.06). The HFPE group had a lipid peroxidation value of 6.08 mg MDA/kg which was significantly greater than the HFSE or HF groups (P < 0.05). Systemic inflammatory status of mice in each group was determined by measuring the plasma acute phase CRP level at the termination of the 12-week study (Table 6.3). CRP plasma levels were at least 9.2% significantly (P < 0.05) higher in the HF group (26.1 ng/mL) compared to the C, HFSE, and HFPE groups (22.2, 23.7, and 22.6 ng/mL, respectively). The result suggests that the HF group had a higher degree of
chronic low-grade inflammation which has been associated with obese populations (40).

These results indicate that the higher levels of plasma CRP inflammatory biomarker present after consuming a high fat diet for at least 12-weeks can be suppressed by the supplementation of GSE or GPE to levels observed in a lower fat diet.

Discussion

This high fat feeding study was designed to allow for the natural progression of the effects of the diet for investigating obesity and type 2 diabetes. Our previous in vitro and acute animal investigations have demonstrated that grape derived extracts had both antioxidant and anti-postprandial hyperglycemic effects that may provide dual protection against type 2 diabetes and associated oxidative stress (31, 41). Here we showed that 12-weeks supplementation of grape extracts (250 mg/kg bw per day) exerted a potential antidiabetic effect by ameliorating fasting blood glucose in high fat fed mice. This study also demonstrated that grape extracts moderately improved oxidative stress and inflammatory status in high fat fed mice.

Feeding mice a high fat diet for 12-weeks effected glucose homeostasis in our study. This is in agreement with data regarding high fat feeding studies in which higher fasting blood glucose levels were detected in mice fed a high fat diet compared to a high carbohydrate diet (39). The authors suggested that the fatty acid composition of the diet may be responsible for metabolic regulation associated with glucose and insulin responses. In our study, the fasting blood glucose data suggest that the suppressed glucose response in high fat fed mice by GSE may be a result of the α-glucosidase inhibitory effects of phenolic compounds in grapes. This mechanism is similar to that of acarbose which competitively inhibits brush border α-glucosidase in the small intestine.
Other naturally derived agents have been identified in phenolic extracts from wine administered to rats for 6-weeks to have anti-diabetes activity (43). Similarly, grape seeds rich in procyanidins have been reported to have an antihyperglycemic effect in STZ-induced rats. The authors of this study suggested that this response was a consequence of a delay in intestinal glucose absorption and/or through an insulin signaling pathway (44). Furthermore, 15-weeks GSE supplementation at approximately 880 mg/kg bw/d (approximately 280 mg polyphenols/kg bw) delayed the development of atherosclerosis in hyperlipidemic rabbits (45). Collectively, the evidence is building in support of hypoglycemic agents derived from natural plant sources such as grapes and their potential to improve glucose metabolism associated with diabetes and our results add to this body of literature.

Improvements in insulin sensitivity can help to maintain blood glucose homeostasis and prevent the detrimental effects of hyperglycemia (46). Our study failed to show an effect of the grape derived extract supplementation on insulin response. The antihyperglycemic response at week-12 observed in the HFSE and HFPE groups may be a result of chronic delay of blood glucose absorption independent of insulin sensitivity. However, there are reports of improved insulin sensitivity from the ingestion of phenolic compounds such as cinnamon (47, 48). Grape seed extracts rich in procyanidin phenols have also been reported to have an insulin-like effect in STZ-injected rats (44). The inconsistency of our results and those of others may be attributed to the duration and or dose of GSE and GPE employed, as such future studies are needed to address the influence of grape phenolic extracts on insulin response.
An unexpected result from this study was the weight gain identified in the HFSE group. In particular, there was a 17.9% increase in bodyweight gain in the HFSE group compared to the C, HF and HFPE groups. The mice in the high fat fed groups were estimated to consume similar amounts of dietary calories. However, unless the animals were housed singly we do not definitely know how much food was consumed for each mouse. Another explanation for the weight gain may be attributed to non-extracted sugar molecules from the GSE which may have contributed to a higher caloric value in the diet. If this was not the case, an interesting effect of feeding mice a daily GSE at a dosage of approximately 250 mg/kg on weight gain was observed when using this high fat diet rodent model and this response was not detected in the HFPE group.

The alterations of body and liver organ weights disagree with some feeding studies that administered phenolic extracts or oral-hypoglycemic agents in which no significant increases in bodyweight were detected (49). However, similar to our findings, there are reports of antihyperglycemic agents increasing weight gain. In particular, rats administered green tea extracts (300 mg/kg bw/d) for 6-weeks had significant bodyweight increases as compared to the diabetic control group (50). The authors suggested this effect may be due to improved glycemic control elicited by the green tea extract in the rats. Additionally, mice with diabetes and supplemented daily with an antihyperglycemic aqueous extract from a Vietnamese edible plant (*Cleistocalyx operculatus*) at 500 mg/kg for 8-weeks had increased bodyweight as compared to a diabetic control group (51). When mice were supplemented with blueberry powders and a high fat diet, bodyweight and body fat increased as compared to control animals fed the same high fat diet (52). However, in the same study it was discovered that feeding mice
with anthocyanin extracts from blueberry and strawberry decreased bodyweight gain and body fat (52). In studies using anthocyanins from purple corn and cherries (rich in cyanidin-3-glucoside) a reduction in the bodyweight of mice fed a high fat diet compared to the high fat diet control group was reported (53, 54). Collectively, these studies indicate that phenolic extracts play a role in weight control. In our study, one explanation for the improvement in fasting glycemic response in the HFSE group may have been contributed to an increase in hydrated glycogen storage in the liver, muscles, and fat cells. This would explain in part the increase in body and liver weight gain in the HFSE group due to the fact that in humans every gram of glycogen is approximately equivalent to 3 grams of water (55).

The heavier liver weights measured in HFSE group provide evidence for the potential of liver abnormalities. This contraindication has been observed from use of acarbose along with other gastrointestinal side effects (56). Although some studies have observed liver weight decreases after plant derived polyphenolic supplementation in a diet induced obesity group, other studies have reported increases in liver weights from the use of anti-diabetic agents (57, 58). Influence on glycemic regulation by increasing hepatic glycogen storage was proposed to be a possible mechanism associated with the liver enlargement after the administration of an oral hypo-glycemic agent, tagatose (57). In a study using broiler chicks, supplementation of grape pomace concentrate (60 g/kg) for 42 days did not increase the weight of liver organs when compared to the control group (59). Overall, it is clear that additional chronic feeding studies need to be conducted in order to determine if our GSE is an appropriate oral hypoglycemic agent and to further clarify its effect on body and liver weight.
The GSE was associated with reduced fasting blood glycemia and this may have contributed to enhance antioxidant capacity detected in the HFSE group as determined by the plasma ORAC data. For example, the HFSE associated ORAC value could be attributed to antioxidant properties of the extract to function as peroxyl radical scavengers. Thus, an enhanced antioxidant capacity translates to an improvement in oxidative stress status by detoxify the ensuing reactive products associated with hyperglycemia or diabetes states. Antioxidants from phenolic rich extracts such as chokeberries have been shown to contribute to hypoglycemic response (60, 61). Similar to our results, enhanced ORAC values have been detected in vivo after consuming grape sources. In particular, serum ORAC values in humans were reported to be transient but generally higher in select grape and raisin varieties after 4 weeks of daily consumption of golden raisins, sun-dried raisins, or grapes (62). Studies have shown that grape wine flavonoids can also improve diabetes associated oxidative stress, but failed to identify any effect on glycemia (63, 64). The daily administration of white wine extracts to STZ-induced diabetic rats was also found to improve oxidative stress associated with diabetes (63). A similar finding was reported where acute red wine consumption with a meal (300 ml wine with approximately 300 mg flavonoids) improved oxidative status in subjects with type 2 diabetes (64). Similar to grape pomace, a pomegranate juice byproduct was found to have antioxidant properties (65). In particular, after 8-weeks of pomegranate peel supplementation, cattle gained more weight and had higher blood levels of alphatocopherol than the animals on standard feed. The authors explained that, the pomegranate peels enhanced bodyweight similar to that of antibiotics and hormones without unwanted side effects and could result in antioxidant rich meat (65).
Lipid peroxidation is an indicator of oxidative stress associated with membrane damage (66). Enzymatic antioxidant activity, such as glutathione peroxidase (GPx), is biomarker of lipid peroxidation and reported to be lower in animals with diabetes (67). As such, enhanced antioxidant activity reflects the potential to reduce oxidative deterioration of lipid macromolecules. Our study showed that grape phenolic supplementation did not effect plasma GPx antioxidant enzyme activity, though the HF group had the highest level of GPx activity, the difference was not statistically significant. Increases in antioxidant enzyme activity may indicate that during the early stages of obesity and development of type 2 diabetes, an effort to reduce oxidative stress occurs when reservoirs are still available (68). If ROS surpass the antioxidant defense threshold, oxidative stress may become more prevalent in the mice with the most compromised antioxidant defense systems. Therefore, increases in antioxidant defenses may be upregulated as an initial countermeasure against oxidative stress associated with the establishment of type 2 diabetes. Future studies need to be conducted in which red blood cells and liver GPx are measured as well as other endogenous antioxidant enzymes in order to clarify the effectiveness of grape phenolic extract supplementation on the endogenous enzymatic antioxidant defense system.

Lipid peroxidation end-products can be measured as thiobarbituric acid reactive substances (TBARS) and is reported to be higher in population with diabetes (69). In our study, oxidative stress associated with liver lipid peroxidation was lower in the high fat fed groups compared to the control fed group. Other studies have reported a decrease in liver lipid peroxidation after feeding mice a high fat diet (72% fat) for 4-week as compared to a control diet (70). These authors suggested that lipid peroxidation was not
necessarily related to the development of metabolic condition such as obesity and diabetes (70). Additionally, the authors explained that the high fat diet contributed to the protection of lipid peroxidation as a result of the 90.8% higher vitamin E concentration in the high fat diet (153 µg/g) as compared to the control diet (14 µg/g) (70). In our study, the significantly higher amount of liver lipid peroxidation detected in the control group compared to the high fat fed groups may have been associated with a reduced availability of potent radical scavenging vitamin E antioxidants. In particular, the control diet was comprised of a vitamin mixture incorporated in the feed which included 100 mg/kg of vitamin E compared to the high fat diet which contained 3350 mg/kg of vitamin E.

Highly reactive and promiscuous ROS can wreak havoc to macromolecules such as lipids, proteins, and DNA causing cellular dysfunction and disease. As such, diets containing polyunsaturated fatty acids (PUFA) are susceptible to attacks by free radicals which results in an increase of lipid peroxidation and generation of ROS (71). Similar to other studies implementing high fat and control diets, the control diet in our study had the highest amount of liver MDA value, indicative of lipid peroxidation (70). In addition to the antioxidant contribution from vitamin E concentrations in each diet, this result may have been associated with an increased susceptibility of lipids to peroxidation due to the increased proportion of PUFA available for formation of lipid bilayers. For instance, the significant increase in liver lipid peroxidation in the control group may be related to the high proportion of PUFA (~63%) derived from soybean oil in this diet (Figure 6.4). This may explain for the lower amount of lipid peroxidation detected the HF diets which had less PUFA (~7%) as compared to the control diet.
When comparing only the high fat fed animals, our results showed that GSE supplementation reduced liver lipid peroxidation the most compared to the high fat and HFPE groups (Table 6.3). Similar lipid peroxidation protection in chicken breast meat from grape derived phenols was reported with chickens supplemented for 42 days with grape pomace (60 g/kg) (59). Others have reported that grape seed proanthocyanindins significantly decreased lipid peroxidative damage in liver (72). Additional studies are needed to determine if the lower level of liver peroxidation in the HFSE group is associated with hypoglycemia and concomitant reduction of ROS. The improved ORAC status in the HFSE group, which reflects antioxidant capacity, supports our speculations that GSE may provide a protective property against lipid peroxidation. Although the HFPE group was not shown to have a significantly lower level of liver lipid peroxidation, a protective effect on lipid peroxidation may be detected by increasing treatment dose or duration. The significantly (P < 0.05) higher level of lipid peroxides in HF group as compared to the HFSE group may indicate the gradual and persistent consequence of the unregulated influx of radical species generated due in part to hyperglycemia. This gradual worsening of oxidative status may be a contributing factor in the establishment of type 2 diabetes in populations habitually consuming a high fat Western-like diet.

Obesity may be associated with oxidative stress and persistent inflammation, both of which may be involved in the pathogenesis of type 2 diabetes (23, 73-75). CRP levels have been shown to be significantly higher in obese populations compared to lean humans (24, 40). As expected, mice fed the HF diet had a higher level of the inflammatory CRP biomarker. The supplementation of GSE and GPE into the high fat diet for 12-weeks had an attenuating effect on the inflammatory immune response when
compared to the HF group. The CRP levels in the grape supplemented groups were similar to that of the healthy aged-matched control mice. The significantly lower level of CRP in grape extract supplemented groups may reflect potential dual bioactive properties in regards to anti-hyperglycemic and free radical scavenging activities. Similar to our findings, improvements in inflammatory immune response have been reported from grape seed extracts. For instance, 19-weeks of high fat diet supplementation with daily procyanidins from grape seed (7 mg) resulted in a decrease in plasma CRP. The authors proposed a mechanism where the procyanidins reduced the translation of proinflammatory cytokines such as TNF-α and IL-6 by inhibiting the activity of the NF-kB pathway. Over time, lower glucose concentrations and sustained antioxidant protection may cause a lower systemic inflammatory response by the innate immune system as reflected by lower levels of CRP.

**Conclusion**

This preliminary grape extract long-term feeding study detected a potential beneficial effect of grape extracts for reducing fasting blood glucose. Grape extracts were associated with a developing protective trend of improved oxidative and inflammatory status in mice fed a high fat diet. One possible mechanism underlying such protection may be stemming from the reduced blood glucose levels which delay the induction and degree of ROS associated oxidative and inflammatory stress. In particular, the GSE was associated with lower fasting blood glucose levels, high plasma ORAC, lower lipid peroxidation, and lower CRP inflammation levels compared to the high fat group. These biomarkers indicate that grape phenolics may play a role in the prevention or delay of obesity induced type 2 diabetes. An obvious caveat to the identified anti-diabetes
beneficial effects in the GSE group was a significant increase in body and liver weight, above and beyond that which was measured in all other groups. Additional research is merited in order to establish if grapes can be safely and efficaciously developed as a nutraceutical for addressing type 2 diabetes.

Acknowledgement
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### Table 6.1. Grape derived extracts incorporated into a high fat diet on fasting bodyweight

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Control</th>
<th>High Fat</th>
<th>High Fat + GSE</th>
<th>High Fat + GPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (g)</td>
<td>18.9 ± 1.5</td>
<td>18.7 ± 0.1</td>
<td>18.7 ± 1.7</td>
<td>19.2 ± 1.1</td>
</tr>
<tr>
<td>Week 2 (g)</td>
<td>21.6 ± 1.5</td>
<td>23.8 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.5 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.2 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 4 (g)</td>
<td>23.3 ± 1.7</td>
<td>27.2 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.6 ± 2.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>27.4 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 6 (g)</td>
<td>25.2 ± 2.8</td>
<td>30.12 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3 ± 3.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>29.9 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 8 (g)</td>
<td>25.6 ± 1.7</td>
<td>32.7 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.9 ± 3.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>33.2 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 10 (g)</td>
<td>28.0 ± 2.0</td>
<td>35.9 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8 ± 3.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>35.7 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 12 (g)</td>
<td>30.1 ± 2.2</td>
<td>38.9 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.3 ± 3.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>38.8 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final weight gain (g)</td>
<td>11.2 ± 2.8</td>
<td>20.2 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.6 ± 4.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>19.6 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Four diet treatments included a control, high fat, high fat incorporated with grape skin extract (GSE), and high fat incorporated with grape pomace extract (GPE). Bodyweight (g) values are the mean ± SD, n = 12 animals (C57BL/6NCr mice) in each group.

<sup>a</sup> Significantly different when compared to control group during the same week, P < 0.05.  
<sup>b</sup> Significantly different when compared to high fat and pomace groups during the same week, P < 0.05.
Table 6.1. Effect of grape derived extracts incorporated into a high fat diet on fasting blood glucose (mg/dL)

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Control</th>
<th>High Fat</th>
<th>High Fat + GSE</th>
<th>High Fat + GPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2 glucose (mg/dL)</td>
<td>90.1 ± 11.6</td>
<td>85.3 ± 10.9</td>
<td>94.5 ± 10.8</td>
<td>97.7 ± 18.2</td>
</tr>
<tr>
<td>Week 6 glucose (mg/dL)</td>
<td>84.3 ± 11.4</td>
<td>110.9 ± 12.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.6 ± 11.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.6 ± 19.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week 12 glucose (mg/dL)</td>
<td>110.3 ± 23.2</td>
<td>144.6 ± 17.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>119.3 ± 17.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>134.2 ± 20.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Four diet treatments included a control, high fat, high fat incorporated with grape skin extract (GSE), and high fat incorporated with grape pomace extract (GPE). Fasting blood glucose (mg/dL) values are the mean ± SD, n = 12 animals (C57BL/6NCr mice) in each diet treatment group. <sup>a</sup> Significantly different when compared to control group during same week, P < 0.05. <sup>b</sup> Significantly different when compared to high fat group during same week, P < 0.05.
### Table 6.3. Effect of 12-week supplementation of grape derived extracts incorporated into a high fat diet on antioxidant and inflammatory biomarkers

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>ORAC (TE uMol/mg)</th>
<th>CRP (ng/mL)</th>
<th>plasma cGPx (m mol/min/g)</th>
<th>Liver TBARS (mg MDA/kg)</th>
<th>Insulin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.1 ± 6.1</td>
<td>22.2 ± 0.4</td>
<td>4.0 ± 0.4</td>
<td>8.2 ± 2.0a</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>High Fat</td>
<td>49.8 ± 12.5</td>
<td>26.1 ± 0.8**</td>
<td>4.3 ± 0.7</td>
<td>5.0 ± 1.8c</td>
<td>1.5 ± 1.7</td>
</tr>
<tr>
<td>High Fat + GSE</td>
<td>52.6 ± 3.4*</td>
<td>23.7 ± 0.6</td>
<td>3.9 ± 0.5</td>
<td>4.1 ± 1.4c</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td>High Fat + GPE</td>
<td>43.4 ± 4.4</td>
<td>22.6 ± 0.6</td>
<td>3.7 ± 0.3</td>
<td>6.1 ± 1.9b</td>
<td>0.8 ± 0.6</td>
</tr>
</tbody>
</table>

Four diet treatments included a control, high fat, high fat incorporated with grape skin extract (GSE), and high fat incorporated with grape pomace extract (GPE).

Values are the mean ± SD, n = 10 animals (C57BL/6NCr mice) in each group.

* Significantly different when compared to control and pomace groups for ORAC biomarker measurement, P < 0.05. ** Significantly different when compared to control, skin, and pomace groups for CRP biomarker measurement, P < 0.05. Means for Liver TBARS with different letters are significantly different, P < 0.05.
Figures

Figure 6.1. Percent of kcalories (kcal%) from fat, carbohydrate, and protein macronutrients comprised in each treatment group. Four diet treatments included a control, high fat, high fat incorporated with grape skin extract (2.4 g/kg feed), and high fat incorporated with grape pomace (2.4 g/kg feed).
Figure 6.2. Estimated daily caloric intake (kcal) for each diet treatment (control, high fat only, high fat incorporated with grape skin extract, high fat incorporated with grape pomace extract) conducted for 12 weeks. Extract dose of approximately 250 mg/kg bw per day).
Figure 6.3. Liver to bodyweight ratio following 12 week diet treatments; n = 12 animals (C57BL/6NCr mice) in each group. Diet treatments included a control, high fat only, high fat incorporated with grape skin extract, high fat incorporated with grape pomace extra.
**Figure 6.4.** Fatty acid profile of the control diet (soybean oil) and high fat diet (milk fat and corn oil). The fatty acids include: saturated, monounsaturated and polyunsaturated. Four diet treatment groups included a control, high fat, high fat incorporated with grape skin extract, and high fat incorporated with pomace extract.
CHAPTER 7: SUMMARY AND FUTURE GRAPE EXTRACT RESEARCH

Grapes as Nutraceuticals

The epidemic of type 2 diabetes and the consensus that maintenance of healthy blood glucose levels can reduce morbidity has given precedence for strategies targeting hyperglycemia (1). Hyperglycemia, oxidative stress, and low-grade inflammation are present in this population and are hypothesized to be contributing to the worsening of type 2 diabetes (2, 3). Despite the grape skin phenolic extract’s potent ability to inhibit α-glucosidase in vitro and promising acute oral glucose tolerance test results discovered from our studies, this naturally derived collection of phenolics needs to be further investigated for its efficacy as a successful nutraceutical with multiple bioactive properties such as antihyperglycemic, antioxidant, and anti-inflammatory effects.

Accumulating evidence suggests that chronic and persistent hyperglycemia increases the odds of generating overwhelming oxidative stress to the body which can cause deleterious physiological modification to vital organs and tissue (4). For example, liver, kidney, nerve endings, and eyes of individual with type 2 diabetes are the “hallmark” targets of increased perturbations as a result of the micro-vasculature associated with their biological environment. As such, ROS-mediated DNA, lipid, and protein damage is more likely to increase at these sites during states of poorly regulated blood glucose homeostasis in populations at risk for or with diagnosed type 2 diabetes (5).

Antioxidant rich grape extract provides for an attractive strategy for achieving improved oxidative status in individuals with type 2 diabetes. Furthermore, the dual or even multiple bioactive properties that grape phenols may possess in terms of being
potent glucose metabolizing enzyme inhibitors as well as antioxidant and anti-inflammatory provides a novel and cost-effective nutraceutical potential of this well known and often consumed fruit.

Based on the results from our initial in vitro characterization study on the Virginia native Norton wine grape, future studies are needed to address the effect of vintage, seasonal variation, maturity of the grape, and the influence of vinification on the antioxidant properties and phenolic profile of the red wine Norton grape (Vitis aestivalis). Next, the α-glucosidase findings reported in the in vitro enzyme inhibition study suggest that the Norton grape skin extract (GSE) and 3 of its phenolic components (resveratrol, ellagic acid, and catechin) strongly inhibit yeast α-glucosidase activity. Consequently, these phenolics may be ideal candidates for utility in future mechanism studies regarding hyperglycemia and glycosylation which is present in diabetes. Additionally, to better understand the inhibition of GSE on mammalian α-glucosidases, the inhibiting action of GSE on specific rat α-glucosidases (i.e. sucrase, maltase, and isomaltase) needs to be clarified in future studies. Furthermore, in vivo studies are needed to determine if specific combinations of phenolics in GSE can suppress the absorption of glucose by way of an α-glucosidase inhibitory mechanism in the small intestine. Our acute animal study demonstrated that the administration of Norton grape pomace extract (GPE) significantly reduced postprandial glycemic response when measured by an oral glucose tolerance test (OGTT). Additional animal studies are needed to establish whether grape pomace, the accumulating byproduct of winemaking, can be used as a safe, effective, and less expensive nutraceutical diabetes source. Our long term animal study suggests that grape
phenolic extracts can lower fasting blood glucose concentrations, which may help to sustain antioxidant protection resulting in a lower systemic inflammatory response.

Future animal studies designed to observe the influence of phenolic extracts on specific DNA, protein, or lipid oxidative damage associated with the establishment of type 2 diabetes would be interesting. Another scenario that oxidative stress may contribute to cellular type 2 diabetes is through modulation of inflammatory gene expression (6). In this respect, type 2 diabetes may induce up-regulation of the nuclear binding activities of NF-κB, a transcription factor stimulated by oxidative stress and has been demonstrated to increase proinflammatory cytokine production (3). As such, additional studies that focus on the innate immune response and grape extract supplementation would provide useful inflammatory and immunology information.

It is clear that continued research is needed in order to determine the potential effects of grape extracts on diabetes-related conditions such as cardiovascular disease, neurodegeneration, nephropathy, and retinopathy and thus may lead to new intriguing therapeutic approaches. Collectively, type 2 diabetes provides a framework for many interesting avenues of investigation and merits additional research to clarify our understanding of the role the grape derived phenolic extracts play in blood glucose control, oxidative stress, and inflammation associated with type 2 diabetes.
Reference


