ANTHOCYANINS AND PROANTHOCYANIDINS FROM BLUEBERRY AND BLACKBERRY FERMENTED BEVERAGES TO REDUCE INFLAMMATION AND TYPE-2 DIABETES: A COMPREHENSIVE IN VITRO AND IN VIVO EVALUATION

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutritional Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

Type-2 diabetes is a serious metabolic disease that currently affects 9.3% of the U.S. population and is aggravated by diets low in fruit and vegetable intake leading to postprandial oxidative stress and inflammation [1, 2]. Berries are one of the richest dietary sources of polyphenolic compounds associated with decreased markers of chronic inflammation and decreased risk for type-2 diabetes [3,4], specifically anthocyanins [5] (ANC) and proanthocyanidins (PAC), polymerized forms of ANC and other phenolics [6]. Fermentation is a feasible way to increase phenolic content of berry juice products, and fermented products may be more bioactive than their unfermented counterparts, thus increasing the potential for health benefits [7]. Our long-term goal, as outlined in Chapter 1, was to fill the gap of knowledge in understanding the mechanisms by which dietary bioactives found in fermented berry beverages contribute to the management of type-2 diabetes using in vitro enzymatic kinetics, analytical assays, computational modeling, in vitro cell culture, and in vivo animal feeding trials. A review of the literature focusing on the role of berries to reduce diabetes is presented in Chapter 2.

Our research focused on the evaluation and potential mechanisms of action of alcohol-free blueberry (Vaccinium corymbosum) and blackberry (Rubus spp.) fermented beverages and their bioactive ANC and PAC components to reduce inflammation and type-2 diabetes in vitro and in vivo through the aims introduced in Chapter 3. First we prepared blueberry and blackberry wines that were comparable to those commercially available (Chapter 4), and found that blueberries and their wine, especially when fermented at cold temperature, contain phenolic compounds that act as antioxidants and potential inhibitory factors of starch-cleaving enzymes that could be used to limit glucose absorption (Chapter 5). We then generated ANC- and PAC-enriched fractions and found that blueberry and blackberry ANC and PAC inhibited expression of proteins in the NF-κB-mediated pathway in macrophages, indicating potential to reduce chronic inflammation associated with hyperglycemia (Chapter 6). ANC were found to bind strongly within active pockets of dipeptidyl-peptidase IV (DPP-IV), an incretin-cleaving target of diabetes therapy, to effectively reduce its activity (IC$_{50}$ = 4.0 μM) (Chapter 7). ANC were also able to reduce the inflammatory cross-talk between macrophages and adipocytes (Chapter 8). Further, ANC increased insulin secretion from pancreatic β-cells by 233 and 100 μIU insulin/mL directly and after epithelial transport, respectively, due to reduced DPP-IV expression and up-
regulated insulin-receptor associated genes and proteins (Chapter 9). Lastly, in Chapter 10 we established the role of phenolic compounds in 30:70% blueberry-blackberry alcohol-free fermented beverage to reduce body weight (BW) gain (10.3 vs. 16.7 g), fat mass (18.0 vs. 31.3% BW), and fasting blood glucose (184 vs. 222 mg/dL), in diet-induced obese C57BL/6j mice consuming ANC beverage vs. water, (p < 0.05). There were some adverse effects noted at the highest dose, warranting future studies into the optimal dose of phenolic compounds extracted and concentrated from the alcohol-free fermented blueberry-blackberry beverage. These results, as summarized in Chapter 11, suggest that ANC in blueberry and blackberry fermented beverages are beneficial sources of antioxidants, decrease glucose absorption, and inhibit inflammation and DPP-IV, and therefore could be used in the management of type-2 diabetes.

ACKNOWLEDGMENTS

I would like to thank my doctoral advisor Dr. Elvira de Mejia for her support and motivation to complete the work described in this dissertation, for providing a laboratory experience that spans from food chemistry to disease mechanism, and for being an example of confidence and execution. I would also like to thank the members of my committee, Drs. Erdman, Freund, Juvik, and Lila for their time and contributions to my project, including Dr. William Artz for being on my committee for the first two years of my graduate studies. I would especially like to thank Dr. Lila for welcoming me into her lab at the Plants for Human Health Research Institute in Kannapolis, North Carolina to learn more about blueberries and to complete the phenolic extraction procedures. My research experience was supported by the lab team of all the current and former de Mejia lab members and visiting scholars, of whom I’d like to thank especially Vermont Dia and Jodee Johnson for training and support. I would also like to thank my collaborating visiting scholars Diego Garcia, for work with the adipocytes, and Junfeng Fan for work with the computational modeling. This work would not have been possible without the assistance of my undergraduate mentees in Food Science and Human Nutrition over the years (including Anita Lucius, Tessa Meyer, Mellisa Yu, Ally Meyer, Julia Amador, Valerie Munoz, Starr’Retiece Gibson, Luis M Real Hernandez, Lauren Hall, and Cheng Lu, among all the undergraduates working in our laboratory).

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Finally, I want to thank my family for their unconditional support throughout my life and during grad school, particularly my mom and my sister for editing several of my papers and applications. None of my successes would have been possible without their love and encouragement.
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CHAPTER 1

OVERALL HYPOTHESES AND OBJECTIVES

Long Term Goal

Fill the gap of knowledge in understanding the mechanisms by which dietary bioactives found in fermented berry beverages contribute to the management of type-2 diabetes using \textit{in vitro} enzymatic kinetics, analytical assays, computational modeling, \textit{in vitro} cell culture, and \textit{in vivo} animal feeding trials.

Overall Objective

To evaluate and determine the potential mechanism of action of berry phenolic compounds from dealcoholized blueberry-blackberry fermented beverages for their effect on markers of inflammation, carbohydrate utilization, incretin action and insulin secretion \textit{in vitro} and \textit{in vivo}.

Central Hypothesis

Anthocyanins (ANC) and proanthocyanidins (PAC) present in blueberry-blackberry fermented beverages can reduce dysfunctional glucose metabolism through their antioxidant and anti-inflammatory action and their ability to inhibit the activity of glucosidases and DPP-IV, and therefore improve incretin and insulin function.

Image 1.1. Overall aims graphic.
GENERAL INTRODUCTION

Type-2 diabetes is a serious metabolic disease that currently affects 9.3% of the U.S. population, with 1.7 million new cases diagnosed in 2012 [1]. It occurs when the body loses the capacity to deal with excess glucose as a result of insulin resistance or beta-cell dysfunction. This inability to control blood glucose increases the risk of serious complications such as heart disease, stroke, kidney failure, lower-limb amputations and non-alcoholic fatty liver diseases, among others. As a result, diabetes was the 7th leading cause of death in 2010. The economic burden of diabetes is staggering with $176 billion for direct medical costs and $69 billion due to reduced productivity [1]. Current obesity trends and modern sedentary lifestyle along with diets low in fruit and vegetable intake lead to postprandial oxidative stress and inflammation in the body, contributing to insulin resistance and development of metabolic syndrome and diabetes [8].

Oral medications and interventions such as insulin pumps are needed if healthy diet and exercise are not enough to regulate glucose levels. One therapeutic approach for management of type-2 diabetes involves reducing glucose absorption by inhibiting enzymes α-amylase and α-glucosidase, as well as to increase insulin secretion. The incretin hormone glucagon-like peptide-1 (GLP-1) is able to stimulate insulin release; however, it is rapidly degraded to inactive metabolites by dipeptidyl peptidase-IV (DPP-IV) [2], a current target for type-2 diabetes therapy. Natural sources of inhibitors of these enzymes would be beneficial for those with diabetes to avoid any potential side effects and to reduce the economic cost. Because berries have been found to be one of the best dietary sources of phenolic compounds, they have potential impact for the management of type-2 diabetes [14]. There is a need for new therapeutic interventions
and experimental models to determine the efficacy and health implications of new solutions to manage diabetes.

Increased phenolic intake has been seen to correlate with a decreased incidence of several obesity-associated diseases, including metabolic syndrome [8] and diabetes [3, 9]. Recent studies have shown a link between increased consumption of anthocyanins (ANC) and anthocyanin-containing foods, such as blueberries, with a decreased risk for type-2 diabetes [2-4]. Blueberries and blackberries are a rich source of ANC, the water-soluble pigments which give berries their red, blue, and purple color [5]. Berries also contain proanthocyanidins, the polymerized form of anthocyanidins, and non-hydrolyzable tannins [6]. The consumption of anthocyanins is among the highest of all flavonoids due to their wide distribution in foods [7]. Regardless of the low bioavailability of anthocyanins, clinical trials have shown the anti-diabetic effect of these compounds [10-13]. Data from mechanistic studies supports the role of flavonoids such as anthocyanins to be beneficial on insulin sensitivity, to decrease blood glucose levels, and to improve insulin secretion.

Some fermented juices and wines from berries inhibit inflammatory protein production or expression more than their unfermented counterparts when consumed [18-23]. Fermentation is known to increase the phenolic content of berry juice products through increased extraction of these compounds from the skins, including ANC [15]; this extraction technique may then also increase their antioxidant capacity [16] and potential health benefits. Dietary flavonoids have been linked to anti-inflammatory pathways [17]; therefore anthocyanins have potential for clinical efficacy in prevention and management of type-2 diabetes by inhibiting the inflammatory response. Due to the wide variety of phenolic compounds present in berry fruits as well as other foods, the benefits of bioactive compounds from berries may not be attributable to just one type
but to a combination. The optimal dose and efficiency of well-characterized phenolic extracts from berry products and their potential benefits may lead to specific recommendations to promote consumption of berries and berry products for type-2 diabetes management. Thus, there is a great need to further investigate the health benefits of specific phenolic compounds from berry fruits and fermented berry beverages and elucidate their role in diabetes and inflammation.

To test the central hypothesis that anthocyanins (ANC) and proanthocyanidins (PAC) present in blueberry-blackberry fermented beverages can help to manage type-2 diabetes through their antioxidant and anti-inflammatory action and their ability to alter glucose metabolism, fermented beverages were produced from Illinois-grown blueberry (Vaccinium corymbosum) and blackberry (Rubus spp.) beverages. Our laboratory has shown that wines produced from blueberries and blackberries have high total polyphenolic and anthocyanin concentrations that correlate with their antioxidant capacity [23]. These phenolic compounds isolated from the blueberry-blackberry beverages were further tested and found to reduce LPS-induced inflammatory response via the NFκB-mediated pathway [24], and to inhibit carbohydrate utilizing enzymes alpha-glucosidase and alpha-amylase. Dipeptidyl peptidase-IV (DPP-IV) is a serine aminopeptidase that is a novel target for type-2 diabetes therapy [25]. DPP-IV rapidly cleaves incretin hormones into their inactive metabolites, preventing their activation of insulin secretion. We found that anthocyanins are able to bind within the catalytic site of DPP-IV and strongly inhibit its activity, thus providing a potential mechanism that anthocyanins may reduce hyperglycemia and therefore type-2 diabetes. These in vitro enzymatic kinetics, analytical assays, computational modeling, and cell culture experiments indicated that phenolic compounds from blueberry and blackberry alcohol-free fermented beverages have the potential to reduce complications associated with chronic inflammatory diseases such as type-2 diabetes through
antioxidant and anti-inflammatory mechanisms. The overall objective was to evaluate and determine the potential mechanism of action of phenolic compounds from dealcoholized blueberry-blackberry fermented beverages to affect markers of inflammation, glucose utilization, and insulin secretion both in vitro and in vivo. The goal of my integrated thesis research is to fill the gap of knowledge in understanding the mechanisms by which dietary bioactives found in fermented berry beverages contribute to the management of type-2 diabetes using in vitro enzymatic kinetics, analytical chemical assays, computational modeling, cell culture, and in vivo animal feeding trials, as indicated in the diagram below.

**Figure 1.1: Integration of Aims 1-5, as follows.**

I. Aim 1: Evaluate commercially available blueberry and blackberry juices and wines produced in the state of Illinois.

II. Aim 2: Determine the effect of fermentation on the chemical composition and quality of blueberry and blackberry fermented beverages.

III. Aim 3: Assess the potential of combinations of blueberry-blackberry fermented beverages for antioxidant capacity, and determine their ability to reduce activity of key enzymes related to diabetes and to modulate inflammation using in vitro and computational studies.

IV. Aim 4: Evaluate the insulin secretory effect of phenolic compounds found in fermented berry beverages in vitro using a simulated absorption model of epithelial and physiologically-relevant pancreatic β-cells.

V. Aim 5: Establish the role of anthocyanins in fermented berry beverages to reduce diet-induced obesity and hyperglycemia in C57Bl/6j mice.
## Research Aims

<table>
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<tr>
<th>Type of Study</th>
<th>Aim</th>
<th>Publication</th>
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| *In vitro*, enzymatic & computational analysis of enzyme targets and markers related to inflammation and adipogenesis | **Aim 3**: Assess the potential of ANC and PAC extracted from blueberry-blackberry fermented beverages for:  

  3.1: Antioxidant capacity and inhibition of starch-degrading enzymes, DPP-IV (dipeptidyl-peptidase IV), and inflammation.  

  3.2 Ability to bind to the active sites of DPP-IV to inhibit DPP-IV enzyme activity.  


| *In vitro*, Caco-2 and iNS-1E β-cells                                         | **Aim 4**: Evaluate the insulin secretory effect of phenolic compounds found in fermented berry beverages *in vitro* using a simulated absorption model of epithelial and physiologically-relevant β-cells. | Johnson MH, de Mejia EG. Anthocyanins from fermented berry beverages increased insulin secretion from pancreatic beta cells *in vitro* beyond their ability to reduce dipeptidyl peptidase IV. *Food and Function*. [Submitted for publication, April 2015]. |
| *In vivo*, obesity-induced hyperglycemia in C57BL/6J mice                    | **Aim 5**: Establish the *in vivo* potential of a fermented berry beverage and effective dose of its phenolic compounds to reduce obesity and hyperglycemia utilizing mice fed a high-fat diet. | Johnson MH, Wallig M, Luna Vital DA, de Mejia EG. Alcohol-free fermented blueberry-blackberry beverage phenolics reduce diet-induced obesity and blood glucose in C57BL/6J mice. *Journal of Nutritional Biochemistry*. [In preparation]. |
Summary

Diets insufficient in the consumption of fruits and vegetables and that are low in flavonoids have been associated with higher incidence of inflammatory-related chronic diseases. Worldwide, the number of people with diabetes is projected to increase to over 360 million by 2030. Type 2 diabetes mellitus occurs when the body loses the capacity to deal with excess glucose as a consequence of insulin resistance or beta-cell dysfunction, resulting in high blood glucose levels. Anthocyanins belong to the flavonoid family, a class of compounds considered to provide antioxidant activity. Found naturally in a number of foods, anthocyanins are the pigments that give berries, grapes, pomegranates and kidney beans their rich colors. In addition, anthocyanins are natural pigments that may provide anti-inflammatory and anti-diabetes benefits. The present chapter offers an overview of the effect of anthocyanins on the reduction of inflammation and diabetes complications in human subjects, in animal models, and their potential mechanisms of action.

In this chapter, the botanical definitions of popular berry fruits, their common uses, and the major class of berry pigments, anthocyanins, will be introduced. An overview of absorption, metabolism, and antioxidant capacity of anthocyanins will be presented, followed by an overview of what causes inflammatory diseases and diabetes, and the biological activities associated with anthocyanins that can overcome these health concerns. The objective is to present an up to date overview of science-based evidence on the chemistry and health benefits of anthocyanins from berries and their roles in prevention of inflammation and diabetes. Other health benefits of anthocyanins will be briefly introduced.

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1. Introduction to Berries

The purpose of this section is to clarify the definition of ‘berry’, to evaluate the horticultural significance and uses of major berry crops, and to explain the geographical significance of berries around the world. Berry fruits are of interest due to recent health claims and promotion for increased fruit and vegetable consumption in the human diet. In general, berries are readily available for consumption by many groups of people around the world. Usually consumed fresh or in processed forms, minimal preparation and varied recipe choices make berries a quick and easy addition to daily eating. Native people in many parts of the world have consumed berries throughout history. Today, berries are a common commercial crop with worldwide demand and potential for health benefits. These plants have been used historically for medicinal purposes especially in rural areas, and have traditionally been consumed as treatment for diarrhea or dysentery. Locally harvested fruits are being used in traditional medicine in India, Bangladesh, and China. In the Amazon region, in addition to being used for nourishment, açai berry oil has been used for its anti-diarrheic action, and the root for its anti-malarial activity. The Chilean maqui berry has been used as an anti-inflammatory agent and as treatment for kidney pains, fever, and stomach ulcers and tumors.

There are currently many areas in the world without access to modern pharmaceutical solutions to chronic diseases that would benefit from increased evaluation of the potential for berry fruits to prevent the pathogenesis of chronic diseases. Investigating the composition of berries and their role as antioxidants helps to establish the basis for their potential health benefits. It is important to understand the scientific background that provides the basis to promote berry fruit consumption for the potential health benefits and use of anthocyanins as functional foods.

1.1. Botanical and Common Definitions

In broad terms, a fruit is the structure of a plant that contains its seeds. Figure 2.1 presents maps indicating the cultivated areas and yields of fruits around the world. When investigating the health benefits and claims attributed to berries, it is important to first define which fruits qualify as a ‘berry’. Botanically, true berries are those which have edible pulp produced from a single ovary. Major ‘berry’ crops are defined as those produced on a shrub or vine that have economic importance. For the purpose of this review, berries will be considered as
those in common parlance, as small fruits that can be eaten whole. These include the true berries: black currant, red currant, and gooseberry; the “false” or epigenous berries: cranberry and blueberry; the aggregate berries: blackberry, raspberry, and hybrid boysenberry (*Rubus ursinus x idaeus*, Rosaceae); and the multiple berry mulberry (*Morus atropurpurea* Roxb., Moraceae). Blackberry, black raspberry, blueberry, cranberry, and red raspberry are the most commonly consumed berries in North America. Also, berries commonly consumed in other parts of the world or consumed by traditional tribal communities will be discussed. These include açai (*Euterpe oleracea*, Arecaceae), Andean mora berry (*Rubus glaucus* Benth, Rosaceae), Chilean maqui berry (*Aristotelia chilensis*, Elaeocarpaceae), bilberry (*Vaccinium myrtillus* L., Ericaceae), chokeberry (*Aronia melanocarpa*, Rosaceae), elderberry (*Sambucus canadensis* L. and *nigra* L., Caprifoliaceae), mortiño (*Vaccinium floribundum* Kunth, Ericaceae), sea buckthorn (*Hippophae rhamnoides*, Elaeagnaceae), and wolfberry, also called goji berry (*Lycium barbarum*, Solanaceae). These commonly consumed berries or exotic berry-type fruits have seen an increase in consumption that may have potential to improve human health. Berries positively modulate chronic diseases such as cardiovascular disease, neurodegeneration, cancer, and diseases related to aging because they contain vitamins, minerals, folate, and fiber in addition to several phytochemicals. It is important to first look at the horticultural and geographical significance of berries commonly consumed in North America and around the world before discussing berry composition and biological activity that suggests their benefits to human health.

A. Fruits areas.

**Figure 2.1. Availability of fruit crops worldwide: A. areas and B. yields**
1.2. Horticulture and Geographical Significance: Berries around the World (Figure 2.2)

True berries are simple fruits produced from a single ovary having seeds and an edible pulp, and contain the *Ribes* genus of berries. Generally, the entire ovary wall of the fruit ripens into an edible outer layer. True berries include black currant, red currant and gooseberry, and have been widely adapted to grow in many soil types and regions. They have been used both fresh and processed into juice, jellies, preserves or pies. Black currants (*Ribes nigrum* L., Grossulariaceae) are perennial plants native to central and Northern Europe and Northern Asia. They grow on a small shrub reaching 1-2 m in height. Most commonly, blackcurrants are used in juice, jellies, and purees, and have a distinctive sharp sweet taste. ‘Superfruit’ is a marketing term used to refer to fruits with appealing taste and exceptionally high nutrient richness and antioxidant quality. Black currants have this special status among consumers, with many health benefits attributed to their high antioxidant levels. Red currants (*Ribes rubrum* L., Grossulariaceae) are similar to the black currant but are bright red translucent berries. The red currant is mostly used in juice, jelly and purees. This small deciduous shrub is native to parts of Western Europe, though Poland is a large producer. Gooseberry (*Ribes uva-crispa* L., Grossulariaceae) grows on a bush from 1-3 m tall with sharp spines on the branches. Gooseberries are usually green and slightly hairy, but may also be deep purple in color. They are indigenous to Europe and Western Asia, growing naturally in thickets and rocky woods in many
countries. Though there are many uses in foods and cakes, gooseberries have a slightly sour taste and are recently losing sales. The Indian gooseberry (*Phyllanthus emblica*, Euphorbiaceae) is also a botanically true berry, having light green to yellow fruit that is sour, bitter and fibrous.

The epigenous or false berries include the *Vaccinium* species of cranberry and blueberry. These are termed false berries as they are not true berries botanically speaking due to the fact that there are many seeds inside, rather than just one. Cranberries (*Vaccinium macrocarpon* Ait., Ericaceae) grow on evergreen creeping shrubs or vines growing to 2 m long and 5-20 cm tall in cooler parts of the Northern hemisphere. The berry turns deep red when ripe and is slightly acidic, leading to almost all being processed into products such as juice, sauce, and sweetened dried cranberries. Sales have increased recently due to recognition of the potential for health benefits, making cranberries a commercially important crop grown mostly in the United States and Canada. Blueberry (*Vaccinium corymbosum* L., *angusifolium* Ait., *ashei* Reade, Ericaceae) fruits are round blue berries with flared crowns at the end, and grow on shrubs that vary in size depending on the variety. They are native to North America but are also cultivated in Argentina, Chile, Australia, and New Zealand. American blueberries include the low-bush, or “wild” blueberry and the high-bush or “cultivated” blueberry. Blueberries can be used in many applications due to their sweet taste, including beverages and snack foods, and are perceived to be generally healthy. Cranberries and blueberries were widely used in the New England area for trading between Native Americans and Colonial settlers.

 Aggregate berries include blackberries, raspberries and boysenberries. Aggregate berries are composed of many small individual drupes, called druplets, which are the small fleshy parts forming around individual ovules of the fruit. There are many varieties of blackberries (*Rubus* sp. Rosaceae), which have naturally sharp spines that reach 3-6 m in height, and grow throughout the Northern hemisphere. Blackberries are one of the most widely available and widespread berries, having many species and hybrids, and are becoming one of the most economically important berries following more common strawberry (*Fragaria × ananassa* Duch.), blueberry (*Vaccinium* spp.), and red raspberry (*R. idaeus* L.) (Finn and Clark 2011). Mostly popular for its use in desserts, jams, and jellies, blackberries can also be made into wine. Raspberries (*Rubus idaeus*, Rosaceae) are similar to blackberries but are smaller, softer, and have a different color. They can be easily grown in many parts of the world and are commercially important for the fresh fruit market and for processing into frozen products, juices, or dried fruit. Fruits of the
raspberry plant grow on woody brambles and are usually red in color, but may also be black or yellow. Black raspberries (*Rubus occidentalis* L., Rosaceae) are not the same species as the red raspberry, but are a North American species grown mostly in the state of Oregon and used for juice and jam production. Boysenberries (*Rubus ursinus x idaeus*, Rosaceae) are a hybrid between a red raspberry and a blackberry, which was discovered in California and is now widely produced in the state of Oregon, as well as in New Zealand. The berry has similar fruit to blackberries with purple fruit, though their fruits are usually are larger and have larger druplets.

The last group of berries used in common parlance are multiple fruit berries, which include mulberries (*Morus atropurpurea* Roxb., Moraceae). Black mulberry (*Morus nigra*, Moraceae) is native to western Asia but has been cultivated in Europe for hundreds of years. The fruits are 2-3 cm long and are found on medium sized deciduous trees growing to 10-15 m tall. The fruit is usually eaten fresh. As they are in high demand by birds they may be important when considering the horticultural production of other berry fruits.

The berries just discussed are the most commonly referred to as berries; however, there are many exotic or rare berries less common to North American consumers with unique characteristics and horticultural interest. Some of these berries have become more common to the general public due to the introduction to grocery stores of the popular marketing term ‘superfruits’. One very popular berry in the market today is açai (*Euterpe oleracea*, Arecaceae). This berry is the fruit of the açai palm that grows in tropical Central and South America mainly in floodplains and swamps. The palm is a small bushy evergreen similar to cherry trees growing to 15 m or more, and the berries are dark purple fruits. Known mostly because of its antioxidant properties, açai berries are used mostly in processed form or as juice or preserves because of poor shelf-life.

### 1.3. Berry Composition

In addition to fiber, minerals, and vitamins C, E, and A, berries contain phytochemicals with many potential benefits to prevent inflammation and diabetes. These phytochemicals are the bioactive phenolic compounds, which are categorized chemically into hydrolysable tannins and phenylpropanoids, such as lignins, flavonoids and condensed tannins. Phenolic phytochemicals are ubiquitous in plants and serve many biological functions, including roles in growth and development of the plant, and in defense mechanisms against insects and UV radiation.
<table>
<thead>
<tr>
<th>Black currant (<em>Ribes nigrum</em> L., Grossulariaceae)</th>
<th>Blackberry (<em>Rubus</em> sp. Rosaceae)</th>
<th>Andes berry (<em>Rubus glaucus</em> Benth)</th>
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<tr>
<td>Red currant (<em>Ribes rubrum</em> L.)</td>
<td>Raspberry (<em>Rubus idaeus</em>)</td>
<td>Acerola (<em>Malpighia emarginata</em>)</td>
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<tr>
<td>nutritionkey.wordpress.com</td>
<td>bbcgoodfood.com</td>
<td>bewellbuzz.com</td>
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<tr>
<td>Gooseberry (Indian Gooseberry: <em>Emblica officinalis</em> Gaertn.)</td>
<td>Boysenberry (<em>Rubus ursinus x idaeus</em>)</td>
<td>Bilberry (<em>Vaccinium myrtillus</em>)</td>
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<td>pickyourown.org</td>
<td>gourmetsleuth.com</td>
<td>herbalextracts.net</td>
</tr>
<tr>
<td>Cranberry (<em>Vaccinium macrocarpon</em> Ait.)</td>
<td>Mulberry (<em>Morus atropurpurea Roxb.</em>)</td>
<td>Chokeberry (<em>Aronia spp.</em>)</td>
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<tr>
<td>hunnylovins.blogspot.com</td>
<td>fruitdirectory.com</td>
<td>fruitdirectory.com</td>
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<td>Blueberry (<em>Vaccinium corymbosum</em> L., <em>angusifolium Ait.</em>, <em>ashei</em> Reade)</td>
<td>Elderberry (<em>Sambucus canadensis</em> and <em>S. nigra</em>)</td>
<td>Crowberry (<em>Empetrum spp.</em>)</td>
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<td>wrensoft.com</td>
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<tr>
<td>Maqui berry (<em>Aristolelia chilensis</em>)</td>
<td>Mortiño (<em>Vaccinium floribundum Kunth.</em>)</td>
<td>Marionberry (<em>Rubus hybrid</em>)</td>
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<td>maquiberrytruth.com</td>
<td>my.englishclub.com</td>
<td>fruitsinfo.com</td>
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</table>

Figure 2.2. Images of berries found around the world. Berry (Scientific name) and Image Source.
Polyphenolic structures are characterized by one or more aromatic six-carbon rings and two or more phenolic hydroxyl groups. Anthocyanidins belong to the polyphenolic group of flavonoids, which are secondary metabolites synthesized by plants and are found in fruits along with ellagitannins, flavan-3-ols, procyanidins, flavonols, and hydroxybenzoate derivatives. Anthocyanidins are the aglycones of anthocyanins, which contain a sugar group or groups. Berries are one of the richest sources of anthocyanins, the largest group of water soluble pigments in nature that give fruits and vegetables their distinctive red, blue or purple color. The word ‘anthocyanin’ is derived from the Greek words *anthos* meaning flowers and *kyanos* meaning dark blue (Anderson and Jordheim 2006). Berries also contain proanthocyanidins, the polymerized form of anthocyanidins, along with non-hydrolyzable tannins. Proanthocyanidins, a major group of phenolic compounds occurring in plants, are the second most abundant natural phenolic after lignin (Gu 2004). Berries may also contain other phenolic compounds including phenolic acids, hydroxycinnamic acids, lignans or phytoestrogens, and stilbenoids, as well as terpenes or isoprenoids. The location of phenolic compounds in fruit varies depending on the type: phenolic acids are mostly found in the pulp, anthocyanins and stilbenes are found in the skin and other phenols including catechins and proanthocyanidins are found in the skin and seeds. Anthocyanins are important to plants because their rich colors attract animals, which allows for seed dispersal and pollination.

Compared to other flavonoids, the dietary consumption of anthocyanins and proanthocyanidins is high due to their wide distribution in plants. Dark colored fruits such as blueberries, raspberries and black currants are major sources of anthocyanins in the human diet. Estimated daily intake of anthocyanins in the United States is between 180-215 mg, but may be as low as 12.5 mg per day, according to a USDA report in 2006 (Wu 2006). In addition to fresh fruit consumption, anthocyanins are also used as food pigments in the beverage industry, which increases their daily consumption and availability. Mean daily intake of proanthocyanidins in the U.S. population is estimated to be 57.7 mg/person, with major sources being apples, chocolate, and grapes (Gu 2004). These intake levels support the potential for these phenolic compounds to have health benefits.
1.4. Structure of Main Anthocyanins and Proanthocyanidins in Berries

The biological activity to lower risk of diseases, the structural stability, and the bioavailability of anthocyanins and proanthocyanidins depend on their chemical structures. Polyphenolic flavonoids contain two benzyl rings (known as the A- and B-rings) separated by a heterocyclic ring (the C-ring) (Figure 2.3). Flavonoids, phenolic acids, lignans, and tannins differ structurally by the types and levels of oxidation on their heterocyclic rings and glycosylation by various sugars. The sub-classes of flavonoids, including flavonols, flavanols, anthocyanins, flavanones, flavones, and isoflavones differ based on the presence or absence of double bonds or carbonyl groups on the C-ring. There are more than 600 anthocyanins that have been identified in nature, and though the human diet provides hundreds of anthocyanins (the glycosylated form), only six anthocyanidins (the aglycone forms) are predominant and therefore important to the human diet. The phenolic profile and relative color varies greatly depending on the type of fruit, with cyanidin derivatives being the most common anthocyanin present in berry fruits.

The six main anthocyanidins are cyanidin, malvidin, delphinidin, peonidin, petunidin, and pelargonidin, with diversity from the position and number of hydroxyl and methoxy groups attached at the R1 and R2 position on the B-ring of the anthocyanin skeleton (Figure 2.3). The hydroxyl groups may be substituted to form O-linked conjugates off the C-ring with many sugars in the form of 3-monoglycosides, often with a single sugar linked to the 3-position of the C-ring, and 3,5-diglycosides, with a sugar attached at both the 3- and 5-positions (Miguel 2011). Glycosylation increases water solubility and stability due to hydrogen bonding, so the more unstable aglycone form is rarely found in nature. More than 90% of anthocyanins contain glucose and are the nonmethylated anthocyanidins, which are cyanidin, delphindin and pelargonidin. Anthocyanins have also the ability to form resonating structures in response to changes in pH, which is responsible for their relative color. Anthocyanins are unique from other flavonoid compounds due to their capacity to form flavylium cations (McGhie 2007). In very acidic conditions, this flavylium cation is the predominant form which results in red color formation due to the presence of conjugated double bonds with positive charge, while the blue-purple quinoidal base is formed at pH 7-8 with the loss of protons (Domitrovik 2011). Many of the biological activities reported for anthocyanins and proanthocyanidins, and their metabolites,
are due to their antioxidant activity from the resulting positively charged oxygen atom in the C-ring. The hydrogen-donating antioxidant capacity of anthocyanins and their ability for electron delocalization make them more effective antioxidants than other flavonoid compounds.

![Anthocyanidin Skeleton](image1)

Anthocyanin (Cyanidin-3-O-glucoside)

![Anthocyanin](image2)

**Figure 2.3.** Blueberries, blackberries, and raspberries and their anthocyanidins and anthocyanins.

### 1.5. Main Anthocyanins in Berries

The anthocyanin profile varies greatly depending on the type of fruit, with the most common anthocyanin present in berry fruits being cyanidin derivatives. The relative color of berries is also due to the types of anthocyanins present and in what amounts. **Table 2.1** presents the chemical composition of phenolics, anthocyanins, and proanthocyanidins in different berries as well as their antioxidant capacity. Blueberry, bilberry, and blackcurrant contain a wide array of anthocyanins while red currant and elderberry contain only one type, cyanidin. Malvidin-3-O-arabinosides and 3-O-galactosides of cyanidin, delphinidin, petunidin and malvidin are the main components of blueberry. Major anthocyanins in blackberry and red raspberry are derivatives of cyanidin, with blackberry having much higher levels of anthocyanins than red raspberry, and therefore darker colors (Seeram 2008). Three cyanidin glycosides have been detected in red
currant extracts, while 3-\textit{O}-glucosides and 3-\textit{O}-rutinosides of delphinidin and cyanidin are the dominant anthocyanins in blackcurrants. Cranberries have cyanidin- and peonidin-based anthocyanin compounds. The major anthocyanin pigments in the Colombian Andes berry are cyanidin-3-rutinoside and cyanidin-3-glucoside. Main anthocyanins in the Chilean maqui berry are delphinidin and cyanidin glucoside derivatives. Açai berry contains cyanidin-3-\textit{O}-rutinoside and cyanidin-\textit{O}-glucoside as the main anthocyanin constituents, while acerola has cyanidin-3-\textalpha-o-rhamnoside as its major anthocyanin, and mortiño berries contain predominantly cyanidin-3-glucosides. Anthocyanins are the major class of phenolics used to characterize several types of berries, especially \textit{Vaccinium} and \textit{Ribes} species. The potential health benefits from berries differ based on the specific compounds present in each berry fruit. Therefore, it is important to understand which phenolic compounds are present.

- Color differences seen in different berry types are due to different anthocyanins present.
- Anthocyanins may act as antioxidants due to their ability to form a flavylium cation.
- Predominant anthocyanins from berries are cyanidin derivatives.

Proanthocyanins are polymers of anthocyanins with multiple units of flavan-3-ols such as catechins and epicatechin that are most commonly linked at the 4 and 8 positions. When this carbon-carbon interflavanyl bond is cleaved under strongly acidic conditions, anthocyanidins are formed. Proanthocyanidins can be found in many plants, including apples, grape skin and seeds, as well as berries like bilberry, cranberry, and black currant. Other foods such as green tea, black tea, red wine and cocoa beans, as well as the bark of plants contain polymeric flavonoids. In berries, complex tannin composition is composed of highly polymerized compounds. The size of these compounds is described as degree of polymerization. Blueberries are known to contain, in addition to monomers, proanthocyanidin oligomers and polymers ranging from 2- to 14-units of catechin/epicatechin. Flavanols are able to polymerize due to the nucleophilicity of the A-rings, susceptibility of the heterocyclic rings to cleavage and therefore subsequent rearrangements, and susceptibility to phenol oxidative degradation (Ferreira 2006). Oligomeric proanthocyanidins found in skins of peanuts, grapes, and apples, are the di- and tri-mers of catechins. Hydrolyzable tannins are gallo- or ellagi-tannins, having structure as seen in \textbf{Figure 2.4}, and the term ‘complex tannins’ is used to describe polyphenols where there is a proanthocyanidin connected to a hydrolyzable tannin through carbon-carbon linkage.
2. Absorption, Distribution, Metabolism, and Excretion of Anthocyanins and Proanthocyanidins in Humans

It is helpful to understand the process that anthocyanins follow once they enter the body to exert their biological effects, and to understand the mechanism behind their antioxidant potential. The positive impact of anthocyanins found in berry fruits on both inflammation and diabetes will be presented following a brief introduction of the pathophysiology and biological processes that lead to these states in the body. Bioavailability is the proportion of an ingested substance that reaches the systemic circulation and thus is available to reach a target tissue. The bioavailability of anthocyanins and proanthocyanidins differs from other flavonoids; however, they follow a similar pathway. After consumption of flavonoid-rich foods, chewing starts the release of compounds from the food matrix. Under certain conditions, the absorption of anthocyanins has been determined to start in the stomach, so these compounds may appear rapidly in the blood, although the small intestine is the major site of absorption of parent ANC (McGhie 2007, Matuschek 2006).
Table 2.1. Total phenolics, proanthocyanidins and anthocyanins, and antioxidant capacity of berries based on data available since 2013.

<table>
<thead>
<tr>
<th>Berry (Scientific name)</th>
<th>Total Phenolics</th>
<th>Proanthocyanidin Content</th>
<th>Anthocyanin Content</th>
<th>Antioxidant Capacity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black currant (Ribes nigrum L.)</td>
<td>21.3 ± 8.0 mg CAE/g FW</td>
<td>162.8 – 180.4 mg / 100 g FW</td>
<td>93.5± 0.6 mg /100 g DW</td>
<td>ORAC: 34.5 ± 1.9 mg TE /g FW, DPPH: 25.0 ± 4.1 mg TE /g FW</td>
<td>⁵Lugasi A, Hóvári J, Kádár G, Dénes F. 2011. Acta Alimentaria, 40: 52–64. ⁶Tarbart J, Franck T, Kevers C. 2011. Food Chem, 131: 1116–1122. ⁷Gu L, Kelm MA. 2004. J Nutr, 134: 613–617.</td>
</tr>
<tr>
<td>Black currant (Ribes nigrum L.)</td>
<td>187.7 - 207.8 mg / 100 g FW</td>
<td>93.5± 0.6 mg /100 g DW</td>
<td>354 mg / 100 g fruit</td>
<td>ORAC: 34.5 ± 1.9 mg TE /g FW, DPPH: 25.0 ± 4.1 mg TE /g FW</td>
<td>⁵Lugasi A, Hóvári J, Kádár G, Dénes F. 2011. Acta Alimentaria, 40: 52–64. ⁶Tarbart J, Franck T, Kevers C. 2011. Food Chem, 131: 1116–1122. ⁷Gu L, Kelm MA. 2004. J Nutr, 134: 613–617.</td>
</tr>
</tbody>
</table>

Table 2.1 (contd.)

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Concentration/Effect</th>
<th>Source</th>
</tr>
</thead>
</table>

DPPH: 2,2-diphenyl-1-picrylhydrazyl; ORAC: Oxygen Radical Absorbance Capacity; GAE: Gallic acid equivalents; C3G: cyanidin-3-glucoside equivalents; CE: Catechin equivalents; DW: dry weight; FW: fresh weight; FRAP: ferric reducing antioxidant power; TE: trolox equivalents; TEAC: Trolox equivalent antioxidant capacity.
Recent evidence has indicated that the major metabolites of ANC are produced due to fermentation by the gut microbiota and their subsequent absorption in the colon (Del Rio 2010, McGhie and Walton 2007). Many phenolic compounds that reach the lower intestines intact are then hydrolyzed by the cecal microflora glycosidases and subsequently absorbed. Glucosidases are enzymes involved to release the aglycone form from the sugar attachment, primarily to release glucose. As the free aglycones have sugar groups removed, they are much smaller than the glycosides and are able to be absorbed through the epithelial layer of the intestines passively. The major metabolites have been identified as protocatechuic acid (PCA), along with other phenolic acids that have been identified in plasma following ANC consumption such as vanillic acid, syringic acid, caffeic acid, and ferulic acid (Vitaglione 2007, deFerrars 2014a).

Bioavailability of proanthocyanidins is largely dependent on the degree of polymerization. Oligomeric proanthocyanidins are generally poorly absorbed across the intestine, and may be hydrolyzed to monomers, dimers, and trimers, especially in acidic environment such as the stomach (Ou and Gu 2013). The dimers and trimers of proanthocyanidins may be absorbed intact, or further degraded to their flavan-3-ol monomers in the intestinal lumen. Intact glycosides are absorbed by the sodium-dependent glucose transporter (SGLT1) and in some part by inefficient passive diffusion. Differing from other flavonoids that have their sugar moiety removed before being absorbed, human studies and animal models have shown that anthocyanins in the glycosidic form can be rapidly absorbed in the jejunal portion of the small intestine (Matuschek 2006). Acylated flavonoids do not have a free sugar available to bind glucose transporters, have a lower affinity for the bilitranslocase transporter, and are not recognized for absorption in the small intestine and therefore these unabsorbed flavonoid molecules may be absorbed farther downstream (Passamonti 2005). In the colon, anthocyanins may undergo hydrolysis by colonic bacteria which releases the sugar moiety and the aglycone for marginal absorption (Keppler and Humpf 2005). The aglycone may then be further degraded to phenolic acids, and other conjugated metabolites, which may be absorbed, reenter the jejunum with bile, and enter circulation again. Proanthocyanidins may be partially metabolized by intestinal microbiota to phenolic acids and other derivatives. Following absorption, anthocyanins and proanthocyanidins are transported to the liver via portal circulation and undergo phase II metabolism, where they form glucuronide and sulfate or methyl conjugates.
Unabsorbed flavonoid molecules are then excreted through feces, with intact anthocyanins and aglycone anthocyanidins largely excreted in urine. Overall, the percentage of anthocyanins excreted in urine is low, but the excretion of metabolites that are produced in the colon can be important to producing beneficial effects attributed to anthocyanins. Recent studies utilizing a $^{13}$C-labelled ANC have indicated that the relative bioavailability of C3G may in fact be closer to 12% based on recovery in urine or breath. This indicates that given the degradation of the parent compound that occurs, the low $C_{\text{max}}$ (ranging from 1.4 to 547 nM) and the short $t_{1/2}$ (between 0.5-1.5 h), most observed benefits from consumption of ANC are probably due to the metabolites such as PCA which are present in circulation at much higher concentrations (Czank 2013, de Ferrars 2014b). Many studies suggest anthocyanins are able to cross the blood-brain barrier and the blood-retinal barrier, thereby providing protection to these tissues. Proanthocyanidins may be distributed to tissues including the kidney, lung, spleen, and connective tissue (Ferreira 2006). Low levels of tissue dispersion and accumulation indicate that potential for toxicity is very low, making these polyphenols valuable compounds to exert health benefits. Only intact anthocyanins glycosides have been detected in urine and plasma, possibly due to low detection of the large amount of metabolites that form. Anthocyanins, proanthocyanidins, and their metabolites may be excreted in urine, feces, bile, or via respiration (Netzel 2005, Czank 2013).

- Human consumption of anthocyanins is among the highest of all flavonoids as they are pigments widely found in fruits and vegetables.
- Absorption occurs in the stomach, and mainly in the small intestine with very efficient epithelial tissue uptake.
- Bioavailability of anthocyanins in terms of transport into plasma and excretion appears to be limited and dependent on the chemical nature of the compound.

3. Antioxidant Capacity

One of the most important benefits of consuming berries is the antioxidant capacity of the anthocyanins. Oxidative stress in the body from accumulation of reactive oxidative species leads to the oxidation of necessary enzymes and proteins in the body, which can lead to chronic diseases. Anthocyanins reduce some chronic diseases by scavenging these free radicals and therefore reducing the oxidative stress in the body. This antioxidant activity may occur through several processes: decreasing oxygen concentrations, preventing initiation of reactive oxygen or
nitrogen species, or binding of metal ions, decomposing peroxides, and scavenging intermediate radicals that may form during normal metabolism (Miguel 2011). Significant increases in plasma antioxidant activity have been seen after humans consume berry juices. There are several assays to study the potential antioxidant and free-radical scavenging activity of berries after consumption. One assay to measure the antioxidant capacity is the Trolox equivalent antioxidant capacity (TEAC) assay, which is based on the ability of a compound to quench a long-lived radical as compared to Trolox, a vitamin E analog. Similar is the oxygen radical absorbance capacity (ORAC) assay, based on hydrogen-transfer ability. Another assay is the ferric reducing ability/power assay, otherwise known as FRAP, based on the ability of the compound to reduce $\text{Fe}^{3+}$ to the ferrous form as a marker of reducing ability of an antioxidant. There is also the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, based on electron transfer. The ability of anthocyanins to support an unpaired electron or donate a hydroxyl group to a free radical differs between anthocyanins and is dependent on the structure. Generally, anthocyanins with more hydroxyl groups or fewer sugar units are associated with having a greater antioxidant capacity. Much of the data for the antioxidant capacity is from *in vitro* studies and may not lead to the same conclusions for anthocyanins studied after *in vivo* human consumption, so it is important to consider results from both types of studies. Once the antioxidant activity has been determined using *in vitro* models, feeding trials using animal models, or human studies can determine if there are similar effects after everyday consumption.

Anthocyanins have been able to increase plasma antioxidant levels, though it seems clear that the metabolites rather than the parent compounds are responsible for these effects. However, after elderberry juice consumption, a direct association was found between plasma antioxidant capacity and total phenolics through the detection of unchanged glycosylated anthocyanins in healthy human volunteers vi HPLC spectrophotometry (Netzel *et al.* 2002). Also, an increase in plasma antioxidant capacity 2 h after oral consumption of black currant anthocyanins was observed. Consumption of berry fruits over several weeks has been shown to significantly increase plasma antioxidant capacity in elderly individuals. These human feeding studies that show an increase in plasma antioxidant capacity is also supported by numerous animal studies, and *in vitro* studies of anthocyanin antioxidant capacity. The antioxidant capacity exhibited by berry fruits attributed to their anthocyanins has a positive impact on the overall health. Antioxidant capacity may be especially important for preventing or delaying complications
associated with specific physiological states that occur during inflammation, diabetes and cardiovascular disease through the reduction of oxidative processes.

4. Health Benefits: Anti-inflammatory Effect

Inflammation is a normal part of the immune response to tissue injury and is necessary for defense mechanisms; however, prolonged and uncontrolled inflammation can result in a number of diseases. Reactive oxygen and nitrogen species, which include superoxide anions, hydroxyl radicals, hydrogen peroxides, nitric oxide, and singlet oxygen, are generated by inflammatory cells. In response to inflammatory state, many proteins such as cytokines are released by macrophages to deal with the stress on the cell. These proteins and inflammatory chemicals may injure important molecules such as proteins and lipids and attack normal tissues, resulting in harmful oxidative damage and thus inflammation. Several human diseases have recently been linked with this resulting chronic inflammatory state and include neurological disorders, cancers, cardiovascular diseases, obesity, metabolic diseases, and skeletal or muscular disorders. Chronic inflammatory diseases have been inversely correlated with the consumption of fruits and vegetables that are rich in flavonoids. Many types of flavonoids including anthocyanins may exert anti-inflammatory potential through their effect on molecular mechanisms and signal pathways involved with inflammation.

Through in vitro studies of the inhibition of these inflammatory markers using cell culture, the potential for berries and berry compounds and their mechanism of action can be determined and later used for more in depth studies involving animals or humans. Anthocyanins from berry fruits have been seen to affect both acute and chronic inflammation through exerting their antioxidant effects. Cyclooxygenases convert the polyunsaturated fatty acid arachidonic acid to prostaglandins that stimulate inflammation, so the inhibition of these compounds is very important for decreasing inflammation. Nitric oxide synthases (NOSs) are multidomain metalloproteins responsible for the synthesis of the agent nitric oxide (NO). Highly selective inhibitors of inducible NOS (iNOS) have been identified and some of these have potential in the treatment of inflammatory conditions in which iNOS has been implicated. Anthocyanins have been found to inhibit several of these inflammatory markers including cyclooxygenase-2 (COX-2) expression or inhibiting inducible nitric oxide (iNOS). Açai berries have been found to inhibit
the activity of cyclooxygenases (COX) -1 and -2 *in vitro*, and extracts have been seen to have direct action to inhibit the production of nitric oxide (NO) in cultured cells, with possible involvement on both acute and chronic processes. Incorrect regulation of the nuclear factor kappa enhancer of activated B cells (NF-κB) protein complex pathway, which controls the transcription of DNA, is involved with inflammation and cancers. Human studies with polyphenol-rich foods have shown decreases in plasma concentrations of inflammatory mediators associated with NF-κB activation.

Correlations have been suggested between obesity and inflammation, and the role of berries to improve inflammatory responses associated with obese or overweight humans has also been studied. Increased adipose tissue associated with obesity leads to the secretion of pro-inflammatory factors. Elevated levels of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) synthesized in adipocytes during obese states is also associated with insulin resistance (Tzanavari 2010). Compared to a control group fed no blueberries, fresh blueberries and blueberry puree fed to obese children decreased oxidant species and slightly reduced clinical and chronic inflammatory markers (Giongo 2011). Intervention strategies involving fresh fruit high in antioxidants, such as blueberries, show promise to reduce the potential onset of morbidity in overweight and obese individuals during childhood. Consumption of black currant anthocyanins by healthy individuals before exercise alleviated the exercise-induced oxidative stress, usually resulting in an acute inflammatory response (Lyall et. al. 2009). Berries have been found to reduce inflammatory responses in a variety of obese conditions in humans. Along with obesity, chronic low grade inflammation is a characteristic feature of metabolic syndrome, and is a major risk factor for developing type 2 diabetes.

- Anthocyanins from berries can inhibit markers of inflammation.
- Berries have been seen to reduce inflammatory responses and reduce the potential morbidity associated with inflammation due to obesity.

5. Diabetes Pathology and Current Treatments

Type-2 diabetes mellitus occurs when the body loses the ability to uptake blood glucose as a result of insulin resistance or beta-cell dysfunction, resulting in hyperglycemia. Normally, insulin is synthesized and secreted from the beta-cells in the pancreas that acts to reduce glucose
production and output in the liver. Insulin also facilitates glucose uptake in skeletal muscle and decreases lipolysis in adipose tissues. Both obesity and diabetes contribute to the metabolic syndrome, which is characterized by low-grade inflammation, oxidative stress, hyperlipidemia, impaired glucose tolerance, and insulin resistance. Increases of glucose and fatty acid in skeletal muscle, liver, and adipose tissue occurs due to insulin resistance and results in metabolic disorders such as fatty liver diseases, type-2 diabetes, chronic kidney disease, and heart diseases. Severe end-stage diabetes may result in long-term complications such as poor circulation, diabetic retinopathy, stroke, heart disease, kidney failure, or even death. Glycated hemoglobin that forms due to glycation of proteins during chronic hyperglycemia is a diagnostic marker for type-2 diabetes along with fasting blood glucose and impaired glucose tolerance levels.

There are many currently available pharmacological treatments to correct dysfunctions that lead to type-2 diabetes, insulin resistance and impaired insulin secretion (Cuny 2012). Oral medications include insulin sensitizers (metformin and thiazolidinediones) and insulin secretagogues (sulfonylureas, glinides, and recent incretin mimetics). Digestive enzyme targets include alpha-amylase and alpha-glucosidase; α-amylase is secreted by the pancreas and is involved in starch hydrolysis, while α-glucosidase is an intestinal enzyme involved in the absorption of glucose. Inhibition of glucosidase enzymes results in decreased postprandial hyperglycemia, the increase in blood glucose levels seen after a meal. Because secretion of insulin may be impaired due to the dysfunction of the beta cells, drugs called sulfonylureas act to increase insulin production by binding to ATP-potassium channels on the cell membrane of pancreatic beta cells in addition to lowering the glucose uptake. However, this pharmaceutical treatment can eventually lead to hypoglycemia; therefore it is desirable to search for natural compounds that increase insulin secretion without this adverse recovery effect. Natural inhibitors of enzymes and promoters of insulin secretion from fruits would be able to control postprandial hyperglycemia, the increase in blood glucose levels seen after a meal, without side effects of digestive problems or hypoglycemic incidents that may be associated with current therapeutic drugs. Berries included in a healthy diet are able to affect these clinical targets of diabetes in a non-synthetic way.

Current dietary recommendations for people with diabetes are to follow a healthy diet, as is recommended for a general healthy population. Unfortunately, fruit and vegetable intake among people with diabetes tends to be low due to the misperception of adverse effects from
fruit sugars on blood glucose levels. The American Diabetes Association suggests that people with diabetes incorporate whole grains, vegetables, and fruits into a diet containing moderate salt and sugar and that is low in fat, especially saturated fats and trans-fats. Besides the many vitamins, minerals, and fiber, fruits also contain carbohydrates. These carbohydrates make fruits important to include in meal planning for people with diabetes in exchange for other carbohydrates such as starches, grains or dairy. One serving of fruit, equivalent to ¾ to 1 cup of berries, has about 15 g of carbohydrate. Fruit consumption is encouraged to increase the average consumption of fruits and vegetables by the U.S. consumer who is currently consuming less than 2 cups per day. Due to their convenience and cost, fresh or freshly frozen common berry fruits including blackberries, blueberries, and raspberries are an ideal fruit choice to include in meal planning.

5.1. In Vitro Clinical Targets

Inhibition of starch degrading and glucose absorbing enzymes results in decreased glucose available to the body, making it easier for the body to handle glucose already present in insulin resistance states that occurs with the development of type 2 diabetes. The mechanism of inhibition of alpha-glucosidase by anthocyanins is not clear, but it is thought to be a competition between the normal substrate and the glucosyl groups linked to the anthocyanins. This structural similarity allows anthocyanins to bind to the enzyme, but not be hydrolyzed, and is the method employed by acarbose, a pharmaceutical drug for hyperglycemic management. The effects of berry compounds have been studied and are summarized in *in vitro* models (Table 2.2). Cranberry powders have been shown to inhibit alpha-glucosidase while having a lowered alpha-amylase inhibition, resulting in fewer undesirable gastrointestinal side effects associated with excessive alpha-amylase inhibition. Additionally, there may be a synergistic effect between acarbose and cyanidin-3-galactoside, which could lead to a reduced dose of acarbose needed for the treatment of type 2 diabetes. Anthocyanins from raspberries and other berry fruits have shown potential to improve glucose control in diabetics through interacting with alpha-amylases (McDougall 2005) and through inducing insulin secretion (Jayaprakasam 2005). The anthocyanins abundant in fruits, especially pelargonidin, promote the secretion of insulin by the body and may represent a beneficial role in preventing type 2 diabetes.
Inflammatory markers may have increased expression with the development of type 2 diabetes, and are likely to contribute to the development of insulin resistance. An excess of pro-inflammatory cytokines including CRP, IL-1, IL-6, and TNF-α, can result in beta-cell dysfunction, affecting both insulin secretion and reduced insulin-dependent signaling, resulting in insulin resistance. A study with humans at risk for cardiovascular disease found that plasma concentrations of pro-inflammatory cytokines including CRP, and IL-6, and IL-15, and monokine induced by INF-γ (MIG) were all significantly decreased in those receiving bilberry juice for 4 weeks compared to the group receiving water (Karlsen et al. 2010). As these are all target genes of NF-κB, the transcription factor crucial for initiating inflammatory responses that lead to insulin resistance, consumption of berries can be seen to positively impact inflammation related to diabetes in humans. Flavonoids like anthocyanins interfere with the pro-inflammatory cytokines to decrease their production and their induced beta-cell dysfunction or degradation that leads to reduced insulin output. They also work to up-regulate the insulin-dependent signaling and improve glucose uptake in many cell and tissue types. Severe end-stage diabetes may result in long term complications such as diabetic retinopathy or death. Therefore, it is important to investigate the potential for berry consumption to reduce metabolic disease precursors including obesity and insulin resistance.

5.2. Anti-Diabetic Studies Using Animal Models

There are many studies investigating the anti-diabetic properties of berries in animal studies. The effects of anthocyanins on health have also been studied and are summarized for their anti-inflammatory and anti-diabetic properties in animal models (Table 2.3). One study found decreased whole-body insulin resistance in mice fed blueberry powder. Anthocyanins may protect beta-cells from glucose-induced oxidative effects, as seen by protective effects of boysenberry on diabetic rats. Black chokeberry ingestion has been seen to improve antioxidant status in a rat model of prediabetes, resulting in hypoglycemic actions, and may be a promising supplemental option for the prevention and treatment of metabolic syndrome and related diseases. The lowered blood glucose concentrations in this study were attributed partly to the inhibition of maltase and sucrose enzymes, similar to the action seen by inhibition of the alpha-glucosidase enzyme in several studies.
Table 2.2. Other health beneficial properties or *in vitro* results attributed to berries and berry compounds.

<table>
<thead>
<tr>
<th>Berry</th>
<th>Dose of Treatment</th>
<th>Proposed target</th>
<th>Model</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5–10 mg/mL blackcurrant total polyphenolic extract (BC-T), enriched anthocyanin sub-extract (BC-A) or enriched proanthocyanidin sub-extract (BC-P)</td>
<td>Airway inflammation related to asthma via IL-4-stimulated eotaxin-3 (CCL26)</td>
<td>Human alveolar endothelial cell line (A549)</td>
<td>Procyanidins (but not anthocyanins) in blackcurrant suppressed both IL-4 and IL-13 stimulated CCL26 secretion, a factor in eosinophil recruitment</td>
<td>Hurst SM, McGhie TK, Cooney JM. (2010). <em>Mol Nutr Food Res</em>, 54 Suppl 2:S159-70.</td>
</tr>
<tr>
<td>Cranberry (Vaccinium spp.)</td>
<td>1-200 mg/mL cranberry powders</td>
<td>Postprandial reduction in glucose via inhibition of starch breakdown</td>
<td><em>In vitro</em> assays for inhibition of α-amylase, α-glucosidase, and angiotensin I-converting enzyme (ACE)</td>
<td>High α-glucosidase inhibition with limited α-amylase inhibition at 5 mg/mL, significant ACE inhibition at 100-200 mg/mL</td>
<td>da Silva-Pinto, MS, Shetty K. (2010). <em>American Chemical Society</em>: Washington, DC, Ch. 8.</td>
</tr>
<tr>
<td></td>
<td>Vaccinium arctostaphylos berry anthocyanin</td>
<td><em>In vitro</em> diabetes models</td>
<td>Pancreatic alpha-amylase inhibition</td>
<td>The IC&lt;sub&gt;50&lt;/sub&gt; of alpha-amylase inhibition was 1.91 (1.89-1.94) mg/mL.</td>
<td>Nickavar B, Amin G. (2011). <em>Iranian J Pharm Res</em>. 65:567-70.</td>
</tr>
<tr>
<td>Blueberry (Vaccinium corymbosum)</td>
<td>100, 200, or 300 mg/kg crude alcoholic extract of Vaccinium corymbosum</td>
<td>Anti-inflammatory and antinociceptive properties</td>
<td>Male Wistar rats, n=6 (200-250 g) and male Swiss mice, (40-45 g)</td>
<td>Dose-dependently reduced rat paw edema and inhibited myeloperoxidase activity, related to inhibition of pain and inflammation</td>
<td>Torri E, Lemos M, Caliari V. (2007). <em>J Pharm Pharmacol.</em>, 59:591-6.</td>
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<tr>
<td>Table 2.2 (contd.)</td>
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<tr>
<td><strong>Blackberry (Rubus sp. Rosaceae)</strong></td>
<td><strong>3.8 μmol anthocyanins/g diet</strong>&lt;sup&gt;a&lt;/sup&gt; 5% whole BRB powder, &lt;sup&gt;b&lt;/sup&gt; anthocyanin-rich fraction, &lt;sup&gt;c&lt;/sup&gt; an organic solvent-soluble extract, or &lt;sup&gt;d&lt;/sup&gt; an organic-insoluble fraction (containing 0.02 μmol anthocyanins/g diet)</td>
<td><strong>Esophageal tumors</strong></td>
<td><strong>F344 rats</strong></td>
<td><strong>Anthocyanins in blackberry suppressed tumor growth</strong></td>
<td><strong>Wang SL, Hecht SS, Carmella SG. (2009). Cancer Prev Res (Phila) 2:84-93</strong></td>
</tr>
<tr>
<td><strong>Maqui berry (Aristolelia Chilensis) and Mortiño (Vaccinium floribundum)</strong></td>
<td><strong>100 μmol/L (C3G or epicatechin equivalent) phenolic extracts from both berries, treatment for 24 h</strong></td>
<td><strong>Cardiovascular risk factors such as adiopogenesis and lipid accumulation</strong></td>
<td><strong>3T3-L1 adipocytes and RAW 264.7 macrophage</strong></td>
<td><strong>Inhibited lipid accumulation by 4.0 – 10.8%, decreased production of inflammatory markers NO, PGE&lt;sub&gt;2&lt;/sub&gt;, iNOS, and COX-2</strong></td>
<td><strong>Schreckinger M, Wang J, Yousef G, de Mejia E. (2010). J Agric Food Chem, 58: 8966-8976</strong></td>
</tr>
<tr>
<td><strong>Elderberry (Sambucus nigra L.)</strong></td>
<td><strong>200, 300 or 400 mL of elderberry juice (containing 361, 541 and 722 mg total anthocyanins)</strong></td>
<td><strong>Excretion &amp; biological activity of anthocyanin</strong></td>
<td><strong>Healthy humans (4 men and 4 women)</strong></td>
<td><strong>Most absorption was through the stomach and intestines, skin is able to easily take up anthocyanins, small amounts are excreted in urine.</strong></td>
<td><strong>Netzel M, Strass G, Herbst M. (2005). Food Res Int, 38: 905-910</strong></td>
</tr>
<tr>
<td><strong>Serviceberry (Amelanchier alnifolia), cranberry (Viburnum trilobum), chokeberry (Prunus virginiana) and buffaloberry (Shepherdia arencea)</strong></td>
<td><strong>5 μg/mL of berry samples dissolved in DMSO; frozen berries were lyophilized, dried, and extracted with 80% ethanol</strong></td>
<td><strong>Diabetic microvascular complications, hyperglycemia, pro-inflammatory gene expression, metabolic syndrome symptoms</strong></td>
<td><strong>L6 cells, a cell model for studying insulin action</strong></td>
<td><strong>Polar constituents (phenolic acids, anthocyanins and proanthocyanidins) were hypoglycemic agents and strong inhibitors of IL-1β and COX-2 gene expression</strong></td>
<td><strong>Burns Kraft TF, Dey M, Rogers RB, Ribnicky DM et al. (2008). J Agric Food Chem, 56:654-60.</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Berry</th>
<th>Dose of Treatment</th>
<th>Proposed target</th>
<th>Model</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red currant (Rhus chirindensis)</td>
<td>RCE, 50–800 mg/kg i.p., DIC, 100 mg/kg i.p., distilled water as control for inflammation; STZ, (75 mg/kg, in 0.1 mol/l citrate buffer at pH 6.3), and distilled water as control for diabetes</td>
<td>Inflammation and diabetes</td>
<td>Balb C male and female mice and Wistar rats with induced diabetes mellitus through injections of streptozotocin (STZ).</td>
<td>Pretreatment with high doses of stem-bark extract significantly reduced blood glucose concentrations of fasted normal and fasted diabetic rats.</td>
<td>Ojewole JAO. (2005). J Ethnopharmacol, 113: 338-345.</td>
</tr>
<tr>
<td>Indian Gooseberry (Emblica officinalis)</td>
<td>1 mL/kg/orally/day fruit juice (541.3 mg GAE/1 g extract) for eight weeks</td>
<td>Diabetes-induced myocardial dysfunction</td>
<td>STZ-induced type 1 diabetic male Wistar rats (200-250 g)</td>
<td>Fruit juice treatment decreased serum glucose and myocardial damage associated with type 1 diabetes mellitus (attributed to concentration of polyphenols)</td>
<td>Patel SS, Goyal RK. (2011). Exp Clin Cardiol, 16:87-91.</td>
</tr>
<tr>
<td>Low-fat, high-fat, or high-fat +BB (with 4% wt:wt blueberry (V. ashei and V. corymbosum) powder (14.5 MJ/kg) for 8 wks</td>
<td>Obesity-associated pathology of adipocyte death and inflammation</td>
<td>High fat fed male C57BI/6j mice, n=8/diet</td>
<td>Shift of global upregulation of inflammatory genes (TNF-α, IL-6, MCP1, iNOS) from high fat diet was attenuated or nonexistent with +BB diet</td>
<td>Defuria J, Bennett G, Strissel KJ. (2009). J Nutr, 139:1510-6.</td>
<td></td>
</tr>
<tr>
<td>Acai (Euterpe oleracea)</td>
<td>500, 1000 and 1500 mg/kg (Euterpe oleracea oil) OEO orally</td>
<td>Inflammation and hyperalgesia</td>
<td>Male albino Wistar rats (180-200 g) and male albino Swiss mice (20-25 g)</td>
<td>Significantly inhibited the granuloma test, a chronic inflammatory model, and decreased histamine action, showing the effect on the acute inflammatory process</td>
<td>Favacho Hugo AS, Oliveira Bianca R, Santos Kelem C. (2011). Brazilian J Pharmacognosy, 21: 105-114.</td>
</tr>
</tbody>
</table>
Table 2.3 (contd.)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract</th>
<th>Activity/Method</th>
<th>Animal Model</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chilean wild blackberry (Aristotelia chilensis)</td>
<td>Aristotelia chilensis Mol (Stuntz.) subfractions</td>
<td>Inflammation and carageenan-induced rat paw edema &amp; gastro protection</td>
<td>Adult male Wistar rats, 4-5 wks old, 200-250 g</td>
<td>Extracts have anti-inflammatory, anti-edema, and reduction in gastric lesion number, length, and amounts</td>
</tr>
<tr>
<td>Omija (schizandra chilensis)</td>
<td>Omija pulp/seed extract (OPE) treatment at 0.5 g/kg</td>
<td>Hyperglycemia induced by sucrose loading</td>
<td>Sprague-Dawley rat model and in vitro assays for enzyme inhibition</td>
<td>Potent α-amylase inhibition, potent α-glucosidase inhibition, significant decrease of blood glucose</td>
</tr>
<tr>
<td>Black choke berry (Aronia melanocarpa)</td>
<td>Fruit juice 10 and 20 ml/kg</td>
<td>Glycaemia and lipidemia</td>
<td>Streptozotocin-induced diabetic rats</td>
<td>Significant reduction in plasma glucose and triglycerides</td>
</tr>
<tr>
<td>Bilberry (Vaccinium myrtillis)</td>
<td>0.02% bilberry anthocyanin-rich extract</td>
<td>Inflammation and cardiovascular disease</td>
<td>Apo-E deficient mice</td>
<td>Reduced plasmatic total cholesterol and hepatic triglyceride levels, but the plasmatic antioxidant status remained unchanged.</td>
</tr>
<tr>
<td>Chokeberry (Aronia spp.)</td>
<td>Black chokeberry extract (0.2%) over 4 weeks, added to a standard casein diet with 8% lard and 65% fructose</td>
<td>Antioxidant and hypoglycemic properties</td>
<td>Male Wistar streptozotocin-induced diabetic rats</td>
<td>Decrease in serum ACL level and serum glucose levels by the extract compared to the high-fructose diet without it.</td>
</tr>
<tr>
<td>Turkey berry (Solanum torvum Swartz)</td>
<td>10, 20 and 40 mg/kg</td>
<td>Hyperlipidemia and diabetes</td>
<td>Streptozotocin-induced rats</td>
<td>Methyl caffeate isolate has antihyperglycemic and anti-diabetic effects</td>
</tr>
<tr>
<td>Chinese magnololive (Fructus schisandrae)</td>
<td>200mg/kg BW ethanol extract fractions given orally for 8 weeks</td>
<td>PPARγ activity and glucose stimulated insulin secretion</td>
<td>90% pancreateized male Sprague-Dawley diabetic rats, 231 ± 25 g and 3T3-L1 fibroblasts</td>
<td>Lignan-rich fractions improved glucose dispersal rates as much as rosiglitazone, and enhanced hepatic insulin sensitivity</td>
</tr>
</tbody>
</table>

A side effect of diabetes mellitus is the increased risk of cardiomyopathy and heart failure from the resulting stiffness and impaired contractility of blood vessels due to the increased glycation of interstitial proteins such as collagen. Treatment with fruit juice from Indian gooseberry in type 1 diabetic rats was seen to improve the glycemic response and reduce oxidative stress through increasing antioxidant enzymes in the heart and to decrease blood triglycerides and total cholesterol levels in the rats. The decrease in the glucose level was seen without an increase in insulin output, indicating that the fruit juice polyphenols were able to increase the sensitivity of the peripheral tissue to insulin. These and many other animal studies with beneficial effects due to berry or anthocyanin consumption provide the basis for human feeding trials and clinical studies, and provide a link to the potential mechanism of action determined with in vitro studies.

5.3 Anti-Diabetic Human Studies

While animal feeding trials are much easier to control or design than those involving humans, it is the clinical and epidemiological studies of berry consumption which provide the most relevant information to elucidate the beneficial effects to improve inflammatory diseases such as diabetes. Diabetes is associated with oxidative stress that results from the imbalance of free radical production and antioxidants in the system. Through their antioxidant activity, anthocyanins may be able to reduce oxidative stress that occurs due to the glycation reactions from hyperglycemia, thereby protecting beta-cells from damage. The effects of berry compounds on health have been studied and are summarized for their anti-inflammatory and anti-diabetic properties in humans (Table 2.4). Gooseberry phenolic compounds significantly improved antioxidant defense and diabetic indices in patients with diabetes (Chen 2011). Young healthy adults fed bilberry extract experienced a decrease in postprandial insulin demand, with a larger amount of bilberries consumed decreasing the glycemic response further (Granfeldt 2011). This study supports the thought that bilberries are able to cause an increased uptake of glucose into the peripheral cells, allowing for enhanced insulin sensitivity in type 2 diabetics. Bilberry extract has also been seen to reduce blood glucose levels through activation of an AMP-activated protein kinase, an enzyme that is crucial for the regulation of tissue fuel preference. A similar activation of this kinase was seen following treatment of fermented blueberry juice, having improved
glucose uptake and therefore decreased hyperglycemia. Exotic berries also have the potential to improve inflammation or diabetes management. Açaí berry preparations are able to reduce reactive oxygen species to assist in normalizing metabolic pathways leading to metabolic syndrome, a precursor to diabetes. Following the twice-daily feeding of 100 g of açai pulp for a month, overweight adults had a reduction of fasting glucose and plasma insulin (Udani 2011), warranting more large-scale studies to improve metabolic parameters following berry consumption.

Low glycemic index (GI) fruit consumption by patients with type 2 diabetes, who were taking oral agents to control diabetes, showed significant changes in the measures of glycemic control, including a negative association with berry intake and glucose levels (Jenkins 2011). Two additional daily servings of low GI temperate climate fruit with a GI value of <70, which includes berries, has been associated with a significant benefit in glycemic control by reducing the blood glucose levels after a meal, and such consumption should be encouraged in the management of type 2 diabetes. One study following consumption of a bilberry drink showed the beneficial metabolic properties of a low glycemic diet through lower insulin response or increased insulin sensitivity, where there was a low insulin demand. Consumption of 45 g/day of berries may be considered high for some; however, due to the potential of berries to decrease the risk of obesity, metabolic syndrome, and type 2 diabetes, increasing consumption to this modest amount is extremely important.

- Human feeding trials with berry anthocyanins were able to increase plasma antioxidant status and reduce oxidative stress.
- Anthocyanins may protect beta-cells, increase the secretion of insulin, and reduce the digestion of sugars in the small intestine.
- Anthocyanins have multiple and simultaneous anti-diabetic effects, including reducing blood glucose and preventing free radical production.
- Berry consumption may reduce postprandial blood glucose increase and improve insulin resistance, beneficial for those with type 2 diabetes mellitus.

6. Other Health Benefits

It is worth mentioning some other health beneficial effects of berry or berry components (Table 2.2). Berries have been found to inhibit tumor initiation, promotion, and progression, indicating potential and have been reported to play a major role in prevention of cardiovascular disease. There is also research into other anticancer properties, cardioprotection, and inhibition of lipid peroxidation, neuroprotection, and anti-atherosclerosis ability of berry fruits.
Table 2.4. Anti-inflammatory and/or diabetes-improving properties attributed to berries and berry compounds in clinical and epidemiological studies in humans.

<table>
<thead>
<tr>
<th>Berry</th>
<th>Dose of Treatment</th>
<th>Proposed target</th>
<th>Participants</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black currant (Ribes nigrum L.)</td>
<td>8 g/kg BW either diluted sweetened juice (1239 or 716 mg anthocyanins) or control sugar water drink, or 716 mg drink and a rice cake</td>
<td>Cardiovascular disease</td>
<td>17 healthy normolipidemic females, 19-48 years old, BMI = 23.0 kg/m²</td>
<td>Protective effects of anthocyanins on atherosclerosis development</td>
<td>Nielsen ILF, Dragsted LO, Ravin-Haren G. (2003). J Agric Food Chem, 51: 2813-2820.</td>
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<td></td>
<td>3 wk double-blind crossover study with 48 g of blackcurrant extract or sugar placebo encapsulated in gelatin</td>
<td>Oxidative stress following 30-min row exercise</td>
<td>Exercise, healthy individuals (five males and five females) between 37 and 63 yrs. old</td>
<td>Anthocyanins from the extract were effective antioxidants in reducing oxidative stress following exercise</td>
<td>Lyall KA, Hurst SM, Cooney J. (2009). Am J Physiol Regul Integr Comp Physiol., 297:R70-81.</td>
</tr>
<tr>
<td>Cranberry (Vaccinium macrocarpon Ait.)</td>
<td>Single cross-over design: unsweetened low-calorie CBJ (LCCBJ; 19 Cal/240 mL), carbohydrate sweetened normal calorie CBJ (NCCBJ; 120 Cal/240 mL), isocaloric low-calorie sugar water control (IcC), and isocaloric normal calorie sugar water control (C) interventions</td>
<td>Glycemic response</td>
<td>6 male and 6 female type 2 diabetics, 65.3 ± 2.3 yrs. old, BMI 34.7 ± 1.6</td>
<td>Low calorie interventions had no significant increase in blood glucose or plasma insulin and therefore should be used instead of normal calorie preparations</td>
<td>Wilson T, Meyers SL, Singh AP. (2008). J Food Sci., 73:H241-5.</td>
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<td>One of six treatments: WB (57 g, 160 cal, 1 g fiber), RC (55 g, 21 cal, 1 g fiber), SDC (40 g, 138 cal, 2.1 g fiber), and SDC containing less sugar (SDC-LS, 40 g, 113 cal, 1.8 g fiber + 10 g polydextrose)</td>
<td>Plasma insulin and blood glucose 0, 30, 60, 120, and 180 minutes after consumption</td>
<td>187 healthy participants (149 women and 9 men), non-diabetic; 19.7 ± 0.13 yrs. old, 67.96 ± 1.03 kg BW</td>
<td>Consumption of a low calorie cranberry juice rich in phenolics is associated with a favorable glycemic response</td>
<td>Wilson T, Singh AP, Vorsa N, Goettl CD, Kittleson KM, Roe CM, Kastello GM, Ragsdale FR. (2010). J Med Food, 11:46-54.</td>
</tr>
</tbody>
</table>
Table 2.4 (contd.)

<table>
<thead>
<tr>
<th>Blueberry</th>
<th>375 g/wk fresh blueberries, 375 g/wk fresh blueberry purée, control was no blueberries</th>
<th>Markers of inflammation and oxidative stress</th>
<th>24 overweight and obese children (8-13 years)</th>
<th>Increased consumption of blueberries increased intake antioxidant, and had a positive effect to reduce markers of inflammation and oxidative stress</th>
<th>Giongo L, Bozza E., Caciagli P. (2011). Berry Research, 1: 147-158.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Açai (Euterpe oleracea Mart.)</td>
<td>100 g açai pulp twice daily for 1 month</td>
<td>Metabolic parameters</td>
<td>10 overweight adults (BMI ≥ 25 kg/m² and ≤ 30 kg/m²)</td>
<td>Significant reductions in fasting glucose and insulin levels compared to baseline, ameliorating post-prandial increase in plasma glucose</td>
<td>Udani JK, Singh BB, Singh VJ, Barrett ML. (2011). Nutr J, 12:45.</td>
</tr>
<tr>
<td>Bilberry (Vaccinium Myrtillus)</td>
<td>330 mL bilberry juice/day (diluted to 1 L using tap water) for 4 weeks</td>
<td>Plasma concentration of NF-kB inflammatory markers</td>
<td>Male and female subjects at risk for cardiovascular disease (n=31/group)</td>
<td>Significant decreases in CRP, IL-6, IL-15 and MIG</td>
<td>Karlsen A, Paur I, Bohn S. (2010). Eur J Nutr, 49: 345-355.</td>
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<td></td>
<td>Fermented oatmeal drinks (FOMD), with 10% bilberry (BFOMD, 270.3 g), enriched to 47% bilberry (BBFOMD, 307.7 g), and 70.0 g reference bread</td>
<td>Glycemic and insulminic responses</td>
<td>Young (26.2 ± 4.6 years) healthy adults (7 women and 4 men), BMI 23.5 ± 2.9 kg/m²</td>
<td>Insulin demand and insulin index was significantly lower after the BFOMD, with BBFOMD having a low insulin demand, and lowered glycaemia compared to FOMD</td>
<td>Granfeldt YE, Bjorck IME. (2011). Nutr J 10:57.</td>
</tr>
<tr>
<td></td>
<td>Randomized cross-over study of 4 diets with equivalent doses of 100 g fresh sea buckthorn (SB), extracts (SBo, SBe) or bilberry (BB)</td>
<td>Type 2 diabetes and atherosclerotic cardiovascular diseases</td>
<td>110 overweight and obese (BMI 26-34 kg/m²) female</td>
<td>Small decrease in fasting plasma glucose after SB, TNF-α level decreased with BB, SB, and SBe</td>
<td>Lehtonen HM, Suomela JP, Tahvonen R. (2011). Eur J Clin Nutr, 65:394-401.</td>
</tr>
</tbody>
</table>

In a study of obese men and women with metabolic syndrome, no effect was seen on serum glucose concentration or lipid profiles; however decreases in blood pressure and plasma LDL were greater in the group receiving blueberries. This study by Basu et al. indicated that though human studies are limited, blueberries may improve selected features of metabolic syndrome, and supports the favorable effects of blueberries on metabolic syndrome and type 2 diabetes seen by animal obesity models. Phytochemicals in berries may prevent oxidation of cellular lipids and repair DNA oxidative damage, impact the cell cycle, inhibit oxidant enzymes, induce endogenous antioxidant enzymes to scavenge free radicals, and modulate signal transduction and intercellular communication (Paredes-Lopez 2010). Black currant concentrates and the main anthocyanins of black currant fruit juices have been shown to phosphorylate eNOS via activation of a kinase-signaling pathway. This induction leads to activation of cell signaling molecules relevant to maintaining endothelial function and therefore reducing the risk for cardiovascular diseases. Whole mulberry extracts, historically known for their anti-inflammatory properties, were able to reduce the acute inflammatory response through fluid accumulation indicative of arthritic damage. Effectiveness of whole fruit extracts rather than individual components provide a prediction of the potential effects due to whole fruit consumption. A clinical study of diets with equivalent doses of 100 g fresh sea buckthorn or bilberries found positive effects on the associated variables of obesity-related diseases. Due to the potential of berries to decrease the risk of obesity, metabolic syndrome, and type 2 diabetes, consumption of berry fruits available around the world should be increased.

7. Concluding Remarks

Berry fruits are a major dietary source of anthocyanins. Anthocyanins show promising benefits to general health and for the reduction of inflammation and diabetes. More understanding of the benefits of berry compounds like anthocyanins will provide increased awareness of their health potential and promote consumption of berries as functional foods. Anthocyanins are generally regarded as a safe food component. Toxicological studies on pure anthocyanins are limited; however, mixtures of anthocyanins extracted from different fruits indicated that they are safe and possess very low toxicity to humans (Crozier et al. 2009). Anthocyanins have potential for clinical efficiency in prevention and treatment of type 2 diabetes
and improving the inflammatory response. Regardless of the low bioavailability of anthocyanins, clinical trials have shown the efficacy of these compounds (Nielsen 2003, Lyall 2009, Basu 2010, Giongo 2011, and Udani 2011). It will be helpful for future studies on anthocyanins to identify the specific structural and chemical properties of anthocyanins, which impacts the mechanism of action. Anthocyanins may be isolated and purified from berry fruits to carry out further *in vivo* experiments and clinical evaluations. With the papers available to date, it can be seen that berry fruits and their anthocyanin pigments may have favorable effects on type 2 diabetes. The effects on stimulating insulin secretion and protection of beta cells through reduced oxidative stress improves insulin resistance and leads to decreased postprandial glucose levels. Studies are needed to determine which specific anthocyanins are most appropriate for a particular purpose or target for treatment of diabetes and related inflammation. It would be beneficial for future researchers to analyze the effects and the composition of berries to properly attribute any benefits to a specific compound. Beyond the health benefits studied by researchers, it will also be important to promote the daily consumption of berry crops to consumers. The benefits of anthocyanins from berries may not be attributable to just one type, therefore the wide variety of anthocyanins present in berry fruits and other foods makes them ideal to promote for use in everyday diet. The optimal dose and efficiency of extracts from berries and levels of foods rich in these compounds can determine which berries to promote for consumption. Additionally, improved horticultural techniques and new cultivar development will further the commercial application for berries and increase their availability. As seen by the USDA Horticulture National Berry Crop Initiative, “Berry crops have the potential to improve vision, fight cancer, and treat infections, but more research, education, and extension efforts are needed to determine the potential health benefits of these crops and to help inform producers and consumers about the benefits.” There is a great need to further investigate the health benefits of anthocyanins from berry fruits and their roles in diabetes and inflammation, and to propagate this knowledge to consumers to promote berry fruit consumption as functional foods.

- Berry fruits are potent antioxidants due to the high amounts of anthocyanins present, and anthocyanins may act to increase plasma antioxidant status in humans.
- Berry extracts, or anthocyanin treatment, have been shown to reduce inflammation-related parameters in human studies, animal studies, and *in vitro* cellular models.
- Berry fruits have been studied for parameters related to type 2 diabetes; therefore consumption to gain these beneficial effects should be advocated to people with diabetes.
• More studies are needed to investigate the clinical and epidemiological role of berry consumption and the role of anthocyanins from berries in improving or preventing inflammation-related chronic diseases.

Glossary

Aglycone: the non-sugar compound; the resulting anthocyanidin form after replacement of the glycosyl group by a hydrogen atom in the glycoside anthocyanin form
Acylation: adding an acyl group (the function group derived from removal of one or more hydroxyl groups from an oxygen-containing species) to a compound
Arachidonic acid: also referred to as AA or ARA, a omega-6 fatty acid in phospholipids of membranes in the body
Cardiomyopathy: deterioration of the function of the myocardium, the heart muscle, which puts one at risk for sudden cardiac arrest
Ellagitannins: the class of hydrolysable tannins, a group of polyphenols
Endogenous: originating from within an organism or tissue
Flavonols: a class of flavonoids with a 3-hydroxyflavone backbone containing a ketone group, different from flavanols, or flavan-3-ols
Flavan-3-ols: a class of flavonoids that are the building blocks of proanthocyanidins, and lack the ketone group on the 4-carbon of the C-ring, contain catechin and epicatechin
Flavylum cation: the ion 2-phenylchromenylium cation, containing a positive oxygen
Glycation: non-enzymatic glycosylation, covalent bonding of a protein or lipid with a sugar molecule without the control of an enzyme
Glycemic index: a measure of the effects of carbohydrates on blood sugar levels, high GI carbohydrates break down quickly during digestion and release glucose rapidly into the bloodstream
Glycosylation: a reaction where a carbohydrate is attached to a hydroxyl or another functional group on another molecule
Hydroxybenzoate: a part of parabens, a class of chemicals use as preservatives
Isoprenoids: also called terpenoids, a large diverse class of compounds derived from the combination of several 5-carbon isoprene units
Jejunum: the middle section of the small intestine, lying between the duodenum and the ileum
Lipid peroxidation: the oxidative degradation of lipids in which free radicals take electrons from lipids in cell membranes, resulting in cellular damage.
Nutraceuticals: a food or food product with reported health and medical benefits that can be included in the prevention and treatment of disease, from “nutrition” and “pharmaceutical”
Postprandial: occurring after consuming a meal
Procyanidins: members of the proanthocyanidin class of flavonoids that form from flavan-3-ols molecules
Stilbenoids: hydroxylated derivatives of stilbene, secondary products in plants (a common example is resveratrol)
Terpenes: derived from units of isoprene, oxidized or rearranged into terpenoids

Annotated Bibliography

Burns Kraft TF, Dey M, Rogers RB, Ribnicky DM, Gipp DM, Cefalu WT, Raskin I, Lila MA. (2008) Phytochemical composition and metabolic performance-enhancing activity of dietary berries traditionally used by Native North Americans. *Journal of Agricultural and Food Chemistry* 56(3):654-60. [Included in Table 2 to indicate the hypoglycemic and anti-inflammatory properties of serviceberry, cranberry, chokeberry and buffalo berry phenolic extracts, and a referee article on historical berry uses in North America].


Chen TS, Liou SY, Wu HC. (2011) Efficacy of epigallocatechin-3-gallate and Amla (*Emblica officinalis*) extract for the treatment of diabetic-uremic patients. *Journal of Medicinal Food* 14: 718-723. [A referee journal article that demonstrates anti diabetic properties, included in Table 4 as a reference for gooseberry extract in reducing oxidative stress in uremic patients with diabetes].


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Favacho HAS, Oliveira BR, Santos KC, Medeiros BJL, Sousa PJC, Perazzo FF, Carvalho JCT. (2011) Anti-inflammatory and antinociceptive activities of Euterpe oleracea oil. *Brazilian Journal of Pharmacognosy* 21(1): 105-114. [Used in Table 3 as a reference for Açai oil on inflammation and hyperalgesia in rats and mice].


Garzon GA, Riedl KM. (2009) Determination of anthocyanins, total phenolic content, and antioxidant activity in Andes berry (Rubus glaucus Benth). *Journal of Food Science* 74 (3): C227–C232. [A referee journal article that provides numbers on post-harvest loses, used in Table 1 as a reference for chokeberry total phenolics, total anthocyanins, and antioxidant capacity].

Gavrilova V, Kajdz M. (2011) Separation, characterization and quantification of phenolic compounds in blueberries and red and black currants by HPLC−DAD−ESI-MS. *Journal of Agricultural and Food Chemistry* 59 (8): 4009–4018. [Used in Table 1 as a reference for black and red currant total phenolics and total anthocyanins].

Giongo L, Bozza E, Caciagli P. (2011) Short-term blueberry intake enhances biological antioxidant potential and modulates inflammation markers in overweight and obese children. *Journal of Berry Research* 1 (3):147-158. [A referee journal article that demonstrates modulation of inflammation, used in Table 4 as a reference for fresh blueberry intake in children clinical trial].


Jo SH, Ha KS, Moon KS. (2011) In vitro and in vivo anti-hyperglycemic effects of Omija (Schizandra chinensis) fruit. *International Journal of Molecular Sciences* 12(2):1359-70. [Used in Table 3 as a reference for Omija pulp/seed extract treatment on hyperglycemia in rats].


Koponen JM, Happonen, AM. (2007) Contents of anthocyanins and ellagitannin in selected foods consumed in Finland. *Journal of Agricultural and Food Chemistry* 55, 1612–1619. [Used in Table 1 as a reference for mulberry in vitro trial].


Lee J, Finn C. (2007) Anthocyanins and other polyphenolics in American elderberry (Sambucus canadensis) and European elderberry (*S. nigra*) cultivars. *Journal of the Science of Food and Agriculture* 87(14): 2665-2675. [Used in Table 1 as a reference for elderberry total polyphenols and total anthocyanins].


Lee J, Finn C. (2007) Anthocyanins and other polyphenolics in American elderberry (Sambucus canadensis) and European elderberry (*S. nigra*) cultivars. *Journal of the Science of Food and Agriculture* 87(14): 2665-2675. [Used in Table 1 as a reference for elderberry total polyphenols and total anthocyanins].
Lehtonen HM, Suomela JP, Tahvonen R. (2011) Different berries and berry fractions have various but slightly positive effects on the associated variables of metabolic diseases on overweight and obese women. *Journal of Clinical Nutrition* 65(3):394-401. [Randomized cross-over study used in Table 4 as a reference for fresh sea buckthorn or extracts].

Lima VLAG, Melo EA, Maciel MIS, Lima DES. (2003) Evaluation of total anthocyanins in frozen acerola pulp from fruits of 12 different trees acerola (Malpighia emarginata DC). *Ciência e Tecnologia de Alimentos* 23:101–103. [Used in Table 1 as a reference for acerola total anthocyanins].


Lugasi A, Hővári J, Kádár G, Dénes F. (2011) Phenolics in raspberry, blackberry and currant cultivars grown in Hungary. *Acta Alimentaria* 40(1): 52-64. [Used in Table 1 as a reference for black currant, red currant, blackberry, and raspberry total phenolics and total anthocyanins].


Netzel M, Strass G, Herbst M. (2005) The excretion and biological antioxidant activity of elderberry antioxidants in healthy humans. *Food Research International* 38(8-9): 905-910. [Used in Table 1 as a reference for elderberry total phenolics, total anthocyanins, and antioxidant capacity, used in Table 2 to exhibit elderberry juice anthocyanin excretion in humans].


Patel SS, Goyal RK. (2011) Prevention of diabetes-induced myocardial dysfunction in rats using the juice of the *Emlica officinalis* fruit. *Experimental and Clinical Cardiology* 16(3):87-91. [Used in Table 3 as a reference for Indian gooseberry juice trial in diabetes-induced rats].


Tarbart J, Franck T, Kevers C. (2011) Antioxidant and anti-inflammatory activities of *Ribes nigrum* extracts. *Food Chemistry* 131(4): 1116-1122. [Used in Table 1 as a reference for black currant total phenolics and antioxidant capacity].


Wu X, Liang L, Zou Y. (2011) Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (Morus atropurpurea Roxb.). Food Chemistry 129(2): 443-453. [Used in Table 1 as a reference for mulberry total phenolics and total anthocyanins].


You Q, Chen F, Huang Z. (2011) Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. Food Chemistry 125 (1): 201-208. [Used in Table 1 as a reference for blueberry total phenolics and total anthocyanins].
CHAPTER 3

OBJECTIVES

I. **Aim 1:** Evaluate commercially available blueberry and blackberry juices and wines produced in the state of Illinois. *(Chapter 4).*

**Hypothesis:** Wines made from dark berry fruits contain phenolic compounds beneficial for health.

**Objectives:** Evaluate blueberry and blackberry wines commercially available in Illinois for chemical and quality components relevant to consumers in order to study their potential health benefits.

![Locations of wineries producing fruit wines made from blueberries and blackberries in Illinois.](image)

II. **Aim 2:** Determine the effect of fermentation on the chemical composition and quality of blueberry and blackberry fermented beverages. *(Chapter 5).*

**Hypothesis:** The berry fruits of Highbush blueberry (Vaccinium corymbosum) contain bioactive compounds with potential to reduce glucose absorption that may be affected by fermentation temperature.

**Objectives:** Compare fruits from fifteen blueberry cultivars and wines prepared from several of these blueberry cultivars throughout fermentation at room temperature and cold temperature fermentation for composition and quality parameters, and ability to inhibit starch-degrading enzymes.

III. **Aim 3:** Assess the potential of combinations of blueberry-blackberry fermented beverages for antioxidant capacity, and to determine their ability to reduce activity of key enzymes related to diabetes, and modulation of inflammation using in vitro and computational studies.

1. **Specific Objective 3.1.** Evaluate blends of blueberry-blackberry wines and their isolated phenolic compounds for total phenolics, total anthocyanins, and antioxidant capacity, in-vitro inhibition of inflammatory markers, starch-degrading enzyme inhibition (α-amylase and α-glucosidase), and DPP-IV (dipeptidyl peptidase-4) inhibition. *(Chapter 6).*
**Hypothesis:** Berries and their fermented products are an excellent source of dietary flavonoids anthocyanins (ANCs) and proanthocyanidins which will have high antioxidant capacity and ability to inhibit starch-degrading enzymes and DPP-IV, with the ANC being more effective than the PAC.

2. **Specific Objective 3.2.** Determine the inhibitory effect of berry phenolic compounds on DPP-IV (dipeptidyl peptidase-4) using a luminescence assay and computational modeling. *(Chapter 7)*.

**Hypothesis:** Anthocyanins (ANC) isolated from berry wine will be able to bind to the active sites of DPP-IV, thus inhibiting DPP-IV enzyme activity.

3. **Specific Objective 3.3.** Evaluate combinations of blueberry-blackberry fermented beverages for their effect to decrease markers of inflammation and adipogenesis in a dual cellular system mimicking pathogenic interaction between adipocytes and macrophages. *(Chapter 8)*.

**Hypothesis:** Addition of antioxidant agents such as anthocyanins will lead to a reduction in the inflammatory cross-talk between macrophages and adipocytes and may improve insulin-sensitivity as potential treatment to improve obesity-associated adverse consequences.

**IV. Aim 4:** Evaluate the insulin secretory effect of phenolic compounds found in fermented berry beverages in vitro using a simulated absorption model of epithelial and physiologically-relevant β-cells. *(Chapter 9)*.

**Hypothesis:** Anthocyanins (ANC) and proanthocyanidins (PAC) will be able to reduce DPP-IV activity to improve incretin function and therefore increase insulin secretion.

**V. Aim 5:** Establish the role of anthocyanins in fermented berry beverages to reduce diet-induced obesity and hyperglycemia in C57Bl/6j mice. *(Chapter 10)*.

**Hypothesis:** Consumption of fermented berry beverage and ANC and PAC phenolics will reduce hyperglycemia to aid, in a dose-dependent manner, in reducing diet-induced obesity and hyperglycemia in mice via improved insulin secretion and incretin function.
CHAPTER 4

COMPARISON OF CHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF COMMERCIALLY AVAILABLE BLUEBERRY AND BLACKBERRY WINES IN ILLINOIS

Abstract

Moderate wine consumption may reduce the incidence of certain age-related chronic diseases such as heart disease, hypertension, metabolic disease, and neurodegenerative disease. Blueberry and blackberry wines commercially available in Illinois were evaluated for chemical and quality components relevant to consumers in order to study their potential health benefits. Total polyphenolic content was measured by the Folin-Ciocalteu method, total anthocyanin content by the pH differential test, and in vitro antioxidant capacity by the oxygen radical absorbance capacity (ORAC) method. Color was measured using Hunter colorimetry and quality parameters including pH, °Brix, acid content, glucose-fructose and percent alcohol were measured using Fourier transform infrared (FTIR) spectroscopy. Blackberry wines (n=6) had an average total polyphenolic content of 2212.5 ± 1090.3 mg ellagic acid equivalents (EAE) /L, total anthocyanin content of 75.56 ± 70.44 mg/L, and antioxidant capacity of 26.39 ± 17.95 mmol trolox equivalents (TE)/L. Blueberry wines (n=4) had an average total polyphenolic content of 1623.3 ± 645.5 mg EAE / L, total anthocyanin content of 20.82 ± 12.14 mg/L, and antioxidant capacity of 21.21 ± 7.71 mmol TE/L. Strong positive correlations were found between °Brix and glucose-fructose concentration (r = 0.90), total acid and malic acid (r = 0.90), and between total polyphenols and antioxidant capacity (r = 0.88). The results suggest that fruit

2 (Publication: Johnson MH, de Mejia EG. Comparison of Chemical Composition and Antioxidant Capacity of Commercially Available Blueberry and Blackberry Wines in Illinois. Journal of Food Science. 2012, 77(1): C141-8.) This work has been previously published, with permission. ©2011 Institute of Food Technologists ®. doi: 10.1111/j.1750-3841.2011.02505.x.
wines made from blueberries and blackberries may have potential health applications and therefore could contribute to the economy of the wine industry.

Practical Application: The majority of wines are produced from grapes, but wine can also be produced from other fruits including blueberries and blackberries, which contain phenolic compounds that may contribute to human health. A comparative evaluation was conducted on commercial non-grape fruit wines and parameters related to their health benefits. Fruit wines made from blueberries and blackberries may have potential health applications and therefore could contribute to the economy of the wine industry.

Keywords: Wine, blueberry, blackberry, phenolics, antioxidants.

Abbreviations used: AAPH: 2,2-azobis 2-amidinopropane dihydrochloride; EAE: ellagic acid equivalents; FTIR: Fourier transform infrared; GAE: gallic acid equivalents; TE: Trolox equivalents; TFA: trifluoroacetic acid; ORAC: oxygen radical absorbance capacity.

Introduction

There is accumulating literature indicating the benefits of drinking red wine and of consuming berries containing phenols and anthocyanins, supporting the concept that wines made from dark berry fruits can also be beneficial for health. Wine is a complex mixture of water, alcohol and other minor components such as polyphenolic compounds and it has been established as having positive effects on health with moderate consumption (USDA 2010). Clinical studies have established a clear link between moderate consumption of grape wines and cardiovascular disease through improvements in vascular function (Vauzour and others 2010, Chaves and others 2009, Mellen and others 2010, Dohadwala and Vita 2009), reduction of atherosclerosis (Badimon and others 2010), and potential reduction in total mortality (Theobald and others 2000). Another study has shown an increase in plasma antioxidant capacity and
plasma total polyphenol concentration in humans after consumption of alcohol-free red wine (Serafini 1998), warranting further investigations into the polyphenolic compounds in red wines.

Beyond heart health, polyphenolic compounds in fruit beverages including flavonoids such as anthocyanins and proanthocyanidins have been found to have anticancer or antiproliferative effects (Cilla and others 2009, Mertens-Talcott and others 2008). Decreased lipidemia (Blade and others 2010) and favorable effects of wine consumption by those with metabolic diseases have been seen in previous studies (Burton-Freeman 2010), showing that wine may lead to decreased inflammatory-related disease risk when included as a part of a diet containing fruits and vegetables. Further connections have been proposed for compounds with longevity attributes in wines (Lekli and others 2010), protection against aging diseases such as Alzheimer’s (Singh and others 2008) and memory loss (Abraham and Johnson 2009), and overall brain health (Spencer 2009, Bastianetto 2000). All these potential impacts on health beyond cardiovascular disease call for further evaluation of wines as beneficial beverages.

In addition to the health relevance, the wine industry has great importance to economy and agriculture. For instance, in Illinois there are over 110 wineries separated into six wine trails with most vineyards located in the southern part of the state. Wine must contain 75% Illinois grapes to be labeled Illinois wine therefore wine produced from local grape production contributes to the overall economy of the state. Of the 87 different grape varieties planted by Illinois wineries, thirteen grape varieties account for 85% of the 2006 grape harvest (Campbell and Shoemaker 2007). Further, of the 550,000 gallons of wine produced in Illinois in 2006, 16.3% was non-grape wine (Campbell and Shoemaker 2007). Non-grape fruit wines can be produced from almost any fruit (Table 4.1 and Figure 4.1) and provides diversity to wines commercially available.
Table 4.1 - Fruit Wines Produced in the State of Illinois Separated by Wine Trail. All cities listed in italics are in the state of Illinois.

<table>
<thead>
<tr>
<th>Northern Illinois Wine Trail</th>
<th>Fruit Wine Brands Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>August Hill Winery Utica</td>
<td>Augie’s Blackberry Port Style Dessert Wine</td>
</tr>
<tr>
<td>Cooper's Hawk Winery Burr Ridge, Orland Park, South Barrington, Wheeling, Brookfield, Naperville</td>
<td>Blueberry Fruit Wine, Cherry Fruit Wine, Cranberry Fruit Wine, Pomegranate Fruit Wine, Peach Fruit Wine, Plum Fruit Wine, Raspberry Fruit Wine, Rhubarb Fruit Wine</td>
</tr>
<tr>
<td>Famous Fossil Vineyard &amp; Winery Freeport</td>
<td>Blackberry, Summer Rhubarb, Red Raspberry</td>
</tr>
<tr>
<td>Fox Valley Winery Oswego</td>
<td>Old Glory Red, Old Glory Blue, Sweet Blue (Blueberry and Blackberry)</td>
</tr>
<tr>
<td>Galena Cellars Vineyard &amp; Winery Galena</td>
<td>Blackberry, Blueberry Wine, Cherry, Cranberry Wine, Peach Wine, Red Raspberry, Rhubarb</td>
</tr>
<tr>
<td>Glunz Family Wine &amp; Cellars Grayslake, Long Grove</td>
<td>Raspberry, Black Currant</td>
</tr>
<tr>
<td>Hailey's Winery &amp; Vineyard Byron</td>
<td>Cranberry, Harvest Apple</td>
</tr>
<tr>
<td>Lavender Crest Winery Colona</td>
<td>Jubilee (Cherry), Blossom (Peach), Out of the Blue (Blueberry), Cosmic Bog (Raspberry), Razmatazz (Raspberry)</td>
</tr>
<tr>
<td>Lynfred Winery Roselle</td>
<td>Apricot, Blackberry, Black Raspberry, Blueberry, Cherry, Cranberry, Kiwi Peach, Kiwi Rose, Mango, Peach, Pear, Plum, Pomegranate, Raspberry, Rhubarb, Strawberry, Strawberry Rhubarb</td>
</tr>
<tr>
<td>Massbach Ridge Winery Elizabeth</td>
<td>Berry Sweet Wine (Blackberry), Apple Wine, Cherry Rose</td>
</tr>
<tr>
<td>Prairie State Winery Genoa</td>
<td>Apple, Elderberry, Cranberry, Prairie Berry (Blueberry, Blackberry, Raspberry), Pomegranate, Pumpkin</td>
</tr>
<tr>
<td>Silver Moon Winery Lanark</td>
<td>Cranberry, Cherry, Peach</td>
</tr>
<tr>
<td>The Village Vintner Winery Carpentersville</td>
<td>Autumn Splash (Apple and Cranberry), Black Satin (Blackberry), Blue Moon (Blueberry), Cherish Noir (Black Cherry), Cran Chi (Cranberry), Melon Delight, Orchard Mist (Apple), Peachard (Peach), Pom-A-Berry (Pomegranate), Purple Passion (Acai &amp; Raspberry), Razmatazz (Raspberry), Rhubarb, Sweet Passion (Strawberry), Tropical Dream (Mango and Pineapple), White berry Bog (White Cranberry), Wine-A-Rita (Orange and Grapefruit)</td>
</tr>
<tr>
<td>Waterman Winery and Vineyards Waterman</td>
<td>Apple Wine (Apple and Cranberry), Cherrylicious (Cherry), Harvest Pumpkin, Perfect Pear, Wild Watermelon</td>
</tr>
<tr>
<td>Wild Blossom Meadery &amp; Winery Chicago</td>
<td>Blueberry Mead, Cran-Nectar, Green Apple Riesling, Pom-Nectar, Raspberry Peach, Wild Berry Mead</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Illinois River Wine Trail</th>
<th>Fruit Wine Brands Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill Prairie Winery Oakford</td>
<td>Cranberry Crush, Prairie Berry (Blueberry and Blackberry), Raz-Cherry (Red Raspberry and Cherry), Strawberry</td>
</tr>
<tr>
<td>Willett's Winery and Cellar Manitou</td>
<td>Apple Splash, Blueberry Chill, Midnight Cherry, Strawberry Breeze, Black Raspberry Rush, Red Raspberry Thrill</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wabash Valley Wine Trail</th>
<th>Fruit Wine Brands Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox Creek Vineyards Olney</td>
<td>Framboise (Raspberry), Peach Wine, Pear Wine, Blackberry Cheer</td>
</tr>
<tr>
<td>Lasata Wines Lawrenceville</td>
<td>Blueberry Bliss</td>
</tr>
<tr>
<td>White Owl Winery Birds</td>
<td>Chard-N-Apricot, Cherry Pie, Elderberry, Paw Paw, Persimmon Solera, Pink Grapefruit Sparkel, Spiced Pear, Old Blue Port (Blueberry), Watermelon</td>
</tr>
<tr>
<td>White Owl Winery Birds</td>
<td>Cherry, Summer Strawberry, Royal Raspberry</td>
</tr>
<tr>
<td>Heartland Rivers Wine Trail</td>
<td>Fruit Wine Brands Produced</td>
</tr>
<tr>
<td>Bretz Wildlife Lodge and Winery Carlyle</td>
<td>Wild Life’s Cherry, Strawberry Blonde, Bad Apple, Blackberry Blombshell, Peach</td>
</tr>
<tr>
<td>Forsee Vineyards Coffeen</td>
<td>Winter Blush, Indian Summer (Blackberry and Blueberry)</td>
</tr>
<tr>
<td>Mary Michelle Winery Carrollton</td>
<td>Illinois Cellars Apple Wine</td>
</tr>
<tr>
<td>Orchard View Winery Alma</td>
<td>Blackberry, Apple Starboard, Front Porch (Apple), John James (Apple), French Kiss (Apple), Hay Loft (Apple with Strawberry), Mona Lisa (Peach), Strawberry, Nectarine</td>
</tr>
<tr>
<td>Schorr Lake Vineyards Waterloo</td>
<td>Apple Seasonal, Blackberry, Blackberry Port, Peach, Raspberry</td>
</tr>
<tr>
<td>Vahlings Vineyards &amp; Winery Stewardson</td>
<td>Cherry Wine, Blackberry Wine, Blueberry Wine, Gnome Nectar (Blackberry), Honey Wine (Mead), Rhubarb Wine, Strawberry Wine</td>
</tr>
<tr>
<td>Southern Illinois Wine Trail</td>
<td>Fruit Wine Brands Produced</td>
</tr>
<tr>
<td>Shawnee Winery Vienna</td>
<td>Temptation (Apple), Black Pearl (Blackberry), Razzle Dazzle (Raspberry), Just Peachy</td>
</tr>
<tr>
<td>Shawnee Hills Wine Trail</td>
<td>Fruit Wine Brands Produced</td>
</tr>
<tr>
<td>Alto Vineyards Alto Pass</td>
<td>Cherry, Cherry Berry (Cherry and Raspberry)</td>
</tr>
<tr>
<td>Blue Sky Vineyard Makanda</td>
<td>Framboise (Raspberry)</td>
</tr>
<tr>
<td>Owl Creek Vineyard and Winery Cobden</td>
<td>Framboise (Red Raspberry)</td>
</tr>
<tr>
<td>Pomona Winery Pomona</td>
<td>Jonathan (Apple), Jonathan Oak Aged Reserve (Apple), Golden (Apple), Golden Oak Aged Reserve (Apple), Kir (Black currants and Apple), Orchard Harvest (Apple), Orchard Spice (Apple), Once In A Blue Moon Blueberry Dessert Wine, Peach Dessert Wine, Strawberry Dessert Wine</td>
</tr>
<tr>
<td>Von Jakob Vineyards Pomona, Alto Pass</td>
<td>Honey Peach, Honey Raspberry, Honey Blackberry, Jonathan Apple, She’s A Peach</td>
</tr>
<tr>
<td>Hidden Wine Trail</td>
<td>Fruit Wine Brands Produced</td>
</tr>
<tr>
<td>Black Diamond Vineyards Nashville</td>
<td>Cherry Starboard</td>
</tr>
<tr>
<td>Cameo Vineyards Greenup</td>
<td>Cherry</td>
</tr>
<tr>
<td>Collver Family Winery Barry</td>
<td>Cherry, Barry Berry, Peach, Blueberry Razz, Raspberry Razz</td>
</tr>
<tr>
<td>Grafton Winery &amp; Brewhaus Grafton</td>
<td>Apple Crisp, Blackberry, Raspberry Kiss, Winter Berry (Cranberry), Spring Berry (Blueberry and Blackberry), Summer Peach, Autumn Berry (Raspberry)</td>
</tr>
<tr>
<td>LauNae Winery Red Bud</td>
<td>Olde Tyme Apple, Blossom City Rose (Strawberry, Blackberry, Elderberry), Strawberry, Peach Honey, Elderberry, Blueberry Sparkling, Peach Sparkling, Strawberry Sparkling</td>
</tr>
<tr>
<td>Pheasant Hollow Winery Whittington</td>
<td>Midnight Medley (Strawberry, Raspberry, Black Cherry), Red &amp; Blue (Cherry and Blueberry), Black &amp; Blue (Blackberry and Blueberry), Cherry, Red Razz (Raspberry), Marionberry, Peach, Cracklin’ Cranberry</td>
</tr>
<tr>
<td>Spirit Knob Winery Ursa</td>
<td>Cerise (Cherry)</td>
</tr>
<tr>
<td>Villa Marie Winery Maryville</td>
<td>Sugar Loaf White (Peaches), Summerset Bliss (Strawberry)</td>
</tr>
</tbody>
</table>

*This table was created using information available on the wineries’ websites.*
Figure 4.1- Numbers of Fruits Used in Non-Grape Wines Available in Illinois.

Fruit wines may offer health benefits unique from wine produced from grapes because of different components present in the fruit initially and those formed during the fermentation process. Dark-colored fruit pigmentation results from the presence of a class of water-soluble flavonoids known as anthocyanins. Increased consumption of berries containing anthocyanins has been linked to decreased incidence of inflammatory related diseases in humans (Seeram and others 2008, Erlund and others 2008), as well as increased free radical scavenging capacity, especially from blackberries (Wang and Jiao 2000). Phenolic compounds including anthocyanins found in berries have many of the same potential health benefits as grape wine compounds regarding improvements of cardiovascular disease (Caton and others 2010, Leifert and Abeywardena 2008) and endothelial function and heart health (Mazza 2007).

It is therefore important to look into the biological activity and potential health benefits of fruit wines available in Illinois and thus promote further production and information to the consumer. The objective of this study was to evaluate and compare fruit wines commercially
available in the state of Illinois made from blueberries and blackberries and describe their chemical composition and quality parameters in the context of the wine industry.

**Materials and Methods**

**Chemicals**

Compounds used as standards and their purity were as follows: Trolox (≥ 97%), glucose (≥ 99.5%), gallic acid (≥ 98%) and ellagic acid (≥ 95%). All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified.

**Commercial samples**

Wines were obtained from near Champaign, IL in February of 2011 and are summarized in Table 4.2 and Image 4.1. Blackberry wines purchased were produced by Door Peninsula Winery (Sturgeon Bay, WI), Galena Cellars Winery (Galena, IL), Lynfred Winery (Roselle, IL), St. James Winery (Saint James, MO), Vahling Vineyard (Stewardson, IL) and Von Jakob Vineyard (Pomona, IL). Blueberry wines purchased were produced by Galena Cellars Winery (Galena, IL), Pomona Winery (Pomona, IL), Tomasello Winery (Hammonton, NJ) and Wild Blossom Meadery (Chicago, IL). A blackberry and blueberry blend used for comparison of both groups was Pheasant Hollow Winery Black & Blue (Whittington, IL), and commercial blueberry juice was R.W. Knudsen Family® Just Blueberry™ Juice (Orrville, Ohio). Wines were tested for quality parameters immediately upon opening and stored at 4 °C until further testing.
Table 4.2- Commercial Information of Ten Blackberry and Blueberry Wines Available in Illinois.

<table>
<thead>
<tr>
<th>Winery name</th>
<th>Location</th>
<th>Bottle size (mL)</th>
<th>Price ($)</th>
<th>Berry/juice source</th>
<th>Claims</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blackberry Wines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Door Peninsula Winery</td>
<td>Sturgeon Bay, WI</td>
<td>750</td>
<td>12.99</td>
<td>Fall berry from Door County, WI</td>
<td>Semi-sweet blackberry wine; Alcohol 11.5% by vol.</td>
</tr>
<tr>
<td>Galena Cellars</td>
<td>Galena, IL</td>
<td>750</td>
<td>13.99</td>
<td>Marion blackberry</td>
<td>Dessert wine, serve chilled; Double Gold 2010 Illinois State Fair Bronze; 2009 Indy Wine Competition; Alcohol 10% by vol.</td>
</tr>
<tr>
<td>Lynfred Winery</td>
<td>Roselle, IL</td>
<td>750</td>
<td>12.99</td>
<td>Produced from 100% Blackberries from Cascade Mountains in Washington</td>
<td>Residual sugar 12%; Alcohol 11% by vol.</td>
</tr>
<tr>
<td>St. James Winery</td>
<td>Saint James, MO</td>
<td>750</td>
<td>8.99</td>
<td>Fresh picked blackberries</td>
<td>Sweet wine; Serve chilled; “Award Winning”; Alcohol 10.5% by vol.</td>
</tr>
<tr>
<td>Vahling Vineyard</td>
<td>Stewardson, IL</td>
<td>750</td>
<td>9.99</td>
<td>Tame and wild blackberries</td>
<td>Bronze Medal at the State Fair</td>
</tr>
<tr>
<td>Von Jakob Vineyard</td>
<td>Pomona, IL</td>
<td>750</td>
<td>13.99</td>
<td>75% honey wine (mead), 25% blackberry wine</td>
<td>Enjoy with spicy foods or chocolate; Sweet blush table wine; Alcohol 12% by vol.</td>
</tr>
<tr>
<td><strong>Blueberry Wines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galena Cellars</td>
<td>Galena, IL</td>
<td>750</td>
<td>13.99</td>
<td>Premium blueberries from Eastern Pennsylvania</td>
<td>Serve chilled; Alcohol 10% by vol.</td>
</tr>
<tr>
<td>Pomona Winery</td>
<td>Pomona, IL</td>
<td>375</td>
<td>13.99</td>
<td>Port-style: neutral brandy added to blueberries from HallsBerry Farm in Lick Creek, Illinois</td>
<td>Residual sugar 10%; Alcohol 18%; Silver 2010 Great Lakes; Silver 2010 Illinois, Bronze 2010 Indy International</td>
</tr>
<tr>
<td>Tomasello Winery</td>
<td>Hammonton, NJ</td>
<td>500</td>
<td>9.99</td>
<td>100% Highbush blueberries grown on Atlantic Blueberry Company farms</td>
<td>Alcohol 11% by vol. Taster’s Guild Silver Medal winner</td>
</tr>
<tr>
<td>Wild Blossom Meadery</td>
<td>Chicago, IL</td>
<td>750</td>
<td>15.99</td>
<td>Local Chicagoland wildflower honey fermented with 20% blueberries from the Midwest aged 6-months</td>
<td>“Antioxidant content one of the highest for any beverage known on Earth”; Alcohol 12.5% by vol.</td>
</tr>
</tbody>
</table>
**pH, °Brix, and color**

Samples were measured for pH using both an Accumet AR15 pH meter (Fisher Scientific, Pittsburg, PA) and with OenoFoss™ (FOSS, Hillerod, Denmark) machine and read using the Foss Integrator software. °Brix was measured using a portable refractometer. Color was determined with Hunter colorimetry and measured on a HunterLab Lab Scan XE color measurement instrument (Reston, VA) with Universal V3.71 software (Hunter Lab, Reston, Va., U.S.A.).

**Total phenolic content**

Total phenolic content (TP) was quantified using the Folin-Ciocalteu method described originally by Amerine and Ough (1974) adapted to a micro-assay. Briefly to a 96-well flat bottom plate, 50 μL of 1 N Folin-Ciocalteu’s phenol reagent and 50 μL of either sample diluted 1:20, standard or blank were added; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na₂CO₃. The solution was allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Ellagic acid was used to create a standard curve (40 to 200 mg/L), and results were expressed as mg ellagic acid equivalents (EAE) / L wine.

**Antioxidant capacity**

Antioxidant capacity (AC) was measured by the oxygen radical absorbance capacity (ORAC) assay as described previously (Prior 2003). A 20 μL trolox standard, sample, or blank of 75 mM phosphate buffer pH 7.4 were added in triplicate to a black walled 96-well plate. To each well, 120 μL of 116.9 nM fluorescein was added for a final concentration of 70 nM/well followed by 60 μL of 40 mM AAPH. The plate was read at 485 nm and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Trolox
was used to create a standard curve (4 to 160 µM) and results were expressed as mmol Trolox equivalents (TE)/L wine.

**Total anthocyanin content**

Total anthocyanins were determined using the AOAC Official Method as described previously (Lee 2005). Samples were diluted to a factor of 1:10 using two different buffers (pH 1.0, KCl buffer and pH 4.5, sodium acetate buffer). Diluted solutions at each pH were transferred to a 1 x 1 cm cuvette and the absorbance was read at 520 nm and 700 nm on a spectrophotometer (Beckman DU ® -64 Spectrophotometer, Fullerton, CA). The total anthocyanins were expressed as cyanidin-3-glucoside equivalents in mg/L using the equation below:

\[
\text{Total monomeric anthocyanins (mg/L)} = \frac{A \times MW \times D \times 1000}{\varepsilon \times PL}
\]

Where: 
- \( A = (A_{520}-A_{700}) \text{ pH}_{1.0} - (A_{520}-A_{700}) \text{ pH}_{4.5} \), 
- MW is 449.2 g/mol for cyanidin-3-glucoside, 
- D = dilution factor determined experimentally with pH 1.0 buffer, 
- PL is the constant at path-length 1 cm, 
- \( \varepsilon \) is 26900 L/mol cm, the molar extinction coefficient for cyanidin-3-glucoside, and 
- 1000 as the factor for conversion from g to mg.

**Wine quality**

Finished wine was measured for quality parameters using 600 µL of wine loaded onto the OenoFoss™ (FOSS, Denmark) machine and read using the Foss Integrator software. Glucose-fructose, pH, total acid, malic acid, volatile acid, and percent ethanol were determined by Fourier transform infrared (FTIR) spectroscopy. Global calibrations provide accurate readings for ranges between 0-15 g/L for glucose-fructose, 2.6 - 4.5 for pH, 1.5 – 8.0 g/L for total acid, 0 - 7 g/L for malic acid, 0 - 1.5 g/L for volatile acid (as acetic acid), and between 8 – 18% ethanol.
**Statistical analysis**

Data were expressed as means of at least three independent replicates and were presented as mean ± standard deviation. Statistical analysis was conducted using Tukey's Studentized Range Test groupings using the proc GLM function of SAS version 9.2 (SAS Inst. Inc., Cary, NC). Group means were considered to be significant at p < 0.05 based on minimum significant differences from one-way analysis of variance (ANOVA) with α = 0.05. Pearson’s correlation was used to determine correlation coefficients (r-values).

**Results and Discussion**

**pH, Brix, and Color**

As seen in Table 4.3, commercial blackberry wine had an average pH of 3.4 and ranged from 3.1 to 3.7. The commercial blueberry wines had an average pH of 3.3 and ranged between 2.8 to 3.7. These results for blueberry and blackberry commercial wines agreed with the typical pH range for red table wines between 2.9 to 4.2 (Zoecklein and others 1995). °Brix, a measurement of residual sugars, ranged from 14.6 to 18.0 with an average of 16.4° Brix for the blackberry wines while the commercial blueberry wines had °Brix measurements ranging from 12.1 to 19.0 with an average of 15.3° Brix. Overall the blueberry wines had a slightly lower °Brix than the blackberry wines and a slight but not significantly lower pH. Typical °Brix values for wine produced from mature grapes are around 18° Brix (Zoecklein and others 1995), so the wines produced from blueberries and blackberries are similar to this value. Hunter colorimetric analysis provides a color scale that depicts the lightness, yellowness, and redness of a sample. Analysis of the wines showed an average Hunter L (lightness index) of 1.55 for the blackberry wines and 1.43 for the blueberry wines; the blueberry wines had slightly less lightness than the blackberry wines on average.
Table 4.3- Quality Parameters including pH and Brix of Blackberry and Blueberry Wines Available in Illinois.

<table>
<thead>
<tr>
<th>Commercial Sample</th>
<th>pH</th>
<th>°Brix</th>
<th>Glucose-Fructose (g/l)</th>
<th>Total Acid (g/l)</th>
<th>Malic Acid (g/l)</th>
<th>Volatile Acid (Acetic acid) (g/l)</th>
<th>% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blackberry Wine</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Door Peninsula Blackberry</td>
<td>3.3 ± 0.1&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>15.6 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100.3 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.7 ± 0.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4.0 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Galena Cellars Blackberry</td>
<td>3.1 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.6 ± 0.03&lt;sup&gt;g&lt;/sup&gt;</td>
<td>86.3 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.1 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.7 ± 0.0&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lynfred Blackberry</td>
<td>3.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.0 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>103.5 ± 2.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.9 ± 0.0&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>5.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.0 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>St. James Blackberry</td>
<td>3.6 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.0 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>124.8 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.2 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.6 ± 0.4&lt;sup&gt;ef&lt;/sup&gt;</td>
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<td>Vahling Blackberry</td>
<td>3.3 ± 0.1&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>18.0 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>124.0 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.2 ± 0.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.2 ± 0.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>9.5 ± 0.3&lt;sup&gt;ef&lt;/sup&gt;</td>
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<tr>
<td>Von Jakob Honey Blackberry</td>
<td>3.6 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>16.5 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>99.3 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.9 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.4 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>Mean ± Std. Dev.</strong></td>
<td>3.4 ± 0.2</td>
<td>16.4 ± 1.3</td>
<td>106.3 ± 15.2</td>
<td>5.5 ± 1.5</td>
<td>3.5 ± 2.2</td>
<td>0.3 ± 0.3</td>
<td>10.7 ± 1.2</td>
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<tr>
<td><strong>Blueberry Wine</strong></td>
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<tr>
<td>Galena Cellars Blueberry</td>
<td>3.7 ± 0.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>12.1 ± 0.07&lt;sup&gt;i&lt;/sup&gt;</td>
<td>77.5 ± 0.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.0 ± 0.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pomona Once In A Blue Moon</td>
<td>3.0 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.0 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>107.5 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7 ± 0.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.3 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tomasetto Blueberry</td>
<td>2.8 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.4 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121.6 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.9 ± 0.0&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild Blossom Blueberry Mead</td>
<td>3.6 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.6 ± 0.03&lt;sup&gt;h&lt;/sup&gt;</td>
<td>77.5 ± 0.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.6 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.7 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.0&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Mean ± Std. Dev.</strong></td>
<td>3.3 ± 0.4</td>
<td>15.3 ± 3.5</td>
<td>95.1 ± 23.8</td>
<td>5.6 ± 1.5</td>
<td>2.8 ± 1.5</td>
<td>0.3 ± 0.3</td>
<td>11.3 ± 2.7</td>
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<tr>
<td><strong>Pheasant Hollow Winery</strong></td>
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<tr>
<td>Black &amp; Blue</td>
<td>3.4 ± 0.0</td>
<td>14.7 ± 0.16</td>
<td>93.1 ± 0.8</td>
<td>4.6 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>11.6 ± 0.4</td>
</tr>
</tbody>
</table>

Tukey's MSD: 0.5459 0.1533 4.8152 0.7009 1.0071 0.2952 0.8629

<sup>a</sup>-<sup>i</sup>: Values within a column followed by different letters are significant at $p < 0.05$. Data is represented as the mean ± SD from at least a triplicate analysis.
Hunter $b$ (yellowness index) values were 2.46 for the blackberry wines and 2.27 for the blueberry wines; blackberry wines were more yellow than blueberry. Hunter $a$ (redness index) values were 1.36 for the blackberry wines and 1.19 for the blueberry wines on average; the blackberry wines had more redness than the blueberry wines. The blackberry wines had slightly more lightness, redness, and yellowness than the blueberry wines on average. There was a positive correlation between Hunter $L$ and $b$ values ($r = 0.70$) indicating the wines with more lightness also had more yellowness.

**Quality parameters**

Table 4.3 also lists the means and standard deviations of the glucose-fructose concentration, total acid, malic acid, and volatile acid as acetic acid, all in g/L, as well as the measured percent ethanol. Glucose-fructose concentration ranged from 77.5 g/L to 124.8 g/L. A strong positive correlation between high °Brix and high glucose-fructose was obtained ($r = 0.90$) as both are measures of the sugar content; wines with high residual sugars have high glucose-fructose concentration while those with lower residual sugars contain less glucose-fructose. The blackberry wines had an average concentration of glucose-fructose of 106.3 g/L while blueberry wines had average glucose-fructose slightly lower at 95.1 g/L. Any wine with over 45 g/L may be labeled as ‘sweet’ (European Union 2002), so all the wines classify as a sweet wine, as was indicated on the labels of the products.

Total acids present in wine are extracted from the fruit source during the wine making process and during fermentation. Total acids of all wines ranged from 3.6 g/L to 8.1 g/L. The average total acids for the blackberry wines (5.5 g/L) was not significantly lower than the average total acids of the blueberry wines (5.6 g/L). A typical range of total acid for wines is between 5-8 g/L (Zoecklein and others 1995). Most samples fall within this range, however
mead wines produced mainly from honey rather than grapes or berries would have lower acidity, the reason mead production often requires addition of acids. There was a negative correlation ($r = -0.60$) between pH and total acid content, indicating that the pH is a fair representation of the total acid content of these fruit wines; as the total acid content increased, pH decreased, as expected.

Fixed, or non-volatile, acids vary greatly depending on the amount of acid present in the fruit used to produce the wine and the yeast or bacteria used for fermentation as well as weather and fruit growing conditions. Expected values in red wines for the fixed acids are 1 to 4 g/L tartaric acid, 0-8 g/L malic acid, 0-0.5 g/L citric acid, and 0.5-2 g/L succinic acid (Zoecklein and others 1995). The commercial wines fell in the range of expected values for malic acid with a range between 0.7 to 6.6 g/L. Blackberry wines had an average of 3.5 g/L of malic acid, slightly higher than the average of the blueberry wines at 2.8 g/L. There was a very strong positive correlation ($r = 0.90$) between the total acidity and malic acid content, indicating that most of the total acid may be due to the malic acid present (Table 4.5). Along with malic acid, tartaric acid is another major acid found in wine present in the original fruit with trace amounts of citric acid, while lactic, acetic, and succinic acid are produced during the alcoholic fermentation process (Zoecklein and others 1995). Volatile acid content, expressed as acetic acid, ranged from 0.0 g/L to 0.7 g/L. The average volatile acid content for all blackberry and blueberry wines was 0.3 g/L. Volatile acids are considered markers for spoilage with the legal limit in dessert wines set to 1.2 g/L (Zoecklein and others 1995), therefore all wines tested fall safely below this level. Acidity of wine influences the stability and quality of wine; therefore it is important to measure it. Though the commercial samples are all sweet wines, they have similar amounts of acid present in grapes and wine.
Ethanol concentration is important for wine stability and many flavor properties. Alcohol measured as percent ethanol ranged from 9.0% to 15.2%. Average alcohol concentration for the blackberry wines was 10.7% ethanol and average alcohol for the blueberry wines was slightly higher at 11.3% ethanol. United States regulations define wine as having between 7 to 24% ethanol by volume, depending on the type of wine and the wine making process. There was a strong correlation ($r = 0.83$) between the reported alcohol concentration on the labels and the alcohol measured with the OenoFoss™ software, indicating that the values reported on commercial wine labels were accurate.

**Table 4.4-** Total Polyphenols, Total Anthocyanins, and Antioxidant Capacity of Blackberry and Blueberry Wines Available in Illinois.

<table>
<thead>
<tr>
<th></th>
<th>Total Polyphenols (mg EAE/L)</th>
<th>Total Anthocyanins (mg/L)</th>
<th>Antioxidant Capacity (mmol TE/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blackberry Wine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Door Peninsula Blackberry</td>
<td>1219.2 ± 92.7$^{efg}$</td>
<td>16.61 ± 0.31$^{e}$</td>
<td>14.65 ± 0.80$^{de}$</td>
</tr>
<tr>
<td>Galena Cellars Blackberry</td>
<td>3620.8 ± 165.8$^{b}$</td>
<td>22.90 ± 2.65$^{de}$</td>
<td>36.53 ± 7.86$^{c}$</td>
</tr>
<tr>
<td>Lynfred Blackberry</td>
<td>2805.0 ± 416.1$^{cd}$</td>
<td>115.29 ± 6.48$^{b}$</td>
<td>48.96 ± 3.16$^{b}$</td>
</tr>
<tr>
<td>St. James Blackberry</td>
<td>1494.2 ± 45.6$^{ef}$</td>
<td>88.26 ± 9.40$^{c}$</td>
<td>11.08 ± 0.29$^{de}$</td>
</tr>
<tr>
<td>Vahling Blackberry</td>
<td>3084.2 ± 294.1$^{c}$</td>
<td>191.95 ± 6.16$^{a}$</td>
<td>41.04 ± 11.21$^{bc}$</td>
</tr>
<tr>
<td>Von Jakob Honey Blackberry</td>
<td>1051.7 ± 53.6$^{fg}$</td>
<td>18.35 ± 0.70$^{e}$</td>
<td>6.10 ± 1.00$^{e}$</td>
</tr>
<tr>
<td>Mean ± Std. Dev.</td>
<td>2212.5 ± 1090.3</td>
<td>75.56 ± 70.44</td>
<td>26.39 ± 17.95</td>
</tr>
<tr>
<td><strong>Blueberry Wine</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Galena Cellars Blueberry</td>
<td>1460.0 ± 413.4$^{ef}$</td>
<td>37.29 ± 2.34$^{d}$</td>
<td>17.02 ± 3.31$^{de}$</td>
</tr>
<tr>
<td>Pomona Once In A Blue Moon</td>
<td>2510.8 ± 427.1$^{d}$</td>
<td>10.71 ± 4.16$^{e}$</td>
<td>32.76 ± 3.96$^{c}$</td>
</tr>
<tr>
<td>Tomasello Blueberry</td>
<td>966.7 ± 44.8$^{g}$</td>
<td>12.72 ± 0.37$^{e}$</td>
<td>17.44 ± 6.24$^{d}$</td>
</tr>
<tr>
<td>Wild Blossom Blueberry Mead</td>
<td>1555.8 ± 74.7$^{c}$</td>
<td>22.56 ± 2.18$^{c}$</td>
<td>17.62 ± 5.83$^{d}$</td>
</tr>
<tr>
<td>Mean ± Std. Dev.</td>
<td>1623.3 ± 645.5</td>
<td>20.82 ± 12.14</td>
<td>21.21 ± 7.71</td>
</tr>
<tr>
<td><strong>Pheasant Hollow Winery</strong></td>
<td></td>
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<tr>
<td>Black &amp; Blue</td>
<td>2950.1 ± 168.3</td>
<td>95.12 ± 0.88</td>
<td>29.08 ± 2.2</td>
</tr>
<tr>
<td>Just Blueberry Juice$^\text{TM}$</td>
<td>4160.8 ± 203.8$^{a}$</td>
<td>192.12 ± 13.79$^{a}$</td>
<td>70.94 ± 4.49$^{a}$</td>
</tr>
<tr>
<td><strong>Tukey’s MSD</strong></td>
<td>490.1200</td>
<td>14.5840</td>
<td>10.9360</td>
</tr>
</tbody>
</table>

$^{a-g}$: Values within a column followed by different letters are significant at $p < 0.05$. Data is represented as the mean ± SD from at least two independent studies and at least a triplicate analysis.
Total polyphenols, total anthocyanins and antioxidant capacity

Total polyphenols for the commercial samples ranged between 966.7 mg EAE/L to 3620.8 mg EAE/L as seen in Table 4.4. The average total polyphenol content for commercial blackberry wines was 2212.5 mg EAE/L and for all blueberry wines was 1623.3 mg EAE/L. Total polyphenols can be expressed as either gallic acid equivalents or ellagic acid equivalents as both are phenolic acids. As many literature values are presented in gallic acid equivalents, a standard curve was also created using gallic acid showing the total polyphenols for the commercial blueberry and blackberry wines ranged from 188.1 ± 23.4 to 1115.3 ± 56.2 mg GAE/L, similar to values in typical table wines produced from grapes which range from 190-290 mg GAE/L in aged white wines to between 955-1215 mg GAE/L in aged red wines (Zoecklein and others 1995). Most of these blueberry and blackberry wines fall near the higher end of the total polyphenols seen in red wines. Additionally, there was a strong negative correlation (r = -0.76) seen between the Hunter L (lightness index) and total polyphenolic content; the more lightness of the wine, the lower total polyphenol content, suggesting the polyphenolic compounds contribute to the darkness of wines, as expected (Table 4.5).

A recent study (Kontoudakis and others 2011) on grape phenolics and wine composition found that higher total phenolic index and anthocyanin and proanthocyanidin concentrations also had lower titratable acidity and bitterness, suggesting a higher wine quality. Also, a non-grape wine produced from kiwifruit found wines with higher total phenolics also had higher sensory quality (Towantakavanit and others 2011). Garcia-Ruiz and others (2008) provided evidence that supports the potential for phenolic compounds to be a natural antimicrobial agent during wine production, so wines containing higher total phenolics may also prevent microbial growth.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>°Brix</th>
<th>Glucose-Fructose</th>
<th>Total Acid</th>
<th>Malic Acid</th>
<th>Volatile (Acetic) acid</th>
<th>Ethanol</th>
<th>TP</th>
<th>TA</th>
<th>AC</th>
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<td>-0.02</td>
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<td>-0.01</td>
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</tbody>
</table>

*Presented r-values are Pearson’s correlation coefficients. TP is total polyphenols in (mg EAE/L). TA is total anthocyanins in mg cyanidin-3-glucoside equivalents/L. AC is antioxidant capacity as measured with ORAC in mmol TE/L. Bold values represent those with r > 0.45.

Total anthocyanins measured as cyanidin-3-glucoside equivalents ranged from 10.71 mg/L to 191.95 mg/L. Average total anthocyanins in blackberry wines was 75.56 mg/L and in blueberry wines was 20.82 mg/L. Most blackberry wine fell within a range of previous values seen for red grape wines of 67.0 to 101.54 mg/L (Granato and others 2011). There was a positive correlation (r = 0.47) between total polyphenols and total anthocyanins, indicating that the total phenolic content of wines increases with an increase in anthocyanin content. Because anthocyanins are the compounds that give berry fruits their red, blue, and purple pigmentation, wines with lighter visual color as measured with the Hunter L lightness index overall had lower
anthocyanin content; indeed, there was a negative correlation between Hunter L value and total anthocyanins \( r = -0.44 \).

Other studies agree with the negative correlation seen between the lightness index and total anthocyanins for these commercial blueberry and blackberry wines. A study on elderberry wines found the hue value to be negatively correlated with total anthocyanins (Schmitzer and others 2010), and the effect of processing and storage on both blackberry (Hager and others 2008) and blueberry products (Brownmiller and others 2008) found an increase in polymeric color but a decrease in total anthocyanins of juices stored over time. This also indicated that the colors in the wine may change during storage of the wines through conversion of monomeric anthocyanins to the polymerized form. A study done on various traditional berries used by Native Americans found the anthocyanins that provided most color associated with the berry were the 3-glucosides or the 3-galactosides of cyanidin and delphinidin (Kraft and others 2008). The profile of anthocyanins found in both blueberries and blackberries has been identified (Grace and others 2009, Cuevas-Rodriguez and others 2010), suggesting that the presence of these specific anthocyanins in wines produced from berries may provide more color to the finished wine product.

Antioxidant capacity of wines ranged from 6.10 mmol TE/L to 48.96 mmol TE/L. The comparatively low antioxidant capacity for Von Jakob Honey Blackberry wine is consistent with other mead wines which range from 3.47 to 16.06 mmol TE/L (Gupta 2009). Average antioxidant capacity of blackberry wines was slightly but not significantly higher at 26.39 mmol TE/L than the average of the blueberry wines at 21.21 mmol TE/L (Table 4.4). There was a strong positive correlation \( r = 0.88 \) between total phenolic content and antioxidant capacity as well as a positive correlation \( r = 0.55 \) between total anthocyanin content and antioxidant
capacity indicating that the antioxidant potential of wines is likely due to phenolics and anthocyanins. Further, there was a negative correlation between the Hunter L values for lightness of the wines and antioxidant capacity \((r = -0.63)\) and a positive correlation \((r = 0.53)\) between the Hunter \(a\) values for redness of the wines; darker wines with more redness had a higher antioxidant capacity. Previous studies found average ORAC total antioxidant capacities of 25.7 mmol TE/L for red wine and 20.6 mmol TE/L for blueberry juice (Seeram and others 2008), and between 13.87 to 35.11 mmol TE/L for South American red wines (Granato and others 2011). All of the quality parameters of the blueberry and blackberry wines are comparable to red grape wine as well as the antioxidant capacity, so blueberry and blackberry wines may have the potential health benefits that have been proven for red grape wine. Several foods and beverages are currently marketed based on their antioxidant capacity. From the results of these wines tested both blueberry and blackberry wines commercially available in Illinois have the potential to be marketed for their high antioxidant capacity.

Comparing to Pheasant Hollow Winery Black & Blue, a commercial sample that is a blend of blueberries and blackberries, all values fell between the averages for the blueberry and the blackberry wines tested, as expected (Tables 4.3 and 4.4). Also seen in Table 4.4 are results from the Just Blueberries Juice™ as an example of concentrated blueberry juice. Because all of the commercial wines contained at least 9% alcohol by volume and were produced from fruit juices or fruit themselves rather than fruit concentrate, the non-alcoholic juice should be more concentrated in the phytochemical characteristics. The organic blueberry juice produced from concentrate showed the highest antioxidant capacity, highest (but not significantly higher than Vahling Blackberry) total polyphenolics, and the highest total anthocyanins; though these parameters were higher for the juice than the wines. Drinking fermented beverages is important
due to the conversion of the compounds present and increase anthocyanin and polymeric proanthocyanidins compounds and antioxidant capacity (Martin and Matar 2005), so though the total polyphenolic and total anthocyanin content was higher for the juice, it is not clear whether these compounds will have the same absorption and bioavailability as the compounds in the wines.

Previous studies on the health benefits of red wine have focused on the role of alcohol or in comparison to white wine rather than juices. For example, a study with alcohol-free red wine found an increase in plasma antioxidant capacity after consumption of 113 mL of red wine (Serafini and others 1998), which is the same as half the serving size indicated for the blueberry juice. Evaluation of Austrian red wines for anti-diabetic potential of whole wines and wine compounds found several compounds in red wines that would make them a beneficial natural alternative to drug therapy for diabetes and other metabolic diseases (Zoechling and others 2011). Limited clinical trials with moderate red wine consumption have seen improved hypertensive side effects including higher high density lipoproteins (HDL) levels and lower triglyceride levels as reviewed by Opie and Lecour (2007) despite high sugar and ethanol content in wines. These and other previous studies and clinical trials on red wine are supportive of further research into the potential for non-grape wines to improve overall health and reduce metabolic diseases.

Conclusions

Wines produced from blueberries and blackberries have high total polyphenolics and total anthocyanin contents which correlate with their antioxidant capacity. Comparing the commercial wines produced in Illinois from blueberries or blackberries to those from wineries in other states showed no significant differences overall. It should be noted that though these fruit
wines contain a higher °Brix than most red wines as they are sweet fruit wines, their antioxidant capacity is similar to red wine. Consumers should then take advantage of available blueberry and blackberry fruit wines. Evaluation of commercially available fruit wines produced from blueberry and blackberry found encouraging results to support further investigation into the mechanism and action of compounds present in fruit wines for potential health applications. The current wine industry would benefit from expanded production and marketing of high polyphenol and high antioxidant beverages such as non-grape wine produced from blueberries and blackberries.

Acknowledgement

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References


CHAPTER 5

CULTIVAR EVALUATION AND EFFECT OF FERMENTATION ON ANTIOXIDANT CAPACITY AND IN VITRO INHIBITION OF α-AMYLASE AND α-GLUCOSIDASE BY Highbush Blueberry (Vaccinium corymbosum)³

Abstract

The berry fruits of Highbush blueberry (Vaccinium corymbosum) contain bioactive compounds with potential health benefits. The objective was to evaluate blueberries grown in southern Illinois as well as the effect of fermentation, at two different temperatures, on chemical and physical parameters. Fruits from fifteen blueberry cultivars were analyzed. Fruit diameter ranged from 12.8 mm to 18.7 mm, pH from 2.6 to 3.7, reducing sugars from 6.4 % to 15.2 %, total sugars from 13.9 % to 21.6 %, total polyphenols from 0.39 to 1.00 mg gallic acid equivalents (GAE)/g blueberry and antioxidant capacity from 5.8 to 10.9 µM Trolox equivalents (TE)/g. In vitro α-amylase and α-glucosidase inhibitory capacity relative to the positive control acarbose, a known anti-diabetic drug, showed a range from 91.8 to 103.3 % for α-amylase and from 103.2 % to 190.8 % for α-glucosidase. Wines prepared from several of these blueberry cultivars were analyzed throughout fermentation and compared at room temperature and cold temperature fermentation for pH (3.5 to 6.3), °Brix (13.6 to 29.7), total polyphenols (375.4 to 657.1 µg GAE/mL wine), and antioxidant capacity (4.5 to 25.1 mM TE). The wines were also tested for their in vitro capacity to inhibit α-amylase and α-glucosidase and maintained similar inhibitory action as the berries. Highbush blueberry cultivars and their fermented beverages are good natural sources of antioxidants and starch-degrading enzyme inhibitors important for type 2 diabetes management.

³ (Publication: Johnson MH, Meyer T, Lucius A, de Mejia EG. Cultivar evaluation and effect of fermentation on antioxidant capacity and in vitro inhibition of α-amylase and α-glucosidase by Highbush blueberry (Vaccinium corymbosum). Journal of Agricultural and Food Chemistry. 2011, 59 (16): 8923-8930.) This work has been previously published and reprinted (adapted), with permission ©2011 American Chemical Society; dx.doi.org/10.1021/jf201720z.
Keywords: Highbush blueberry, *V. corymbosum*, fermentation, antioxidant, α-amylase, α-glucosidase.

Abbreviations used: AAPH: 2,2-azobis 2-amidinopropane dihydrochloride; AC: antioxidant capacity; CAE: crude-amberlite extract; CT: Cold temperature (4 °C); EAE: ellagic acid equivalents; GAE: gallic acid equivalents; RT: Room temperature (20-22 °C); TE: Trolox equivalents; TFA: trifluoroacetic acid; TP: total polyphenol; ORAC: oxygen radical absorbance capacity.

Introduction

In the United States there are increasing numbers of teens and adults that have pre-diabetes, and currently, 25.6 million people have diabetes [1]. This condition causes severe complications if blood glucose levels are not monitored properly, making diabetes prevention and management a very important health issue. There is a need to develop strategies for diabetes management in order to decrease its incidence and deaths from diabetes-related complications. One therapeutic approach for management of type 2 diabetes is to reduce glucose absorption by inhibiting α-amylase and α-glucosidase involved in starch degradation. Various berries have been investigated for their potential use in the management of diabetes by inhibiting starch-degrading enzymes after the consumption of soft fruits [2], Brazilian native fruits [3], strawberries [4] and raspberries [5]. In clinical trials, consumption of whole blueberries and freeze-dried blueberry beverages have been found to exhibit anti-diabetic properties by reducing blood glucose concentration [6, 7] in both healthy subjects and those with metabolic syndrome. For those with diabetes, natural sources of inhibitors of α-amylase and α-glucosidase would be beneficial in order to avoid potential gastrointestinal side effects [8] caused by commercial α-glucosidase inhibitors.

Berries in general have been noted for having positive health effects due to their high amount of phenolic compounds and antioxidants [9, 10]. The phenolic profiles and composition of many fruits and vegetables have been investigated and blueberries have been found to be rich
sources of antioxidants. The high antioxidant activity of blueberries is well documented and it has been correlated to their anthocyanin \[11\] and total phenolic content \[12, 13\]. High concentrations of phenolic compounds, such as resveratrol and anthocyanins found in grapes, have shown potential for reducing hyperglycemia \[14\].

Specific cultivars of blueberries and their composition have been studied \[15\], but not much research explains the compositional changes from processing, including fermentation. Studies have shown that during ripening of berries, especially blueberries, anthocyanins accumulate after harvest and even during storage \[16\]. Since the bioavailability of anthocyanins is relatively low compared to other flavonoids \[17\], and because fermentation is known to increase antioxidant capacity of blueberry juice \[18\], wine processing techniques that maximize the amount of skin contact are viable ways to increase the anthocyanin content and antioxidant capacity of berry juice products \[18, 19\]. In addition, ellagic acid, a major sedimentation product formed during fermentation, has been previously shown to have greater losses due to precipitation at higher temperatures of storage for wines produced from muscadine grapes \[20\]. One specific hypothesis of this study is that fermenting blueberries at colder temperatures will cause less ellagic acid precipitation, having important implication to wine quality.

To our knowledge, no research has been conducted on the effect of berry fermentation temperature on the inhibition of starch-degrading enzymes. Therefore, there is a need to further investigate different genotypes of berries and the effects of fermentation for their potential use to decrease starch degradation. This research aimed to perform an evaluation of Highbush blueberries, \textit{V. corymbosum}, grown at the Dixon Springs Agricultural Center (DSAC) in southern Illinois, including their physical descriptions, total polyphenols, antioxidant capacity, and \textit{in vitro} enzymatic inhibition of \(\alpha\)-amylase and \(\alpha\)-glucosidase. In addition, the effect of
fermentation of blueberries at two different temperatures on these parameters and the in vitro potential of the resulting wine for inhibiting starch breakdown were also assessed.

**Materials and Methods**

**Chemicals.** Compounds used as standards and their purity were as follows: Trolox (≥ 97%), glucose (≥ 99.5%), gallic acid (≥ 98%), and ellagic acid (≥ 95%). All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified.

**Blueberry cultivars.** All berries were grown at the same location under the same environmental conditions and all of them were harvested when fully ripe, independently of their diameter, during June 2010 from the Dixon Springs Agricultural Center (DSAC) in Simpson, IL. They were immediately washed, dried, and stored in plastic bags at -4 °C. All blueberries grown at DSAC were of the species *Vaccinium corymbosum*. Cultivars studied were Berkley, Blue Chip, Blue Haven, Blue Jay, Bluecrop, Bluemont, Blue ray, Bluetta, Collins, Coville, Darrow, Earliblue, North Country, Patriot, Spartan, and Stanley (*Image 5.1*). The results obtained from the blueberries grown at DSAC were compared to a fresh fully ripe commercial blueberry sample (Naturipe Farms, Naturipe Farms LLC, Martinez, GA).

*Image 5.1. Blueberry cultivars evaluated.*
**Physical characterization.** One hundred blueberries were randomly picked from each cultivar. The cross-diameter of each berry was measured in duplicate immediately after harvesting using a pair of Vernier calipers (Bel-Art Products Scienceware, Pequannock, NJ 07440-1992). Twenty grams of blueberries from each cultivar listed above were blended in 50 mL double distilled (dd) water and filtered using Whatman #4 filter paper. The filtrates were stored at 4 °C in test tubes covered with Parafilm before analysis. The filtrates were taken out of refrigeration 10 min before the experiment to allow the samples to reach room temperature, 26.3 °C. A pH meter (Mettler Toledo SevenEasy™, Columbus, OH) and pH probe (Mettler Toledo InLab® Expert 2m, Columbus, OH) were used to measure the pH of each blueberry extract. Reducing sugars were determined using a modified method originally described by Lindsay [21]. Briefly, 0.5 mL of each extract was transferred into a clean, dry test tube and 0.5 mL of 1.5 N H₂SO₄ were added, boiled in a water bath (100 °C) for 20 min with occasional mixing. Tubes were cooled down and 0.75 mL of 2 N sodium hydroxide added, then 1 mL of dinitrosalicylic acid reagent and 2 mL of dd water. The test tubes were heated again (100 °C) in a water bath for 5 min and then cooled down, 16 mL of dd water were added and the absorbance read at 570 nm in a spectrophotometer (Beckman DU ®-64 Spectrophotometer, Fullerton, CA) using glucose as a standard.

**Extraction and removal of reducing sugars.** Berry extraction and fractionation were performed based on procedures developed by Grace et al. [22]. Twenty grams of frozen berries were blended with 40 mL repeat washings of 100% methanol acidified with 0.3% trifluoroacetic acid (TFA) and filtered through Whatman # 4 filter paper until no further color was detected. The collected hydro-alcoholic extract was concentrated using a rotary evaporator (40 °C). Frozen berry extract or wine sample (5 mL) was loaded onto an amberlite XAD-7 column (30 × 3 cm)
preconditioned with acidified water, (0.3% TFA). The aqueous layer and resin was washed thoroughly with acidified water (0.3% TFA, ~600 mL) to remove free sugars, pectins, and phenolic acids. The polyphenolic mixture was then eluted with acidified methanol (0.3% TFA) and concentrated on a rotary evaporator (40 °C) to yield a crude-amberlite extract (CAE). Samples were then freeze-dried and stored at -80 °C.

**Fermentation of berry juice.** Upon collection of the fruits from the various cultivars, the berries were stored frozen at -18 °C until use. Berry wines were prepared, as indicated in Figure 5.1A, using about 90.7 kg of roughly even mixtures of cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Bluery, Blueta, Collins, Coville, Darrow, Earliblue, Elliot, Jersey, Late Blue, and Spartan. After thawing frozen berries overnight at room temperature (20 – 22 °C), 180 mL SO₂ solution (50 ppm), 3 g pectic enzyme solution (dissolved in water), 11.3 kg sucrose solution, and 180 g potassium bicarbonate in water were added. This mixture was then divided into four evenly divided lots, about 22.7 kg each. Dry wine yeast Lavin EC 1118 (*Saccharomyces bayanus*) was obtained from Presque Isle Wine Cellars (North East, PA). Yeast EC 1118 was rehydrated in potable water at 40 °C for 20 min with occasional gentle stirring to remove clumps prior to inoculation of the juice. Per manufacturer’s recommendations, yeast EC 1118 was inoculated at 40 mg dry yeast/100 mL and 35 mL yeast (23 °C) was added to each lot of fruit (22 °C). Fifty g fermaid was dissolved in water and 15 mL of this nutrient was added to each lot. Lots 1 and 2 were held at room temperature (RT, 20 – 22 °C); lots 3 and 4 were held at cold temperature (CT, 4 °C) for two weeks until pressed (skin removed); RT ready to press 14 days after yeast was added, CT ready 5 days later. Juice from all treatments was tested each week for pH, °Brix (Figure 5.1B) and phenolics. Fermented wine has been stored at 0 °C. Results
presented are averages of the independent duplicates lots 1 and 2 as room temperature (RT) or 3 and 4 as cold temperature (CT), unless otherwise specified.

A.

**Figure 5.1. A.** Flow diagram of the fermentation of berry juice. B. Fermentation curves for each batch of wine throughout time. Batch 1 and 2 were fermented at room temperature (RT) while 3 and 4 were fermented at cold temperature (CT).
**Total phenolic content.** Total phenolic content (TP) was quantified using the Folin-Ciocalteu method described originally by Amerine and Ough [23] adapted to a micro-assay. Briefly, to a 96-well flat bottom plate, 50 µL of 1 N Folin-Ciocalteu’s phenol reagent and 50 µL of either sample, standard or blank were added; this mixture was allowed to stand for 5 min before the addition of 100 µL of 20% Na₂CO₃. The solution was then allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Results were expressed as µg gallic acid equivalents (GAE) / g blueberry or µg ellagic acid equivalents (EAE) / mL wine.

**Total anthocyanins.** Total anthocyanins were determined using the AOAC Official Method as described previously by Lee [24]. Samples were diluted 1:10 using two different buffers (pH 1.0 KCl buffer and pH 4.5 sodium acetate buffer) and the absorbance was read at 520 nm and 700 nm on a spectrophotometer (Beckman DU ®-64 Spectrophotometer, Fullerton, CA). The results were expressed as total monomeric anthocyanins as cyanidin-3-glucoside equivalents in mg/L using the equation below:

\[
\text{Total monomeric anthocyanins (mg/L)} = \frac{(A \times MW \times D \times 1000)}{(\varepsilon \times PL)}
\]

Where: 
- \(A = (A_{520}-A_{700}) \text{ pH 1.0} - (A_{520}-A_{700}) \text{ pH 4.5}\)
- \(MW = 449.2 \text{ g/moL for cyanidin 3-glucoside}\)
- \(D = \text{dilution factor}\)
- \(PL = \text{the constant at path-length1 cm}\)
- \(\varepsilon = 26900 \text{ L/moL cm, the molar extinction coefficient for cyanidin-3-glucoside}\)
- \(1000 = \text{the factor for conversion from g to mg}\)

**Antioxidant capacity.** Antioxidant capacity (AC) was measured by the oxygen radical absorbance capacity (ORAC) assay as described by Prior et al. [25] using 20 µL trolox standard, samples, or blank (75 mM phosphate buffer pH 7.4), 120 µL of 116.9 nM fluorescein (final concentration 70 nm/well), and 60 µL of 40 mM AAPH per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multi-well
plate reader (Biotek, Winooski, VT). Results were expressed as mmoL Trolox equivalents (TE) or µmol TE/g blueberry.

**α-glucosidase and α-amylase inhibition.** The methods for enzymatic inhibition assays were adapted from Apostolidis et al. [26]. Briefly for the α-glucosidase assay, to a 96-well plate 50 µL of sample or positive control (1 mM acarbose) were added to 100 µL of a 1 U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 µL aliquot of a 5 mM p-nitrophenyl-α-D-glucopyranoside solution (in 0.1 M sodium phosphate buffer pH 6.9) were added briefly to each well and incubated at 25 °C for 5 min before reading the absorbance at 405 nm. For the α-amylase assay, 500 µL of sample or positive control (1 mM acarbose) were added to 500 µL of 13 U/mL α-amylase solution (Type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 µL of 1% soluble starch solution (dissolved in sodium phosphate buffer and boiled for 10 - 15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color reagent was added and the tubes were placed in 100 °C water for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100% inhibition.

**Wine quality.** Finished wine was measured for quality parameters using 600 µL of wine loaded onto the OenoFoss™ (FOSS, Denmark) machine and read using the Foss Integrator software. Glucose/fructose, pH, total acid, malic acid, volatile acid, and percent ethanol were determined by Fourier transform infrared (FTIR) spectroscopy. Global calibrations provide accurate readings for ranges between 0-15 g/L for glucose/fructose, 2.6 - 4.5 for pH, 1.5 – 8.0
g/L for total acid, 0 - 7 g/L for malic acid, 0 - 1.5 g/L volatile acid (as acetic acid), and 8 – 18% ethanol.

**Statistical analysis.** Data were expressed as means of at least three replications. Statistical analysis was conducted using Tukey's Studentized Range Test groupings using the proc GLM function of SAS version 9.2 (SAS Inst. Inc., Cary, NC). Group means were considered to be significant at $p < 0.0001$ based on minimum significant differences from one-way analysis of variance (ANOVA) with alpha = 0.05.

**Results and discussion**

**Physical characterization of the blueberry cultivars.** The physical and chemical characteristics of the different cultivars are presented in Table 5.1.

<table>
<thead>
<tr>
<th>V. corymbosum Cultivar</th>
<th>pH of juice</th>
<th>Average Diameter (mm)</th>
<th>% Total sugar</th>
<th>% Reducing sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berkley</td>
<td>2.8</td>
<td>16.57 ± 1.76bcde</td>
<td>16.9 ± 2.9bc</td>
<td>11.6 ± 0.2abc</td>
</tr>
<tr>
<td>Blue Chip</td>
<td>2.7</td>
<td>17.58 ± 2.74b</td>
<td>17.7 ± 0.3b</td>
<td>12.9 ± 0.5abc</td>
</tr>
<tr>
<td>Blue Haven</td>
<td>2.9</td>
<td>17.17 ± 1.36bc</td>
<td>17.4 ± 0.1b</td>
<td>13.6 ± 1.0abc</td>
</tr>
<tr>
<td>Blue Jay</td>
<td>2.9</td>
<td>16.63 ± 1.96de</td>
<td>15.3 ± 0.7bc</td>
<td>12.5 ± 1.5abc</td>
</tr>
<tr>
<td>Bluercrop</td>
<td>2.8</td>
<td>16.50 ± 1.14de</td>
<td>16.1 ± 0.3bc</td>
<td>13.7 ± 1.7ab</td>
</tr>
<tr>
<td>Blueray</td>
<td>3.0</td>
<td>16.84 ± 2.16cd</td>
<td>15.3 ± 0.1bc</td>
<td>10.8 ± 0.6bcd</td>
</tr>
<tr>
<td>Bluetta</td>
<td>3.0</td>
<td>15.20 ± 2.58f</td>
<td>16.7 ± 0.5bc</td>
<td>9.3 ± 0.1cde</td>
</tr>
<tr>
<td>Collins</td>
<td>2.8</td>
<td>16.44 ± 1.61de</td>
<td>16.3 ± 0.4bc</td>
<td>6.4 ± 0.1de</td>
</tr>
<tr>
<td>Coville</td>
<td>3.0</td>
<td>16.74 ± 2.13cde</td>
<td>15.6 ± 0.2bc</td>
<td>7.1 ± 0.0de</td>
</tr>
<tr>
<td>Darrow</td>
<td>3.0</td>
<td>18.49 ± 3.43a</td>
<td>16.7 ± 0.6bc</td>
<td>15.2 ± 2.2a</td>
</tr>
<tr>
<td>Earliblue</td>
<td>3.4</td>
<td>15.49 ± 2.46ef</td>
<td>17.8 ± 1.0b</td>
<td>8.9 ± 0.1cde</td>
</tr>
<tr>
<td>North Country</td>
<td>3.0</td>
<td>12.80 ± 0.99g</td>
<td>21.6 ± 0.4a</td>
<td>14.6 ± 1.4ab</td>
</tr>
<tr>
<td>Patriot</td>
<td>2.6</td>
<td>17.46 ± 2.72bc</td>
<td>15.6 ± 0.5bc</td>
<td>12.7 ± 1.4abc</td>
</tr>
<tr>
<td>Spartan</td>
<td>2.8</td>
<td>18.69 ± 1.84a</td>
<td>13.9 ± 0.1c</td>
<td>12.7 ± 0.8abc</td>
</tr>
<tr>
<td>Stanley</td>
<td>3.3</td>
<td>15.00 ± 1.38f</td>
<td>14.5 ± 0.2bc</td>
<td>12.0 ± 0.4abc</td>
</tr>
<tr>
<td>Commercial Sample</td>
<td>3.7</td>
<td>16.28 ± 3.79e</td>
<td>14.4 ± 0.3bc</td>
<td>7.3 ± 0.3de</td>
</tr>
</tbody>
</table>

* The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Values within a column followed by different letters are significant at $p < 0.05$. Diameter minimum significant difference = 0.5003; % Total sugar minimum significant difference = 3.4178; % Reducing sugar minimum significant difference = 4.0038.
**Diameter.** Darrow and Spartan cultivars were found to have the largest average diameters (18.49 ± 3.43 and 18.69 ± 1.84 mm, respectively). North Country, on the other hand had the smallest diameter (12.80 ± 0.99 mm). Eleven of the cultivars grown at DSAC were found to have higher average diameters than the commercial sample (16.28 ± 3.79 mm). The commercial sample showed the greatest variability in size. Despite variation in average diameters, all blueberries sampled were categorized as ‘large’ [27] since all of the average diameters fell above 12 mm. It was found that diameter and total sugar had a strong negative correlation (r = - 0.568); as the diameter increased in these Highbush blueberry cultivars, the total sugar content decreased.

**pH.** The commercial sample was found to have the highest pH (3.7), while Patriot showed the lowest (2.6). The pH among all cultivars ranged from 2.6 to 3.4 as seen in Table 5.1. This range is close to that found in the literature, from 2.85 to 3.49 [28].

**Reducing sugars.** The range of reducing sugars across the blueberry cultivars was found to be as low as 6.4 ± 0.1% in Collins to 15.2 ± 2.2% in Darrow (Table 5.1). All other cultivars grown at DSAC were found to have higher reducing sugars than the commercial sample. The reducing sugars content of commercial blueberries was not significantly different from the value of 7.1% reported in the literature [28].

**Total sugars.** Similarly, total sugar content varied across the blueberry cultivars from 13.9 ± 0.1% in Spartan to 21.6 ± 0.4% in North Country, (Table 5.1). With the exception of Spartan, fourteen out of the fifteen cultivars grown at DSAC had comparable or higher content of total sugars than the commercial sample. The total sugar content in the commercial sample was approximately twice the value reported in the literature of 7.3% [29].
**Total polyphenol content.** Total polyphenols ranged from 0.39 ± 0.03 mg GAE /g berry in Collins to 1.00 ± 0.02 mg GAE /g blueberry in North Country (Table 5.2). The phenolic content could be affected by the diameter of the blueberry, as most polyphenolic compounds are located in the skins of the fruits; however no significant correlation was found (results not shown). Overall, most of the TP values for the blueberry cultivars were slightly below the reported average of 0.95 mg GAE/g blueberry [13] with exception of Collins.

### Table 5.2. Total Polyphenols, Antioxidant Capacity, Alpha-Amylase Inhibition and Alpha-Glucosidase Inhibition of Blueberry Cultivars Grown in Southern Illinois, Dixon Springs Agricultural Center.

<table>
<thead>
<tr>
<th>V. corymbosum Cultivar</th>
<th>Total Polyphenols (mg GAE / g berry)</th>
<th>Antioxidant Capacity (µmol TE / g blueberry)</th>
<th>CAE % α-amylase inhibition (%*)</th>
<th>CAE % α-glucosidase inhibition (%*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berkley</td>
<td>0.75 ± 0.03^b</td>
<td>8.98 ± 0.31^b</td>
<td>97.67 ± 0.28^cd</td>
<td>147.07 ±0.04^bcd</td>
</tr>
<tr>
<td>Blue Chip</td>
<td>0.48 ± 0.01^cd</td>
<td>10.86 ± 0.22^a</td>
<td>103.32 ± 0.34^a</td>
<td>181.66 ±0.05^ab</td>
</tr>
<tr>
<td>Blue Haven</td>
<td>0.55 ± 0.01^bcd</td>
<td>5.81 ± 0.04^g</td>
<td>96.59 ± 0.21^b</td>
<td>157.62 ± 0.02^abc</td>
</tr>
<tr>
<td>Blue Jay</td>
<td>0.47 ± 0.06^d</td>
<td>7.50 ± 0.26^cd</td>
<td>101.70 ± 0.62^g</td>
<td>157.76 ± 0.02^cde</td>
</tr>
<tr>
<td>Bluecrop</td>
<td>0.47 ± 0.04^d</td>
<td>7.64 ± 0.33^c</td>
<td>91.79 ± 0.82^de</td>
<td>129.07 ± 0.39^abc</td>
</tr>
<tr>
<td>Blueraay</td>
<td>0.48 ± 0.03^ed</td>
<td>6.14 ± 0.03^fg</td>
<td>92.06 ± 0.34^g</td>
<td>184.70 ± 0.01^ab</td>
</tr>
<tr>
<td>Bluettia</td>
<td>0.54 ± 0.01^ed</td>
<td>6.74 ± 0.12^ef</td>
<td>95.78 ± 0.21^ef</td>
<td>133.12 ± 0.07^cde</td>
</tr>
<tr>
<td>Collins</td>
<td>0.39 ± 0.03^d</td>
<td>7.02 ± 1.55^de</td>
<td>96.95 ± 0.00^de</td>
<td>193.61 ± 0.02^a</td>
</tr>
<tr>
<td>Coville</td>
<td>0.54 ± 0.31^bcd</td>
<td>10.76 ± 0.13^a</td>
<td>97.08 ± 0.49^de</td>
<td>190.78 ± 0.01^a</td>
</tr>
<tr>
<td>Darrow</td>
<td>0.69 ± 0.14^bc</td>
<td>10.77 ± 0.45^a</td>
<td>97.49 ± 0.49^ed</td>
<td>155.24 ± 0.14^abc</td>
</tr>
<tr>
<td>Earliblue</td>
<td>0.75 ± 0.04^b</td>
<td>9.06 ± 0.32^b</td>
<td>98.29 ± 0.84^c</td>
<td>103.22 ± 0.15^f</td>
</tr>
<tr>
<td>North Country</td>
<td>1.00 ± 0.02^a</td>
<td>9.07 ± 0.04^b</td>
<td>94.84 ± 0.43^f</td>
<td>113.91 ± 0.09^def</td>
</tr>
<tr>
<td>Patriot</td>
<td>0.65 ± 0.03^bcd</td>
<td>6.77 ± 1.90^ef</td>
<td>101.12 ± 0.40^b</td>
<td>161.04 ± 0.07^abc</td>
</tr>
<tr>
<td>Spartan</td>
<td>0.69 ± 0.01^bc</td>
<td>6.86 ± 0.27^de</td>
<td>97.67 ± 0.68^cd</td>
<td>184.03 ± 0.01^ab</td>
</tr>
<tr>
<td>Stanley</td>
<td>0.66 ± 0.00^bc</td>
<td>7.07 ± 0.71^de</td>
<td>101.39 ± 0.93^b</td>
<td>161.99 ± 0.11^abc</td>
</tr>
<tr>
<td>Commercial Sample</td>
<td>0.47 ± 0.03^d</td>
<td>5.88 ± 0.26^g</td>
<td>86.80 ± 0.39^h</td>
<td>75.54 ± 0.19^f</td>
</tr>
</tbody>
</table>

*Positive control acarbose was considered as 100% inhibition; CAE = crude amberlite extract; contains no reducing sugars that would interfere with assay a–h. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Values within a column followed by different letters are significant at p < 0.05.

**Antioxidant capacity.** AC varied from 10.86 ± 0.22 µmol TE / g blueberry in Blue Chip to 5.81 ± 0.04 µmol TE / g blueberry in Blue Haven (Table 5.2). Most blueberry cultivars grown
at DSAC showed higher AC values than the commercial sample (5.88 ± 0.26 µmol TE / g blueberry), except Blue Haven. Factors that have been known to impact antioxidant capacity include the total anthocyanin content, total phenolic content, fruit maturity, as well as growing and post-harvest storage conditions [30]. There was a strong positive correlation (r = 0.70) between total polyphenolics and antioxidant capacity, suggesting the antioxidant activity is due to phenolic compounds found in the berries.

**Blueberry cultivar and enzyme inhibitory capacity.**

*α-amylase.* The effectiveness of α-amylase inhibition of the blueberry fruits from the different cultivars was measured using acarbose, an anti-diabetic drug, as a positive control having 100% inhibition. In comparison to acarbose, all blueberry extracts showed similar α-amylase inhibition capabilities, ranging from 91.79 ± 0.82% for Bluecrop to 103.32 ± 0.34% for Blue Chip, (Table 5.2). All blueberries grown at DSAC were found to have higher percent enzyme inhibition than the commercial sample (86.80 ± 0.39% inhibition). It is expected that the α-amylase inhibitory activity increases as the total polyphenolic content rise in a fruit as previously seen for small fruits [2]; there was a strong positive correlation between total polyphenolic content and α-amylase inhibitory activity (r = 0.85). Our preliminary studies with *Vaccinium* showed that a proanthocyanidin-enriched fraction had the lowest IC$_{50}$ value (IC$_{50}$ = 31.4 µg/mL) for α-amylase, suggesting a higher enzyme inhibitory potential, in comparison to the anthocyanin-enriched fraction (IC$_{50}$ >100 µg/mL).

*α-glucosidase.* As seen in Table 5.2, all blueberry cultivar extracts were found to have higher α-glucosidase inhibitory capabilities than acarbose. Additionally, most blueberry cultivars grown at DSAC had significantly higher percent inhibition than the commercial sample (75.54 ± 0.19%) with the highest inhibition seen by Collins (193.60 ± 0.02% inhibition) and Coville
It is desirable for starch degrading inhibitory agents to have a high α-glucosidase inhibition and a moderate α-amylase inhibition to avoid gastrointestinal side effects [7]. This makes Highbush blueberry cultivars a good natural alternative for inhibiting starch degradation. There was a positive correlation (r = 0.47) between total polyphenolic content and α-glucosidase inhibition. Regarding α-glucosidase, our preliminary studies with Vaccinium showed that a proanthocyanidin-enriched fraction had the lowest IC50 value (IC50 = 25.0 µg/mL), suggesting again a higher enzyme inhibitory potential, in comparison to the anthocyanin-enriched fraction (IC50 > 100 µg/mL).

Combining all results from cultivar evaluation, after sugar elimination, blueberries grown at DSAC have hypoglycemic effects comparable to acarbose, a known anti-diabetic drug. Tannins normally present in fruits of red color such as red grapes, red wines, raspberries, and strawberries are not present in as high amounts in blueberries [2]. Because tannins contribute to the α-amylase inhibition, the effectiveness of the blueberry extracts to inhibit α-amylase is lower than the effectiveness to inhibit α-glucosidase. It was found that smaller V. corymbosum berries contained higher levels of total phenolics, anthocyanins, and flavonoids than larger genotypes [31], although we found non-significant correlations.

To summarize our findings on the blueberry cultivars, out of the fifteen studied, Blue Chip and Coville exhibited the most hypoglycemic potential having high antioxidant capacity, high total polyphenol content, high α-glucosidase inhibition, and moderate α-amylase inhibition. Overall, all cultivars had high antioxidant capacity measured as µmol trolox equivalents and high total polyphenol content measured as mg gallic acid equivalents. The commercial sample showed considerably lower antioxidant capacity, α-amylase inhibition and α-glucosidase inhibition. A lower antioxidant capacity for the commercial sample could be due to
postharvest handling, storage, and transportation conditions which may have resulted in an oxidation of compounds having antioxidant activity. The phenolic profile of the blueberry cultivars needs to be further studied in order to attribute the enzyme inhibitory activity to a certain polyphenolic compound.

**Berry wine analysis: changes of berry juice due to fermentation**

**Quality.** Finished blueberry wine samples fermented at room temperature (RT) had a pH of 3.8 ± 0.13, total acid content of 6.2 ± 0.25 g/L, malic acid content of 4.5 ± 0.07 g/L, volatile acid content measured as acetic acid of 0.2 ± 0.11 g/L, and alcohol content of 11.2 ± 0.07 %. The blueberry wine samples that were fermented at cold temperature (CT) had a pH of 3.8 ± 0.14, total acid content of 5.3 ± 0.18 g/L, malic acid content of 4.2 ± 0.18 g/L, volatile acid content measured as acetic acid of 0.3 ± 0.12 g/L, and alcohol content of 9.5 ± 0.32 %. Comparing the temperature of fermentation, the RT samples had a slightly higher total acid content and higher alcohol content, indicating that CT fermentation of blueberries caused less acid and ethanol production than RT. Because the volatile acid content is actually lower for RT than CT and the malic acid contents varied only slightly, the larger acid content in the RT samples must be due to another acid present, most likely ellagic acid, a major sedimentation product formed during the production of berry wines.

**Brix.** °Brix was measured daily during the fermentation process as a measure of total sugar content; the fruit was pressed following a rapid decrease in Brix. As seen in Table 5.3, Brix for RT decreased by 16.1 °Brix from week 0 to the fermented wine stage, and for CT it decreased by 6.3 °Brix, showing a greater decrease in Brix during RT fermentation than during CT fermentation. A similar decrease in Brix was previously seen for fermented lowbush blueberries [18], but the values of Brix for our Highbush blueberry wines were much higher than
the previously reported range. This difference could be due to higher initial sugar content in the blueberries used or an incomplete fermentation resulting in more sugar in our final fermented wine.

**pH.** pH was also measured daily throughout the fermentation process. Because of the addition of potassium bicarbonate to the blueberry lot, the initial pH was much higher than expected for blueberry juice. As seen in Table 5.3, the pH for RT samples decreased by 2.5 from week 0 to the fermented wine stage, and for CT the pH decreased only 0.8.

Table 5.3. Chemical Characteristics of Blueberries Throughout Fermentation

<table>
<thead>
<tr>
<th>Time points</th>
<th>pH</th>
<th>°Brix</th>
<th>Total polyphenols (µg GAE/mL wine)</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% inhibition *</td>
<td>% / g GAE</td>
</tr>
<tr>
<td>RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>6.3</td>
<td>29.7a</td>
<td>362.3 ± 6.9c</td>
<td>103.11</td>
<td>4.35a</td>
</tr>
<tr>
<td>Week 1</td>
<td>3.5b</td>
<td>19.1cd</td>
<td>563.3 ± 7.4ab</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pressed</td>
<td>3.6c</td>
<td>15.5e</td>
<td>657.1 ± 6.1a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fermented</td>
<td>3.8c</td>
<td>13.6de</td>
<td>590.0 ± 26.7ab</td>
<td>92.21</td>
<td>3.81b</td>
</tr>
<tr>
<td>CT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>4.5c</td>
<td>26.3abc</td>
<td>357.4 ± 27.7c</td>
<td>102.89</td>
<td>4.19a</td>
</tr>
<tr>
<td>Week 1</td>
<td>3.5c</td>
<td>22.5abc</td>
<td>524.2 ± 13.4c</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pressed</td>
<td>3.6c</td>
<td>20.6cd</td>
<td>378.5 ± 7.9c</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fermented</td>
<td>3.8c</td>
<td>20.0bc</td>
<td>423.3 ± 35.0c</td>
<td>91.71</td>
<td>3.74b</td>
</tr>
</tbody>
</table>

*Positive control acarbose is 100% inhibition.

Comparing the samples from the two fermentation temperatures, there was a greater decrease in pH for the RT samples than the CT samples, possibly due to a lower production of
acids such as ellagic acid during CT fermentation, although both fermentation temperatures resulted in the same final pH of 3.8. There was no difference in final pH due to temperature of fermentation. Overall, this trend of pH change due to fermentation is consistent with that seen previously [18] in that there was an initial decrease of pH and then a gradual increase throughout fermentation.

**Total polyphenol content.** Figure 5.2A presents the TP content for the wines. TP increased from week 0 to the fermented wine by 228.0 µg GAE / mL (926.0 µg EAE / mL) for RT and increased by 65.9 µg GAE / mL (292.6 µg EAE / mL) for CT. The blueberry wine showed a significantly greater increase in TP from week 0 to fermented wine at RT than at CT fermentation (p < 0.0023) (Figure 5.3A). Since TP was measured in EAE, higher production of ellagic acid at RT could explain the higher total acid content measured in the finished wine for RT compared to CT. This indicated that the temperature of fermentation may affect the ellagic acid production in blueberry wine production. The TP results throughout time (Figure 5.2A) are consistent with those seen by Martin and Matar [19], in that there was a significant increase in the total phenolic content initially. Results from this previous study on red wines made from grapes indicated lower concentrations of ellagic acid at higher temperature fermentation (37 °C compared to 20 °C), but our results indicated lower total polyphenols measured as ellagic acid equivalents at the cold temperature of 4 °C compared to 20-22 °C. Figure 5.3B presents the total anthocyanin content for the blueberry fermented wine at RT (71.4 ± 10.4 mg cyanidin-3-glucoside equivalents /L) and for the blueberry fermented wine at CT (57.7 ± 8.2 mg cyanidin-3-glucoside equivalents /L); these values were not statistically different.

**Antioxidant capacity.** The AC trend is seen in Figure 5.2B and the change from week 0 to the fermented wine is seen in Figure 5.3C. AC for RT samples increased by 0.86 mM TE
from week 0 to the fermented wine stage, and for CT samples AC increased by 6.36 mM TE. RT fermentation showed a smaller increase in AC while CT fermentation showed a larger increase in AC for blueberry wine from the week 0 time point, although this was not significant between RT and CT ($p = 0.172$), as seen in Figure 5.3C.

**Figure 5.2.** A. Total polyphenol trend throughout fermentation: room temperature vs. cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). B. Antioxidant capacity trend throughout fermentation: room temperature vs. cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). Time points and treatments: Week 0 = day yeast was added: no fermentation occurred; Week 1 = 7 days after yeast added; Pressed (skin removed): RT ready to press 14 days after yeast, CT ready 5 days later; Fermented = stored in cold room at 0 °C: Values are averages of independent duplicates for each temperature treatment: RT= Room temperature (20-22 °C); CT= Cold temperature (4 °C).
Increases in antioxidant activities during the beginning of fermentation are associated with the production of phenolic compounds having antioxidant capacity. A decrease in AC throughout fermentation could be due to oxidation or degradation of compounds with antioxidant activity. A loss of antioxidant activity towards the end of fermentation has been attributed to the alteration of phenolic compounds but not a loss in total phenolics [18], which explains why we did not find a correlation between TP content and AC. The chemical profile of these wines will have to be further investigated in order to attribute the antioxidant activity to specific phenolic composition.

**Figure 5.3.**

A. Changes due to fermentation in total polyphenols as ellagic acid equivalents for room temperature vs. cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences (p < 0.05). 

B. Total anthocyanins of fermented wines, room temperature (RT) vs. cold temperature (CT). Results are expressed as monomeric anthocyanins as cyanidin-3-glucoside equivalents. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences (p < 0.05). 

C. Change in antioxidant capacity due to fermentation of blueberry wine at room temperature vs. cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences (p < 0.05). Blueberry wine was produced by fermentation of Highbush blueberries (*Vaccinium corymbosum*, cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetta, Collins, Coville, Darrow, Earlilblue, Elliot, Jersey, Late Blue, and Spartan) collected from Dixon Springs Agricultural Center in Simpson, IL Summer 2010 at RT= Room temperature (20-22 °C) or CT= Cold temperature (4 °C).
Berry wine enzyme inhibitory capacity

*α-amylase*. Due to the fact that reducing sugars interfere with the accuracy of this assay, the wine samples were run through an amberlite XAD-7 column to remove sugars along with pectins and phenolic acids. The crude amberlite extract (CAE) was then tested for enzyme inhibition. Results, expressed as a percentage of inhibition compared to the positive control of acarbose at 100% inhibition, are presented in Table 5.3 for both temperatures of the initial
samples and fermented wine samples. At week 0, the blueberry juice averaged between fermentation temperatures showed an α-amylase inhibition of 103%. Correcting for the TP content in the CAE used this was 4.27% inhibition/g GAE. The fermented wine, averaging RT with CT, showed 92% inhibition of α-amylase; correcting for the TP content in the CAE this was 3.77% inhibition/g GAE. The blueberry wines at both stages of fermentation showed inhibitory capacities that were similar to the positive control acarbose; although there was a significant decrease in inhibition from week 0 to fermented wine ($p < 0.0001$), which was not correlated to any measured specific fermentation changes.

**α-glucosidase.** The blueberry juice before fermentation showed 314.3% inhibition of α-glucosidase compared to acarbose’s 100% inhibition, and the fermented blueberry wine had 308.4% inhibition. Correcting for the TP content of the CAE used, this was 13.2%/g GAE and 12.7%/g GAE for the blueberry juice and fermented wine, respectively. There was no significant difference in α-glucosidase inhibition at any time point or temperature of fermentation. This shows that fermentation maintains the α-glucosidase inhibitory capacity of blueberry juice. When considering natural sources for inhibition of starch degrading enzymes, it is desirable to have high α-glucosidase inhibition and moderate α-amylase inhibition to avoid unwanted side effects from undigested starch [8]. Our results show almost twice as high inhibition of α-glucosidase than α-amylase for both unprocessed and fermented blueberry samples, indicating that both blueberry juice and fermented wines have desirable inhibition capacity for these two starch degrading enzymes. A previous study [31] found that wine had one of the highest α-glucosidase inhibitory activities and this activity was linked to specific phenols including rosmarinic acid and resveratrol. Wines high in these compounds may also have a high α-glucosidase inhibition relevant to diabetes management.
In summary, for the wine produced from blueberries at two different temperatures, we found that the cold temperature fermentation had significantly lower total polyphenolic content and no statistical difference in antioxidant capacity. It was found that fermentation retained the *in vitro* starch degrading enzyme inhibitory capacity of the blueberries. Exploring the phenolic profile of the blueberry wine would explain why different trends in activity were seen and is a goal for future studies. Blueberries and their fermented products are good natural sources of polyphenols, antioxidants, and *in vitro* inhibition of α-amylase and α-glucosidase.

**Acknowledgments**

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**References**


Abstract

Berries are an excellent source of dietary flavonoids which have several health benefits. We evaluated well-characterized anthocyanins and proanthocyanidins from fermented blueberry-blackberry beverages. Wines were produced from Highbush blueberries and blackberries grown in Illinois and blended to create ratios ranging from 100% blueberry to 100% blackberry. Total anthocyanins of the wine were strongly correlated to total phenolics ($r = 0.99, p < 0.05$) and to antioxidant capacity ($r = 0.77, p < 0.05$). Anthocyanin (ANC) and proanthocyanidin (PAC) enriched fractions were purified from each wine blend and a phenolic profile was generated. Anthocyanins increased with more blackberries from 1114 to 1550 mg cyanidin-3-glucoside (C3G) equivalents/L. Hydrolysable tannins were identified in the PAC enriched fraction. Both ANC and PAC enriched fractions inhibited starch-degrading enzyme α-glucosidase and dipeptidyl peptidase-IV activity. Computational docking demonstrated that delphinidin-3-arabinoside effectively inactivated DPP-IV by binding with the lowest interaction energy (-3228 kcal/mol). ANC and PAC (100 µM C3G and epicatechin equivalents, respectively) from blueberry-blackberry blends reduced LPS-induced inflammatory response in mouse macrophages.

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macrophages via the NFκB-mediated pathway. Anthocyanin and proanthocyanidin (including hydrolysable tannins in blackberry) enriched fractions from blueberry and blackberry fermented beverages are beneficial sources of antioxidants, inhibitors of carbohydrate-utilizing enzymes, and potential inhibitors of inflammation.

**Abbreviations:** DPP-IV, dipeptidyl peptidase-IV; EAE, ellagic acid equivalents; ORAC, oxygen radical absorbance capacity; TE, trolox equivalents; C3G, cyanidin-3-O-glucoside; LPS, lipopolysaccharide; DMEM, Dulbecco’s Modified Eagle Medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PES, phenazine ethosulfate; p-p65 NFκB, phosphorylated p-65 subunit of nuclear factor kappa B; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NO, nitric oxide; ANC, anthocyanin enriched fraction; PAC, proanthocyanidin enriched fraction

**Keywords:** Anthocyanin/ Proanthocyanidin/ Berry/ Diabetes/ Inflammation/

**Introduction**

Blueberries and blackberries are a rich source of anthocyanins, the water-soluble pigments which give berries their red, blue, and purple coloring [1]. Berries also contain polymerized phenolic compounds including hydrolysable tannins such as ellagitannins and gallotannins and condensed tannins known as proanthocyanidins [2]. The consumption of anthocyanins is among the highest of all flavonoids due to their wide distribution in foods [3]. Positive health effects have been attributed to the high antioxidant capacity of these phytochemicals. Anthocyanins have been established as having a high antioxidant capacity by scavenging free radicals and reducing oxidative stress [4]. Proanthocyanidins may also reduce oxidative stress and inflammation [5]. Increased phenolic intake has been seen to correlate with health benefits and is linked with decreased incidence of several diseases, including metabolic syndrome [6] and diabetes [4].

Fermentation is known to increase the phenolic content of berry juice products by increasing extraction from the skins, including anthocyanins [7], and therefore may increase the
antioxidant capacity [8] and potential for health benefits. Moderate wine consumption as part of a healthy diet may reduce the risk of certain chronic diseases linked to inflammatory processes [6, 9]. The health benefits and sensory properties attributed to fermented berry products may be due to the high amounts of polyphenolic compounds present [10-12].

Type 2 diabetes occurs when the body is unable to provide a proper insulin response to deal with glucose from the diet, leading to excessive blood glucose and insulin resistance. Several studies have been conducted to evaluate the reduction of absorption of glucose by berries and berry compounds to inhibit α-glucosidase and α-amylase [13-15]. One potential drug therapy for diabetics targets these carbohydrate-utilizing enzymes; natural sources of inhibitors of these enzymes would be beneficial to avoid undesirable side effects that occur with drug therapy [16]. Dipeptidyl peptidase-IV (DPP-IV) is another current pharmaceutical target for diabetes treatment. Inhibitors of this enzyme are emerging as anti-diabetes therapy and allow for stimulation of insulin secretion to consequently decrease blood glucose levels [17]. However, the inhibitory potential on DPP-IV by berry compounds needs to be further studied.

Chronic inflammation due to abnormal circulating levels of pro-inflammatory cytokines is associated with the development of diabetes and other metabolic diseases. A stimulated inflammatory cascade may be mediated by nuclear-factor κB (NFκB) signaling pathway to upregulate pro-inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2). Dietary flavonoids have been linked to anti-inflammatory pathways [3], and many berries or berry compounds have been investigated for their anti-inflammatory properties and antioxidant capacity or radical scavenging activity [4, 18-21]. Fermented juices and wines from berries have been investigated [11, 22], and in several studies they were found to
inhibit inflammatory protein production or expression more than their unfermented counterparts [12, 23-25].

The objective of this study was to evaluate well-characterized anthocyanins enriched fraction (ANC) and proanthocyanidins enriched fraction (PAC) present in blueberry-blackberry fermented beverages for their effect on markers of inflammation in RAW 264.7 macrophages and carbohydrate utilization in vitro. We hypothesized that blends of ANCs and PACs present in blueberry-blackberry fermented beverages can enhance their antioxidant capacity, their ability to reduce carbohydrate-utilizing enzymes activity, and reduce inflammation.

**Materials and methods**

Scheme 6.1. **Methodology.** Anthocyanins (ANC) and proanthocyanidins (PAC) were extracted from blueberry-blackberry fermented beverages following this scheme. Freeze-dried ANC and PAC were used for Aim 3 and Aim 4 (in vitro); Freeze-dried PAE was used for Aim 5 (in vivo).
Materials

Wines used were produced from Highbush blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus* spp.) grown at Dixon Springs Agricultural Center in Simpson, IL. Blueberry wine was fermented as previously described [14]. Blueberry (*Vaccinium corymbosum*) cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetta, Collins, Coville, Darrow, Earliblue, Elliot, Jersey, Late Blue, and Spartan were collected from Dixon Springs Agricultural Center in Simpson, IL during the ripening season of 2010. Blueberry juice from crushed frozen blueberries were fermented with *Saccharomyces bayanus* at 23 °C until alcohol content reached 5-6%; finished wine was filtered through cheesecloth. Blackberry wine was prepared at the same time using a similar procedure [14]. Blackberries (*Rubus fruticosus*) cultivars A-1937, A-2215, A-2241 Natchez, A-2315, APF 27, APF 40, APF 41, and Prime Jan were collected from Dixon Springs Agricultural Center in Simpson, IL during the ripening season of 2010. Upon collection, the berries were stored frozen at -18°C until use. Wine was prepared using 90.7 kg blackberries, with roughly even mixtures of all cultivars listed above. To the blackberry lot, 180 mL SO₂ solution (50 ppm), 3 g pectic enzyme solution (dissolved in water), and 10 kg sucrose solution were added. Dry wine yeast Lavin EC 1118 (*Saccharomyces bayanus*) obtained from Presque Isle Wine Cellars (North East, PA) was rehydrated at 40 °C for 20 min with occasional gentle stirring to remove clumps prior to inoculation of the juice. Per manufacturer’s recommendations, EC 1118 was inoculated at 40 or 50 mg dry yeast/100 mL, and held at 21 ± 1 °C during fermentation. Juice was sampled once each week to measure pH, Brix and phenolic composition. Blends ranging from 100% blueberry to 100% blackberry were made using room temperature fermented wines. Blends are presented as a ratio of %blueberry:%blackberry. The ratios were 100:0, 70:30, 50:50, 30:70, and 0:100 of blueberry:blackberry wine blends, respectively.
All solvents used for phenolic extraction were HPLC-grade and were purchased from Fisher Scientific (Pittsburg, PA). Amberlite XAD-7 was purchased from Sigma-Aldrich (St. Louis, MO), Sephadex LH-20 was purchased from GE Life Sciences (Buckinghamshire, UK). Murine macrophage RAW 264.7 cell line and Dulbecco’s Modified Eagle Medium with L-glutamine (DMEM) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Fetal bovine serum was from Invitrogen (Grand Island, NY, USA). CellTiter 96® AQueous One Solution Proliferation assay kit (MTS/PES) was purchased from Promega Corporation (Madison, WI, USA). Mouse monoclonal antibodies (actin, iNOS, COX-2) and rabbit polyclonal antibody (p-p65 NFκB) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antimouse and antirabbit IgG horseradish peroxidase conjugated secondary antibodies were from GE Healthcare (Buckinghamshire, UK). Compounds used as standards and their purity were as follows: Trolox (≥ 97%), glucose (≥ 99.5%), gallic acid (≥ 98%), ellagic acid (≥ 95%), (-)-epicatechin (≥ 99%), and cyanidin-3-O-glucoside (≥ 99%). All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified.

**Total phenolic content**

Total phenolic content (TP) was quantified using the Folin-Ciocalteu method adapted to a micro-assay [26]. Briefly, to a 96-well flat bottom plate, 50 μL of 1 N Folin-Ciocalteu’s phenol reagent and 50 μL of either sample, standard or blank were added; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na₂CO₃. The solution was then allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Results were expressed as μg ellagic acid equivalents (EAE) / mL wine.
**Antioxidant capacity**

Antioxidant capacity was measured by the oxygen radical absorbance capacity (ORAC) assay as described by Prior et al. [27] using 20 µL trolox standard, samples, or blank (75 mM phosphate buffer pH 7.4), 120 µL of 116.9 nM fluorescein (final concentration 70 nm/well), 60 µL of 40 mM AAPH per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Results are expressed as mmol Trolox equivalents (TE) /L.

**Total anthocyanin content**

Total anthocyanins from wine blends were determined using the AOAC Official Method adapted from Lee [28] for micro-assay. Samples were diluted to a factor of 1:10 using two buffers (pH 1.0, KCl buffer and pH 4.5, sodium acetate buffer). Diluted solutions at each pH were transferred to a 96-well plate and the absorbance was read at 520 and 700 nm on a Synergy 2 multiwell plate reader (Biotek, Winooski, VT). The total anthocyanins were expressed as cyanidin-3-O-glucoside (C3G) equivalents in milligram per liter using the equation below:

\[
\text{Total monomeric anthocyanins (mg/L)} = \frac{A \times MW \times D \times 1000}{\varepsilon \times PL} \times 0.45,
\]

where: \(A = (A_{520}-A_{700})\) pH 1.0 - \((A_{520}-A_{700})\) pH 4.5, \(MW = 449.2\) g/mol for C3G, \(D = \) dilution factor determined experimentally with pH 1.0 buffer, \(PL\) is the constant at path-length 1 cm, \(\varepsilon\) is 26900 L/mol cm, the molar extinction coefficient for C3G, and 1000 as the conversion factor from gram to milligram, and 0.45 as the conversion factor from the established method [27] to the plate reader method. The AOAC official method [28] has been standardized and validated and uses cyanidin-3-glucoside to express anthocyanin as equivalents as it is the most common anthocyanin pigment found in nature. Similarly, \((-\text{-epicatechin})\) was used to quantify proanthocyanidins due to common use as a standard. The pH differential method utilizes C3G to
calculate total monomeric anthocyanins and referred them as C3G equivalents, and therefore this was used as the quantification standard for the HPLC peaks.

**Phenolic extraction and preparation of enriched fractions**

Phenolic extraction was adapted from the method by Grace *et al.* [29]. To each blend, 0.1% TFA was added to stabilize the anthocyanins during isolation. A rotary evaporator with temperature not exceeding 40 °C was used to remove the alcohol completely and most of the water from 600 mL of each blend. The dealcoholized and concentrated beverage blends were mixed with amberlite XAD-7 resin preconditioned with acidified water, (0.1% TFA), settled at room temperature for 20 min and water was decanted. This process was repeated three more times until the supernatant became clear where sugars, pectins, phenolic acids, and other impurities were removed from samples. XAD-7 is a moderately polar resin and is used to remove relatively polar compounds from non-aqueous solvents, and to remove non-aromatic compounds from polar solvents. Acidified water was used to precondition the resin and was acidified to protect the anthocyanins from degradation. The sample was allowed to get into the resin pores for 20 min before the washing with acidified water removed any compounds that did not get into the resin pores, which included phenolic acids. The sample with resin was loaded onto a column (30 × 3 cm) and washed thoroughly with 1.5 L acidified methanol (0.1% TFA) to elute the polyphenolic mixture from resin. The polyphenolic mixture was concentrated to remove any methanol on a rotary evaporator (not exceeding 40 °C) to yield a crude extract which contained ANC and PAC phenolics present in wine. This was then partitioned with ethyl acetate to remove any non-polar compounds, and the polar eluate was then concentrated again on a rotary evaporator (not exceeding 40 °C). Samples were then stored at -80 °C and subsequently lyophilized on a Labconco freeze-dryer (Kansas City, MO).
To generate enriched ANC and PAC enriched fractions, approximately 4 g of the freeze dried material dissolved in 10 mL of acidified water:methanol (80:20) (0.1% TFA) was loaded on a Sephadex LH-20 column (30 × 3 cm) preconditioned with water:methanol (80:20) (0.1% TFA). With an isocratic elution using the same solvent ratio, fractions were collected starting when colored material began to elute to generate ANC enriched fractions 1-4, and then 50% methanol was used to remove any remaining ANC as enriched fraction 5. Next, 70% acetone was used to elute the PAC, and enriched fractions were collected as PAC 1-4. The solvents were evaporated on a rotary evaporator (not exceeding 40 °C), and fractions were freeze dried, weighed, and stored at -80 °C until further use as indicated below:

**Scheme 6.2.** Phenolic extraction and fractionation procedure to prepare ANC and PAC enriched fractions, indicating the methods conducted.
ANC 1-5 and PAC 1-4 were collected from each blend; in this manuscript we will use ANC and PAC to indicate ANC enriched fraction and PAC enriched fraction, respectively. The following enriched fractions were used for further analysis because they had the highest ANC or PAC concentration based on HPLC analysis: ANC-3 for 100% blueberry and also for 50:50%; ANC-2, for 100% blackberry. PAC-1, for 100% blueberry; PAC-4 for 50:50%; PAC-2 for 100% blackberry. These fractions were enriched, so most phenolic acids should have been removed. The other fractions were not studied beyond HPLC analysis.

**Anthocyanin and proanthocyanidin analysis**

ANC analysis was conducted on a 1200 HPLC (Agilent Technologies, Santa Clara, CA) using a Supelcosil LC-18RP column (250 \( \times \) 4.6 mm, 5 µM) (Supelco, Bellefonte, PA). Samples were prepared by dissolving 2 mg of each fraction in 1 mL of methanol and filtering through 0.2 µM PTFE syringe filters (Fisher Scientific, Pittsburg, PA) before injection. The mobile phase consisted of 5% formic acid in H\(_2\)O (A) and 100% methanol (B). The flow rate was held constant during the HPLC analysis at 1 mL/min with a step gradient of 10%, 15%, 20%, 25%, 30%, 60%, and 10% of solvent B at 0, 5, 15, 20, 25, 45, and 60 min, respectively. For PAC analyses, the same instrumentation was used but with mobile phase consisting of 95% H\(_2\)O: 5% acetonitrile with 0.1% formic acid (A) and of 95% acetonitrile: 5% H\(_2\)O with 0.1% formic acid (B). The flow rate was held constant at 1 mL/min with a step gradient of 0%, 5%, 30%, 60%, 90%, and 0% of solvent B at 0, 5, 40, 45, 50, and 55 min, respectively. ANC were detected at 520 nm and PAC at 280 nm using a diode array detector (DAD). Chemstation software (Agilent Technologies, Santa Cruz, CA) was used for both sample analysis protocol and data processing. A reference blueberry extract (post-amberlite extract, PAE 22%) was always run as an internal standard, during HPLC analysis, to account for any variation in the retention time among runs. For qualitative peak
identification of specific anthocyanins, we used pure standards that were already incorporated into our HPLC library, and then we did comparisons to references in the literature. MS was generated and identification of anthocyanins was performed; accurate determination was accomplished using our own reference library of peaks generated from the phenolic analysis of many blueberry cultivars on the same equipment and under exactly the same analytical conditions. ANC were identified based on comparison to standards as our previously published data indicated [20, 29].

Using the peak areas as measured by HPLC at 520 nm, total ANC were quantified from a standard curve generated from 0.125, 0.25, 0.5, and 1.0 mg/mL of cyanidin-3-O-glucoside and are presented as C3G equivalents. In the same way, total PAC was calculated as epicatechin equivalents from the peak areas measured at 280 nm. Fractions containing the highest total anthocyanins and proanthocyanidins were considered ANC and PAC, respectively. To further elucidate the composition of the PAC, samples were analyzed with HPLC-ESI-MS using the same protocol above except at a flow rate of 0.4 mL/min and Eclipse XDB-C18 RP column (250 mm × 3 mm × 5 μm, Agilent Technology, Inc., Wilmington, DE). Liquid chromatography mass spectrometer (LC/MS) Accurate–Mass 6220 model (Agilent Technology, Inc., Wilmington, DE) was used for PAC analysis. Mass analyses conditions were: gas temperature at 350 °C, gas flow 10 L/min, nebulizer 45 psi, ion source dual ESI, positive mode, and injection volume was 10 μL (2mg/ml sample). MassHunter Workstation Software for data acquisition version 2.B.00 and qualitative analysis version B.03.01 (Agilent Technology, Inc., Wilmington, DE) was used to run samples and analyze the total ion chromatogram (TIC) data at 150-3000 m/z range.
**Enzymatic studies with α-glucosidase and α-amylase**

The methods for enzymatic inhibition assays were adapted from Kwon et al. [16]. For the α-glucosidase assay, to a 96-well plate, 50 μL of sample (0.1 mg freeze dried product/mL) or positive control (1 mM acarbose) were added to 100 μL of a 1 U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. Fifty μL of a 5 mM p-nitrophenyl-α-D-glucopyranoside solution (in 0.1 M sodium phosphate buffer pH 6.9) were added briefly to each well and incubated at 25 °C for 5 min before reading the absorbance at 405 nm. For the α-amylase assay, 500 μL of sample or positive control (1 mM acarbose) were added to 500 μL of 13 U/mL α-amylase solution (Type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 μL of 1% soluble starch solution (dissolved in sodium phosphate buffer and boiled for 10 - 15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color reagent was added and the tubes were placed in 100 °C water for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm. Results were presented as percent inhibition relative to the current diabetes drug acarbose as the positive control, which was set at 100% inhibition.

**DPP-IV inhibition and computational docking study**

Measurements of the activity and potential inhibition of DPP-IV (EC 3.4.14.5), a type II membrane glycoprotein, were done using the DPP-IV Glo™ Protease Assay (Promega, Madison, WI) following the manufacture’s protocol. Briefly, 50 μL of DPP-IV Glo™ reagent was added to a black-walled 96-well plate containing 50 μL of blank, positive control, or treatment. The blank contained the vehicle control only while positive control contained the vehicle and purified DPP-IV enzyme at a final concentration of 1 ng/mL. Treatments used were
enriched ANC or known inhibitor, diprotin A, and the purified DPP-IV enzyme at a final concentration of 1 ng/mL. The content of the wells was gently mixed using a plate shaker at 400 rpm for 30 s. DPP-IV cleavage of the provided substrate generated a luminescent signal by luciferase reaction with the amount of DPP-IV enzyme available to bind Gly-Pro-aminoluciferin proportional to relative light units (RLU) produced. This signal in RLU was measured after 30 min in an Ultra Microplate Reader (Biotek Instruments, Winooski, USA) and was compared to the inhibitory action of diprotin A. Diprotin A linear standard curve (y = -50.41x + 86.35, R² = 0.94) where y is the % maximum activity of the enzyme and x is the log₁₀ of the concentration (µM) of known inhibitor diprotin A was used to calculate IC₅₀ values.

A computational docking study to predict the affinity of main ANC present in the blends as cyanidin-3-glucoside (PDB: 10099), malvidin-3-galactoside (PDB: 671499), malvidin-3-arabinoside, delphinidin-3-glucoside (PDB: 13563) and delphinidin-3-arabinoside and diprotin A with DPP-IV enzyme (PDB: 1R9M) were conducted with the protein-ligand docking program SwissDock (http://swissdock.vital-it.ch/, accessed July 2012). The PDB format files of ANC and diprotin A were drawn by chemical drawing software MarvinSketch (ver 5.10.3, ChemAxon). The OpenBabel GUI chemical expert system was used for converting chemical file format (PDB file) of ANC (malvidin-3-galactoside and delphinidin-3-arabinoside) and diprotin A to Mol2 files which can be read by the docking program. Docking results predicting binding modes were visualized with UCSF Chimera1.6.2, for displaying, animating and analyzing large biomolecular systems using 3-D graphics and built-in scripting. At the end, the best conformation was selected based on the lowest FullFitness [30]. The FullFitness is the sum of the total energy of the system and includes a solvation term: \[ \text{FullFitness} = E_{\text{ligand}}^{\text{intra}} + E_{\text{recept}}^{\text{intra}} + E_{\text{inter}} + \Delta G_{\text{elecsolv}} + (\sigma \ast \text{SASA}). \]
Where $E_{\text{ligand}}^{\text{intra}}$ and $E_{\text{recept}}^{\text{intra}}$ are the internal energy of anthocyanin and DPP-IV, respectively; $E_{\text{inter}}$ is the interaction energy between the anthocyanin and DPPI-IV, and is equal to the sum of the van der Waals and the electrostatic interaction energies; $\Delta G_{\text{elec,solv}}$ is electrostatic potential of solvent and calculated using the analytical Generalized Born Molecular Volume (GB-MV2) model implemented in CHARMM; SASA is the solvent accessible surface area; $\sigma$ is 0.0072 kcal/(mol Å$^2$).

**Cell proliferation and treatment**

Murine macrophage cell line RAW 264.7 was cultured in DMEM supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO$_2$/95% air using a CO$_2$ Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). The cell proliferation assay was conducted using the CellTiter 96® AQueous One Solution Proliferation assay kit containing a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega, Madison, WI). Briefly, $1 \times 10^4$ cells were seeded in a 96-well plate and the total volume was adjusted to 200 μL with growth medium. After 24 h incubation, the cells were treated with 5, 50, or 100 μM C3G (for ANC) or epicatechin (for PAC) equivalents, and incubated for an additional 24 h. After this time, the growth medium was replaced with 100 μL fresh growth medium and 20 μL MTS/PES were added to each well. The plate was incubated for 2 h at 37 °C in 5% CO$_2$/95% air and the absorbance was read at 515 nm in an Ultra Microplate Reader (Biotek Instruments, Winooski, USA). The percentage of viable cells was calculated with respect to untreated cells in complete media.

For treatments, RAW 264.7 macrophages were seeded at $2 \times 10^5$ in a six-well plate, and the total volume was adjusted to 2 mL with growth medium and incubated for 48 h at 37 °C in
5% CO₂/95% air. After 48 h incubation, the cells were treated with 25, 50, or 100 µM C3G (for ANC) or epicatechin (for PAC) equivalents, stimulated with 1µg/mL lipopolysaccharide (LPS) and incubated for an additional 24 h. After 24 h, spent medium was collected for nitric oxide production measurement and whole cell lysates were collected for western blot analysis of inflammatory markers, as follows.

**Nitric oxide (NO) measurement in supernatant of RAW 264.7 cell culture**

Nitrite measurement was performed using Griess reaction. Briefly, 100 µL of the growth medium diluted 1:5 were plated in 96-well plate and an equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl)ethylenediamine-diHCl in 2.5% H₃PO₄) was added. The plate was incubated for 5 min at room temperature and the absorbance was measured at 550 nm in an Ultra Microplate Reader (Biotek Instruments, Winooski, USA). The amount of NO was calculated using sodium nitrite standard curve (y = 0.14x + 0.05, R² = 0.997)

**Western blot analysis**

Treated cells were washed twice with ice cold DMEM and twice with ice cold PBS and the cells were lysed with 200 µL of Laemmli buffer (BioRad, Hercules, CA) containing 5% β-mercaptoethanol. The cell lysates were sonicated for 30 s and then boiled for 5 min. Protein was quantified using the RCDC Assay (BioRad, Hercules, CA), and 30 µg protein was loaded in 4–20% Tris–HCl gels (BioRad) for protein separation. The resolved proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) and blocked with 3% milk in 0.1% TBST for 1 h at room temperature. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min each) and incubated with primary antibody (1:500) at 4°C overnight. The membrane was washed again and incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (1:2500) for 1 h at room temperature. After incubation and repeated
washing, the expression of proteins was visualized using chemiluminescent reagent (GE Healthcare, Pittsburgh, PA) following manufacturer’s instructions. The membrane picture was taken with a GL 4000 Pro Imaging system (Carestream Health Inc., Rochester NY).

**Statistical analyses**

Data were expressed as means of independent duplicates with at least three replications. Statistical analysis was conducted using the proc GLM procedures of SAS version 9.3 (SAS Inst. Inc., Cary, NC). Group mean comparisons were conducted using LSD means and were considered to be significant at \( p < 0.05 \) based on minimum significant differences from one-way analysis of variance (ANOVA) with alpha = 0.05. Correlations were done using Pearson’s correlation values with \( p < 0.05 \).

**Results**

**Blackberry increased total anthocyanins, total phenolics and antioxidant capacity of wine blends.**

Blueberry-blackberry wine blends had total anthocyanins ranging from 76.8 (100% blueberry) to 137.2 mg C3G equivalents/L (100% blackberry); total polyphenols ranging from 2196.7 (100% blueberry) to 4029.0 mg EAE/L wine (100% blackberry); and antioxidant capacity from 24.5 (70:30 blend) to 37.4 mmol TE/L (100% blackberry), as can be seen in Table 6.1. Both total polyphenols and total anthocyanins increased consistently with a higher percent of blackberry in the blend, with the highest values for the 100% blackberry wines. Antioxidant capacity increased with 70% blackberry in the blend, with the highest antioxidant capacity seen again for the 100% blackberry blend. Table 6.1 also shows that based on our previous study [14], and for comparative purposes, blueberry juice decreased 16.1 brix, pH decreased by 2.5 and total polyphenols increased by 625.4 \( \mu \)g/ml EAE from week 0 to fermented wine at room
temperature. Blackberry juice decreased 17.5 brix, pH increased only slightly (by 0.3) and total polyphenols increased 643.8 µg/ml EAE from week 0 to fermented wine at room temperature.

**Table 6.1.** Total anthocyanins (TA) and total phenolics (TP) of wine blends and antioxidant capacity (AC) of wine and ANC and PAC.¹

<table>
<thead>
<tr>
<th>Blend ratio (%Blueberry : %Blackberry)</th>
<th>Total anthocyanins (mg C3G-equivalents/L wine)</th>
<th>Total phenolics (mg EAE²/L wine)</th>
<th>Antioxidant capacity (mmol Trolox Eq. /L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ANC³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PAC³</td>
</tr>
<tr>
<td>100:0</td>
<td>76.8 ± 2.6d</td>
<td>2196.7 ± 42.7c</td>
<td>25.3 ± 2.2b</td>
</tr>
<tr>
<td>70:30</td>
<td>94.3 ± 6.2ed</td>
<td>2611.2 ± 76.6d</td>
<td>24.5 ± 0.82b</td>
</tr>
<tr>
<td>50:50</td>
<td>105.8 ± 6.3bc</td>
<td>2932.3 ± 28.5c</td>
<td>25.3 ± 1.0b</td>
</tr>
<tr>
<td>30:70</td>
<td>121.7 ± 8.7ab</td>
<td>3403.4 ± 50.5b</td>
<td>26.5 ± 0.1b</td>
</tr>
<tr>
<td>0:100</td>
<td>137.2 ± 9.6a</td>
<td>4029.0 ± 68.4a</td>
<td>37.4 ± 1.4a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Correlations ⁵</th>
<th>TA_wine</th>
<th>TP_wine</th>
<th>AC_wine</th>
<th>AC_ANC</th>
<th>AC_PAC</th>
<th>Total ANC ⁴</th>
<th>Total PAC ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.00</td>
<td>0.99</td>
<td>0.77</td>
<td>0.61</td>
<td>-0.35</td>
<td>0.93</td>
<td>0.78</td>
</tr>
</tbody>
</table>

¹Values are means ± SEM. Means with different letters in each column are significantly different across columns (p < 0.05). ²EAE: Ellagic acid equivalents. ³ANC and PAC at 0.1mg freeze dried product/mL. ⁴Total ANC and PAC are based on HPLC data presented in Table 6.2. ⁵Correlation values are Pearson’s r-values (p < 0.05). Note based on our previous results [14]: For blueberries: Wk0 (Unfermented): Total polyphenols (µg/mL EAE), 993.8 ± 6.9. For blackberries: Wk0 (Unfermented): Total polyphenols (µg/mL EAE), 2141.3 ± 101.1. Week 0 = no fermentation occurred; Values are averages of independent duplicates.

**Main anthocyanins and proanthocyanidins in the blueberry and blackberry enriched fractions.**

ANC identified for each blend are presented in **Table 6.2.** Chromatographic analysis revealed up to seventeen anthocyanins present in blueberry and blackberry blend fermented beverages. Malvidin-3-galactoside was the main anthocyanin present in 100% blueberry, while delphinidin-3-arabinoside was the predominant anthocyanin in the blends containing blackberry.
Total ANC, using total peak areas at 520 nm as quantified with HPLC in comparison with the commercial standard cyanidin-3-O-glucoside, increased with more blackberry in the blend and ranged from 1112.3 (70:30 blend) to 1550.4 mg C3G equivalents /L (100% blackberry), confirming the increase in total ANC seen by the wines before phenolic separation. The identification of anthocyanins and proanthocyanidins were based on their mass charges (m/z), UV absorption (520 nm for ANC, and 280 nm for PAC), and our previously published data on blueberry and blackberry species [19, 20, 29]. Total PAC were quantified using peak areas at 280 nm and ranged from 560.3 (100% blueberry) to 1249.0 mg (-)-epicatechin equivalents/L (100% blackberry) (Table 6.2).

**Table 6.2. Anthocyanin identification and quantification by HPLC, and total proanthocyanidins determined at maximum absorption of 520 and 280 nm, respectively.**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Anthocyanin ID</th>
<th>100:0</th>
<th>70:30</th>
<th>50:50</th>
<th>30:70</th>
<th>0:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.44</td>
<td>Delphinidin-3-galactoside</td>
<td>10.8</td>
<td>11.2</td>
<td>9.1</td>
<td>6.9</td>
<td>nd</td>
</tr>
<tr>
<td>25.98</td>
<td>Delphinidin-3-glucoside</td>
<td>26.8</td>
<td>51.0</td>
<td>34.7</td>
<td>12.3</td>
<td>8.8</td>
</tr>
<tr>
<td>27.33</td>
<td>Cyanidin-3-galactoside</td>
<td>6.5</td>
<td>6.3</td>
<td>nd</td>
<td>17.7</td>
<td>23.8</td>
</tr>
<tr>
<td>28.86</td>
<td>Delphinidin-3-arabinoside</td>
<td>11.9</td>
<td>nd</td>
<td>993.3</td>
<td>1079.3</td>
<td>1244.7</td>
</tr>
<tr>
<td>29.69</td>
<td>Cyanidin-3-glucoside</td>
<td>23.4</td>
<td>564.1</td>
<td>12.1</td>
<td>10.2</td>
<td>10.1</td>
</tr>
<tr>
<td>30.82</td>
<td>Cyanidin-3-arabinoside</td>
<td>140.2</td>
<td>16.7</td>
<td>8.0</td>
<td>nd</td>
<td>116.8</td>
</tr>
<tr>
<td>31.62</td>
<td>Petunidin-3-glucoside</td>
<td>7.3</td>
<td>79.6</td>
<td>45.7</td>
<td>100.9</td>
<td>9.6</td>
</tr>
<tr>
<td>32.69</td>
<td>Peonidin-3-glucoside</td>
<td>39.8</td>
<td>6.7</td>
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<tr>
<td>33.95</td>
<td>Petunidin-3-arabinoside</td>
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<td>23.7</td>
<td>14.3</td>
<td>9.7</td>
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<tr>
<td>34.39</td>
<td>Malvidin-3-galactoside</td>
<td>292.1</td>
<td>32.7</td>
<td>11.9</td>
<td>38.4</td>
<td>6.7</td>
</tr>
<tr>
<td>35.19</td>
<td>Malvidin-3-glucoside</td>
<td>11.1</td>
<td>69.3</td>
<td>22.6</td>
<td>49.6</td>
<td>6.4</td>
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<tr>
<td>37.14</td>
<td>Malvidin-3-arabinoside</td>
<td>278.9</td>
<td>127.2</td>
<td>111.5</td>
<td>44.7</td>
<td>7.5</td>
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<tr>
<td>39.03</td>
<td>Delphinidin-6-acetyl-3-glucoside</td>
<td>7.3</td>
<td>12.6</td>
<td>10.3</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>40.55</td>
<td>Cyanidin-6-acetyl-3-glucoside</td>
<td>7.8</td>
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<td>6.1</td>
<td>6.1</td>
<td>43.9</td>
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<td>41.70</td>
<td>Malvidin-6-acetyl-galactoside</td>
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<td>42.25</td>
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<td>nd</td>
<td>11.9</td>
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<tr>
<td>44.40</td>
<td>Malvidin-6-acetyl-3-glucoside</td>
<td>10.8</td>
<td>19.4</td>
<td>14.6</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Total ANC: 1113.7, 1112.3, 1403.1, 1448.2, 1550.4

PAC total: 560.3, 1166.7, 1244.7, 1240.5, 1249.0

Bold values indicate the concentration of the predominant ANC in 100% blueberry and 100% blackberry. nd = Peak not detected.
Further HPLC-ESI-MS analyses for PAC revealed complex tannin composition with highly polymerized compounds. In the fraction mostly composed of blueberry matrix (100:0, 70:30, 50:50; blueberry: blackberry), in addition to monomers, proanthocyanidin oligomers and polymers were the predominant compounds ranging from 2- to 14-units of catechin/epicatechin. This was consistent with published data on complex PAC in blueberry and cacao extracts [31-34]. Fractions derived mostly from blackberry matrix/blend (0:100, 30:70, 50:50; blueberry: blackberry) contained in addition to PAC, a complex chemical composition of hydrolysable tannins (HTs). Basic structures for anthocyanins, proanthocyanidins, and HTs are presented in Figure 6.1. Figure 6.2 shows the composition of a mixture of blueberry: blackberry, with a blend of 50% each. Based on the relative abundance of the ESI chromatogram, HTs are the predominant polymerized compounds in the matrix obtained from mostly blackberry wine. Proanthocyanidins (condensed tannins) were polymeric forms of catechin and/or epicatechin molecules (m/z 291.0234 each, [M+H]+), while HTs were more complex, diverse, and with larger molecular size [20]. Based on LC-MS data across all PAC, blueberry contributed proanthocyanidins while blackberry contributed mostly HTs (Figure 6.2).

The HTs in blackberry included gallic acid (m/z : 170.2129) and ellagic acid (m/z : 303.0307) which form gallotannins and ellagitannins, respectively, in diversified polymers ranging from 465.269 to 2486.2511 m/z [M+H]+ present as shown in Figure 6.2. More than 1000 HTs have been identified from simple glucogallin to pentameric ellagitannins with molecular weight ranging from 332 to 5000 [35]. LC-MS/MS fragmentation of HTs in blackberry samples led to the identification of several major compounds based on their m/z, UV absorption at 280 nm, and published data such as ellagic acid pentoside (m/z: 435.2345), ellagic acid glucose (m/z: 465.0272), galloyl-HHP glucose (m/z: 633.0259), sanguim H-6 (m/z:
1871.2658, nobotanin A/ malabathrin B (m/z: 1736.1203), and lambertianin isomers A, C, and D (m/z: 1871.1403.5335, and 1870.9546, respectively). These identifications were in agreement with previous publications [20, 36-40]. A comprehensive structural elucidation of HTs in blackberry and other plant species was recently reviewed [41].

![Basic chemical structures for bioactive phytochemicals found in blueberry and blackberry: anthocyanins (cyanidin, peonidin, delphinidin, petunidin), proanthocyanidin (catechin, epicatechin), and hyrolyzable tannins (gallic acid, ellagic acid).](image)

**Figure 6.1.** Basic chemical structures for bioactive phytochemicals found in blueberry and blackberry: anthocyanins (cyanidin, peonidin, delphinidin, petunidin), proanthocyanidin (catechin, epicatechin), and hyrolyzable tannins (gallic acid, ellagic acid).
Additions of glycoside(s) from different sugar types to anthocyanidins results in formation of anthocyanins listed in Table 6.2. Addition of glucoside(s) and/or polymerization of gallic/ellagic acids results in the formation of hydrolysable tannins shown in Figure 6.2. Proanthocyanin polymers are multi-units of catechins and/or epicatechins.

**Figure 6.2.** HPLC-ESI-MS chromatograph for PAC from 50:50 blend of blueberry: blackberry. The large bold numbers refer to average degree of polymerization for proanthocyanidins, consisting of dimers (2 units of catechin/epicatechin) to 14-mers, with peaks contained two bold numbers indicated the presence of a doubly charged ion and the presence of both degrees of polymerization (i.e. 7/14 heptamer and 14-mers). Mass ions tagged with HT refer to polymerized hydrolysable tannins contributed only by blackberry. Hydrolysable tannins included gallic acid \((m/z : 170.2129)\) and ellagic acid \((m/z : 303.007)\) that formed gallotannins and ellagitannins in diversified polymers shown in LC-MS spectrum ranging from of 465.268 to 2486.2511 m/z [M+H]+.
The HTs in blackberry included gallic acid \((m/z : 170.2129)\) and ellagic acid \((m/z : 303.0307)\) which form gallotannins and ellagitannins, respectively, in diversified polymers ranging from 465.269 to 2486.2511 \(m/z [M+H]^+\) present as shown in Figure 6.2. More than 1000 HTs have been identified from simple glucogallin to pentameric ellagitannins with molecular weight ranging from 332 to 5000 [35]. LC-MS/MS fragmentation of HTs in blackberry samples led to the identification of several major compounds based on their \(m/z\), UV absorption at 280 nm, and published data such as ellagic acid pentoside \((m/z : 435.2345)\), ellagic acid glucose \((m/z : 465.0272)\), galloyl-HHP glucose \((m/z : 633.0259)\), sanguinim H-6 \((m/z : 1871.2658)\), nobotanin A/ malabathrin B \((m/z : 1736.1203)\), and lambertianin isomers A, C, and D \((m/z : 1871.1403.5335, \text{ and } 1870.9546, \text{ respectively})\). These identifications were in agreement with previous publications [20, 36-40]. A comprehensive structural elucidation of HTs in blackberry and other plant species was recently reviewed [41].

Freeze-dried ANC had an antioxidant capacity ranging from 407.3 \((100\%\text{ blueberry})\) to 758.8 mmol TE/L \((50:50\text{ blend})\), with an increase in all blends containing blackberry compared to 100\% blueberry (Table 6.1), however there was no difference in the antioxidant capacity of PAC, having an average antioxidant capacity of 605.6 mmol TE/L. Correlations between components of the wines and enriched-fractions are shown in Table 6.1.

**Anthocyanins significantly inhibited activity of \(\alpha\text{-glucosidase and DPP-IV.}\)**

While \(\alpha\)-amylase was not inhibited by either the ANC or the PAC (data not shown), \(\alpha\)-glucosidase was significantly inhibited (Table 6.3). The ANC concentration needed to inhibit \(\alpha\)-glucosidase by 50\% \((\text{IC}_{50})\) in C3G equivalents ranged from 27.6 \(\mu\text{M}\) for 100\% blackberry to 36.2 \(\mu\text{M}\) for 100\% blueberry, with the 50:50 blend having an IC\(_{50}\) value in between the 100% blends. When compared to the IC\(_{50}\) values for PAC, which ranged from 54.3 \(\mu\text{M}\) in epicatechin
equivalents for 100% blackberry to 115.1 µM for 100% blueberry, it can be seen that less ANC was needed to reduce α-glucosidase than PAC. Correlations between IC₅₀ values needed to inhibit the enzyme are shown in Table 6.3. There was a strong negative correlation ($r = -0.97, p < 0.05$) between the amount of ANC required to inhibit α-glucosidase by 50% (IC₅₀) and the total ANC present as determined by HPLC. Similarly, there was a strong negative correlation ($r = -0.93, p < 0.05$) between PAC needed to inhibit α-glucosidase by 50% (IC₅₀) and the total PAC present as determined by HPLC.

**Table 6.3.** Determination of IC₅₀ values (mean ± standard error) of ANC and PAC needed to inhibit enzymes involved in diabetes management.¹

<table>
<thead>
<tr>
<th>Enriched fraction</th>
<th>100% Blueberry</th>
<th>50%:50% Blackberry</th>
<th>100% Blueberry</th>
<th>α glu ANC</th>
<th>α glu PAC</th>
<th>DPP-IV ANC</th>
<th>DPP-IV PAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANC-</td>
<td>36.2 ± 13.2b</td>
<td>33.7 ± 9.8b</td>
<td>27.6 ± 9.7b</td>
<td>1.00</td>
<td>0.90</td>
<td>0.98</td>
<td>-0.86</td>
</tr>
<tr>
<td>PAC-</td>
<td>115.1 ± 28.5a</td>
<td>71.3 ± 28.9ab</td>
<td>54.3 ± 30.8ab</td>
<td>1.00</td>
<td>-0.99</td>
<td>-1.00</td>
<td></td>
</tr>
<tr>
<td>ANC-</td>
<td>15.9 ± 0.7a</td>
<td>4.2 ± 1.5b</td>
<td>5.5 ± 0.4b</td>
<td>1.00</td>
<td>-0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAC-</td>
<td>16.6 ± 1.1a</td>
<td>19.3 ± 1.1a</td>
<td>18.2 ± 3.2a</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹IC₅₀ values were determined from at least two independent duplicates done in triplicate. Values are means ± SEM. Means with different letters in each column are significantly different for each enzyme ($p < 0.05$).

²Positive control of inhibition for α-glucosidase was acarbose having an IC₅₀ value of 500 µM.

³The positive control of inhibition for DPP-IV was diprotin A (Ile-Pro-Ile) at 0.1 mg/mL, and had an IC₅₀ value of 4.3 ± 1.6 mg/mL. ⁴Correlation values are Pearson’s r-values ($p < 0.05$).

Compared to a standard curve of diprotin A, a known inhibitor of DPP-IV containing an Ile-Pro-Ile sequence, ANC and PAC all had the ability to inhibit DPP-IV activity at concentrations of 20 and 40 µM in C3G and epicatechin equivalents, respectively. ANC were more effective at inhibiting DPP-IV activity than the PAC, as seen by IC₅₀ values in Table 6.3.
Overall, the 50:50 blend was the most effective at reducing the activity of DPP-IV with an IC₅₀ value of 4.0 µM C₃G, comparable to the known inhibitor’s IC₅₀ value of 4.3 ± 1.6 mg/mL. Correlations between IC₅₀ values needed to inhibit the enzyme are shown in Table 6.3, as well as between IC₅₀ values to inhibit both DPP-IV and α-glucosidase. A strong negative correlation between amount of ANC (r = -0.90, p < 0.05) and IC₅₀ values to inhibit DPP-IV indicated that less of the compound was needed to inhibit the activity of the enzyme. Conversely, there was a strong positive correlation between PAC and the IC₅₀ value to inhibit DPP-IV, indicating that higher PAC was required in the sample to inhibit the enzyme activity. Results from the computational docking study showed that the FullFitness of the anthocyanins with the DPP-IV had no obvious differences (from -3228 to -3052 kcal/mol) (Table 6.4). Moreover, they were as low as those of diprotin A (-3208 kcal/mol), which is a potent DPP-IV inhibitor. These results suggest that anthocyanins contained in blueberry and blackberry have strong DPP-IV inhibiting effect. Among the four chains in DPP-IV, chain C bound each anthocyanin with the lowest interaction energy. Malvidin-3-galactoside in blueberry and delphinidin-3-arabinoside in blackberry, present in the highest concentrations, were presumed to be the major active components of blueberry and blackberry. The interactions of chain C with the two anthocyanins are shown in Figure 6.3. These results indicate that delphinidin-3-arabinoside and malvidin-3-galactoside could effectively inhibit the activity of the enzyme.

Markers of inflammation were inhibited in the NFκB-mediated pathway

Proliferation assays showed that none of the ANC or PAC from 100% blueberry, 50% blueberry: 50% blackberry, or 100% blackberry were cytotoxic to RAW 264.7 mouse macrophages at concentrations up to 100 µM in C₃G or epicatechin equivalents, respectively.

---

5 The work shown in Table 6.4 and Figure 6.3 were completed by Dr. Junfeng Fan.
<table>
<thead>
<tr>
<th>Chain of DPP-IV</th>
<th>FullFitness (kcal/mol)</th>
<th>SimpleFitness* (kcal/mol)</th>
<th>H Bond Position</th>
<th>Distance of H Bond (Å)</th>
<th>△G (kcal)</th>
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C3G, M3GA, M3A, D3G, D3A and DA represent cyanidin-3-glucoside, malvidin-3-galactoside, malvidin-3-arabinoside, delphinidin-3-glucoside, delphinidin-3-arabinoside and diprotin A, respectively. Other abbreviations indicate the three-letter amino acid code in the binding site. *The SimpleFitness is equal to the total energy of the system calculated with the CHARMM22 molecular mechanics force field, and neglects the effect of solvent known to have an important contribution to the binding free energy.

This work was conducted by Dr. Junfeng Fan.
Figure 6.3. Computational visualization of the most optimal docking conformation of (A) delphinidin-3-arabinoside, (B) malvidin-3-galactoside, and (C) diprotin A, with chain C of the DPP-IV enzyme. This work was conducted by Dr. Junfeng Fan.
None of the ANC or PAC (100 µM C3G or epicatechin equivalents) increased production of any of the inflammatory proteins tested when not stimulated with LPS. All three (100:0, 50:50, and 0:100) blueberry-blackberry blend ANC at 100 µM were able to lower total p-p65 NF-κB subunit following LPS-stimulation by 68.9, 88.6, and 80.8%, respectively (Figure 6.4A). ANC from the blends containing 50% or 100% blackberry were able to significantly inhibit the activation of the p-p65 NF-κB subunit at 50 µM by 88.6 and 72.0%, respectively. PAC from the 100% blueberry and the 50:50 blends were also able to significantly decrease the p-p65 NF-κB subunit at 100 µM epicatechin equivalents by 55.7 and 40.0%, respectively; however PAC from the 100% blackberry blend did not significantly decrease this inflammatory protein (Figure 6.4A). This can be attributed to the fact that, unlike blueberry with more polymeric proanthocyanidins or condensed tannins, blackberry contains HTs.

COX-2 expression was decreased by all ANC at 100 µM C3G equivalents; 52.4% for 100:0, 67.8% for 50:50, and 72.2% for 0:100 (Figure 6.4B). PAC from 100% blackberry and the 50:50 blends were also able to decrease COX-2 expression significantly by 64.3 and 65.2%, respectively. PAC at 100 µM epicatechin equivalents from the 100% blueberry blend decreased COX-2 expression by 36.8%, however this decrease was not significant. iNOS expression was decreased by ANC with the 100% blueberry and 50:50 blends at 100 µM C3G equivalents, with ANC from the 100% blueberry the most effective with 75.7% inhibition compared to 40.2% for 50:50 (Figure 6.5A). PAC from all blends were able to decrease iNOS expression in mouse macrophages following LPS-stimulation at 100 µM epicatechin equivalents, with the 50:50 blend the most effective to decrease iNOS expression by 57.4% compared to 53.0% inhibition by 100:0 and 42.3% by 0:100. iNOS protein produces the inflammatory marker nitric oxide (NO) when active, so NO production was also measured.
Figure 6.4. Effect of ANC (cyanidin-3-O-glucoside equivalents) and PAC (epicatechin equivalents) at 50 and 100 μM on NFκB (A), and COX-2 expression (B). Western blots are shown above with p-p65 subunit of NFκB detected at 65 kDa, COX-2 at 70-72 kDa, and actin at 43 kDa from whole cell lysates. Values are relative expression of protein of interest over actin; means ± SEM with different letters are significantly different (n = 4, p < 0.05).
As seen in Figure 6.5B, ANC at 100 µM were able to decrease NO production with blackberry in the blend by 37.9% for 50:50 and 48.1% for 0:100; however the 100% blueberry ANC was unable to show a significant decrease in NO production. PAC from 100:0, 50:50, and 0:100 at 100 µM epicatechin equivalents also decreased NO production by 16.4, 4.8, and 14.7%, respectively; however, these decreases were not significant \((p > 0.05)\).

![Figure 6.5](image)

**Figure 6.5.** Effect of ANC (cyanidin-3-O-glucoside equivalents) and PAC (epicatechin equivalents) at 50 and 100 µM on (A) iNOS expression. Western blots are shown above with iNOS detected at 130 kDa and actin at 43 kDa from whole cell lysates. Values are relative expression of protein of interest over actin. (B) NO assay presents data from treatments at 100 µM cyanidin-3-O-glucoside (C3G) equivalents (ANC) or epicatechin equivalents (PAC) and was measured from spent medium. Means ± SEM with different letters are significantly different \((n = 4, p < 0.05)\).
Discussion

Fermented berry beverages have been established as having an increased phenolic content and higher antioxidant capacity than their non-fermented counterparts [7-9, 23]. Beneficial effects of berries in the diet have been attributed to their high phenolic content, especially their anthocyanin content [40]. Wines were tested for their total polyphenols, total anthocyanin content, and antioxidant capacity, and these levels increased with more blackberry in the blend associated with higher ANC concentration. To our knowledge, this is the first reported analysis of anthocyanins from fermented blueberry-blackberry blends; however, the results from blueberry and blackberry wines were comparable to previously reported values [24]. In this study, we show that the anthocyanin profile from fermented berry beverages may be altered from profiles previously generated for blackberry fruits [20] or even phenolic-rich blueberry extracts [29]. Malvidin contains two methoxy side chains on its B ring, while delphinidin has two hydroxyl groups, which may contribute to the higher antioxidant capacity seen for the blackberry blends. Delphinidin (45.0 - 74.9 mg/100 g FW) and malvidin (37.1 - 62.2 mg/100 g FW) were the predominant anthocyanins in the varieties tested in Highbush and lowbush blueberries [42], similar to the findings from the fermented products.

During wine processing from berries, phenolic compounds may be effectively extracted into the wines; delphinidin-3-glucoside has been identified as a major component in red wine and had the most variation among all anthocyanins [43]. Although cyanidin 3-glucoside has been identified as the major anthocyanin in different blackberry species, cyanidin 3-rutinoside was the major component in the wines from Andean blackberry (Rubus glaucus Benth.) [44]. These results contrast with the major anthocyanin identified in our study as delphinidin-3-arabinoside, though many unknown compounds in their study were only tentatively identified. Authors agree that fermentation causes an absolute and relative change in anthocyanin content, allowing for
new pigments to be generated or formed during the fermentation process or during wine maturation. While the process of anthocyanin transformation is not clear, it is supported by the changes seen in wines during aging as native blackberry anthocyanins are transformed during and after alcoholic fermentation.

Our results showed inhibition of α-glucosidase activity; other studies on red and black currants and red and green gooseberries have shown strong inhibition of intestinal glucose absorption by inhibiting α-glucosidase activity [13]. Anthocyanins present in blueberry fruits and their fermented products appear to be more potent at inhibiting α-glucosidase than the proanthocyanidins. While a previous study in our laboratory evaluated blueberry wines and found strong inhibition of α-amylase in vitro [14]. During the phenolic extraction process phenolic acids were removed indicating these may be the bioactive compounds with ability to inhibit α-amylase activity rather than the ANC or PAC. Grussu et al. [45] demonstrated that it is not the anthocyanins that are required to inhibit α-amylase because both red and yellow raspberries showed similar inhibitory capacity.

A study of rowanberry proanthocyanidin-rich fraction had a low level of inhibition compared to the whole extract [46], suggesting that tannin and ellagitannin components are not influential or are poor inhibitors of α-glucosidase. Our results indicate that there was some inhibition of the enzyme by the PAC-enriched fraction, but the ANC-enriched fraction was more potent. The efficacy of the anthocyanins to inhibit DPP-IV enzyme activity at a rate comparable to a known inhibitor (diprotin A) indicated that anthocyanins may be able to act as naturally-occurring DPP-IV inhibitors of DPP-IV. If DPP-IV is inactivated by drugs or natural compounds, the degradation of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) is prevented, and these hormones are able to inhibit glucagon
release and stimulate insulin release, respectively, thereby lowering blood glucose levels [17].

All of the anthocyanins tested showed similar FullFitness energies from the computational modeling study; therefore, they have no major difference in anti-DPP-IV activity. Due to the higher amounts of malvidin-3-galactoside in blueberry and delphinidin-3-arabinoside in blackberry, these ANC are likely the major active components. To confirm these results, the SwissDock computational docking study demonstrated that delphinidin-3-arabinoside and malvidin-3-galactoside were able to interact with DPP-IV through ligand interaction, and therefore potentially inactivate the activity of the enzyme. The most optimal conformation happened in chain C of DPP-IV indicated by the lowest interaction energies of -3228 kcal/mol (for delphinidin-3-arabinoside) and -3107 kcal/mol (for malvidin-3-galactoside), which are comparable to the interaction energy of the known inhibitor diprotin A with the enzyme of -3208 kcal/mol. Due to the lowest interaction energy of chain C, we concluded that the chain C was the major binding site of these anthocyanins.

Our results agree with previous studies that reported the ability of berries to reduce inflammation through decreased expression of enzymes iNOS and COX-2 [19-20]. The upstream mediator in the NFκB-mediated pathway, the p-p65 subunit of NFκB that lead to the induction of these inflammatory markers was inhibited by ANC 50:50% blend and 100% blackberry. The strong anti-inflammatory effect was considered to be exerted by a combination of various polyphenol compounds rather than any single compound.

Berry extracts can inhibit α-glucosidase at concentrations less than 50 µg GAE/mL, an amount that can be easily reached in the gastrointestinal tract after consumption of berries or juices [47]. Additionally, a previous study reports that a moderate drink of 140 mL of wine made
from Highbush blueberry would correspond to an intake of 2.42 mmol of TE of ORACPE/day [43].

**Concluding remarks**

ANC and PAC at 100 µM C3G/ epicatechin equivalents obtained from blueberry-blackberry blends affected LPS-induced inflammatory response in RAW 264.7 mouse macrophages. These ANC and PAC reduced the phosphorylation of the p65-subunit NFκB, decreased iNOS and COX-2 expression, and decreased NO production. Along with the high antioxidant capacity and phenolic content and ability to decrease enzyme activity related to improved glucose utilization, bioactive ANC and PAC compounds present in fermented berry beverages may act synergistically to reduce risk factors for chronic inflammatory diseases, especially type 2 diabetes.

**Acknowledgements**

Thanks to Dixon Springs Agricultural Center in Simpson, IL for providing materials for the fermentation. Special thanks to Yan Wei and Christina Alcaraz for their initial work with computational docking, and to the State scholarship fund for J.F. from Beijing Forestry University and China Scholarship Council, China. The Office of Research (University of Illinois at Urbana-Champaign) provided funds for this research.

The authors have declared no conflict of interest.
References.


CHAPTER 7

BERRY AND CITRUS PHENOLIC COMPOUNDS INHIBIT DIPEPTIDYL PEPTIDASE IV: IMPLICATIONS IN DIABETES MANAGEMENT

Abstract

Beneficial health effects of fruits and vegetables in the diet have been attributed to their high flavonoid content. Dipeptidyl peptidase IV (DPP-IV) is a serine aminopeptidase that is a novel target for type 2 diabetes therapy due to its incretin hormone regulatory effects. In this study, well-characterized anthocyanins (ANC) isolated from berry wine blends and twenty-seven other phenolic compounds commonly present in citrus, berry, grape, and soybean, were individually investigated for their inhibitory effects on DPP-IV by using a luminescence assay and computational modeling. ANC from blueberry-blackberry wine blends strongly inhibited DPP-IV activity (IC\textsubscript{50}, 0.07 ± 0.02 to >300 μM). Of the twenty-seven phenolics tested, the most potent DPP-IV inhibitors were resveratrol (IC\textsubscript{50}, 0.6 ± 0.4 nM), luteolin (0.12 ± 0.01 μM), apigenin (0.14 ± 0.02 μM), and flavone (0.17 ± 0.01 μM), with IC\textsubscript{50} values lower than diprotin A (4.21 ± 2.01 μM), a reference standard inhibitory compound. Analyses of computational modeling showed that resveratrol and flavone were competitive inhibitors which could dock directly into all three active sites of DPP-IV, while luteolin and apigenin docked in a noncompetitive manner. Hydrogen bonding was the main binding mode of all tested phenolic

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7 (Publication: Fan J, Johnson MH, Lila MA, Yousef G, de Mejia EG. Berry and citrus flavonoids inhibit dipeptidyl peptidase IV, implications in diabetes management. Evidence-based Complementary and Alternative Medicine. Hindawi Publishing Corporation. Volume 2013, Article ID 479505. Academic Editor: Mohd Roslan Sulaiman) Copyright © 2013 Junfeng Fan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited (as above). My role for this chapter was to prepare and provide the ANC isolated from the berry wine blends, to conduct and assist with the DPP-IV luminescent assays, and to assist with the data analysis and manuscript preparation.
compounds with DPP-IV. These results indicate that flavonoids, particularly luteolin, apigenin, and flavone, and the stilbenoid resveratrol can act as naturally occurring DPP-IV inhibitors.

**Abbreviations used:**

ANC: Anthocyanins  
C3G: Cyanidin-3-glucoside  
DPP-IV: Dipeptidyl peptidase IV  
GLP-1: Glucagon-like peptide-1  
GIP: Glucose-dependent insulino tropic polypeptide  
IC\textsubscript{50}: Concentration to inhibit 50% enzyme activity  
\(K_i\): Enzyme inhibitor constant.

**Introduction**

Type 2 diabetes is characterized by excessive blood glucose and insulin resistance due to an improper insulin response of the body to manage glucose from the diet [1]. Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a serine peptidase, is one of the newest pharmaceutical targets for type 2 diabetes treatment [1]. On the other hand, incretin-based therapy has several potential sites of action for the treatment of type 2 diabetes ranging from increasing insulin secretion, reducing glucagon secretion, and regulating glucose control [2]. It is well known that glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP) are major human incretin hormones that stimulate insulin release in a glucose-dependent manner in healthy individuals [3, 4]. However, DPP-IV rapidly transforms these two gut incretin hormones after secretion by cleavage of the penultimate proline or alanine at N-terminus, and thus forms their inactive metabolites [5–7]. Both hormones have very short half-lives (approximately 2 min) due to the rapid degradation by DPP-IV [8]. Among the several peptide substrates of DPP-IV, GLP-1 is one of the well-characterized physiological and pharmacological substrates of the enzyme. GLP-1, which is secreted in a nutrient-dependent manner, stimulates glucose-dependent
insulin secretion and regulates glycemia. However, the actions of GLP-1 do not last long due to degradation by DPP-IV. For this reason, DPP-IV inhibition is expected to result in elevated plasma insulin levels by inhibiting the degradation of active GLP-1 after oral glucose intake. This in turn leads to the suppression of blood glucose elevation. Therefore, development of DPP-IV inhibitors is being actively conducted worldwide, and control of blood glucose levels by enhancement of GLP-1 action is a new option for the treatment of diabetes.

In recent years, protein-ligand docking has become a powerful tool for drug development, and is also a method to be able to identify binding modes with high accuracy. For DPP-IV, computational docking analyses have been commonly used for designing inhibitors [9], screening of potential inhibitors [10], and explaining the differences in activity of drugs with different structures [11]. However, most of the previously investigated inhibitors of DPP-IV have been synthetically derived. As for naturally occurring flavonoids, the binding modes with DPP-IV are still not yet established.

Phenolic compounds, such as flavonoids, widely abundant in fruits and vegetables, have been suggested as important compounds for diabetes reduction [9, 10]. However, so far only a few phenolic compounds have been investigated to inhibit DPP-IV activity. These include procyanidin from grape seeds [12] and naringin from orange peel [13]. Therefore, it is necessary to further elucidate the modulating effect on DPP-IV activity of phenolic compounds from other natural sources.

In epidemiological studies, berries were the most important contributors to lowering risk for type 2 diabetes [14]. Additionally, an inverse relationship between intake of flavonoids, specifically those from berries, and risk of type 2 diabetes was found [15]. However, there is lack of evidence for the role of specific phenolics in clinical trials, and there is not yet sufficient data
to confirm that anthocyanins have a protective effect against the risk of type 2 diabetes [16]. Additionally, anthocyanins found in berries have been found to have a beneficial effect on glucose metabolism; however, stronger scientific evidence is needed.

Anthocyanins (ANC) from blueberry-blackberry wine blends have been evaluated for DPP-IV and carbohydrate-utilizing enzymes inhibitor studies in our laboratory, and they have exhibited potent DPP-IV and α-glucosidase inhibitory activities [17]. Thus, the aim of the present study was to further characterize the ANC-rich fractions from blueberry-blackberry wine blends by HPLC and analyze their DPP-IV inhibitory effect in vitro. Furthermore, a variety of other phenolic compounds commonly present in berries, citrus, and other plant foods were studied for their DPP-IV inhibitory activity. We hypothesized that berry and citrus phenolics could bind to the active sites of DPP-IV, thus inhibiting DPP-IV enzyme activity. For the most potent compounds, kinetic and computational docking analyses were used to elucidate the binding modes with the DPP-IV enzyme.

Materials and Methods

Materials

Wines were produced from Highbush blueberry (Vaccinium corymbosum) cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetta, Collins, Coville, Darrow, Earliblue, Elliot, Jersey, Late Blue, and Spartan and blackberry (Rubus fruticosus) cultivars A-1937, A-2215, A-2241 Natchez, A-2315, APF 27, APF 40, APF 41, and Prime Jan, collected from Dixon Springs Agricultural Center in Simpson, IL, USA during the ripening season of 2010. Blueberry wine and blackberry wine were separately fermented using Saccharomyces bayanus as previously described [17]. After the fermentation, blends ranging from 100% blueberry to 100%
blackberry were made using room temperature fermented wines. Blends were prepared with different ratios of % blueberry : % blackberry. The ratios were 100 : 0, 75 : 25, 25 : 75, and 0 : 100 of blueberry: blackberry wine blends, respectively.

All solvents used for phenolic extraction were HPLC-grade and were purchased from Fisher Scientific (Pittsburg, PA). Amberlite XAD-7 was purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 was purchased from GE Life Sciences (Buckinghamshire, UK). Porcine kidney DPP-IV enzyme (88% sequence homology with human; both are homodimers with a subunit molecular mass of ~30 kDa) and diprotin A were purchased from Sigma-Aldrich. DPP-IV Glo™ Protease Assay kits were purchased from Promega (Madison, WI). Flavonoids with high purities that were purchased from Sigma-Aldrich included luteolin (>98%), apigenin (>95%), quercetin (>98%), kaempferol (>97%), rutin hydrate (>94%), naringenin (>95%), neohesperidin (>90%), flavone (>97%), naringin (>90%), hesperidin (>80%), cyanidin-3-glucoside (>95%), cyanidin (>95%), malvidin (>95%), resveratrol (>99%), protocatechuic acid (>97%), catechin (>98%), epicatechin (>90%), epigallocatechin gallate (EGCG, >95%), gallic acid (>97.5%), caffeic acid (>98%), and chlorogenic acid (>95%). Hesperetin (>95%) was purchased from Sigma-Aldrich (Wicklow, Ireland) and limonin (>90%) from MP BioMedicals (Solon, OH). Narirutin (>93.9%) and eriocitrin (>97.4%) were purchased from Chromadex (Irvine, CA). Genistein (>90%) and genistin (>90%) were kindly donated by Dr. Mark Berhow, USDA. All other reagents were of analytical grade.

**Phenolic extraction and preparation of ANC fractions**

Phenolic extraction and preparation of ANC fractions were conducted as previously described [17]. Briefly, each wine was firstly acidified, dealcoholized, and then mixed with amberlite XAD-7 resin to remove sugars and phenolic acids. After nonpolar compounds were
further removed from the crude polyphenolics, the polar eluate was loaded onto a Sephadex LH-20 column to generate ANC-enriched fractions. With an isocratic elution using water : methanol (80 : 20, containing 0.1% TFA) and then 50% methanol, five anthocyanin-rich fractions (ANC 1–5) were obtained. ANC 2–5 from each blend of blueberry and blackberry were analyzed by HPLC to determine their ANC composition.

**Anthocyanin analysis**

ANC analyses were conducted as previously published [17] using a 1200 HPLC (Agilent Technologies, Santa Clara, CA) with a Supelcosil LC-18 RP column (250 × 4.6 mm, 5 μM) (Supelco, Bellefonte, PA). ANC were detected at 520 nm using a diode array detector (DAD). Specific anthocyanins were identified based on comparison to our previously published data [18, 19]. A previously well-characterized blueberry extract [19] was included with each sample run to verify compound separation and identification. Using the peak areas as measured by HPLC at 520 nm, total ANC were quantified from a standard curve generated from 0.125, 0.25, 0.5, and 1.0 mg/mL of cyanidin-3-glucoside (C3G) and ANC amounts are presented as C3G equivalents.

**DPP-IV inhibition**

Measurement of the activity and potential inhibition of DPP-IV, a type II membrane glycoprotein, was done using the DPP-IV Glo™ Protease Assay following the manufacture’s protocol (Promega, Madison, WI). Briefly, 50 μL of DPP-IV Glo™ reagent was added to a white-walled 96-well plate containing 50 μL of blank, positive control, or treatment. The blank contained the vehicle only while positive control contained the vehicle and purified DPP-IV enzyme (at a final concentration of 1 ng/mL). Treatments used were enriched ANC fractions (0.5, 5, 20, and 40 μg/mL), phenolic compounds (0.5, 5, 20 and 40 μg/mL) or known inhibitor, diprotin A (1, 2, 12, 24, 125, and 250 μM), and the purified DPP-IV enzyme at a final
concentration of 1 ng/mL. The content of the wells was gently mixed using an Ultra Microplate Reader (Biotek Instruments, Winooski, VT) at medium intensity for 4 s. DPP-IV cleavage of the provided Gly-Pro-amino methyl coumarin (AMC) substrate generated a luminescent signal by luciferase reaction, with the amount of DPP-IV enzyme available to bind Gly-Pro-AMC proportional to relative light units (RLU) produced. This signal in RLU was measured after 30 min in the Ultra Microplate Reader and then compared to the blank. Diprotin A linear standard curve ($y = 41.936x + 27.294$, $R^2 = 0.91$), where $y$ was the % inhibitory activity of diprotin A and was the log10 of the concentration ($\mu$M) of known inhibitor diprotin A, was used to calculate IC$_{50}$ value: the concentration needed to decrease the activity of the enzyme by 50% of its original activity. IC$_{50}$ values were calculated based on the molecular mass of each compound or C3G as the equivalent for ANC-enriched fractions.

**Inhibitory kinetics study**

Porcine kidney DPP-IV activity was measured at various concentrations of three flavonoids (5 and 10 mg/mL for luteolin, apigenin, and flavone; and 0.25 and 0.5 mg/mL for resveratrol). Each concentration was evaluated in the presence of various concentrations of Gly-Pro-AMC (0–60 $\mu$M). DPP-IV activity was measured using the DPP-IV Glo Protease Assay as mentioned above. The inhibition pattern was evaluated utilizing the Lineweaver-Burk plot. Enzyme-inhibition constant $K_i$ was determined by plotting the reciprocal of the initial luminescence versus the reciprocal of the initial substrate concentration.

**Molecular modeling and computational docking study**

The DPP-IV enzyme exists as a dimer in the crystal form, and each monomer consists of 726 amino acids [20]. The docking studies were conducted with the monomeric unit of the enzyme, as the active site of the enzyme resides deep within each monomer of the receptor.

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8 Dr. Junfeng Fan conducted all the molecular modeling and computational modeling performed in this chapter.
protein and not on the enzyme surface [21]. The molecular docking analysis of flavonoids was carried out using AUTODOCK 4.2 (CCDC, UK; http://www.ccdc.cam.ac.uk/products/csd/) [22]. The crystal structure of the DPP-IV enzyme (Protein Data Bank (PDB) ID: 2I03) was obtained from the protein data bank (http://www.rcsb.org/pdb), and the protein structure was prepared using Accelrys Discovery Studio 3.5 program (Accelrys Software Inc., San Diego, CA). For the computational docking study, the energies of diprotin A and flavonoids were minimized by applying a CHARM22 force field, using the Accelrys Discovery Studio 3.5 program. After removing water molecules and adding all the hydrogen atoms, Gasteiger-Hückle charges were assigned to the enzyme. The ligand conformers were treated as flexible and protein structures were treated as rigid during the docking process. The docking was carried for 100 genetic algorithm runs, which was optimum to validate the crystal structure of the ligand. Most of the other genetic algorithm parameters such as the population size were maintained at their default values. The best docking results were considered to be the conformation having the lowest binding energy ($\Delta G$) using the equation:

$$\Delta G = \Delta G \text{ (intermolecular)} + \Delta G \text{ (internal)} + \Delta G \text{ (tor)} - \Delta G \text{ (Unbound extended)}$$

Where $\Delta G$ (intermolecular) denotes the sum (kcal/mol) of Van der Waals energy, hydrogen bond energy, electrostatic energy and desolvation energy; $\Delta G$ (internal) is final total internal energy (kcal/mol); $\Delta G$ (tor) denotes torsional free energy (kcal/mol); and $\Delta G$ (Unbound extended) is the unbound system’s energy (kcal/mol). In the context of Autodocking, inhibition constant ($K_i$) is directly related to the binding energy: $K_i = e^{[\Delta G/(RT)]}$

Where e is the base number of natural logarithm (approximately equals to 2.72), R is gas constant (kcal/mol), and T is the absolute temperature. Smaller $K_i$ and more negative $\Delta G$ mean tighter binding.
**Statistical analyses**

Data were expressed as means of independent duplicates with at least three replicates. The dose-response analysis of each compound on DPP-IV activity was performed using nonlinear or linear regression (curve fit) using EXCEL Microsoft (e.g., see Supplementary Information available online at http://dx.doi.org/10.1155/2013/479505). Statistical analysis was conducted using the proc GLM procedures of SAS version 9.3 (SAS Inst. Inc., Cary, NC, 2009). Group mean comparisons were conducted using Duncan means and were considered to be significant at $P < 0.05$ based on the least significant differences (LSD) from one-way analysis of variance (ANOVA) with alpha = 0.05. Correlations were made using Pearson’s correlation values with $P < 0.05$.

**Results**

*Blackberry wine presented high concentrations of delphinidin-3-arabinoside*

Relative distribution of ANC in the extracts of blueberry-blackberry wine blends are shown in Table 7.1. Chromatographic analyses revealed up to seventeen ANC present in blueberry-blackberry blends. Malvidin-3-galactoside and cyanidin-3-glucoside were the main ANC present in the blueberry wine, while delphinidin-3-arabinoside was the predominant ANC present in the blackberry wine. Total ANC ranged from 1653.8 mg C3G equivalents/L for blueberry wine to 3267.8 mg C3G equivalents/L for blackberry wine. It was also observed that there was an obvious difference between ANC amounts of different fractions generated as ANC 2–5.

*Anthocyanins from blackberry wine potently inhibited DPP-IV*

ANC-enriched fractions (ANC 1–5) isolated from blueberry-blackberry wine blends were analyzed for their DPP-IV inhibitory effect. Table 7.2 shows the IC$_{50}$ values of ANC from blueberry-blackberry wine blends needed to inhibit DPP-IV enzyme.
Table 7.1. Anthocyanin (ANC) identification and quantification by HPLC at maximum absorption of 520 nm.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>ANC ID</th>
<th>Anthocyanins (mg C3G equivalents/L) per blend (%Blueberry : %Blackberry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.44</td>
<td>Delphinidin-3-galactoside</td>
<td>ANC2 10.8 10.0 6.6 11.2 6.9 7.0 nd nd nd nd nd</td>
</tr>
<tr>
<td>25.98</td>
<td>Delphinidin-3-glucoside</td>
<td>75:25 45.5 nd 9.0 51.0 7.7 12.3 17.7 nd nd 8.8 nd nd</td>
</tr>
<tr>
<td>27.33</td>
<td>Cyanidin-3-galactoside</td>
<td>25:75 6.5 nd nd 6.3 nd 17.7 nd nd 6.5 23.8 nd 6.9 7.0</td>
</tr>
<tr>
<td>28.86</td>
<td>Delphinidin-3-arabinoside</td>
<td>0:100 nd 11.9 30.9 nd nd nd nd nd 1079.3 716.1 30.1 16.8 1244.7 794.1 35.8 17.8</td>
</tr>
<tr>
<td>29.69</td>
<td>Cyanidin-3-glucoside</td>
<td>100:0 6.4 23.4 nd nd 60.6 564.1 29.7 9.3 10.2 12.2 nd 10.1 6.5 6.1 nd</td>
</tr>
<tr>
<td>30.82</td>
<td>Petunidin-3-glucoside</td>
<td>nd 140.2 7.4 nd 11.0 16.7 8.0 7.2 nd 20.2 10.0 nd 116.8 nd 10.8 7.0</td>
</tr>
<tr>
<td>31.62</td>
<td>Petunidin-3-arabinoside</td>
<td>nd 6.0 39.8 nd nd 8.3 6.7 nd 8.0 8.4 nd 13.9 7.0 10.1 7.1 18.8 8.0</td>
</tr>
<tr>
<td>32.69</td>
<td>Malvidin-3-arabinoside</td>
<td>nd 5.9 178.5 22.5 nd 34.5 9.1 nd 14.3 8.3 10.4 6.5 9.7 nd 9.7 9.9</td>
</tr>
<tr>
<td>33.95</td>
<td>Malvidin-3-galactoside</td>
<td>34.39 266.1 nd 3.2 14.5 6.4 38.4 nd 15.5 nd 6.7 6.3 15.9 nd</td>
</tr>
<tr>
<td>35.19</td>
<td>Malvidin-3-glucoside</td>
<td>63.1 11.1 6.1 266.3 69.3 18.3 7.4 49.6 11.8 11.2 6.4 6.4 11.7 10.7 10.4</td>
</tr>
<tr>
<td>37.14</td>
<td>Malvidin-3-arabinoside</td>
<td>5.9 278.9 19.7 8.3 91.5 127.2 24.5 9.2 44.7 150.2 30.6 6.5 7.5 nd 37.1 7.0</td>
</tr>
<tr>
<td>39.03</td>
<td>Delphinidin-6-acetyl-3-glucoside</td>
<td>nd 7.3 17.9 6.7 6.3 12.6 11.4 6.4 nd nd 6.8 nd 7.2 nd 6.2</td>
</tr>
<tr>
<td>40.55</td>
<td>Cyanidin-6-acetyl-3-glucoside</td>
<td>41.70 7.8 6.7 nd 8.9 nd 6.6 nd 6.1 nd 7.3 6.9 43.9 nd nd 7.1</td>
</tr>
<tr>
<td>42.25</td>
<td>Malvidin-6-acetyl-galactoside</td>
<td>nd 26.7 7.1 nd 13.6 nd 7.7 nd 6.3 nd nd nd nd 7.2 6.6</td>
</tr>
<tr>
<td>44.40</td>
<td>Petunidin-6-acetyl-arabinoside</td>
<td>42.25 6.0 nd 11.9 nd nd nd nd nd nd nd nd nd nd</td>
</tr>
<tr>
<td></td>
<td>Malvidin-6-acetyl-glucoside</td>
<td>10.8 10.5 nd 11.5 19.4 8.4 nd 8.9 8.1 6.4 nd 14.2 9.3 5.9</td>
</tr>
<tr>
<td>Sub-total ANC</td>
<td>190.5 1113.7 2756.7 74.0 864.0 1112.3 183.8 81.6 1448.2 1073.3 239.2 146.8 1550.4 1204.8 302.8 229.8</td>
<td></td>
</tr>
<tr>
<td>Total ANC</td>
<td>1653.8 2241.7 2907.5 3267.8</td>
<td></td>
</tr>
</tbody>
</table>

*Bold numbers indicate the dominant flavonoids in that particular ANC fraction.

nd = Peak not detected.
Compared to a standard curve of diprotin A (IC$_{50}$, 4.21 ± 2.01 μM), a known DPP-IV inhibitor with an Ile-Pro-Ile sequence, ANC 2–5 tested at concentrations of 0.5, 5, 20, and 40 μM in C3G equivalents obtained from each blend had IC50 values ranging from 2.64 ± 1.40 μM in ANC 2 from blueberry wine to 0.07 ± 0.02 μM in ANC 3 from blackberry wine (Table 7.2). Table 7.2 also shows that ANC from blackberry wine were the most effective of the blends at reducing the activity of DPP-IV (with IC$_{50}$ values of no more than 0.22 μM C3G).

Table 7.2: Anthocyanin (ANC) concentration (μM) from blueberry-blackberry wine blends needed to inhibit DPP-IV enzyme activity by 50%$^{1,2}$.

<table>
<thead>
<tr>
<th>Blend ratio (%blueberry:%blackberry)</th>
<th>Fractions</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Blueberry</td>
<td>ANC-1</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>ANC-2</td>
<td>2.64±1.40a</td>
</tr>
<tr>
<td></td>
<td>ANC-3</td>
<td>0.64±0.33bc</td>
</tr>
<tr>
<td></td>
<td>ANC-4</td>
<td>1.37±0.58abc</td>
</tr>
<tr>
<td></td>
<td>ANC-5</td>
<td>0.72±0.25bc</td>
</tr>
<tr>
<td>25%:75%</td>
<td>ANC-1</td>
<td>NA$^3$</td>
</tr>
<tr>
<td></td>
<td>ANC-2</td>
<td>2.02±0.56ab</td>
</tr>
<tr>
<td></td>
<td>ANC-3</td>
<td>0.41±0.11c</td>
</tr>
<tr>
<td></td>
<td>ANC-4</td>
<td>0.22±0.05c</td>
</tr>
<tr>
<td></td>
<td>ANC-5</td>
<td>0.36±0.16c</td>
</tr>
<tr>
<td>75%:25%</td>
<td>ANC-1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ANC-2</td>
<td>0.34±0.10c</td>
</tr>
<tr>
<td></td>
<td>ANC-3</td>
<td>0.33±0.08c</td>
</tr>
<tr>
<td></td>
<td>ANC-4</td>
<td>0.52±0.18c</td>
</tr>
<tr>
<td></td>
<td>ANC-5</td>
<td>0.20±0.10c</td>
</tr>
<tr>
<td>100% Blackberry</td>
<td>ANC-1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ANC-2</td>
<td>0.22±0.03c</td>
</tr>
<tr>
<td></td>
<td>ANC-3</td>
<td>0.07±0.02c</td>
</tr>
<tr>
<td></td>
<td>ANC-4</td>
<td>0.18±0.07c</td>
</tr>
<tr>
<td></td>
<td>ANC-5</td>
<td>0.20±0.09c</td>
</tr>
</tbody>
</table>

1 IC$_{50}$ values were determined from at least two independent duplicates done in triplicate and calculated in C3G equivalents. Values are means ± SEM. Means with different letters in the IC$_{50}$ column are significantly different ($p < 0.05$). 2 The positive control of inhibition for DPP-IV was diprotin A (Ile-Pro-Ile) at 0.1 mg/mL, and had an IC$_{50}$ value of 4.21±2.01 μM. 3 NA: No activity detected at > 300 μM.
Resveratrol, a stilbenoid, luteolin, apigenin, and flavone, flavonoids commonly present in fruits, have strong DPP-IV inhibitory activity

Twenty-seven phenolic compounds commonly present in citrus, berries, grape, soybeans, and other plants were tested for DPP-IV inhibitory effect (Table 7.3). Sixteen phenolic compounds demonstrated DPP-IV inhibitory activity with IC\(_{50}\) values ranging from 0.6 ± 0.4 nM (resveratrol) to 10.36 ± 0.09 μM (erocitrin). Eleven compounds did not have DPP-IV inhibitory activity including rutin, narirutin, naringin, hesperidin, limonin, neohesperidin, genistin, catechin, epicatechin, chlorogenic acid, and protocatechuic acid (data not shown).

**Table 7.3:** DPP-IV inhibition\(^1\) by flavonoids (IC\(_{50}\)), their number of hydroxyl groups (OH), binding energy, inhibition constant (K\(_i\))\(^2\), H bonds involved, and π interactions.\(^9\)

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC(_{50}) (μM)</th>
<th>Number of OH groups</th>
<th>Binding energy (kcal/mol)</th>
<th>K(_i) (μM)</th>
<th>H Bonds (^3)</th>
<th>π interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td></td>
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<tr>
<td>Berry flavonoids</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cyanidin</td>
<td>1.31 ± 0.34 (^{c})</td>
<td>5</td>
<td>-5.95</td>
<td>43.43</td>
<td>TRP563:HN-UNK:O15 ALA564:HN-UNK:O18 UNK:H7-TYR48:OH UNK:H11-GLY741:O</td>
<td>π-π UNK:B:TRP629 UNK:B:TRP629 UNK:B:TRP629 UNK:B:TRP629</td>
</tr>
</tbody>
</table>

\(^{9}\) All computational work was conducted by Dr. Junfeng Fan.
<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>Luteolin</th>
<th>Apigenin</th>
<th>Quercetin</th>
<th>Kaempferol</th>
<th>Flavone</th>
<th>Hesperetin</th>
<th>Naringenin</th>
<th>Eriocitrin</th>
<th>Soy isoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Citrus flavonoids</strong></td>
<td>0.12 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>2.92 ± 0.68</td>
<td>0.49 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.28 ± 0.07</td>
<td>0.24 ± 0.03</td>
<td>10.36 ± 0.09</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td><strong>Genistein</strong></td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td><strong>Ho</strong></td>
<td>25.83</td>
<td>31.77</td>
<td>23.03</td>
<td>13.99</td>
<td>13.57</td>
<td>9.57</td>
<td>9.9</td>
<td>225.96</td>
<td>17.31</td>
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<td><strong>π-cation</strong></td>
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<tr>
<td><strong>UNK-ARG669:NH2</strong></td>
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<td><strong>π-σigma</strong></td>
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<td><strong>UNK-PHE357:C</strong></td>
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<tr>
<td><strong>UNK-ARG669:OH</strong></td>
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<tr>
<td><strong>UNK-PHE357:CB</strong></td>
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</table>

*Table 7.3 (contd.)*
<table>
<thead>
<tr>
<th>Genistein contd.</th>
<th>UNK:H4-ARG358:O</th>
<th>UNK-ARG356:NH1</th>
<th>UNK-ARG358:NH1</th>
<th>UNK-ARG669:NH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>0.0006 ± 0.0004</td>
<td>3</td>
<td>-6.54</td>
<td>15.96</td>
</tr>
<tr>
<td>EGCG</td>
<td>10.21 ± 0.75</td>
<td>8</td>
<td>-4.39</td>
<td>604.9</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>4.65 ± 0.99</td>
<td>3</td>
<td>-3.96</td>
<td>1.25</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3.37 ± 0.14</td>
<td>3</td>
<td>-5.23</td>
<td>147.6</td>
</tr>
</tbody>
</table>

Table 7.3 (contd.)

1 IC$_{50}$ values were determined from at least two independent duplicates done in triplicate for each of the concentrations tested. Concentrations (μM) were calculated based on the molecular mass of each pure compound. Values are means ± SEM. Means with different letters in each column are significantly different for DPP-IV ($p < 0.05$). 2 $K_i$ values were obtained from computational docking as indicated in Materials and Methods section. 3 UNK refers to phenolic compound or diprotin A.

Of the sixteen effective phenolic compounds, three had IC$_{50}$ values higher than diprotin A (4.21 ± 2.01 μM) including eriocitrin (IC$_{50}$ value of 10.36 ± 0.09 μM), EGCG (10.21 ± 0.75 μM), and gallic acid (4.65 ± 0.1 μM). However, IC$_{50}$ values of the other thirteen compounds were lower than that of diprotin A, indicating that less of these compounds was needed to inhibit DPP-IV. These thirteen phenolics could be divided into three categories according to the results of statistical differences on their DPP-IV inhibitory effect: less active with high IC$_{50}$ values (1.31–3.37 μM), intermediate activity with IC$_{50}$ values of 0.24–0.74 μM, and very high activity with low IC$_{50}$ values (0.0006–0.17 μM). The phenolic compounds with high IC$_{50}$ values were
cyanidin, quercetin, and caffeic acid; the ones with intermediate activity were naringenin, hesperetin, cyanidin-3-glucoside, kaempferol, and malvidin. The four phenolics with very high activity included resveratrol, luteolin, apigenin, and flavone. IC$_{50}$ value of resveratrol had the highest DPP-IV inhibitory activity among all of the compounds tested ($P < 0.05$).

*Resveratrol and flavone inhibited DPP-IV activity in a competitive manner, while luteolin and apigenin inhibited noncompetitively*

To examine whether the most potent phenolic compounds, resveratrol, luteolin, apigenin and flavone, inhibited DPP-IV through interaction with the active site of the enzyme, we tested the enzyme kinetics. The inhibitory manner of the flavonoids was determined through generating a Lineweaver-Burk plot (Figure 7.1). As noted in Figures 7.1(a) and 7.1(d), both the slope and the $x$-intercept were changed by the addition of inhibitors, but there was no effect on the $y$-intercept. This is the definition of linear competitive inhibition. Therefore, resveratrol (Figure 7.1(a)) and flavone (Figure 7.1(d)) inhibited DPP-IV activity in a competitive manner. The values were calculated to be $0.2 \pm 0.01 \, \mu M$ for resveratrol and $18.6 \pm 0.3 \, \mu M$ for flavone. As for luteolin and apigenin (Figures 7.1(b) and 7.1(c)), both the slope and the $y$-intercept were changed by the added inhibitors, but there was no effect on the $x$-intercept. Therefore, luteolin and apigenin noncompetitively inhibited the enzyme, with $K_i$ values at $4.9 \pm 0.2 \, \mu M$ and $7.9 \pm 1.4 \, \mu M$, respectively.

*Diprotin A and natural phenolic compounds inhibit DPP-IV activity by binding tightly into the active site of the enzyme*

Binding pose of diprotin A, resveratrol and flavone in the DPP-IV active site is indicated in Figures 7.2 and 7.3, showing that these three compounds interact closely with key residues of sites S1, S2 and S3 within the active pocket.
Figure 7.1: Inhibition kinetics of porcine dipeptidyl peptidase-IV (DPP-IV) by resveratrol (a), luteolin (b), apigenin (c), and flavone (d). Different concentrations of the flavonoids (0, 5, and 10 μg/mL for luteolin, apigenin, and flavone and 0, 0.25, and 0.5 μg/mL for resveratrol) were incubated in the presence of various concentrations of Gly-Pro-AMC (0–60 μM) as substrate. Initial rates of the reaction were measured, and the results are expressed as a Lineweaver-Burk plot. Data are expressed as the mean of four independent experiments.

Figure 7.2: Key interactions of diprotin A (A₁, A₂) with active sites of DPP-IV enzyme. Binding of diprotin A (A₁, grey) in the DPP-IV active site is indicated (surface view: blue), wherein it interacts closely with key residues of active sites S1, S2, and S3. Residues with pink circles indicate hydrogen bond, or ionic or polar interactions; residues with green circles indicate Van der Waals interactions. The arrows indicate hydrogen bonds to side chain residues in blue and backbone residues in green.
Figure 7.3: Key interactions of resveratrol (A1, A2), flavone (A1, A3), luteolin (B1, B2), apigenin (B1, B3), quercetin (C1, C2), and genistein (C1, C3) with active sites of DPP-IV enzyme. Binding pose of resveratrol (A1, green) and flavone (A2, yellow) in the DPP-IV active site is indicated (surface view: blue), wherein two compounds interact closely with key residues of active sites S1, S2, and S3. Binding pose of luteolin (B1, green), apigenin (B1, yellow), quercetin (C1, green) and genistein (C1, yellow) in the DPP-IV binding site is indicated, wherein these flavonoids interact closely with the key residues of sites S2, and S3. Residues with pink circles indicate hydrogen-bond, or ionic or polar interactions, residues with green circles indicate Van der Waals interactions. The arrows indicate hydrogen bonds to side chain residues in blue and backbone residues in green.

Diprotin A is a potent DPP-IV inhibitor with Ile-Pro-Ile sequence commonly used as a reference compound. Figure 7.2 shows the binding mode of diprotin A with DPP-IV. The binding site of diprotin A is located at the S1, S2 and S3 sites (Figure 7.2(A1)). In the S2 site (Figure 7.2(A2)), the N-terminal amino group of diprotin A is hydrogen-bonded to the carboxyl oxygens of two Glu residues (Glu205 and Glu206). Furthermore, the N-terminal amino group forms a π interaction to the Tyr666. The carbonyl oxygen of Ile-1 of diprotin A forms an electrostatic interaction with Tyr662, Arg125, and Asn710 residues. Pro-2 of diprotin A is
located in the S1 site and forms a hydrophobic interaction with the phenol rings of Tyr666, and Tyr547. The carbonyl oxygen of Ile-3 of diprotin A also forms double hydrogen bonds to Tyr547 and Tyr666. In the S3 site, Van der Waals interactions are also seen between diprotin A and Ser209 and Phe357 residues of DPP-IV. These observations agree with the reported results obtained from X-ray crystal structure complex of DPP-IV and diprotin A [20].

The overlay of binding poses of resveratrol (green) and flavone (yellow) in the DPP-IV active site is shown in Figure 7.3(A). As observed in Figure 7.3(A1), resveratrol and flavone dock very well into all three active sites S1, S2, and S3 of DPP-IV. Resveratrol showed hydrogen bonding of 4′-OH-, 3′-OH-, and 5′-OH-group with hydroxyl of side chain of Ser630 (S1 pocket) and Ser209 (S3 pocket). Hydrogen bonds were also seen between 5′-OH of resveratrol, NH2-group of side chain of Arg 669 residues, and C=O groups of side chains of Glu206 (S2 pocket) (Figure 7.3(B2)). At the same time, electrostatic interactions were also observed between resveratrol and S1 pocket (His740, Tyr631, Ser630, His125), S2 pocket (Glu205, Glu206), S3 pocket (Ser209), and Arg669 of DPP-IV.

No hydrogen bonds were seen between flavone and amino acids in the pockets of DPP-IV (Figure 7.3(A3)). However, electrostatic interactions between flavone and amino acid residues in S1 pocket (Tyr547, Ser630, Asn710, and His740), S2 pocket (Arg125, Glu205), and Van der Waals interactions between flavone and amino acid residues in S1 pocket (Tyr631, Val656, Tyr666, Val711), S2 pocket (Glu206) and S3 pocket (Phe357), allowed flavone to anchor in the active sites of DPP-IV.

The overlay of binding poses of luteolin (green) and apigenin (yellow) in the DPP-IV active site is also shown in Figure 7.3(B). As shown in Figure 7.3(B1), luteolin and apigenin had almost identical binding modes with the active sites of DPP-IV with each having ring B and C
docked into sites S2 and S3. Three common features of binding with DPP-IV exist between both flavonoids (Figures 7.3(B2) and 7.3(B3)). Firstly, hydrogen bonds and π-interactions played important roles in docking both the flavonoids into the active pockets S2 and S3 of DPP-IV enzyme. In the S2 pocket, the B ring 5′-hydroxyl of luteolin formed a hydrogen bond with hydroxyl group of side chain of Ser209 (S3 pocket), while the 4′-hydroxyl group on B ring of apigenin forms a similar hydrogen bond within the S3 pocket. Luteolin also showed H-bonding by B ring 4′-hydroxyl with C=O groups of side chains of Glu205 (S2 pocket). Secondly, in the S3 pocket, both compounds formed a hydrogen bond of the C ring 1′-oxygen with the NH of Arg358’s guanidine side chain. H-bonding of the A ring 8′-hydroxyl with C=O groups of side chains of Glu361 favors strong binding of both flavonoids to the DPP-IV active site. A third common feature of both flavonoids was shown by π-cation interactions of ring B and the NH2 of Arg669. Additional features of each flavonoid added to their unique docking within the active site of DPP-IV. A hydrogen bond between the A ring 5′-hydroxyl of apigenin and the NH of Arg361’s guanidine side chain also enhanced the docking of apigenin and DPP-IV. For luteolin, a π-sigma interaction was also seen between the side chain of Phe357 and C ring of luteolin (S3 pocket).

Quercetin and genistein had a comparable binding position to luteolin and apigenin (Figure 7.3(c)). Hydroxyl groups in A and B rings were also important for quercetin and genistein to bind into the S2 and S3 sites (Figures 7.3(C2) and 7.3(C3)). At the same time, π-interactions between these flavonoids and Arg358 and Arg669 also contributed to the tethering of the two flavonoids to the active sites. All hydrogen bonds formed between phenolic compounds and DPP-IV are indicated in Table 7.3.
The binding energies obtained by computational docking analyses were compared among the compounds tested (Table 7.3). Gallic acid had the highest binding energy (−3.96 kcal/mol), while diprotin A had the lowest binding energy (−7.31 kcal/mol). IC_{50} values of the phenolic compounds that were found to inhibit DPP-IV activity correlated with their binding energies \((r = 0.67, p < 0.05)\). Both a lower IC_{50} value and lower binding energy indicate stronger inhibitory potency. The inhibition constant (K_i) obtained by computational docking analyses is also shown in Table 7.3. The K_i values of these phenolic compounds varied from 1.25 μM for gallic acid to 604.91 μM for EGCG. A highly significant correlation existed between K_i values and IC_{50} values \((r = 0.82, p = 0.0002)\). Significant correlations were also found between K_i values and binding energies \((r = 0.56, p < 0.05)\), and between K_i values and number of hydroxyl group \((r = 0.56, p < 0.05)\).

**Discussion and conclusions**

This study showed that ANC from berry wine and a variety of other phenolic compounds commonly present in fruits and vegetables had strong DPP-IV inhibitory effect *in vitro* and in silico. Computational docking analyses also showed for the first time that these natural phenolics could inhibit DPP-IV activity by binding tightly into the active sites of the enzyme. The biological activities, stability, and bioavailability of anthocyanins depend on their chemical structures. Blends were created to generate a mixture of potentially bioactive compounds commonly present in both blueberries and blackberries after fermentation, which can be optimized based on the characterization and potential benefit.

Previous studies on wine compounds and biological activity indicated that it is not the presence of a single compound that is responsible for beneficial effects such as antioxidant
capacity or ability to reduce inflammation, but rather involves several phenolic compounds. Major contributions are from compounds such as transresveratrol as well as minor contributions from cinnamic and hydroxycinnamic acids, cyanidin, and some phenolic acids [23]. The combination of these phenolic compounds within the blends produced from fermented blueberry and blackberry provided a unique potential for inhibition of DPP-IV. Therefore, while the inhibitory effects demonstrated by the anthocyanin-enriched blends are primarily due to the major anthocyanin components, the presence of other compounds also influenced the demonstrated potency.

In general, anthocyanins may protect beta-cells, increase the secretion of insulin, reduce the digestion of sugars in the small intestine, and thereby have multiple and simultaneous antidiabetic effects. Inhibitors of DPP-IV have been found to prevent pancreatic beta cell destruction in mice [24]. Extracts enriched in flavonoids have been seen to inhibit plasma DPP-IV [25].

The primary anthocyanin in the blackberry blends was delphinidin, which has previously shown potency to inhibit enzymatic activity of a glyoxalase I, which is being investigated as a target for prevention of cancer. Compared to other anthocyanins found in berries, (cyanidin and pelargonidin), delphinidin had the most potent DPP-IV inhibitory effect, suggesting the importance of interactions of the hydroxyl groups on the B ring of anthocyanins. Further, binding modes indicated that the hydroxyl groups located at the R1 position greatly contribute to inhibitory potency and specificity to the binding site [26]. This previous study, along with the results from our research, indicated that the anthocyanin delphinidin can form several hydrogen bonds to several amino acids due to its hydroxyl groups at R1 position.
Our previous study also showed that the blueberry-blackberry wine contained high amounts of total anthocyanin [17]. However, correlation was not seen between DPP-IV inhibitory effect and anthocyanin concentration in ANC fractions ($P > 0.05$) from berry wines. For example, ANC3, ANC4, and ANC5 from blackberry wine were of similar IC$_{50}$ values to inhibit DPP-IV, while the anthocyanin concentration was almost 4 times higher in ANC3 than in ANC4 and ANC5. Additionally, ANC4 and ANC5 had the same IC$_{50}$ values and ANC concentration, but their anthocyanin compositions differed. These results indicate that delphinidin-3-arabinoside, as the major anthocyanin identified in blackberry blends, could contribute to the DPPIV inhibition, however, other ANC may also play an important role in DPP-IV inhibition. Therefore, DPP-IV inhibitory effect of ANC could depend on not only the concentration but the composition and structures of flavonoids present. More research should be conducted to clarify the relationship between DPP-IV inhibitory effect and anthocyanin structure of ANC from berries.

Phenolic compounds are widely recognized for their ability to improve diabetic conditions by decreasing blood glucose levels [27]. It is interesting that most of the ANC fractions showed potent DPP-IV inhibitory activity, with the lowest IC$_{50}$ value from blackberry wine. Grape seed-derived procyanidins (GSPE) were also able to inhibit recombinant human DPP-IV activity, achieving around 70% inhibition at 200 mg/L of GSPE [12]. In order to compare with the GSPE, the percentage inhibition was given in this study as IC$_{50}$ values. The concentrations of all ANC from blackberry wine for achieving the same inhibitory effect on DPP-IV were less than 200 mg/L. Especially for ANC3 from blackberry wine, the concentration of 41.9 mg/L could lead to around 70% inhibition of DPP-IV activity. These results suggest that ANC from blueberry and blackberry wine have strong DPP-IV inhibitory activity. The efficacy
of the ANC to inhibit DPP-IV enzyme activity at a rate comparable to diprotin A and GSPE indicated that ANC may be able to act as naturally occurring DPP-IV inhibitors.

Many kinds of natural flavonoids exist in plants but only a few have been reported for DPP-IV inhibitory effect [12, 13]. In the present study of twenty-seven phenolic compounds commonly present in berries, citrus, soybeans, and other plant commodities, most flavonoids were determined to have DPP-IV inhibitory effect. It is interesting that most of the flavonoids tested in the present study showed lower IC₅₀ values and therefore were more potent than the reference inhibitor standard diprotin A. Resveratrol, luteolin, apigenin and flavone showed the most potent DPP-IV inhibitory activity due to their lowest IC₅₀ values. In particular, this study demonstrated that resveratrol was the most potent DPP-IV inhibitor with IC₅₀ value at 0.6 nM exhibiting even lower values than sitagliptin (18 nM) and vildagliptin (3.5 nM) [10], two current pharmacologic drug inhibitors of DPP-IV. A summary of current foods and food components in the prevention of diabetes by Thomas and Pfeiffer [16] has indicated that the potential evidence for phenolic compounds is not conclusive; however, resveratrol was found to have a beneficial effect on protecting beta cells, which may be due to its ability to modulate the activity of DPP-IV.

DPP-IV has three binding pockets/active sites (S1, S2 and S3). The specificity pocket S1 is composed of the side chains of catalytic triad (Ser630, Asn710, and His740), which are involved in strong hydrophobic interactions [10]. The cavity near Glu205, Glu206 and Tyr662 residues is referred to as the S2 pocket. The S3 pocket of DPP-IV consists of Ser209, Arg358, and Phe357 [21]. The outside position of the S3 pocket in DPP-IV allows larger groups access to the site; on the other hand, the inside position of the S3 pocket favors smaller groups [28]. The four most potent compounds, resveratrol, luteolin, apigenin and flavone, had low Kᵢ values to
inhibit DPP-IV, which indicated that they had high affinity to the active sites of DPP-IV. The kinetic analysis showed that resveratrol and flavone inhibited DPP-IV activity in a competitive manner, while luteolin and apigenin were in a noncompetitive manner. Further computational docking analyses are consistent with the tested inhibitory manner of the phenolic compounds. Docking analysis showed that resveratrol and flavone bound well into all the three sites S1, S2 and S3 of DPP-IV, while luteolin and apigenin could only bind into S2 and S3 pockets. Although luteolin and apigenin could dock into S2 and S3 pockets, the kinetic analysis showed that they inhibited DPP-IV in a noncompetitive manner. We presume that the binding of luteolin and apigenin into S2 and S3 may lead to DPP-IV conformational changes, or changes in the side chain of amino acid residues of DPP-IV, and the catalytic activity will be decreased when the substrate is also bound.

We found that apigenin had a similar effect as resveratrol to directly inhibit DPP-IV activity, and genistein also exhibited a potent DPP-IV inhibitory effect. In the present study, most of the glycosylated flavonoids with two sugar groups, including naringin, rutin, narirutin, hesperidin, and neohesperidin, had no DPP-IV inhibitory effect. One explanation is that conjugation of bulky sugar groups to the flavonoid core structure could sterically hinder binding to the active sites within DPP-IV, thus resulting in no inhibitory capacity of the tested flavonoids. The computational docking analyses further supported this phenomenon. However, cyanidin-3-glucoside, which has been identified as the major ANC in different blackberry species [29], showed no statistical difference (P > 0.05) on DPP-IV inhibitory activity (IC$_{50}$, μM) than cyanidin (IC$_{50}$, ) and malvidin (IC$_{50}$, ). Considering ANC-enriched fractions from blueberry and blackberry wines contain a mixture of flavonoids with only one sugar group,
flavonoids with monosaccharide groups may have better DPP-IV inhibitory effects than flavonoids with more sugar groups due to less steric hindrance.

Flavone, luteolin, and apigenin have the same flavone core structure. However, flavone could dock into all three active sites of DPP-IV, while luteolin and apigenin could dock into only two of them. Computational docking showed comparably strong binding of luteolin and apigenin due to hydrogen bonds of ring B hydroxyls with residue Ser209 in the S3 pocket, for ring C 1′-oxygen with the NH of guanidine side chain of Arg358 in the S3 pocket, and for ring A 8′-hydroxyls with C=O groups of side chains of Glu361. These features also exist in the binding of other citrus flavonoids (including kaempferol, quercetin, hesperetin, and naringenin) to DPP-IV, which have the same flavone core structure. Even the binding of genistein, a soy isoflavone, to DPP-IV also had these features. Therefore, hydroxyls in these flavonoids are important to dock into active sites of DPP-IV with the same binding modes. Furthermore, the formation of π-interaction between A or B ring of citrus flavonoids and Arg669 or Arg358 also favors the binding of citrus flavonoids into S2 and S3 sites. Flavone has no hydroxyl residues capable of hydrogen bonding with residues in S2 and S3 pockets with the same binding modes as flavonoids like luteolin. Therefore, although it could dock into all the three pockets of DPP-IV, flavone had a higher $K_i$ value due to absence of hydroxyl groups.

Significant correlations were seen between IC$_{50}$ values of these flavonoids and their binding energies and values determined computationally in the present study. In the docking studies, if a compound shows lower binding energy compared to the standard, it proves that the compound has higher activity [30]. These results indicated that more negative binding energy and smaller $K_i$ result in tighter binding, and then more potent inhibitory effect. Meanwhile, a significant correlation also exists between the $K_i$ values determined in silico and the number of
hydroxyl groups of flavonoids (r = 0.56, p < 0.05), which indicated that more hydroxyl groups of flavonoids can result in higher inhibition constant and therefore higher IC₅₀ value, indicating less affinity to bind the active site. This could explain why quercetin with five hydroxyls has a higher IC₅₀ value (less potent) than the other citrus compounds, despite sharing the same flavone core structure. IC₅₀ values of citrus compounds were also found to be significantly correlated with their numbers of hydroxyls.

We obtained Kᵢ values using the computational analyses as well as experimentally. Kᵢ values determined with the computational analyses were calculated from the binding energy. However, the binding energy is designed to score and rank conformations of ligand and protein and not designed to give accurate binding energy. Therefore, Kᵢ values generated from Autodock correlated with free binding energies significantly (r = 0.56, p < 0.05) but differed from the experimental Kᵢ values.

In conclusion, our study demonstrated that ANC isolated from blueberry-blackberry wine blends and a variety of other phenolic compounds commonly present in citrus, berry, soy, and other plants could strongly inhibit DPP-IV activity. Resveratrol and flavone were competitive inhibitors which could dock into all the three active sites, while luteolin and apigenin bound to DPP-IV in a noncompetitive manner. Results obtained from this study further support the efficacy of flavonoids as naturally occurring DPP-IV inhibitors.

**Conflict of interest**
The authors have declared no conflict of interest.

**Acknowledgments**
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References


CHAPTER 8

ANTHOCYANINS FROM FERMENTED BERRY BEVERAGES INHIBIT INFLAMMATION-RELATED ADIPOSYT RESPONSE IN VITRO

Abstract

Increased adiposity has been associated with macrophage infiltration into the adipose tissue, which in turn leads to obesity co-morbidities, including type 2 diabetes. The objective of this study was to evaluate the effect of anthocyanin (ANC)-enriched fractions from blackberry: blueberry beverages on inflammation and adipogenesis in an in vitro model of inflammation mimicking the pathologic interaction between adipocytes and macrophages. Blends ANCs inhibited secretion of nitric oxide (17.5%), TNF-α (89.4%) and phosphorylated p65 NFkB (52.1%) in lipopolysaccharide-induced RAW 264.7 macrophages after 24 h. Blends reduced intracellular fat accumulation (28.2%) when applied during 3T3-L1 adipocyte differentiation, and inhibited isoproterenol-induced lipolysis (18.6%) of mature 3T3-L1 cells. In addition, Blend ANCs restored adiponectin-blunted gene expression induced by TNF-α treatment (18.2%), and reduced the glycerol release (15.9%) induced by LPS-induced macrophage conditioned media (CM) in adipocytes. Furthermore, blends slightly restored the insulin-induced glucose uptake of adipocytes, blunted by the CM treatment. In conclusion, anthocyanins from blueberry and blackberry dealcoholized fermented beverages are potential inhibitors of inflammation-related adiposity response and sensitizers of insulin signaling in adipocytes.

Keywords: adiposity, anti-inflammatory, anthocyanins, diabetes, fermentation.

10 (Publication: Garcia-Diaz D, Johnson MH, de Mejia EG. (2015) Anthocyanins from blueberry-blackberry fermented blends inhibit the pathogenic interaction between adipocytes and macrophages. Journal of Medicinal Foods 18(4):489-96. doi:10.1089/jmf.2014.0039.) Reprinted with permission from Mary Ann Liebert, Inc. My roles were to prepare and provide the anthocyanin blends for cell culture, to conduct the detection of secreted inflammatory proteins with Western blot and ELISA, assist with the analysis and interpretation, and help to prepare and revise the manuscript.
**Introduction**

Obesity is an important health issue negatively affecting our society [1]. Excessive body fat accumulation initiates several associated clinical manifestations, such as type 2 diabetes (T2D) and cardiovascular disease, among others [2].

Obesity is often accompanied by a low-grade inflammation in the adipose tissue [3]. Adipokines, cytokines, and other factors produced by this tissue during adipose hyperplasia could be responsible for the presence and prevalence of inflammation [4]. Several inflammatory proteins derived from adipose tissue, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and nitric oxide (NO) correlates with increased body adiposity [5]. This may lead to inflammatory macrophage infiltration into the adipose tissue. Since the crosstalk between macrophages and adipocytes may negatively influence cellular insulin sensitivity [6], this consequently aggravates the obesity state [7]. Therefore, a reduction in inflammatory status by anti-inflammatory agents could constitute a potential approach to reduce adverse obesity-associated consequences.

Dietary flavonoids have been linked to anti-inflammatory pathways [8], and several berries or berry compounds have been investigated for their anti-inflammatory properties [9-11]. Blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus spp.*) are important sources of anthocyanins; water-soluble pigments responsible for the colors of fruits and vegetables [12]. It has been reported that the consumption of these phytochemicals exerts several positive health effects, particularly due to their high antioxidant capacity [8,13]. Research has shown that fermented juices and wines from berries inhibit inflammatory protein production or expression more than their unfermented counterparts [14-17]. The role of phenolic compounds, such as anthocyanins, in the interaction between the adipose and inflammatory state is not yet fully
understood. Therefore, it is important to identify and evaluate specific bioactive phenolic compounds from fermented berry beverages on obesity-related inflammation.

The aim of the present study was to evaluate the effect of anthocyanins extracts, from combinations of blueberry-blackberry dealcoholized fermented beverages, on the levels of markers of inflammation and adipogenesis in a dual cellular system, mimicking the pathogenic interaction between adipocytes and macrophages. The hypothesis was that the addition of antioxidant agents such as anthocyanins would lead to a reduction in the inflammatory crosstalk between macrophages and adipocytes and thus improve insulin-sensitivity as a potential approach to improve obesity-associated adverse consequences.

Materials and methods

Materials

Enriched ANC fractions stored from previous work of our group were utilized for all experiments [18]. The rationale for using fermented blends ANCs was that fermentation has been seen to increase the phenolic content of fruit juices and their anti-inflammatory effects [14-17]. These fractions were obtained from the following blends of blackberry (Bck) and blueberry (Blue) wines: 100%Bck:0%Blue, 70%Bck:30%Blue, and 30%Bck:70%Blue. These blend ANCs were selected based on their high antioxidant and anti-inflammatory properties and chromatographic results at both 520 nm and 280 nm to determine composition of both anthocyanins and other phenolic compounds [18]. The blends were made from wines produced from Highbush blueberries (Vaccinium corymbosum) and blackberries (Rubus spp.) grown at Dixon Springs Agricultural Center in Simpson (Illinois), and fermented with Saccharomyces bayanus, as previously described [18,19]. One hundred µM cyanidin-3-glucoside (C3G) equivalents were used for cell culture.
Murine macrophage RAW 264.7 and pre-adipocyte 3T3-L1 cell lines and Dulbecco’s Modified Eagle Medium with L-glutamine (DMEM) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Fetal bovine serum and bovine serum were from Invitrogen (Grand Island, NY, USA).

Rabbit polyclonal antibody (p-p65 NFκB) and mouse monoclonal antibody (actin) and their control lysates were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse IgG horseradish peroxidase conjugated secondary antibodies were from GE Healthcare (Buckinghamshire, UK).

**Cell culture**

Mouse pre-adipocytes 3T3-L1 and RAW264.7 mouse macrophages (ATCC, Rockville, USA) cell lines were maintained at 37 ºC in a humidified atmosphere containing 5% CO₂. Experiments were conducted as follows:

I. RAW264.7 mouse macrophages were seeded in 6-well plates at 70,000 cells/cm² density, pre-treated with each blend ANC (100 µM C3G) for 1 h, and then stimulated with lipopolysaccharides (LPS; 1 µg/mL) for 24 h. Cells were collected and processed immediately according to western blot sample preparation. Supernatants were collected and stored at -80ºC for their use as conditioned media (CM) or until secretion analyses were performed.

II. Pre-adipocytes 3T3-L1 cells were cultured in 6-well plates in DMEM containing 4.5 g/L glucose and 10% calf serum. Two days after full confluence, cells were differentiated by incubation with 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, and 10 µg/mL insulin in 4.5 g/L mM glucose DMEM supplemented with 10% fetal bovine serum, using a

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11 For this publication, Dr. Diego Garcia was responsible for the initial design of the co-culture experiment, cell culture maintenance and conducted all of the experiments involving the adipocytes.
previously described protocol [20]. To determine the impact on adipogenesis, blend ANCs were added at each media re-feed until adipocytes reached 8-10 days of differentiation. Fully differentiated adipocytes were fixed and stained for triglyceride determination as described below, or were incubated for 24 h in the presence or absence of 10 nM isoproterenol (IP) and each blend ANC for lipolysis assays. IP was used as a model of catecholamine-induced lipolysis. Cells were collected for gene expression assays and supernatants were collected and stored at -80ºC for glycerol determination.

III. Fully differentiated adipocytes were incubated for 24 h in presence or absence of macrophage CM (obtained as described in I) for 24 h or 4 ng/mL TNF-α (Sigma-Aldrich Company, St. Louis, USA). Cells were pre-treated for 1 h with each blend ANCs and then incubated with TNF-α. Cells and supernatants were collected and stored at -80ºC until further analysis for glycerol release, gene expression and secretion of MCP-1 and adiponectin.

IV. Fully differentiated adipocytes were incubated for 96 h (replacing media every 24 h) with CM (obtained as described in I) for evaluating modulation of insulin-sensitivity (by incubating with 100 nM insulin). Supernatants were collected and stored at -80ºC for determination of glucose uptake.

Cell viability assay

Cell viability was measured with the lactate dehydrogenase (LDH) Cytotoxicity Assay at 24 h according to manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, USA).

Western blot

Western blotting was performed by loading 20 µg protein from cell lysates in 4–20% Tris–HCl gels (BioRad, Hercules, USA) for SDS-PAGE as described previously [18]. Proteins were transferred to a PVDF Hybond-P membrane (GE Healthcare, Buckinhamshire, UK),
blocked, washed, and subsequently incubated with either p-Akt or p-p65 NFκB primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were washed again and incubated with anti-mouse (for p-Akt) or anti-rabbit (for p-p65 NFκB) IgG horseradish peroxidase conjugate secondary antibody. The expression of proteins was visualized using chemiluminescent reagent (GE Healthcare, Pittsburgh, PA) following manufacturer’s instructions using a GL 4000 Pro Imaging system (Carestream Health Inc., Rochester NY).

**Adipogenesis assay**

Adipogenesis was determined by measuring triglyceride accumulation through Oil-Red-O staining, according to a modified protocol [21]. Briefly, the staining solution was prepared by dissolving Oil-Red-O (Sigma-Aldrich, St. Louis, USA) in isopropanol (0.5% w/v), then filtered and diluted 40% with distilled water. Adipocyte layers were washed with PBS, fixed with 2.7% formaldehyde, washed with 60% isopropanol, allowed to dry, and then stained for 30 min with Oil-Red-O solution. Wells were washed 4 times with distilled water before quantifying the accumulated lipid by eluting the stain from cells with 100% isopropanol and reading the absorbance at 540 nm.

**Glycerol release**

Glycerol production, as a measure of adipocyte lipolysis, was calculated as the increase of glycerol in the medium from adipocytes after 24 h using a glycerol assay (RANDOX Laboratories, Crumlin, United Kingdom) according to manufacturer indications.

**Nitric oxide release measurement**

The amount of nitrite in supernatant was measured using Griess reagent according to manufacturer’s protocol (range of standards = 0.43-65 µM Nitrite) (Sigma-Aldrich Company, St. Louis, USA).
Gene expression assays\textsuperscript{12}

All procedures were as previously described with modifications [20]. Briefly, total RNA was isolated from samples using the RNeasy Mini Kit according to manufacturer instructions (QIAGEN, Valencia, USA). First strand cDNA was then obtained using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). The resultant cDNA was amplified with specific probes for mouse MCP-1 and adiponectin (ADIPOQ) in a total volume of 10 μL. Real-time PCR was performed in an ABI PRISM 7000 HT Sequence Detection System following manufacturer's recommendations: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s, and 60 ºC for 1 min. RT-PCR products were analyzed with the SDS 2.3 and the RQ Manager 1.2 software (Applied Biosystems). Expression levels of target genes studied (FAS, HSL, MCP-1, ADIPOQ) were normalized with the expression of cyclophilin as the selected internal control (probe supplied by Applied Biosystems). Fold change between groups was calculated using the $2^{-\Delta\Delta Ct}$ method.

MCP-1, adiponectin and TNF-α secretion analyses

MCP-1 secretion into culture media was measured following manufacturer’s instructions using the MCP-1 Mouse ELISA Kit (Invitrogen, Grand Island, NY, USA), adiponectin using the Adiponectin Mouse ELISA Kit (Invitrogen) and TNF-α using the TNF alpha Murine ELISA Kit (Abcam Inc., Cambridge, USA).

Glucose uptake\textsuperscript{13}

Glucose utilization by cells was assessed by measuring the concentration of glucose in the medium with the Autokit Glucose from Wako Chemicals (Richmond, USA). After 100 nM

\textsuperscript{12} Dr. Diego Garcia conducted the RNA extraction and gene expression assays.

\textsuperscript{13} Dr. Diego Garcia conducted the glucose uptake assays.
insulin treatment for 24 h, the amount of glucose in the medium was then subtracted from the initial concentration of glucose with previously reported calculations [22].

**Statistical analyses**

Results are expressed as mean ± standard deviation (SD). All data were evaluated using one-way ANOVA, followed by Tukey’s post-hoc test. Association analyses were performed using the Pearson correlation coefficient. Significance was determined at p < 0.05. Statistical analyses were performed using the GraphPad Prism Software 6.0 (GraphPad Software Inc., San Diego, USA).

**Results**

**Inhibition of macrophage activation**

The 100% Bck and 30%Bck:70%Blue blend ANCs treatment effectively reduced LPS-induced macrophage activation as measured by NO release (Figure 8.1A). Likewise, LPS-induced TNF-α release was also significantly inhibited compared to the LPS-stimulated control in these same treatment groups (Figure 8.1B), with the greatest reduction by the 30%Bck:70%Blue treatment (90%). NO and TNF-α release were highly correlated (r = 0.897, p < 0.001). Moreover, the expression of the p-p65 subunit of nuclear factor kappa-B (NFκB) was decreased due to treatments with all blend ANCs in comparison to the non-treated LPS-stimulated control (53.4 ± 25.6%, 47.9 ± 19.5%, 56.8 ± 33.2% for 100%Bck, 70%Bck:30%Blue and 30%Bck:70%Blue, respectively, vs 100% LPS control group) (Figure 8.1C). The analysis of the relative expression of p-Ser473-Akt is presented in Figure 8.1D; no statistical differences were observed between LPS-control and blend ANCs treated groups.
Figure 8.1. LPS-stimulated macrophage response. The level of macrophage activation by LPS (1 µg/mL) treatment was determined by NO release (A), TNF-α secretion (B), relative p-p65 expression (C), and relative p-Akt expression (D) after 24 h, in the presence or absence of each blend ANCs (100 µM C3G equivalents). Data (n = 3–6) are expressed as mean – SD. One-way ANOVA followed by Tukey’s post hoc test was performed to identify statistical differences. Different letters represent statistical differences of at least p < 0.05. ANC, anthocyanin; Bck, blackberry; Blue, blueberry; C3G, cyanidin-3-O-glucoside; C, Control; LPS, lipopolysaccharide; NO, nitric oxide; phosphorylated-p65, p-p65; SD, standard deviation; TNF-a, tumor necrosis factor-α.
Modulation of fat accumulation in adipocytes

The blend ANCs were tested for modulation of triglyceride accumulation in adipocytes during the differentiation period (Figure 8.2A). Cells that were treated with 30%Bck:70%Blue blends ANCs had higher lipid accumulation after 14 days than the differentiated control group (128.2 ± 12.9% vs. 100 ± 2.5%). Blend ANCs were then analyzed for their effect on IP-induced lipolysis of adipocytes as measured by amount of glycerol released (Figure 8.2B). As expected, IP treatment caused higher glycerol release. Blend ANCs treatment following IP treatment caused a protective effect, with significant reduction of glycerol release by 100%Bck and 30%Bck:70%Blue (85.3 ± 4.3% and 81.4 ± 6.3%, respectively, vs 100.0 ± 3.2% IP group). No significant differences were observed among groups regarding IP-induced FAS mRNA expression (Figure 8.2C). In addition, treatment with the 30%Bck:70%Blue blend showed highest HSL gene expression in IP-induced adipocytes (124.9 ± 13.2% vs. 100.0 ± 0.9% IP group) (Figure 8.2D).

Figure 8.2. Modulation of adipocyte fat accumulation. (A) The level of lipid accumulation was determined at the final stage of differentiation (8-10 days) of adipocytes that had previously been treated daily with each blend ANC (100 µM C3G equivalents) since day 1. Glycerol release (B), and FAS (C), and HSL gene expression (D), were determined in fully differentiated adipocytes after a 24 h treatment with isoproterenol (IP) (10 nM), in presence or absence of each blend ANC. Data (n=3–6) are expressed as mean ± SD. One-way ANOVA followed by Tukey’s post-hoc test were performed to identify statistical differences. Different letters represent statistical differences of at least p < 0.05. C, Control; D, Differentiated; IP, isoproterenol; Bck, Blackberry; Blue, Blueberry.
Figure 8.2 (contd.)

Figure 8.3 shows modulation of lipolysis in adipocytes treated with TNF-α for 24 h, measured as glycerol release. A slight but not statistically significant trend toward inhibition of TNF-α induced lipolysis was observed by treatment with 30%Bck:70%Blue blend ANCs (90.7 ± 4.5% vs. 100.0 ± 6.6% TNF-α group), (Figure 8.3A). Adipocytes were also treated for 24 h with CM from macrophages that were previously treated with LPS and/or each blend ANCs. Treatment with 100%Bck and 70%Bck:30%Blue blends significantly reduced the LPS-induced glycerol release (86.1 ± 1.2% and 84.1 ± 3.7%, respectively, vs. 100.0 ± 2.7% CM-LPS group) (Figure 8.3B).

Figure 8.3. Modulation of lipolysis by inflammatory inducers and fermented blend ANCs of fully differentiated adipocytes. The lipolysis modulation, measured by glycerol release, was determined after 24 h treatment in fully differentiated adipocytes by 4 ng/ml TNF-α (A) and activated macrophage conditioned media (CM-LPS) (B), in presence or absence of each blend ANC (100 µM C3G equivalents). Data (n=3-6) are expressed as mean ± SD. One-way ANOVA followed by Tukey’s post-hoc test were performed to identify statistical differences. Different letters represent statistical differences of at least p < 0.05.
Inhibition of inflammatory processes involved in obesity

Figure 8.4 presents the effects of TNF-α and blend ANCs treatments on MCP-1 and adiponectin mRNA expression and protein secretion by adipocytes. Regarding MCP-1 gene expression, surprisingly 70%Bck:30%Blue group presented significant higher mRNA expression (p < 0.05), compared to control (108.8 ± 5.8% vs. 100.0 ± 3.4% TNF-α group) (Figure 8.4A). Both gene expression and secretion of MCP-1 were highly correlated (r = 0.919; p < 0.001); however, there was no significant difference detected for MCP-1 secretion (Figure 8.4B). Treatment with the 30%Bck:70%Blue blend ANCs induced higher ADIPOQ gene expression than TNF-α treated cells (118.2 ± 9.7% vs. 100.0 ± 8.3% TNF-α group) (Figure 8.4C). Although an important significant correlation was observed between the gene expression and secretion of this adipokine (r = 0.722, p < 0.001); however, when adiponectin secretion was evaluated, no differences were observed among TNF-α treated groups (Figure 8.4D). The same analysis was performed utilizing CM from macrophages previously treated with LPS and/or each berry blend; no differences were observed among groups (data not shown).

Effects on insulin sensitivity of adipocytes

Glucose uptake of adipocytes after a chronic treatment of macrophage CM-LPS is presented in Figure 8.5. As expected, insulin (INS) induced higher glucose uptake. LPS treatment reduced the capability of INS to induce glucose uptake in adipocytes. While blend ANCs did not have an insulin-sensitizing effect (p > 0.05), from either INS or LPS groups; however, when independent t-test was performed among specific groups, significant differences were observed between INS control group (100.0 ± 9.5%) vs. INS-CM-LPS (56.0 ± 23.1%) (p < 0.05), vs. INS-CM-LPS 70%Bck:30%Blue (67.9 ± 10.6%) (p < 0.05), and vs. INS-CM-LPS 30%Bck:70%Blue (64.0 ± 7.7%) (p < 0.01).
Figure 8.4. Gene expression and secretion of inflammatory markers after the blend ANCs treatment. mRNA expression (A) and secretion (B) of MCP-1, and mRNA expression (C) and secretion (D) of adiponectin were measured in adipocytes after 24 h of treatment with 4 ng/ml TNF-α and/or each fermented blend (100 µM C3G equivalents). Data (n=3-6) are expressed as mean ± SD. One-way ANOVA followed by Tukey’s post-hoc test were performed to identify statistical differences. Different letters represent statistical differences of at least p < 0.05. C, Control; Bck, Blackberry; Blue, Blueberry. MCP, monocyte chemoattractant protein-1.
Figure 8.5. Insulin sensitivity as determined by glucose uptake of adipocytes. Fully differentiated adipocytes were treated with activated macrophage conditioned media (CM-LPS) and/or each blend ANC (100 µM C3G equivalents) for 4 days. After this period, the glucose uptake for 24 h was determined by measuring the concentration of glucose in the medium as induced by 100 nM insulin, and then subtracting it from the initial concentration of glucose. Data (n=3-6) are expressed as mean ± SD. One-way ANOVA followed by Tukey’s post-hoc test were performed to identify statistical differences. Different letters represent statistical differences of at least p < 0.05. C, Control; INS, insulin; CM, conditioned media; LPS, lipopolysaccharides; Bck, Blackberry; Blue, Blueberry.

Discussion

In the current study, ANCs from dealcoholized fermented blueberry-blackberry beverage blends were applied to macrophages and adipocytes in order to determine their potential to counteract the interaction between these two cells in order to reduce the self-perpetuating cycle of inflammation and obesity.

Our results show inhibition of NO and TNF-α secretion from macrophages following treatment with 30%Bck:70%Blue ANC, which consists of C3G as its major anthocyanin [18]. NO and TNF-α secretion were highly correlated, consistent with LPS-activation of macrophages. The fact that the 70%Bck:30%Blue and 100%Bck treatments did not reduce TNF-α secretion may be due to their anthocyanin composition; these blends contain mainly delphindin-3-
arabinoside, with minor amounts of C3G [18]. Previous studies show that C3G is related with down-regulation of pro-inflammatory cascades [23]. A decrease in the phosphorylation of p65 NFκB in macrophages was also observed. In this regard, polyphenols such as resveratrol have been able to reduce the phosphorylation of p65 NFκB in adipocytes [24]. Moreover, C3G has been reported to have the capability to inhibit LPS-induced expression of inflammatory mediators through decreasing I-kappa-B-alpha phosphorylation in human macrophages [23]. No differences were observed regarding the relative expression of p-Ser^{473}-Akt among LPS-treated groups, suggesting that the inflammatory mechanism of activation is most probably not driven through the PI3K pathway. These previous reports, along with the results of the present work, indicate that phenolic compounds from berry fruits have the ability to reduce the low grade inflammatory state associated with obesity due to inhibition of the NFκB-mediated pathway in both macrophages and adipocytes.

A previous study using phenolic-enriched extracts from berries showed their ability to inhibit adipogenesis, and that the highest inhibition of lipid accumulation was observed in adipocytes treated with proanthocyanidin-enriched fraction from *Vaccinium floribundium* [21]. Another study indicated that fermented blueberry juice decreased triglyceride accumulation to levels comparable to the control; however, the non-fermented juice did not affect adipogenesis [17]. On the other hand, the higher triglyceride content induced by the 30%Bck:70%Blue ANC treatment in our study could be related to an upregulation of PPAR-γ activity, since it is a key molecule involved in adipogenesis [25] and inhibitor of NFκB signaling [26].

In obese subjects, visceral adipose tissue lipolysis is known to be upregulated [27]. The increase in free fatty acids (FFA) levels has been directly correlated with the establishment of insulin-resistance. Even though the present study was *in vitro*, some blend ANCs were able to
modulate the fat accumulation in the adipocytes by exerting a protective effect on glycerol release. This fact is remarkable regarding adipose tissue inflammation; less FFA release has potential to cause a reduction in the insulin-resistant environment. C3G has been previously related with anti-lipolytic effect in 3T3-L1 cells via decreased expression of adipose triglyceride lipase [28]. Furthermore, it has been described that anthocyanin extracts (68.3% cyanidin-3-O-glucoside, 25.2% delphinidin-3-O-glucoside, and 6.5% petunidin-3-O-glucoside) from black soybean inhibit basal lipolysis [29]. As expected, no effects of IP treatment regarding FAS and HSL expression were observed, since both proteins are post-transcriptionally activated by catecholamines [30]. Our experiment found that only IP and 30%Bck:70%Blue ANC increased HSL expression. This apparently contradicts the obtained lipolysis results, however, this contradiction is explained by post-transcriptional modifications of HSL following stimuli. Finally, the fact that only delphinidin-enriched ANCs inhibited CM-LPS induction of glycerol release indicated prevention of lipolysis by a specific ANC in a more physiological model of obesity-related inflammation.

It has been reported that adiponectin production is reduced in subjects with visceral fat accumulation [31]. Hypoadiponectinemia induced by visceral fat accumulation has also been closely associated with T2D, lipid disorders, hypertension and certain inflammatory diseases [31]. ANC from dealcoholized fermented beverages did not inhibit MCP-1 induction in TNF-α-induced or LPS-induced-CM adipocytes. However, TNF-α-induced ADIPOQ gene expression inhibition was significantly reduced by 30%Bck:70%Blue ANC treatment. This result correlated significantly with the TNF-α-induced glycerol release. To our knowledge, this is the first time that a C3G-enriched berry blend from a fermented and dealcoholized beverage has been related
to the reduction of the blunted effect of adiponectin gene expression caused by an inflammatory inducer.

Our results indicate that glucose uptake was stimulated following insulin treatment. INS-CM-LPS effectively reduced glucose uptake, thus adipocytes were significantly insulin-resistant. The fact that no significant difference was observed between INS and INS-CM-LPS 100%Bck, indicate that this treatment induced some insulin-sensitizing improvement. In a previous study it was observed that amelioration of glucose uptake in the presence of insulin by blueberry juices was highest at 6 h following LPS-stimulation [17]. In this regard, ANC interventions have potential to induce reduction of T2D incidence through modulation of insulin sensitivity and glucose utilization [32].

In conclusion, anthocyanin enriched fractions from blueberry-blackberry dealcoholized fermented beverages presented a significant positive effect on an in vitro adipose tissue inflammatory model, in relation to several markers of adipogenesis and inflammation. Accordingly, more research is needed in order to unveil the promising features of these fermented beverages and their bioactive compounds in vivo on inflammatory-related insulin-sensitivity.

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Disclosure statement

No competing financial interests exist.
References


CHAPTER 9

ANTHOCYANINS FROM FERMENTED BERRY BEVERAGES INCREASED INSULIN SECRETION FROM PANCREATIC Β-CELLS IN VITRO BEYOND THEIR ABILITY TO REDUCE DIPEPTIDYL PEPTIDASE IV

Abstract

Berries are a rich source of phenolic compounds that can inhibit the enzyme dipeptidyl peptidase IV (DPP-IV), a target for type-2 diabetes therapy. The objectives were to determine the role of berry phenolic compounds to modulate incretin-cleaving hormone DPP-IV and its substrate GLP-1 in order to increase insulin secretion from pancreatic β-cells, and to investigate the modulation of genes and proteins involved in insulin signaling pathway. iNS-1E pancreatic β-cells were used to elucidate the role of phenolic compounds to act as insulin secretagogues both directly and following simulated absorption in a dual-cell system with Caco-2 cells. Anthocyanins (ANC) from 50%blueberry- 50%blackberry (Blu-Bla) and 100% blackberry (Bla) fermented beverages at 50 µM cyanidin-3-glucoside equivalents were able to increase glucose-stimulated insulin secretion directly and after Caco-2 transport. ANCs increased the protein expression of IGF-II, IGFBP-2 and 3, and VEGF in iNS-1E cells. ANC50%Blu-Bla and ANC100%Bla up-regulated incretin hormone gene for GLP-1 and genes in the insulin secretory pathway including iGF1R. PTP1B gene expression was down-regulated by ANC100%Bla, a type-2 diabetes therapeutic target. In conclusion, anthocyanins from fermented berry beverages have the potential to increase insulin secretion due to reduced DPP-IV expression and up-regulated expression of mRNA of insulin-receptor associated proteins.

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**Abbreviations:** ANC, anthocyanin; 100%Bla, 100% blackberry fermented beverage; 50%Blu-Bla, 50%blueberry-blackberry fermented beverage; DPP-IV, dipeptidyl peptidase IV; EMEM Eagle’s Minimum Essential Medium; GLP-1, glucagon-like peptide-1; GSIS, glucose stimulated insulin secretion; KRBH, Krebs-Ringer-bicarbonate-HEPES buffer; PAC, proanthocyanidins; RLU, relative light units, Res, resveratrol; Sitag, sitagliptin; TEER, transepithelial electrical resistance; VB, VitaBlue®

**Keywords:** anthocyanin, diabetes, DPP-IV, insulin, pancreatic β-cell

**Introduction**

Type-2 diabetes is a serious metabolic disease that affects over 9.3% of the United States (U.S.) population, with 1.7 million new cases diagnosed in 2012 [1]. The inability to control blood glucose increases the risk of serious complications such as heart disease, stroke, blindness, kidney failure, and lower limb amputations. As a result, diabetes was the 7th leading cause of death in the U.S. in 2010. The economic burden of diabetes in the U.S. is staggering with $176 billion for direct medical costs and $69 billion due to reduced productivity in 2012 [1]. One strategy to manage type-2 diabetes is to increase insulin secretion. The incretin hormone glucagon-like peptide-1 (GLP-1) is able to stimulate insulin release from pancreatic β-cells; however, it is rapidly degraded to inactive metabolites by dipeptidyl peptidase-IV (DPP-IV) [2]. DPP-IV is a serine aminopeptidase, a current target for type-2 diabetes therapy since its inactivation by drugs or natural compounds prevents the degradation of GLP-1 [3]. Our previous results using computational and enzymatic studies indicated that anthocyanins (ANC) are strong inhibitors of purified DPP-IV [4]. However, there is lack of information on the efficacy of ANC from fermented berries to inhibit DPP-IV in β-cell function.

In addition to fiber, minerals, and vitamins, blueberries and blackberries are rich sources of polyphenolic ANC as well as proanthocyanidins (PAC), the polymerized forms of ANC and other phenolic compounds like epicatechin [5-7]. Berry fermentation increases contact with berry
skins rich in phenolic compounds thus becoming a viable way to increase the phenolic content of berry juice products, and consequently increasing the potential for health benefits [8]. Clinical studies have shown the potential of bioactives from blueberries to improve insulin sensitivity or insulin resistance [9, 10]. Furthermore, an increased consumption of ANC and ANC-containing foods results in decreased risk for type-2 diabetes [11]. Nevertheless, the role of berry phenolics to increase insulin secretion is not fully understood.

Current cell-based assays used to investigate β-cell function lack mechanistic data needed to translate and promote new interventions to improve insulin secretion. Human-derived Caco-2 cells are a well-studied model of epithelial transport and are commonly used for permeability, transport, or absorption studies, and in addition they express DPP-IV [12-14]. Rat insulinoma iNS-1E β-cells are a clone of the commonly used iNS-1 cell line that better mimic human responses to glucose and insulin secretion at physiological levels [15, 16]. Further, these cells are able to be cultured over long periods of time without losing their response to glucose [17]. Caco-2 and iNS-1E cells have been previously cultured using transwell inserts to study the transport of plant extracts [18]. Transport of materials across the insert membrane allows only absorbed phenolic compounds and metabolites to impact the insulin secretion of pancreatic β-cells growing on the plates below them, thus simulating their gastrointestinal absorption.

This study applied current cellular absorption and more physiologically-relevant β-cell models to investigate the role of phenolic compounds following simulated absorption to improve insulin secretion. The objectives were to demonstrate the role of ANC and PAC, from fermented blueberry and blackberry, to modulate incretin-cleaving hormone DPP-IV and its substrate GLP-1 in order to increase insulin secretion from pancreatic β-cells, and in addition, to investigate the corresponding modulation of potential genes and proteins involved in the insulin signaling
pathway. Using a simulated absorption model and a physiologically-relevant β-cell line allows for an assessment of the role of phenolic compounds to act as insulin secretagogues. The hypothesis was that ANC and PAC from fermented berry beverages will improve glucose-stimulated insulin secretion in vitro using Caco-2 epithelial and iNS-IE pancreatic β-cells through reduction of DPP-IV activity and expression, and modulation of genes and proteins involved in insulin secretion and signaling.

**Materials and methods**

**Materials**

Eagle’s Minimum Essential Medium (EMEM), RPMI-1640, and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Penicillin-streptomycin was purchased from Corning Inc. (Corning, NY, USA), fetal bovine serum (FBS) from Hyclone (Thermo Scientific Hyclone, Logan, UT, USA). Primary mouse monoclonal antibodies GADPH (sc-47724), DPP-IV (sc-9153), and GLP-1R (sc-66911) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Controls used were the commercial DPP-IV inhibitor Sitagliptin (Sitag) at 0.5 and 5 μM based on the IC$_{50}$ value to inhibit DPP-IV, and purified phenolic compound resveratrol (Res) at 5 μM as a control of a purified stilbenoid. VitaBlue® (VB) 20% anthocyanin blueberry extract (N208.2) was provided by FutureCeuticals, Inc. (Momence, IL, USA) and used as a commercial semi purified ANC-enriched extract from whole blueberry (ethanol-water extraction 70-30) at 50 μM considering 20% anthocyanins by weight. Phenolic extracts were previously characterized [15]; ANC- and PAC- enriched fractions extracted from a 50% blueberry-blackberry fermented
beverage (50%Blu-Bla) and from a 100% blackberry fermented beverage (100%Bla), were also used at 50 μM.

**Caco-2 and iNS-1E cell proliferation**

Caco-2 cells (HTB-37 from ATCC, Manassas, VA) were subcultured using EMEM media supplemented with 20% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate. iNS-1E cells were kindly provided by Dr. Maechler [17] and were maintained in RPMI-1640 media containing 10 mM HEPES, 2 mM L-glutamine, 1mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 4500 mg/L glucose, supplemented with 10% FBS, and 1% Amphotericin B. Cells were maintained at 37°C in 5% CO₂/95% air using a CO₂ Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN). Cells were grown at 5000 cells/well in a 96-well plate for 24 h (Caco-2) or 5,000 cells/well in a 96-well plate for 3 d (iNS-1E) and proliferation was measured using a CellTiter 96® AQueous One Solution Proliferation assay kit (Promega, Madison, WI); viability of neither cell line was significantly affected compared to the media-only treated cells (p > 0.05) by treatments of sitagliptin at 0.5 μM, resveratrol at 5 μM, or by VB, ANC 50%Blu-Bla, ANC 100%Bla, PAC 50%Blu-Bla, and PAC 100%Bla at 50 μM in cyanidin-3-glucoside or epicatechin equivalents, respectively. The concentration of 50 μM was chosen based on efficacy in previous studies [4, 21]. While much higher concentrations than what could be achieved in plasma for these compounds were used, the proposed cell culture system is a simulated absorption and the *in vitro* concentration of 50 μM of ANC in C3G equivalents translates to consumption of 3 mg of ANC by a human, assuming their intestinal fluid volume is 150 mL [18] and that 1% of this would reach their β-cells.
**Dual-layered simulated absorption**

Phenolic compounds were applied to the apical side of human epithelial Caco-2 cells grown in a monolayer inserts above iNS-1E pancreatic β-cells to determine the effect of transported ANC or PAC compounds or their metabolites on insulin secretion (Figure 9.1).

![Diagram of the dual-cell simulated absorption system. Caco-2 cells were seeded on the apical side of 24-well cell culture inserts at a density of 5*10^4 cells /cm^2 alone, with volume of culture media at 0.4 mL. Only those cells that successfully formed a monolayer, as determined by TEER measurement to insure the integrity of the monolayer were used for the experiment. On day 18, pancreatic iNS-1E cells were seeded onto 24-well plates at a density of 1*10^5 cells /cm^2, with 1 mL of culture medium. After 20 days, Caco-2 cells reached confluence and treatments (sitagliptin, VB, ANC 50%Blu-Bla and 100%Bla, or PAC 50%Blu-Bla and 100%Bla) were added to the apical side for 24 h before cells were layered by transferring the inserts into the 24-well plates containing the iNS-1E cells. Diagram adapted from [16].

**Figure 9.1** Diagram of the dual-cell simulated absorption system.
This dual-cell system is a combination of both human and rat cells as there is currently no appropriate human β-cell line that is physiologically relevant [15]; however, they are not growing as a co-culture adjacent to each other which should avoid any cross-species concerns. Hanging cell culture inserts contained a polystyrene membrane with a 0.4 µM pore that allows transport of phenolic compounds [19]. Caco-2 cells were seeded on the apical side of 24-well cell culture inserts at a density of 5*10⁴ cells/cm² alone, with volume of culture media at 0.4 mL. Only those cells that successfully formed a monolayer, as determined by Transepithelial Electrical Resistance (TEER) measurement to insure the integrity of the monolayer were used for the experiment. TEER measurement with Millicel-ERS volt-ohm-meter (Millipore, Billerica, MA) was taken daily and before and after the experiment to insure the integrity of the monolayer in each well. On day 18, pancreatic iNS-1E cells were seeded onto 24-well plates at a density of 1*10⁵ cells/cm², with 1 mL of culture medium. After 20 days, Caco-2 cells reached confluence (average TEER for all wells was 300.9 ± 4.8 Ω*cm²); only compounds that passed through the Caco-2 cells would be able to affect the pancreatic β-cells growing on the inserts below. Treatments (sitagliptin, VB, ANC 50%Bla and 100%Bla, or PAC 50%Bla and 100%Bla) were added to the apical side for 24 h, and cells were layered by transferring the inserts into the 24-well plates containing the iNS-1E cells. After 24 h, the media from both the apical and basolateral side and both cell lysates were collected, centrifuged at 4°C for 10 min at 1000 g to remove cell debris, and stored for analysis of insulin as described previously or for GLP-1 as follows. While this model will not address the metabolism of the extracts before they reach the part of the gut that is represented by the Caco-2 cells nor the effect that gut bacterial may have on the metabolism of the extracts, it is an attempt to go a step beyond direct treatment.
of the extracts to the cell line that is a limiting factor to translation of in vitro results to in vivo effects.

**Glucose stimulated insulin secretion (GSIS)**

Measurement of insulin release from INS-1E cells was performed according to Asfari et al. [20], with modifications. INS-1E cells were plated 8 x 10^4 cells/well in 24-well plates and incubated for 3 days at 37 °C. On the day of the experiment, the media was replaced by RPMI 1640 without glucose supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% HEPES, 1% sodium pyruvate, and 50 μM β-mercaptoethanol and incubated for 2 h as starvation. The cells were then gently washed 3 times with modified Krebs-Ringer-bicarbonate-HEPES buffer (KRBH), containing 135 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 10 mM HEPES, 2 mM NaHCO₃, and 1.5 mM CaCl₂. Cells were incubated in 0-glucose modified KRBH or treatments dissolved in the 0-glucose modified KRBH buffer, and incubated for 30 min. The KRBH was replaced by KRBH with 0.1% bovine serum albumin (BSA) containing 0, 2.5, or 20 mM glucose for glucose stimulation, and incubated for another 30 min. The 0-glucose KRBH treated wells were used as the negative control.

For the dual-cell system, INS-1E cells were plated (8 x 10^4 cells/well in 24-well plates) on day 18 and incubated for 3 days at 37 °C, in a humidified 95% air, 5% CO₂ atmosphere. Following the 24 h layered-cell treatment, INS-1E media was changed to 0-glucose RPMI media for 2 h before experiment (as starvation) before stimulation with 0 or 20 mM glucose modified-KRBH.

In both direct and dual-layered experiments, the medium was collected and cleared by centrifugation at 15,000 g for 1 min, and stored at -20 °C until assayed by Insulin ELISA (Abcam, Cambridge, MA). To determine insulin content, each standard (recombinant human
insulin, 0-300 μIU/mL) or sample was loaded into the supplied wells and allowed to adhere overnight at 4°C with gentle shaking. After, the solution was discarded and the wells were washed of non-adherent material. Then, a biotinylated insulin detection antibody was added to each well and incubated for 1 h at room temperature with gentle shaking, followed by additional washing. Next, HRP-Streptavidin solution was added to each well, incubated for 45 min at room temp, and wells washed again. Finally, the provided substrate reagent was added to each well, incubated for 30 min at room temperature in the dark with gentle shaking until the stop solution was added and the absorbance was read at 450 nm on an ELx808™ Microplate Reader (Biotek Instruments, Winooski, VT). Insulin content was calculated using the standard curve y = 0.0014x - 0.0068, R² = 0.986.

**Dipeptidyl peptidase-IV inhibition**

Activity of DPP-IV was measured using DPP-IV Glo ™ Protease Assay (Promega, Madison, WI) following manufacturer’s instructions. Cell lysates collected from Caco-2 cells, plated at 2*10⁵ cells/well in 6-well plates following direct treatment with samples for 4, 8, 24, and 48 h were collected, normalized for protein content, and diluted to a final concentration of 10 ng of protein per well. The provided Glo-substrate was added, and the content of the wells was gently mixed using an Synergy2 multi-well plate reader (Biotek, Winooski, VT) at medium intensity for 4 s. DPP-IV cleavage of the provided Gly-Pro-amino methyl coumarin (AMC) substrate generates a luminescent signal by luciferase reaction, with the amount of DPP-IV enzyme available to bind Gly-Pro-AMC proportional to relative light units (RLU) produced. This signal in RLU was measured after 30 min, and treatments were compared to wells containing cell lysates treated with media only as control of no DPP-IV enzyme inhibition.
**Adipokine array**

The effect on adipokine expression were carried out on non-glucose stimulated INS-1E cells without using the dual cell system in order to determine the direct action of the compounds tested. Pancreatic iNS-1E cells plated at 2*10^5 cells/well in 6-well plates were treated with 50 µM ANC 50%Blu-Bla or ANC 100%Bla, sitagliptin (0.5 µM), or media only for 24 h. Cells were washed twice with DPBS before collecting cell lysates using a lysis buffer containing 1% Igepal CA-630, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% SDS, 2 mM EDTA, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, and 10 µg/mL Pepstatin. Cell lysates were diluted to 200 µg protein, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the Proteome Profiler Rat Adipokine Array nitrocellulose membranes (R&D Systems, Minneapolis, MN) containing capture and control antibodies spotted in duplicate. The membrane was then washed to remove unbound material before application of streptavidin-HRP and chemiluminescent detection reagents. The chemiluminescent signal produced was captured using a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY) and analyzed for pixel intensity.

**Gene expression**

Gene expression analyses were carried out on non-glucose stimulated INS-1E cells without using the dual cell system in order to determine the direct action of the compounds tested. iNS-1E cells growing at 1*10^5 cells/well in a 6-well plate were treated with 50 µM ANC50%Blu-Bla and ANC100%Bla to determine modulation of genes involved in insulin signaling based on the potential of these two treatments to have the greatest effect in the insulin secretion analysis. After treatment for 24 h, RNA was collected using an RNAeasy® Kit (Qiagen, Germantown, MD), quantified, and checked for quality. cDNA was synthesized using a
Qiagen RT² First Strand synthesis kit. Quantitative RT-PCR was performed using an 7900HT Fast Real-Time PCR System Cycler (Applied Biosystems, Foster City, CA) and a targeted array (PARN-030ZA RT² Profiler PCR Array, Qiagen, Germantown, MD) that profiles the expression of 84 insulin-responsive genes. Thermocycling conditions were: 1 cycle at 95°C for 10 min and then 40 cycles at 95°C for 15 s followed by 60°C for 1 min before a dissociation stage. The relative mRNA expression levels of each gene were calculated using the $2^{-\Delta\Delta C_t}$ method in reference to β2 microglobulin, present in all nucleated cells.

**Active glucagon-like peptide 1 measurement**

The active form of GLP-1 was quantified from both the apical and basolateral media using an ELISA (EGLP-35K, EMD Millipore, Billerica, MA). Per manufacturer’s instructions, the active GLP-1 present in media was captured in a 96-well plate by a monoclonal antibody that binds the N-terminal region of the active GLP-1 molecule. The wells were washed to remove unbound materials, and anti-GLP-1 alkaline phosphatase detection conjugate was added to bind the immobilized GLP-1. Next, the unbound conjugate was washed, and bound detection conjugate was quantified by addition of methyl umbelliferyl phosphate, which forms a fluorescent signal proportional to the concentration of active GLP-1 in the unknown sample. Fluorescence was read using a FL600 Microplate Fluorescence Reader (Bio-Tek, Winooski, VT) with excitation/emission wavelength of 360/460 nm and quantified using GLP-1 standards ranging from 2-100 pM with equation of $y = 0.44x - 2.52$, $R^2 = 0.95$.

**Western blot analysis**

To measure the effect of phenolic compounds on DPP-IV inhibition and resulting active GLP-1, equal amounts of Caco-2 or iNS-1E cell lysate protein (20 µg) were loaded in each well of 4-20% gradient SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to
polyvinylidene difluoride membranes and blocked with 3% non-fat dry milk in 0.1% TBST for 1 h at 4°C. After blocking, the membranes were washed with 0.1% TBST (5 times, 5 min each) and incubated with primary antibodies (1:500) overnight at 4°C. The membranes were washed again and incubated with anti- IgG horseradish peroxidase conjugate secondary antibodies (1:2500) for 2 h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare Biosciences, Pittsburgh, PA). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). Relative expression of DPP-IV and GLP-1R were normalized to GAPDH.

Statistical analysis

Means of at least three replicates of independent duplicates were generated and adjusted with Tukey’s post hoc comparisons using Statistical Analysis System Software, version 9.3 (Cary, NC). Significant differences were reported at p-values < 0.05.

Results and discussion

Glucose-stimulated insulin secretion was increased by anthocyanin treatments when directly applied to iNS-1E cells

Direct treatment of the phenolic compounds tested did not affect the proliferation of iNS-1E pancreatic β-cells. Cell viability was 104.2 ± 5.8%, 104.1 ± 7.7%, 108.8 ± 5.5%, 87.8 ± 3.6%, 100.4 ± 8.8%, 100.0 ± 14.0%, and 100.4 ± 1.7% for sitagliptin, resveratrol, VB, ANC50%Bla, PAC50%Bla, ANC100%Bla and PAC100%Bla, respectively (p < 0.05). Insulin secretory response of iNS-1E cells following glucose stimulation and pre-treatment by sitagliptin, VB, or ANC and PAC from 50%Bla or 100%Bla was measured to determine
their ability to act as insulin secretagogues in cell culture. When iNS-1E cells were not stimulated with glucose (0 mM), there was an increase of insulin secretion seen by all treatments (Figure 9.2); this increase was significantly higher than media-only treated cells (106.9 ± 4.3 µIU/mL) for sitagliptin (289.3 ± 18.9 µIU/mL), VB (254.6 ± 41.4 µIU/mL), ANC50%Blu-Bla (232.7 ± 30.0 µIU/mL), and PAC100%Bla (272.7 ± 36.7 µIU/mL). Sitagliptin was able to enhance the secretion of insulin even without any glucose or GLP-1 present. The fact that the ANC and PAC treatments are able to stimulate insulin secretion even without the presence of glucose can be attributed to the glycosidic side chains of these compounds allowing them to act as glucose mimetics [21]. Previous studies have indicated that procyanidins, part of the proanthocyanidin class of flavonoids, may play a role to modify insulin synthesis and secretion leading to decreased insulin production [22], while proanthocyanidins in this study were seen to increase insulin secretion without glucose stimulation. This difference could be due to the degree of polymerization of these compounds from grapes in the previous study rather than those present in blackberries used in this study. Glucose-stimulation at 2.5 mM did not alter insulin secretion from iNS-1E cells among treatments (Figure 9.2A). As expected [17,19], stimulating the β-cells with 20 mM glucose induced insulin secretion in media-only treated cells (301.0 ± 33.3 µIU/mL). Within treatment, the ability to increase insulin secretion in response to glucose was most increased by the ANC 50%Blu-Bla (452.7 ± 105.1 µIU/mL) and ANC 100%Bla (376.0 ± 29.6 µIU/mL). The glucose-stimulated insulin secretion from iNS-1E cells was only increased when stimulated with 20 mM glucose compared to media-only treated cells following 30 min pretreatment by ANC 50%Blu-Bla. The fact that the increase in insulin secretion by treatments, compared to media-only, seen without glucose stimulation was lost when stimulated with 20 mM glucose is an interesting finding, and suggests the mechanism may not be directly through
improved glucose-stimulation. Previously, anthocyanins present in fruits were shown to increase the insulin secretion following 4 and 10 mM glucose in pancreatic β-cells *in vitro*, with the most effect seen by cyanidin-3-glucoside and cyanidin-3-galactoside [21]. The primary anthocyanin present in the ANC100%Bla was delphinidin-3-arabinoside, which has two hydroxyl groups in its B-ring, while the ANC50%Blu-Bla contained higher amounts of cyanidin derivatives [4]. The difference in ability of these anthocyanins to act as insulin secretagogues may be attributed to the difference in the concentration of different anthocyanins present and their structure [23].

*Dipeptidyl peptidase-IV expression and activity in Caco-2 cells treated with blueberry extracts for 24 h were reduced*

Due to limited bioavailability, the ability of anthocyanins to affect glucose homeostasis in the body may be mediated by the effects of the compounds within the gastrointestinal tract. As such, Caco-2 cells were treated with media only, sitagliptin, resveratrol, a commercial berry extract (VB), and anthocyanin and proanthocyanidin extracts from a fermented berry beverage, and the activity and amount of DPP-IV protein expression was measured. The greatest reduction of DPP-IV activity was seen in Caco-2 cells after treatment for 24 h by 23.6% ± 2.6 for Res and 10.7% ± 1.5 for VB, respectively. Following dual-layering with treatments for 24 h, DPP-IV protein expression was reduced by VB (69.5%), ANC 50%Blu-Bla (48.7%) and PAC 50%Blu-Bla (54.0%) in Caco-2 cells (*Figure 9.2B*). There was no reduction seen by the DPP-IV inhibitor sitagliptin on expression of DPP-IV as the mechanism of inhibition is competitive binding within the active site rather than an inhibition of the enzyme’s expression; the IC$_{50}$ of DPP-IV for sitagliptin was reported as 0.06 ± 0.03 μM [24]. Resveratrol was evaluated as a control of a purified stilbenoid, which may be present in the PAC fractions and was previously seen to be the most potent inhibitor of DPP-IV activity using computational modeling [25]. In this study we
found no reduction in the expression of DPP-IV by resveratrol indicating that the inhibition is purely through competitive inhibition rather than reduction in protein expression. As VB (extract standardized to 20% anthocyanins) is an ANC-enriched extract from blueberry that contains both ANC and PAC, and since no reduction was seen by the 100%Blu extracts, this effect to reduce DPP-IV activity and expression appears to be due to compounds present only in blueberries.

**Figure 9.2** A) Glucose-stimulated insulin secretion from iNS-1E cells following 30 min pretreatment by anthocyanin (ANC) or proanthocyanidins (PAC) from a 50% blueberry-50% blackberry blend or from 100% blackberry (50%Blu-Bla or 100%Bla, respectively, sitagliptin (Sitag), or VitaBlue® (VB). Values are means ± SEM, n=4-8. Lowercase letters indicate significant differences (p < 0.05) between 0 mM treatments, uppercase letters between 20 mM treatments; 2.5 mM treatments did not differ among groups (p > 0.05); * indicates significant difference within treatments. B) Relative DPP-IV expression in Caco-2 cells following treatments for 24 h. Values are means ± SEM. Means with different letters are significantly different (p < 0.05).
Our previous studies have indicated that phenolic compounds, especially anthocyanins, are strong inhibitors of the enzyme DPP-IV [25]. Grape seed procyanidins have been found previously to inhibit Caco-2 expressed DPP-IV activity by about 20%, with longer treatment leading to inhibition not seen by higher concentrations for 24 h [26], which agrees with the results seen in our study. While a reduction in the protein expression would lead to a reduction in the cleavage of GLP-1, inhibition through competitive binding of the active site rather than a reduction of protein expression would allow the Caco-2 cells expressing DPP-IV to maintain their normal function, however there is limited data regarding intestinal DPP-IV activity.

**Proteins of iNS-1E cells related to insulin growth factor binding were increased with anthocyanins from 50% blueberry-50% blackberry treatment**

Following treatment with anthocyanins from 50%Blu-Bla or anthocyanins from 100%Bla, the relative expression of adipokines in iNS-1E proteins was measured to determine the impact of anthocyanins on proteins in pancreatic β-cells (Figure 9.3 and Figure 9.4). It is well established that cytokines secreted from adipose have an effect on the function of the pancreas to secrete insulin, and that their secretion from adipocytes may be modulated by anthocyanin treatment [27]; and it is also known that the pancreas itself can secrete these compounds [28]. The proteins with the highest pixel intensity that were significantly increased by treatment with ANC 50%Blu-Bla in comparison to the control were insulin-like growth factor binding proteins 3 (IGFBP-3, fold-change 1.58 ± 0.01), insulin-like growth factor 2 (IGF-II, fold-change 1.40 ± 0.19), vascular endothelial growth factor (VEGF, fold-change 1.53 ± 0.06), and insulin-like growth factor binding proteins 2 (IGFBP-2, fold-change 1.26 ± 0.01) (Figure 9.3A). IGFBP-2 binds to IGF-I and IGF-II, and these proteins are involved in insulin-signaling pathway and in the cellular response to various stimuli. Since ANC 50%Blu-Bla increased the
pixel intensity of these proteins more than ANC 100%Bla, anthocyanins from blueberries may contribute more than those from blackberries to the increased expression of these proteins. An increase of VEGF by anthocyanins is supported by previous research that purified C3G protected against endothelial dysfunction in mice [28]. The role of VEGF in inhibiting apoptosis indicates a potential mechanism by which anthocyanins may protect β-cells from loss of mass of the pancreas.

Figure 9.3  Rat adipokine array of thirty proteins detected in iNS-1E after 24 h treatment by anthocyanins from a 50% blueberry-50% blackberry blend or from 100% blackberry (ANC 50%Blu-Bla or ANC 100%Bla, respectively. A) The pixel intensity of the most expressed proteins indicated in membranes and B) pixel intensity of proteins less highly expressed sorted by largest to smallest pixel intensity based on the ANC 50%Blu-Bla treatment. Different letters in A) indicate significant differences for each protein (p < 0.05); while in B) * indicates significant difference from the media-only control and # indicates significant differences between ANC treatment and the control.
Other adipokine proteins that were less highly expressed were also modulated by treatment with anthocyanins (Figure 9.2B), with the trend being an increase in protein expression by ANC 50%Blu-Bla and a slight decrease by ANC 100%Bla treatment relative to the control.

![Figure 9.4](image)

**Figure 9.4.** Blots that were generated using the Rat Adipokine Array and the location of the thirty proteins detected. The array was conducted using iNS-1E cells after 24 h treatment by anthocyanins from a 50% blueberry-50% blackberry blend or from 100% blackberry (ANC 50%Blu-Bla or ANC 100%Bla, respectively.

Adipokines that were affected differentially between ANC from either 50%Blu-Bla or 100%Bla control include leukemia inhibitory factor (LIF), IGFBP-5 and -6, fibroblast growth factor 21 (FGF-21), monocyte chemoattractant protein-1 (MCP-1), and Lipocalin-2. LIF is a secreted cytokine with growth factor activity, related to the role of IGFBP-5, which plays a key role in growth and development.
role in growth factor binding as well as signal transduction and cellular response to cAMP [37]. IGFBP-6 also is involved in cell growth and has a higher affinity for IGF-II than IGF-I, which also agrees with the observed increased expression of IGF-II over IGF-I. Another adipokine that is a member of the growth factor family, FGF-21, is involved in regulation of glucose import beyond its activities related to cell and tissue growth and may lead to increases in proliferation or mass of β-cells as well as increases in insulin transcription [29]. Lipocalin-2 is involved in apoptosis and inflammatory response, indicating that the reduced expression of this adipokine by the ANC from 100%Bla would be more protective against inflammatory-mediated cell death than ANC from 50%Blu-Bla. Finally, MCP-1 is involved in cellular calcium ion homeostasis, response to ATP, and VEGF signaling, indicating the compounds present in ANC50%Blu-Bla may be more responsive to intracellular signaling than ANC100%Bla.

Circulating adipokine proteins DPP-IV, TNFα, IL-6, and IGFBP-1 were not affected by either anthocyanin treatment compared to the control (data not shown). As these are secreted proteins, any effect may not have been detected due to screening of the whole cell lysate rather than secreted proteins. It is important to note that the adipokines that have been reported to have the most significant deleterious effects on the function and proliferation and eventual failure of β-cells, including TNFα and DPP-IV, were not increased in our study [29]. These results from the array also agree with our results from the western blot in that there was no change by the ANC treatments to affect the expression of DPP-IV. Overall, an increase in these cytokines produced by the β-cell may have a protective role in defense against infection and eventual loss of pancreatic function; however, the physiological relevance of these results may be impacted by the presence of circulating adipokines from adipose, and should be considered as only direct effects on protein expression within the pancreatic β-cell.
Genes affected in specific pathways related to insulin signaling were up-regulated following anthocyanin treatment

Beyond the ability of ANC from 50%Blu-Bla or 100%Bla, with no glucose stimulation, to act as insulin secretagogues, the effect on gene expression of genes involved in insulin signaling was compared to media-only treated iNS-1E cells. Genes linked to insulin signaling that were most up-regulated by either anthocyanin treatment are shown in Table 9.1, while Table 9.2 indicates the genes that were most down-regulated. Overall, ANC from 50%Blu-Bla were able to up-regulate 28 genes in the array (fold-change >1.5), while ANC from 100%Bla up-regulated 14 genes. Of genes most up-regulated, there was an effect on insulin, insulin receptors and insulin binding including Ins1 and Ins2, as well as Dok3, Igfbp1, and Igf1r. Dok3 (docking protein 3) plays a key role in insulin binding and Ras protein signal transduction, and was upregulated by 8.5 and 6.5-fold for ANC 50%Blu-Bla and 100%Bla, respectively. Glucagon (Gcg) induces glucose production, regulates carbohydrate and protein metabolism, and also induces transcription of GLP-1, an important inducer of insulin secretion following food intake. There was an increase of Gcg by 3.0 and 2.0-fold compared to the control for ANC 50%Blu-Bla and 100%Bla, respectively. This increase in the gene that induces GLP-1 may be the reason these anthocyanins were able to increase insulin secretion following glucose stimulation. Igf1r (insulin-like growth factor 1 receptor) is the receptor for Igf-1 which is involved in the induction of cell cycle progression and survival, and therefore the induction of this gene by 2.3 ± 0.6 for ANC 50%Blu-Bla and 1.6 ± 0.3 by ANC 100%Bla may allow for an increase in survival of pancreatic β-cells in response to excess glucose. Igfbp1 (insulin-like growth factor binding protein 1) is a modulator of insulin growth factor (IGF) bioavailability and was also up-regulated by both ANC treatments.
<table>
<thead>
<tr>
<th>Gene Symbol (ID)</th>
<th>Official Full Name</th>
<th>Cell action</th>
<th>ANC 50%Blu-Bla</th>
<th>ANC 100%Bla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dok3 (306760)</td>
<td>docking protein 3</td>
<td>Insulin binding and Ras protein signal transduction</td>
<td>8.58 ± 0.34</td>
<td>6.52 ± 4.31</td>
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<tr>
<td>Gcg/GLP-1 (24952)</td>
<td>glucagon</td>
<td>Glucose production; regulates carbohydrate and protein metabolism</td>
<td>3.03 ± 1.45</td>
<td>2.00 ± 0.31</td>
</tr>
<tr>
<td>Ras/p23 (361568)</td>
<td>related RAS viral (r-ras) oncogene homolog</td>
<td>GDP, GTP, and protein binding involved in catabolic processes and Ras protein signal transduction</td>
<td>2.66 ± 0.79</td>
<td>2.03 ± 0.60</td>
</tr>
<tr>
<td>Igf1r (25718)</td>
<td>insulin-like growth factor 1 receptor</td>
<td>Involved in the induction of cell cycle progression and survival, ATP and insulin binding</td>
<td>2.30 ± 0.63</td>
<td>1.61 ± 0.26</td>
</tr>
<tr>
<td>Akt2 (25233)</td>
<td>v-akt murine thymoma viral oncogene homolog 2</td>
<td>Involved in phosphatidylinositol 3-kinase (PI3-K) mediated signaling and ATP binding</td>
<td>2.10 ± 0.47</td>
<td>1.71 ± 0.73</td>
</tr>
<tr>
<td>Ins2 (24506)</td>
<td>insulin 2</td>
<td>Encodes pre-pro-insulin, involved in hormone activity</td>
<td>2.17 ± 0.47</td>
<td>-1.05 ± 0.17</td>
</tr>
<tr>
<td>Irs2 (29376)</td>
<td>insulin receptor substrate 2</td>
<td>Insulin receptor binding, involved in insulin-stimulated fetal liver growth and PI3-K binding</td>
<td>2.13 ± 0.61</td>
<td>1.09 ± 0.37</td>
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<tr>
<td>Fos (314322)</td>
<td>FBJ osteosarcoma oncogene</td>
<td>Encodes a nuclear protein involved in signal transduction, involved in DNA binding and cellular response to calcium ion</td>
<td>1.87 ± 0.30</td>
<td>2.20 ± 0.52</td>
</tr>
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<td>Ldlr (300438)</td>
<td>low density lipoprotein receptor</td>
<td>Calcium ion binding and LDL particle binding</td>
<td>1.80 ± 0.20</td>
<td>1.05 ± 0.15</td>
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<tr>
<td>Insr (24954)</td>
<td>insulin receptor</td>
<td>Receptor for insulin, involved in intracellular signaling and ATP binding</td>
<td>1.74 ± 0.61</td>
<td>-1.41 ± 0.04</td>
</tr>
<tr>
<td>Igfbp1 (25685)</td>
<td>insulin-like growth factor binding protein 1</td>
<td>Modulator of insulin growth factor bioavailability and insulin receptor signaling, and positive regulation of cell growth</td>
<td>1.53 ± 0.81</td>
<td>1.95 ± 0.08</td>
</tr>
</tbody>
</table>

iNS-1E cells were treated for 24 h with anthocyanins at 50 µM from either 50% blueberry-50% blackberry (ANC 50%Blu-Bla) or 100% blackberry (ANC 100%Bla). Fold-change was calculated using the $2^{-ΔΔCT}$ method. Data are expressed as the mean + SEM compared to media-only treated cells. Cell actions from [37].
### Table 9.2 Fold-change of the genes most down-regulated in iNS-1E cells following anthocyanin treatment.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Official Full Name</th>
<th>Cell action</th>
<th>ANC 50%Blu-Bla</th>
<th>ANC 100%Bla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpd1 (60666)</td>
<td>glycerol-3-phosphate dehydrogenase 1 (soluble)</td>
<td>Mitochondrial G3P dehydrogenase, functions in NADH oxidation and gluconeogenesis</td>
<td>1.42 ± 0.60</td>
<td>-2.40 ± 0.06</td>
</tr>
<tr>
<td>Ins1 (24505)</td>
<td>insulin 1</td>
<td>Involved in glucose metabolism chaperone binding and hormone activity</td>
<td>-2.11 ± 0.14</td>
<td>1.48 ± 0.55</td>
</tr>
<tr>
<td>Ins13 (114215)</td>
<td>insulin-like 3</td>
<td>Ligand for the LGR8 relaxin receptor, functions in G-protein coupled receptor binding</td>
<td>-1.44 ± 0.02</td>
<td>-1.95 ± 0.08</td>
</tr>
<tr>
<td>Pklr (24651)</td>
<td>pyruvate kinase, liver and RBC</td>
<td>L-isozyme of pyruvate kinase that catalyzes the conversion of ATP and pyruvate to ADP and phosphoenolpyruvate in glycolysis</td>
<td>-1.36 ± 0.15</td>
<td>-1.92 ± 0.28</td>
</tr>
<tr>
<td>Shc1 (85385)</td>
<td>SHC (Src homology 2 domain containing) transforming protein 1</td>
<td>Involved in Ras-dependent transformation and differentiation, interacts with growth factor and insulin receptor binding</td>
<td>-1.41 ± 0.42</td>
<td>-1.92 ± 0.05</td>
</tr>
<tr>
<td>Ptnp1 (24697)</td>
<td>protein tyrosine phosphatase, non-receptor type 1</td>
<td>Negative regulator of insulin signaling, involved in the ER unfolded protein response</td>
<td>-1.35 ± 0.33</td>
<td>-1.92 ± 0.08</td>
</tr>
<tr>
<td>Fbp1 (24362)</td>
<td>fructose-1,6-bisphosphatase 1</td>
<td>Catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate in gluconeogenesis</td>
<td>1.01 ± 0.34</td>
<td>-1.91 ± 0.07</td>
</tr>
<tr>
<td>Jun (24516)</td>
<td>jun proto-oncogene</td>
<td>Transcription factor that acts as a proto-oncogene and may be involved in activated TLR4 signaling and DNA binding</td>
<td>-1.01 ± 0.02</td>
<td>-1.88 ± 0.03</td>
</tr>
<tr>
<td>Slc2a1 (24778)</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>Transporter for glucose and other hexoses, may be involved in response to stress</td>
<td>-1.34 ± 0.20</td>
<td>-1.76 ± 0.04</td>
</tr>
<tr>
<td>Pparg (25664)</td>
<td>peroxisome proliferator-activated receptor gamma</td>
<td>Ligand-activated transcription factor, mediates expression of genes involved in lipid metabolism</td>
<td>-1.77 ± 0.13</td>
<td>1.84 ± 0.04</td>
</tr>
<tr>
<td>G6pc (25634)</td>
<td>glucose-6-phosphatase, catalytic subunit</td>
<td>Catalyzes the conversion of D-glucose 6-phosphate and water to D-glucose and phosphate, involved in calcium ion binding and cation channel activity</td>
<td>-1.75 ± 0.22</td>
<td>-1.66 ± 0.09</td>
</tr>
</tbody>
</table>

iNS-1E cells were treated for 24 h with anthocyanins at 50 µM from either 50% blueberry-50% blackberry (ANC 50%Blu-Bla) or 100% blackberry (ANC 100%Bla). Fold-change was calculated using the $2^{-\Delta\Delta CT}$ method. Data are expressed as the mean ± SEM compared to media-only treated cells. Cell actions from [37].
Other genes that were up-regulated by both ANC treatments included Rras (related RAS viral, r-ras, oncogene homolog), Akt2 (v-akt murine thymoma viral oncogene homolog 2) that is related to phosphatidylinositol 3-kinase (PI3-K) mediated signaling, Hras (Harvey rat sarcoma virus oncogene), and Frs2 (fibroblast growth factor receptor substrate 2).

Ins2 and Irs2 were only up-regulated by ANC 50%Blu-Bla, indicating that the presence of malvidin or cyanidin structures in the blueberry may be able to affect these genes, rather than delphinidin from blackberry. Ins2 (insulin 2) gene encodes pre-pro-insulin, which is processed to form the insulin hormone involved in glucose and lipid metabolism while Irs2 encodes the insulin receptor substrate necessary for insulin signaling to occur.

Table 9.2 indicates the genes that were most down-regulated by either anthocyanin treatment. Overall, ANC from 50%Blu-Bla were able to down-regulate with fold-change < -1.5 only 7 genes, while ANC from 100%Bla down-regulated the expression of 16 genes in the array. G6pc (glucose-6-phosphatase, catalytic subunit) was down-regulated by both anthocyanin treatments by -1.7 for both ANC 50%Blu-Bla and ANC 100%Bla. This enzyme plays a role in gluconeogenesis through catalyzing the conversion of D-glucose 6-phosphate and water to D-glucose and phosphate. Pparg (peroxisome proliferator-activated receptor gamma) and Ins1 (insulin 1) were down-regulated only by ANC50%Blu-Bla (-2.11 ± 0.14 and -1.77 ± 0.13, respectively). PPARγ is a ligand-activated transcription factor that mediates expression of genes involved in lipid metabolism. Other studies have indicated that cyanidin is an agonistic ligand of PPARα [30], and therefore the binding by cyanidin present in the 50%Blu-Bla blend that was not seen by ANC 100%Bla could be due to this similarity in binding ligand properties of cyanidin. Other studies have found that cyanidin-3-glucoside up-regulated PPARγ activity and mRNA levels in adipocytes [31, 32]; however, in this study we found a down regulation of the gene for
this protein. This difference could be due to diverse roles or regulation of PPARγ by various cell types in response to anthocyanins. The ANC 50%Blu-Bla treatment did not strongly down-regulate many genes, though down-regulation of several genes involved in insulin signaling was seen following treatment by ANC 100%Bla. This could be due to the ability of the primary anthocyanin present, delphinidin-3-arabinoside, to down-regulate genes more than other cyanidin or malvidin derivatives contributed by ANC from fermented blueberries [4]. Genes most strongly down-regulated by the ANC 100%Bla treatment were Gpd1, Insl3, Pklr, Shc1, Ptpn1, and Fbp1. Gpd1 (glycerol-3-phosphate dehydrogenase 1, soluble) is involved in carbohydrate and lipid metabolism and the glycerol-phosphate shuttle. Insl3 (insulin-like 3) is a ligand for the LGR8 relaxin receptor that leads to stimulation of adenylate cyclase and increase of cAMP. Pklr (pyruvate kinase, liver and RBC) catalyzes the conversion of ATP and pyruvate to ADP and phosphoenolpyruvate in glycolysis. These three down-regulated genes are all involved in energy metabolism, and increasing ATP through down-regulation of Pklr, for example, would allow for the glucose-stimulated increase of insulin secretion resulting from the increased ATP to ADP ratio to occur. Ptpn1 (protein tyrosine phosphatase, non-receptor type 1) has phosphotyrosine phosphatase activity and is a negative regulator of insulin-signaling, and is therefore a current target of type-2 diabetes therapy. Other flavonoids from licorice or botanical roots have been identified as natural inhibitors of PTPN1 [33, 34], however there is a need for studies with flavonoids from fermented berries. Slc2a1, a transporter for glucose and other hexoses that may be involved in response to osmotic and metabolic stress, and Jun, a transcription factor that acts as a proto-oncogene and may be involved in activated TLR4 signaling, were down-regulated, indicating that ANC from blackberry may affect sensing of pancreatic β-cells to the extracellular state of the body. Figure 9.5 illustrates the primary predicted downstream pathways affected
including glucose metabolism by ANC from 50%Blu-Bla (A) or from 100%Bla (C) and cell viability 50%Blu-Bla (B) or from 100%Bla (D).

Figure 9.5 Gene expression results as interpreted using Ingenuity® Pathway Analysis at FDR <0.3. Genes differentially expressed are listed surrounding the predicted affected pathway. A) illustrates the up or down-regulation of genes surrounding pathways related to glucose metabolism affected by the 50%Blu-Bla ANC while B) illustrates the genes that lead to a predicted decrease of cell death and apoptosis with a predicted increase in cell viability by 50%Blu-Bla ANC; C) illustrates the downstream predicted effect on glucose metabolism by 100%Bla ANC, and D) illustrates the downstream prediction for 100%Bla ANC to increase cell viability while decreasing apoptosis.
This analysis utilizing the IPA software takes into consideration all of the genes that were affected in the array indicating that both ANC treatments would lead to an improvement of glucose metabolism and cell vitality indicating the primary ways that ANC may act upon pancreatic β-cells to lead to an improvement in insulin secretion.

**Glucose-stimulated insulin secretion following treatment in the simulated absorption model of Caco-2 cells on inserts over iNS-1E cells was attenuated by treatment with anthocyanins from 100% blackberry**

To simulate absorption, Caco-2 cells that had formed a confluent monolayer on cell culture inserts were treated with compounds that could cross into the basolateral side only if transported through the epithelial cells. After treatment for 24 h, iNS-1E cells on the wells below were evaluated for response to glucose stimulation. As seen with the direct treatment, all groups were able to increase the amount of insulin secreted without any glucose stimulation, compared to the media-only, except ANC 100%Bla (Figure 9.6).

![Figure 9.6](image_url) **Figure 9.6** Insulin secretion from iNS-1E cells at 0 or 20 mM glucose stimulation after dual-layering Caco-2 cells with treatments for 24 h. Values are means ± SEM. Lowercase letters indicate significant differences \((p < 0.05)\) between 0 mM glucose, uppercase letters between 20 mM glucose; * indicates significant differences within treatments.
When cells were stimulated with 20 mM glucose, insulin secretion was increased by VB (291.1 ± 7.6 µIU/mL), ANC 50%Blu-Bla (274.4 ± 37.9 µIU/mL) and ANC 100%BluA (300.8 ± 5.1 µIU/mL) compared to media-only treated cells. In the simulated absorption model, the polymerized compounds were no longer able to act as insulin secretagogues, or may have been cleaved into their smaller components such as procyanidins that have previously been shown to decrease insulin secretion [22].

**GLP-1 and GLP-1R were increased following proanthocyanidins treatment from 100% blackberry, but this increase did not correlate directly with insulin secretory results**

To measure the effect of inhibiting DPP-IV in order to increase biologically active GLP-1 in the media that is able to stimulate insulin release from pancreatic β-cells, GLP-1 was measured in both the apical and basolateral sides of the dual-layered system. Total GLP-1 was not measured as this takes into account the inactive metabolites of GLP-1 after cleavage by DPP-IV, and thus these other forms of GLP-1 would not lead to stimulation of insulin secretion. Total active GLP-1 in the supernatant of Caco-2 (apical side) and of iNS-1E cells (basolateral side) was increased for only the PAC 100%BluA treatment (Figure 9.7A). Active GLP-1 of the apical side only was increased from media-only treated cells following treatment with sitagliptin (39.1 ± 13.3 pM) that was mirrored by VB (53.1 ± 13.1 pM), PAC 50%Blu-Bla (51.4 ± 12.9 pM), and PAC 100%BluA (50.8 ± 8.7 pM), suggesting these compounds were acting through a DPP-IV inhibitory mechanism to increase GLP-1. Further, because VB contains a mixture of ANC and PAC, and acted similarly to the PAC-only treatments rather than the ANC treatments, it seems as if the more complex polyphenolic compounds were needed to have an effect to increase GLP-1 on the apical side.
Figure 9.7 A) Total active GLP-1 in the supernatant of dual-layered Caco-2 and iNS-1E cells and B) Expression of GLP-1R in iNS-1E cells with treatments for 24 h. Means ± SEM with different uppercase letters indicate significant \((p < 0.05)\) differences for total active GLP-1. * indicates significant differences between apical side compared to control of media-only. Different lowercase letters are significantly different \((p < 0.05)\) for the relative intensity of GLP-1R protein expression.
Previous studies have indicated the importance of the GLP-1 receptor to regulate endogenous glucose production [35] and to enhance insulin secretion [36], therefore the relative amount of the GLP-1R protein in the pancreatic β-cells following the dual-cell experiment was measured. There was an increased expression of GLP-1R in iNS-1E cells following the treatment for 24 h by PAC100%Bla (relative intensity of 3.0 ± 1.9); however, other treatments did not differ from the media-only treated cells (Figure 9.7B). This increase in the GLP-1R for PAC 100%Bla correlates with the increase seen in the total active GLP-1 in the media of the dual-cell system, however, these results do not correlate with the modulation of insulin secretion seen. Therefore, we concluded that ANC and PAC from fermented berry beverages may be acting through a mechanism beyond DPP-IV inhibition to increase incretin-mediated insulin secretion in pancreatic β-cells. Based on all of these results, a proposed mechanism of action is presented in Figure 9.8.

Conclusions

We demonstrated the role of phenolic compounds (such as ANC and PAC) from fermented berry beverages to modulate incretin-cleaving hormone DPP-IV and its substrate GLP-1 for increasing insulin secretion from pancreatic β-cells. Bioactive compounds (ANC and PAC) from fermented berry beverages reduced incretin-cleaving hormone DPP-IV protein activity and protein expression in Caco-2 cells, increased insulin secretion of iNS-1E pancreatic β-cells both directly and following a dual-cell layered model of simulated absorption. Of the genes that were affected by both anthocyanin treatments, Dok3 was the most up-regulated (average of 7.5 fold change for both ANCs). Additionally, the ANC treatments up-regulated genes of insulin-receptor associated proteins and GLP-1, and down-regulated PTP1B in iNS-1E cells.
Figure 9.8 Proposed mechanism of action by which phenolic compounds from berries will affect genes (blue boxes, green outline indicates up-regulation while red outline indicates down-regulation) and proteins (light green boxes) due to GLP-1 mediated or glucose stimulated insulin secretion with the pancreatic β-cell.
Our results indicated that the ability of ANC to act as insulin secretagogues in cell culture beyond their DPP-IV inhibitory action is possibly linked to enhanced insulin receptor signaling and up-regulation of proteins IGFBP-2 and -3, IGF-II, and VEGF. These effects, however, should be further evaluated in vivo, in a more complete physiological model system.

**Author contributions**

MJ and EDM proposed the project and designed the experiments; MJ developed and wrote the manuscript and conducted the experiments. EDM provided scientific guidance throughout the research, gave suggestions for the development of the manuscript and revised and edited the manuscript. Authors read and approved the manuscript.

**Acknowledgement**

To Mr. Diego Luna for his help with figures related to gene expression.

*The authors have declared no conflict of interest.*

**References**


CHAPTER 10

ALCOHOL-FREE FERMENTED BLUEBERRY-BLACKBERRY BEVERAGE PHENOLICS REDUCE DIET-INDUCED OBESITY AND BLOOD GLUCOSE IN C57BL/6J MICE

Abstract

The purpose of this research was to determine the potential of phenolic compounds from a fermented 70:30% blackberry-blueberry beverage to reduce obesity and hyperglycemia in three week old male diet-induced obese C57BL/6j mice. Mice were randomized into six groups (n=12/group) to drink (ad libitum) an alcohol-free fermented beverage (AFFB), three doses of a phenolic extract (PAE) generated from AFFB to consume 0.1X, 1X and 2X where X contains the amount of anthocyanins (ANC) in the AFFB (65.1 ± 1.6 mg cyanidin-3-glucoside (C3G) equivalents/L); sitagliptin as positive control of DPP-IV inhibition (30 mg/kg body weight (BW)/day, or d); water as the negative control. After a week of treatments, mice started on a 60% high fat diet (HFD) to induce obesity and hyperglycemia. The diet-induced obesity and fat mass gain was attenuated by mice receiving the highest doses of the phenolic extract (11.7 and 10.3 g BW gained for 1X and 2X, respectively, compared to water control 16.7 ± 1.1, p < 0.05). There was also a reduction (p < 0.05) in the percent fat mass, epididymal fat pad weights, adipocyte diameters, and plasma triglycerides and cholesterol by the PAE treatments. All groups became hyperglycemic due to the HFD, but by the end of the study (week 12), fasting blood glucose for mice receiving 9 (1X) or 19 (2X) mg ANC/kg BW/d was significantly lower (167.5 and 183.7 mg/dL, respectively) than the water (221.8 mg/dL) and the sitagliptin group (216.9 mg/dL, p <

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Follow-up histochemical analysis observed an unexpected adverse event of enlarged livers and lipid deposition in the livers of mice fed ANC at the levels of 1X or 2X that was associated with increased plasma alanine aminotransferase and alkaline phosphatase levels, increased hepatocyte proliferation, but not increased oxidation, indicating that there was potential toxicity at these doses. The PAE also induced the most gene expression changes, including down-regulation of the lipoprotein lipase gene and up-regulation of zinc-a2-glycoprotein 1(AZGP1) gene. This indicated a highly significant predicted downstream effect to reduce D-glucose concentrations for all three doses of the PAE with significant z-scores of -2.71, -2.12 and -2.12 for 0.1X, 1X, and 2X respectively. Overall, phenolic compounds from a fermented blueberry-blackberry beverage reduced HFD-induced BW gain, fat mass accumulation, plasma triglycerides and fasting blood glucose in C57BL/6j mice. However, the dose of the phenolic extract should not exceed 9 mg/kg BW/d to avoid adverse effects on the liver.

**Abbreviations:** AFFB, alcohol-free fermented beverage; BW, body weight; C3G, cyanidin-3-glucoside; DIO, diet-induced obesity; EGCG, epigallocatechin-3-gallate; FBG, fasting blood glucose; HFD, high-fat diet; PAE, post-amberlite extract.

**Keywords:** Anthocyanins, phenolics, berries, diet-induced obesity, hyperglycemia, C57Bl/6j mice

**Introduction**

Type-2 diabetes is a serious metabolic disease that currently affects over 9.3% of the U.S. population, with 1.7 million new cases diagnosed in 2012 [1]. Current obesity trends and modern sedentary lifestyle along with diets low in fruit and vegetable intake lead to postprandial oxidative stress and inflammation in the body that contribute to insulin resistance and development of diabetes [2]. Diets rich in whole grains, fruits, vegetables, legumes, nuts, and moderate alcohol have been linked to a reduced risk of type-2 diabetes [3,4]. Specific fruits such
as blueberries, grapes, apples [5], as well as specific compounds present in these fruits such as anthocyanins [6], flavones, and flavan-3-ols [7] have been attributed to these effects. Blueberries and blackberries contain high amounts of water-soluble phenolic anthocyanin (ANC) pigments [8] in addition to other phenolic compounds such as catechins and gallic or ellagic acid that can polymerize to form proanthocyanidins (PAC) [9]. Data from mechanistic studies supports the role of flavonoids such as anthocyanins to be beneficial on insulin sensitivity, to decrease blood glucose levels, and to improve insulin secretion.

Previous studies have shown that dietary blueberries may have a role in reducing hyperglycemia in high-fat fed C57BL/6 mice, a model that mimics pathological development of obesity-induced hyperglycemia [10-14], compared to other chemically-induced or genetically altered models of diabetes. However, the mechanisms by which berry phenolics reduce the risk of type-2 diabetes are not yet clear. There is then need to identify and evaluate the effects and mechanism of action of fermented berry phenolics to reduce obesity and hyperglycemia. Previous studies conducted in C57BL/6j mice evaluating the role of anthocyanins on diet induced obesity at doses of 0.2 and 0.6 mg ANC/d [15] showed an improvement of β-cell function with purified ANC at levels of 0.2 mg/mL [16]. Also, fasting blood glucose and glucose tolerance improved at 12.5 mg ANC/mouse/d [17] without any adverse effects indicating that there may be a specific dose at which purified ANC might be most effective. There are several studies on purified ANC to reduce obesity outcomes in C57BL/6 mice fed a HFD [18-20]; however, information is needed on the effect of a fermented berry beverage to reduce obesity or hyperglycemia.

We found in vitro that ANC and PAC from fermented blueberries and blackberries were able to inhibit enzymes related to glucose absorption and incretin cleavage [21], reduce
inflammatory response in macrophages [22], and improve insulin secretion in pancreatic β-cells. Fermentation increases the phenolic content of berry juice products through increased extraction from the skins and creates a more stable environment for ANC pigments and converts naturally present sugars into ethanol. An alcohol-free fermented beverage thus has increased potential for health benefits [23-27]. Our hypothesis was that consumption of a fermented blueberry-blackberry beverage and its phenolic compounds will reduce obesity and hyperglycemia, in diet-induced obese mice.

The objective of this research was to understand the effect of phenolic compounds from fermented berry beverages, using mice fed a high fat diet, on hyperglycemia and obesity in vivo. To explain any observed changes in obesity and hyperglycemia induced by a high-fat diet due to the phenolic compounds treatments, we also evaluated body composition, histomorphological changes and genes affected.

**Materials and Methods**

**Preparation of fermented beverage and phenolic extracts.** Fermented berry beverages made from blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus spp.*) grown at the Dixon Springs Agricultural Research Station in Illinois [21, 22] were used. The alcohol was removed from these fermented beverages through rotoevaporation and the removed volume was replaced with water to yield the resulting alcohol-free fermented beverage (AFFB) that was 70% blackberry-30% blueberry and contained 65.1 ± 1.6 mg cyanidin-3-glucoside (C3G) equivalents/L. This AFFB was eluted through Amberlite XAD-7HP to remove the sugars and phenolic acids in order to generate the post-amberlite extract (PAE). The PAE was then concentrated, freeze dried (Labconco) and adjusted to 6.4 (0.1X), 59.6 (1X) and 192.4 (2X) mg
C3G eq./L dissolved in water. Total anthocyanins of the AFFB and PAE groups were determined using the AOAC official method [28] and total polyphenols using the Folin-Ciocalteu method adapted for micro-assay as previously performed [21, 22].

**Identification of anthocyanins using HPLC-MS/MS.** Analysis of phenolic compounds was conducted by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS) using a Q-tof Ultima mass spectrometer (Waters Corporation, Milford, USA), equipped with an Alliance 2795 HPLC system. Samples were prepared by dissolving freeze-dried AFFB and PAE from both blueberry and blackberry and the 70% blackberry-30% blueberry mixture in 2% formic acid-H₂O at 10 mg dry weight/mL. The separation of the components was performed using a mobile phase of solvent A, 2% formic acid in H₂O and solvent B (100% Methanol) at a constant flow rate of 0.5 mL/min. The elution was in a linear step of 100%, 75%, 50%, 100% and 100% of solvent A at 0, 15, 40, 41, and 60 min, respectively. ANC were detected at 520 nm, PAC and hydroxycinnamic acids at 280 nm, and flavanols or flavonoids at 320 and 360 nm, respectively. To further elucidate the composition of the phenolic compounds present, a Q-tof Ultima mass spectrometer equipped with a Z-spray ion source with a positive ion electrospray mode (+ESI) was used. The Q-tof was operated at capillary voltage of 3.5 kV and a cone voltage of 35V. MassLynx 4.1V software (Waters, Milford, USA) was used to control the instruments and to process the data.

**Animal care and diet**

**Animal husbandry and care.** Animal use was conducted in accordance with Institutional Animal Care and Use Committee approved protocols (#14060) at the University of Illinois at Urbana-Champaign. Male C57BL/6J mice 3 weeks old were purchased from Jackson Laboratory (Bar Harbor, Maine), housed in standardized conditions with a reversed 12 h light/dark cycle,
kept in a room maintained at 23°C in individual caging systems (singly) in order to accurately determine feed and water intake, and acclimated for one week upon arrival. Power analysis indicated that a sample size of 12 animals/group would provide at least 95% power to detect a 1-standard deviation effect of blood glucose levels at P = 0.05, α = 0.05.

**Diet and treatments.** The experimental design can be seen in Figure 10.1. All treatment groups received a standard 22/4 rodent diet (TD.8640) for weeks 1 and 2 before switching to a high-fat diet (TD.06414: 60% fat, 21% carbohydrate, 18% protein, Harlan Laboratories, Madison, WI) at week 3. The composition of the HFD was (in g/Kg): casein, 265.0; L-cystine, 4.0; maltodextrin, 160.0; sucrose, 90.0; lard, 310.0; soybean oil, 30.0; cellulose, 65.5; mineral mix, AIN-93G-MX (94046), 48.0; calcium phosphate, dibasic, 3.4; vitamin mix, AIN-93-VX (94047), 21.0; choline bitartrate, 3.0; blue food color, 0.1.

**Figure 10.1. Experimental Design.** Three week old male diet-induced obese C57BL/6j mice (n=12/group) were randomized into six groups to drink: an alcohol-free fermented beverage (AFFB) containing 65.1 ± 1.6 mg cyanidin-3-glucoside (C3G) equivalents/L; three doses of a phenolic extract where AFFB was eluted through Amberlite XAD-7HP and adjusted to 6.4 (0.1X), 59.6 (1X) and 192.4 (considered as 2X based on consumption) mg C3G eq./L; sitagliptin (30 mg/kg BW/d) as positive control of DPP-IV inhibition, or water as negative control. After a week of baseline measures, mice were randomized into one of these six treatment groups. After an additional week of measurements to determine the effect of the treatment, all mice were started on a 60% fat diet (HFD). After about three weeks of the HFD, the mice began to develop the obesity and hyperglycemia phenotype.
**Food and treatment intake.** Food was provided *ad libitum* and intake was recorded bi-weekly. After 1 week of baseline blood glucose measures, the mice were randomized to receive one of 6 treatments in place of water (*ad libitum*), including an alcohol-free fermented beverage (AFFB), three doses of phenolic compounds from AFFB (PAE), and a commercial DPP-IV inhibitor as an admix also provided in the water, as follows: (n = 12/group): (a) AFFB (70%blackberry-30% blueberry); (b) dose 0.1X PAE; (c) dose 1X PAE; (d) dose 2X PAE; where x is the equivalent dose of ANC found in fermented beverage group (AFFB); (e) water only as the negative control; or (f) the group receiving sitagliptin phosphate at 150 mg/L in water as the positive control. All treatments were replenished daily and were covered in foil to reduce degradation or loss of the phenolic compounds due to heat or light, and adjusted for sucrose content based on intake so that all mice were consuming the same amount of sucrose as naturally present in the AFFB (10% w/v). After starting on the HFD, mice received treatments for 9 more weeks based on the time of phenolic compounds to reduce hyperglycemia in previous studies [20, 29].

**Body weight.** Body weight was evaluated at least bi-weekly by transfer to a clean empty weighing cage using a calibrated scale.

**Body composition analysis.** Echo-MRI scans for body composition were conducted on weeks 1, 4, 7, 10, and 12 to determine lean mass versus fat mass using the EchoMRI 700 system from Echo Medical Systems. The EchoMRI-700 Body Composition Analyzer for animals takes direct measurements of total body fat, lean mass, free water, and total body water without anesthesia, sedation, or restraint; animals were allowed free movement within the limited space of the scanning tube.
Blood glucose. Blood was collected under red-light during metabolically-active hours for the mice from the tail vein of conscious animals [30] that had been morning fasted for 4-6 h, during which time their treatments were replaced with plain water containing no sucrose. Briefly, the tail was lanced with a sterile scalpel blade and blood was measured directly onto glucose testing strips with a handheld glucometer (FreeStyle Freedom, Abbott Laboratories, Abbott Park, IL) [31].

Plasma analysis

Plasma collection. At the end of the study, animals were deeply anesthetized with isoflurane and a larger volume of blood was collected into a cryovial by cardiac puncture. Blood was processed by centrifugation for 10 min at 2500xg and 4°C, and plasma was then isolated from whole blood, aliquoted, and frozen at -80°C before the following analyses.

Insulin content and HOMA-IR calculation. Serum insulin was measured using a sandwich ELISA assay (Abcam). HOMA-IR was calculated from these measurements as a degree of insulin resistance using the following equation: \[ \frac{\text{blood glucose (mmol/L)} \times \text{serum insulin (mU/L)}}{22.5} \]
and HOMA-β calculated as a degree of the percent beta-cell function with the following equation: \[ \frac{20 \times \text{serum insulin (mU/L)}}{\text{blood glucose (mmol/L)}} - 3.5 \] % as previously reported [32].

Plasma triglycerides. Triglyceride content of plasma was quantified using a commercial colorimetric kit following the manufacturer’s instructions (Abcam).

Plasma DPP-IV activity. Dipeptidyl peptidase IV (DPP-IV) activity in 5uL plasma was measured using a commercial DPP-IV Glo® kit (Promega, Madison, WI) after 30 min and a standard curve \( y = 115.81x + 727.15, R^2 = 0.9985 \) was produced using human DPP-IV enzyme
(Sigma-Aldrich) as previously reported [33]. Percent of the maximal activity of DPP-IV was calculated considering the water mice as 100% activity.

**Plasma analysis of liver function.** To determine liver function, plasma was screened for enzymes alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), and total bilirubin using a Beckman Coulter AU 680 Chemistry System (Beckman Coulter, Inc., Brea, CA) [34].

**Tissue analyses.** Immediately following euthanasia, tissues were collected and weighed including pancreas, liver, and adipose (both epididymal and retroperitoneal fat pads), and a portion of each tissue was fixed with 10% formalin. A separate portion of the pancreas and liver were preserved in RNAlater® (Life Technologies, Carlsbad, CA) for genetic analysis.

**Immunohistochemical analysis.** Formalin-fixed unprocessed tissue of pancreas, liver, and adipose tissue (n=12/group) was used for hematoxylin and eosin (H&E) staining to determine histomorphological features. In liver, a standard non-alcoholic fatty liver disease (NAFLD) lipid scoring tool was used to determine the size and distribution of lipid vacuoles. In adipose, the macrophage infiltration was determined as the number of macrophages per total cell and adipocyte diameter was measured using ImageJ to measure the diameter of 50 adipocytes per animal (total 500 adipocytes/group) [35]. Histomorphologic features of the pancreas such as presence or absence of inflammation, apoptosis, necrosis, and fibrosis were evaluated. Pancreatic islet diameters were measured using ImageJ (n > 24/group). Prior to immunohistochemical (IHC) analyses, paraffin-embedded tissues (n=12/group) were cut at 5 µm thickness and transferred to a slide. Liver and pancreas were stained with rabbit polyclonal antibodies for proliferating cell nuclear antigen (PCNA) (ab2426, Abcam) as a marker of cell proliferation and malondialdehyde (MDA) (ab6463, Abcam) as a marker of oxidation to assess free radical
damage. For assessment of islet cell function, IHC analyses for insulin and glucagon were performed. The total number of cells in each islet was counted, then just the number of insulin-positive (beta) cells to obtain the proportion of β-cells in each islet. Glucagon-positive (alpha) cells were counted and related to the total cell population in the same manner. Insulin (and glucagon) content was semi-quantified on a scale from 0-4 as follows: 0- no positive staining for insulin; 1- weak diffuse staining; 2- sparse granular staining; 3- strong granular staining in most cells; 4- intense granular staining in most or all cells. The mean scores were analyzed using non-parametric statistics. Analyses were performed at the Veterinary Diagnostic Laboratory at the University of Illinois under the supervision and training of Mathew Wallig DVM, PhD. Stained slides were visualized using Nanozoomer Digital Pathology (Olympus Hamamatsu, Bridgewater, NJ).

**Gene expression analysis.** RNA was extracted from RNA later®-stabilized frozen pancreatic tissue using a RNeasy Mini Kit (Qiagen, MD, USA) following manufacturer’s protocol to measure changes in expression of all mouse genes and was analyzed using an Illumina MouseWG-6 v2.0 Expression BeadChip. Microarray data pre-processing and statistical analyses were done in R [36] (v 3.0.1) using the limma package [37] (v 3.16.7). Pairwise comparisons between treatments and control were pulled as contrasts from the model; along with the equivalent of a one-way ANOVA test for the treatments. Raw p-values were adjusted separately for each comparison using the False Discovery Rate method [38]. Gene annotation for each probe, including Gene Ontology terms and KEGG pathways, were taken from the package maintained by Bioconductor [39]. The cut-off used for reporting significant changed in gene expression was FDR p-value <0.3 for pancreatic tissue. Similarities and differences on the genes affected by the treatments were analyzed by GeneVenn [40]. Genes were annotated and
biological processes were analyzed using the DAVID v 6.7 [41, 42]. As an alternative approach to identify biological processes and functions that are modulated after exposure to the treatments, exploratory functional analysis was performed using Ingenuity® Pathway Analysis (IPA) v 9.0 (Ingenuity® Systems). A Downstream Effect Analysis was performed using IPA, which identifies biological functions that are expected to be increased (z > 0) or decreased (z < 0) given the observed gene expression changed in a data-set uploaded to the software. The z-score statistic is a calculated quantity to reflect the overall predicted activation state of a biological function. A particular function has more increased than decreased predictions if z ≥ 0 or vice more decreased than increased predictions if z ≤ 0. If an absolute z-score is large enough it will fall into the tail of the Gaussian distribution of the possible calculated z-score statistic, thus, it would be unlikely to obtain that value of z by chance. In practice, z-scores less than -2 or greater than 2 can be considered significant in order to reduce the chance that random data will generate significant predictions. Therefore, z-scores ≤ -2 indicate a significant decrease while z-scores ≥ 2 indicate a significant increase of that biological function.

**Statistical analysis.** Group mean comparisons were conducted using one-way analysis of variance (ANOVA) and LSD post-hoc comparison using the proc GLM procedures and non-parametric Mann-Whitney test of SAS version 9.3 (SAS Inst. Inc., Cary, NC). Significance was considered at p < 0.05, and data are presented as means ± SEM, n=12 mice/group unless stated.

**Results**

**Identification of anthocyanins using HPLC-MS/MS.** The main peaks of the 70%blackberry-30%blueberry AFFB or PAE were detected at maximal absorbance at 280, 320, 360, and 520 nm (Figures 10.2 and 10.3). The primary peak present in both AFFB and PAE was

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16 Gene expression analysis using IPA was conducted by Diego Luna Vital.
seen at retention times of 38.9 min. This peak was maximally absorbed at 280 nm, indicating that it was a phenolic acid or hydrolysable tannin that was tentatively identified as ellagic acid with MS/MS analysis. After passing the AFFB through Amberlite resin to generate the PAE, the concentration of phenolic acids and anthocyanins was increased, while the flavonols and hydroxycinnamic acids were filtered out (Figure 10.3). The relative proportion of the peak at 38.9 min was reduced in the PAE relative to the AFFB due to the increase of peaks at 22.9 and 27 min. The peak at 27 min was identified as a derivative of the anthocyanin cyanidin, while the peak at 22.9 was identified as the flavonol gallic acid 3-O-gallate. Total polyphenolic content in the PAE groups was determined to be 65.1, 631.6, and 2033.6 mg gallic acid equivalents (GAE) per mL leading to a consumption of phenolic compounds at 0.41, 2.64, and 6.38 mg GAE/mouse/d for 0.1X, 1X, and 2X, respectively.

**Diet and treatments.** The consumption of anthocyanins for the AFFB group (8.35 mg/kg BW/d), was almost the same and not statistically different (p > 0.05) as the amount consumed by the 1X group (9.04 mg/kg BW/d.). The 0.1X group consumed 1.11 mg/kg BW/d, which was 12% of the ANC content present in the AFFB and 1X PAE group. The highest PAE group consumed twice as much (18.9 mg/kg BW/d), therefore is referred to as 2X. This is equivalent to 0.23, 0.032, 0.24, and 0.47 mg C3G/mouse/d for AFFB, 0.1X, 1X, and 2X, respectively. Consumption of sucrose was not significantly different between groups (p > 0.05) (Table 10.1). While food intake was lower for the 2X group compared to the water control (p > 0.05), the metabolic efficiency was not. The metabolic efficiency was determined as the ratio of BW gained compared to the amount of diet consumed. This only differed between the 1X PAE and sitagliptin mice compared to the 0.1X PAE and water-only mice (p < 0.05).
Figure 10.2. HPLC chromatogram of AFFB with RT (retention time, in min) indicating the peaks present at maximum absorbance of 360 nm (flavonols), 320 nm (hydroxycinnamic acids), 280 nm (phenolic acids and hydrolysable tannins), and 520 nm (anthocyanins) and the full diode array. AFW.BB: Alcohol-free fermented beverage.
Figure 10.3. HPLC chromatogram of the phenolic extract PAE with RT (retention time, in min) indicating the peaks present at maximum absorbance of 360 nm (flavonols), 320 nm (hydroxycinnamic acids), 280 nm (phenolic acids and hydrolysable tannins), and 520 nm (anthocyanins) and the full diode array. PAE.BB: Post-amberlite extract; both are 70% blackberry – 30% blueberry.
Table 10.1. Averages of food intake, sucrose consumption, body weight gain, fat and lean mass, and fasting blood glucose, throughout the entire study or changes in parameters from baseline to week 12 of the experiment.

<table>
<thead>
<tr>
<th>Value Group</th>
<th>Avg. total Food Intake (g)</th>
<th>Ratio (g BW/g HFD consumed)</th>
<th>Avg. sucrose consumption (g/day)</th>
<th>Weight gain (g)</th>
<th>Week 12 % BW Fat, (Fat Mass Gain in g)</th>
<th>Lean Mass Gain (g)</th>
<th>Week 12 FBG and (change due to HFD) (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>187.8 ± 5.0 a</td>
<td>0.10 ± 0.006 b</td>
<td>0.30 ± 0.02 a</td>
<td>16.7 ± 1.1 a</td>
<td>31.1 ± 1.2 (19.3 ± 1.2 b)</td>
<td>4.9 ± 0.5 ab</td>
<td>221.9 ± 7.7 a (109.2 ± 18.4 ab)</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>188.0 ± 6.0 ab</td>
<td>0.11 ± 0.005 a</td>
<td>0.30 ± 0.02 a</td>
<td>14.9 ± 1.4 ab</td>
<td>27.4 ± 2.1 (15.9 ± 2.0 ab)</td>
<td>4.7 ± 0.4 ab</td>
<td>216.9 ± 10.5 a (119.8 ± 15.2 a)</td>
</tr>
<tr>
<td>AFB</td>
<td>181.4 ± 4.2 abc</td>
<td>0.10 ± 0.006 ab</td>
<td>0.35 ± 0.02 ab</td>
<td>12.6 ± 0.8 bc</td>
<td>23.8 ± 1.5 (12.2 ± 1.4 bc)</td>
<td>4.5 ± 0.3 ab</td>
<td>228.6 ± 6.6 a (107.9 ± 17.9 ab)</td>
</tr>
<tr>
<td>0.1X</td>
<td>180.9 ± 4.9 ab</td>
<td>0.09 ± 0.003 b</td>
<td>0.31 ± 0.01 a</td>
<td>14.7 ± 0.9 ab</td>
<td>25.6 ± 1.5 (13.4 ± 1.6 b)</td>
<td>5.4 ± 0.5 ab</td>
<td>185.0 ± 9.8 b (49.9 ± 18.7 c)</td>
</tr>
<tr>
<td>1X</td>
<td>179.0 ± 3.3 bc</td>
<td>0.11 ± 0.007 a</td>
<td>0.32 ± 0.02 a</td>
<td>11.7 ± 0.4 c</td>
<td>21.3 ± 1.6 (9.2 ± 1.5 cd)</td>
<td>4.0 ± 0.4 b</td>
<td>167.5 ± 9.4 b (66.3 ± 19.7 bc)</td>
</tr>
<tr>
<td>2X</td>
<td>171.7 ± 2.4 c</td>
<td>0.10 ± 0.005 ab</td>
<td>0.34 ± 0.02 a</td>
<td>10.3 ± 0.3 c</td>
<td>18.1 ± 0.8 (6.8 ± 0.9 d)</td>
<td>4.5 ± 0.2 ab</td>
<td>183.7 ± 7.3 b (40.3 ± 17.8 c)</td>
</tr>
</tbody>
</table>

HFD, high-fat diet; BW, body weight; FBG, fasting blood glucose. All values are mean ± standard error of the mean over the course of the experiment. “Gain” indicates a change from week 0. Different letters in each column indicate significant differences between groups for that column (p < 0.05). ^Change is from week 3 when the HFD began until the end of the study.
**Body weight.** The body weight of all groups did not differ at baseline (p > 0.05) nor at the first week when mice were randomized into treatment groups. After groups were started on the high-fat diet for about three weeks they started to gain weight (Figure 10.4A). At the end of the experiment, the water group had gained the most weight (16.7 g), though this was not significantly different than the weight gained by the mice in the sitagliptin group or the mice receiving the lowest ANC concentration (0.1X PAE) (Figure 10.4B). There was, however, a marked difference (p < 0.05) in the body weights of the mice receiving the 1X and 2X PAE (11.7 and 10.3 g BW gained, respectively) compared to the other groups. This attenuation of diet-induced obesity could also be observed phenotypically comparing mice receiving only water (Figure 10.4C) to those receiving the highest dose of PAE (Figure 10.4D). It was found that mice in the group consuming the most anthocyanins (9 or 19 mg C3G/kg BW/d) had the lowest body weight gain.

A.

![Graph showing body weight gain over time and differences between groups.](image)

**Figure 10.4.** Body weight in grams throughout time (A), and body weight gain from baseline (B). (C) indicates a representative mouse from the water group, and (D) a representative mouse from the 2X PAE group at week 12. n=12/group. Different letters indicate significant differences (p < 0.05).
**Body composition analysis.** Free and total body water did not differ between the groups throughout the study (p > 0.05). The percent body weight for free water decreased for all mice throughout the study from 0.62 ± 0.10% at baseline to 0.55± 0.15% at week 12, while total body water decreased from 63.8 ± 1.1% at baseline to 51.2 ± 1.3% at week 12. Percent fat mass for all groups increased over the course of the study (Figure 10.5A) with clear differences due to treatments observed at week 10 and week 12 of treatments, with an inverse association observed for percent lean mass (Figure 10.5C). The percent of fat mass for all groups at week 10 was lower compared to the water group (p < 0.05). Fat mass gain in absolute terms was also significantly (p < 0.05) lower in mice drinking AFFB and all three doses of PAE compared to
water (Figure 10.5B). At week 12, the lowest percent fat mass was seen for the 2X PAE group, followed by the 1X PAE mice, the AFFB group, the 0.1X, and finally the sitagliptin group which did not differ from the water group (p > 0.05). The absolute fat mass followed the same trend as the percent fat mass; mice in the 2X group gained the least amount of fat (6.75 g) compared to the water group (19.33 g). This trend was mirrored for the percent lean mass in that the percent lean mass was highest for the mice receiving the 2X PAE, and mice consuming water had the lowest percent lean mass. The percent lean mass was significantly higher for all groups compared to the water control mice at week 12 (Figure 10.5C). However, there was no difference in the gain in lean mass over time between the groups, except for the mice in the 1X group who gained slightly less lean mass than the other groups (Figure 10.5D); the differences seen between groups for percent lean mass over time is due to gained fat mass, rather than a change in absolute lean mass. Overall, mice in both groups consuming the most anthocyanins attenuated the HFD-induced weight gain seen by the water control mice, and also had the lowest gain in fat mass (18.0 ± 0.8 % BW).

Blood glucose. There was no difference in baseline fasted (4-6 h) blood glucose (FBG) due to the treatments for one week before beginning the HFD (Figure 10.6A). After being on the HFD for three weeks the mice began to develop the hyperglycemic phenotype, and at week 6, all groups had FBG above 120 mg/dL. There was no difference among the groups until week 11, when the FBG for both, the 1X and the 2X PAE mice, had a lower FBG than the water group. At week 12, all three PAE groups had a lower FBG (p < 0.05) than the water group (221.8 mg/dL) at 184.9, 167.4, and 183.4 mg/dL for 0.1X, 1X and 2X, respectively.
Figure 10.5. Fat mass as a percent by week (A), and as an absolute change (B) was significantly lower in mice drinking AFFB and all three doses of PAE compared to water. Percent lean mass was significantly higher for all groups compared to the water control mice at week 12 (C), but there were no significant differences in terms of absolute lean mass (D). Different letters indicate significant differences ($p < 0.05$) for that week (A and C) and in (B and D) different letters indicate significant differences for absolute mass gain ($p < 0.05$). Mice were scanned with an EchoMRI-700 Body Composition Analyzer. n=12/group.
Considering the change in blood glucose that was induced by consumption of the HFD, all three doses of the phenolic extract attenuated the HF-driven hyperglycemia with the 0.1X and the 2X mice the most effective (Figure 10.6B). FBG for 1X and 2X groups was also significantly lower than the sitagliptin group (216.9 mg/dL, p < 0.05). The increase in FBG due to the HFD for the group receiving 1X PAE was also not different from either the AFB or the sitagliptin group. Further, AFFB and sitagliptin were not able to attenuate the induction of hyperglycemia due to the consumption of the HFD compared to water.

**Figure 10.6.** Fasting blood glucose induced by the HFD was attenuated by the PAE. Mice (n=12/group) were morning fasted 4-6 h and treatments were replaced with plain water until tail blood was measured for glucose using an Alpha-Trak2 handheld glucometer. *Indicates significant (p < 0.05) difference from water group for that week. Different letters for gains indicate significant (p < 0.05) differences between groups (change from week 3 to week 12).
**Insulin content and HOMA-IR calculation.** Fasting plasma insulin did not differ due to treatments; all mice became hyperinsulinemic (Table 10.2), as expected for this strain of mice. At the end of the study (week 12), the range of insulin was from 2.43 to 2.95 ng/mL. When considering the glucose along with the plasma insulin all three PAE groups had a calculated HOMA-IR lower than the water group, while the HOMA-β for the PAE groups were all higher than the water group (Table 10.2). HOMA-IR is a calculated value that provides the degree of insulin resistance, while HOMA-β is a calculated value indicating the percent beta cell function. This takes into account both the plasma glucose and insulin that was confirmed with immunohistochemical detection of insulin and glucagon content in pancreatic islets (results shown in the histochemical assessment of the pancreas section below).

**Plasma triglycerides.** Plasma triglycerides were highest for the mice receiving water, sitagliptin, or the AFFB that was attenuated by the mice receiving the highest amount of phenolic compounds (1X and 2X) (Table 10.2). There was no difference (p > 0.05) between the 1X and 2X mice, similar to the results seen for the reduction in fat mass.

**Plasma DPP-IV activity.** Plasma DPP-IV was evaluated to determine the effect on prolonging incretin action by reducing the activity of this incretin-cleaving hormone. As expected, sitagliptin, used as the control of DPP-IV inhibition, was able to decrease the maximal activity of DPP-IV seen in the water group by 36.6% activity. There were also decreases of 16.7 and 11.5 % for AFFB and 0.1X PAE, respectively. There was a trend to decrease, but was not significant, the DPP-IV activity in the mice receiving the higher doses of the phenolic extract.

**Tissue analysis.** Table 10.3 indicates the weights of the tissues collected at the end of the study. There was no effect on the weights of the spleens or the weight of the pancreas (when corrected for BW) and no major differences in the weights of the lungs.
<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen</th>
<th>Pancreas</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lungs</th>
<th>Heart</th>
<th>Pericardial adipose</th>
<th>Total Visceral Adipose^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.072 ± 0.002</td>
<td>0.227 ± 0.013</td>
<td>1.081 ± 0.035</td>
<td>0.342 ± 0.008</td>
<td>0.132 ± 0.005</td>
<td>0.113 ± 0.015</td>
<td>0.026 ± 0.006</td>
<td>2.431 ± 0.158</td>
</tr>
<tr>
<td></td>
<td>(0.22 ± 0.01)</td>
<td>(0.71 ± 0.03)</td>
<td>(3.37 ± 0.09)</td>
<td>(1.06 ± 0.02)</td>
<td>(0.41 ± 0.01)</td>
<td>(0.35 ± 0.04)</td>
<td>(0.06 ± 0.02)</td>
<td>(7.52 ± 0.37)</td>
</tr>
<tr>
<td>Sitag</td>
<td>0.074 ± 0.006</td>
<td>0.224 ± 0.017</td>
<td>1.026 ± 0.035</td>
<td>0.332 ± 0.013</td>
<td>0.132 ± 0.008</td>
<td>0.127 ± 0.005</td>
<td>0.030 ± 0.010</td>
<td>2.088 ± 0.282</td>
</tr>
<tr>
<td></td>
<td>(0.24 ± 0.02)</td>
<td>(0.72 ± 0.03)</td>
<td>(3.34 ± 0.08)</td>
<td>(0.98 ± 0.09)</td>
<td>(0.44 ± 0.04)</td>
<td>(0.41 ± 0.01)</td>
<td>(0.09 ± 0.02)</td>
<td>(6.46 ± 0.56)</td>
</tr>
<tr>
<td>AFB</td>
<td>0.073 ± 0.012</td>
<td>0.175 ± 0.018</td>
<td>1.071 ± 0.093</td>
<td>0.332 ± 0.020</td>
<td>0.130 ± 0.004</td>
<td>0.121 ± 0.003</td>
<td>0.015 ± 0.002</td>
<td>1.438 ± 0.191</td>
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<td></td>
<td>(0.27 ± 0.04)</td>
<td>(0.65 ± 0.07)</td>
<td>(3.99 ± 0.35)</td>
<td>(1.24 ± 0.08)</td>
<td>(0.48 ± 0.02)</td>
<td>(0.45 ± 0.01)</td>
<td>(0.04 ± 0.01)</td>
<td>(5.12 ± 0.54)</td>
</tr>
<tr>
<td>0.1X</td>
<td>0.067 ± 0.002</td>
<td>0.209 ± 0.013</td>
<td>1.326 ± 0.046</td>
<td>0.326 ± 0.007</td>
<td>0.135 ± 0.004</td>
<td>0.124 ± 0.003</td>
<td>0.024 ± 0.005</td>
<td>1.773 ± 0.188</td>
</tr>
<tr>
<td></td>
<td>(0.23 ± 0.01)</td>
<td>(0.72 ± 0.05)</td>
<td>(4.52 ± 0.14)</td>
<td>(1.11 ± 0.03)</td>
<td>(0.46 ± 0.02)</td>
<td>(0.42 ± 0.01)</td>
<td>(0.06 ± 0.01)</td>
<td>(5.88 ± 0.43)</td>
</tr>
<tr>
<td>1X</td>
<td>0.070 ± 0.004</td>
<td>0.191 ± 0.007</td>
<td>1.871 ± 0.140</td>
<td>0.356 ± 0.009</td>
<td>0.149 ± 0.003</td>
<td>0.109 ± 0.013</td>
<td>0.016 ± 0.003</td>
<td>1.125 ± 0.065</td>
</tr>
<tr>
<td></td>
<td>(0.26 ± 0.01)</td>
<td>(0.72 ± 0.02)</td>
<td>(7.02 ± 0.52)</td>
<td>(1.33 ± 0.02)</td>
<td>(0.56 ± 0.01)</td>
<td>(0.41 ± 0.05)</td>
<td>(0.05 ± 0.01)</td>
<td>(4.19 ± 0.19)</td>
</tr>
<tr>
<td>2X</td>
<td>0.065 ± 0.002</td>
<td>0.165 ± 0.005</td>
<td>2.004 ± 0.056</td>
<td>0.340 ± 0.007</td>
<td>0.132 ± 0.005</td>
<td>0.110 ± 0.007</td>
<td>0.009 ± 0.001</td>
<td>0.820 ± 0.073</td>
</tr>
<tr>
<td></td>
<td>(0.27 ± 0.01)</td>
<td>(0.67 ± 0.03)</td>
<td>(8.17 ± 0.13)</td>
<td>(1.39 ± 0.04)</td>
<td>(0.54 ± 0.02)</td>
<td>(0.45 ± 0.03)</td>
<td>(0.02 ± 0.01)</td>
<td>(3.30 ± 0.24)</td>
</tr>
</tbody>
</table>

^Total visceral adipose includes both the epididymal and retroperitoneal fat pads. Data are mean ± standard error of the mean, n = 9-12. Different lowercase letters indicate significant differences for that organ by weight and different uppercase letters by % of BW; no letters indicate there were no differences (p > 0.05). BW, body weight.

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Mice in the 2X group had the smallest hearts in terms of absolute mass that were largest as a percent of BW. There was a significant reduction in the percent weight of pericardial fat by the AFFB, 1X and 2X PAE compared to the water as well as a dramatic reduction in total visceral adipose in both absolute weight and when corrected for BW. There was an unexpected increase in the weights of the livers by both the 1X and 2X PAE, which prompted further histomorphological investigation.

**Histomorphologic features of the pancreas.** Staining with Hematoxylin and Eosin (H&E) indicated no presence of apoptosis, inflammation, necrosis or fibrosis in any of the groups (Figure 10.7A1-A6). The islet diameter for all groups fell within the functional range; they were the largest for the sitagliptin treated mice (151 ± 23 µm), with no difference from the 1X and 2X PAE groups (p > 0.05), while mice in the AFFB group had the smallest islet diameters (111 ± 17 µm). The relative insulin and glucagon content indicated that most islets stain strongly for glucagon on the periphery of the islets (α-cells) while the staining for insulin occurs in the majority of the islet, indicating β-cells (Figure 7B). Results of insulin and glucagon content of the pancreatic islets in consideration with the total number of islet cells determine any difference in the functionality of the pancreatic β-cells. As most of the mice were hyperinsulinemic, many of the β-cells stained only lightly for insulin as much of the insulin had been secreted already, however there was stronger staining present for glucagon. The amount of proliferating cells in pancreatic islets was also determined by staining with PCNA; the more cells that are proliferating in the pancreatic islets may indicate apoptosis of the currently existing β-cells. There was a trend to increase the number of proliferating cells per islet in the sitagliptin group (1.8 ± 0.2 cells/islet) that was only significantly different (p < 0.05) than the 2X mice that had the fewest proliferating cells per islet (1.1 ± 0.2 cells/islet).
Table 10.3. Insulin, calculated insulin resistance and β-cell function, triglycerides, DPP-IV, and liver functional enzymes analyzed in fasting plasma.

<table>
<thead>
<tr>
<th>Value Group</th>
<th>Insulin (ng/mL)</th>
<th>HOMA-IR</th>
<th>HOMA-β (%)</th>
<th>Triglycerides (mmol/μL)</th>
<th>[DPP-IV] (% of water)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>AST:ALT ratio</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2.95 ± 0.28a</td>
<td>3.75 ± 0.4a</td>
<td>15.8 ± 1.5b</td>
<td>1.23 ± 0.39a</td>
<td>100.0 ± 5.4a</td>
<td>69 ± 25b</td>
<td>324 ± 138a</td>
<td>3.8 ± 0.6ab</td>
<td>53 ± 2c</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>2.50 ± 0.19a</td>
<td>3.30 ± 0.4ab</td>
<td>14.5 ± 1.1bc</td>
<td>1.13 ± 0.34a</td>
<td>63.4 ± 4.1c</td>
<td>35 ± 5b</td>
<td>194 ± 36a</td>
<td>5.0 ± 0.6a</td>
<td>54 ± 2c</td>
</tr>
<tr>
<td>AFB</td>
<td>2.57 ± 0.19a</td>
<td>3.37 ± 0.3ab</td>
<td>13.0 ± 1.0c</td>
<td>1.20 ± 0.10a</td>
<td>83.3 ± 2.7b</td>
<td>91 ± 36b</td>
<td>250 ± 48a</td>
<td>4.2 ± 0.7ab</td>
<td>63 ± 2bc</td>
</tr>
<tr>
<td>0.1X</td>
<td>2.55 ± 0.17a</td>
<td>2.69 ± 0.2bc</td>
<td>18.7 ± 2.1ab</td>
<td>1.03 ± 0.08ab</td>
<td>88.5 ± 6.9b</td>
<td>71 ± 19b</td>
<td>259 ± 72a</td>
<td>3.8 ± 0.3ab</td>
<td>77 ± 4bc</td>
</tr>
<tr>
<td>1X</td>
<td>2.43 ± 0.21a</td>
<td>2.29 ± 0.2c</td>
<td>21.6 ± 2.9a</td>
<td>0.65 ± 0.07c</td>
<td>90.8 ± 3.4ab</td>
<td>52 ± 7b</td>
<td>171 ± 23a</td>
<td>3.4 ± 0.3bc</td>
<td>219 ± 7a</td>
</tr>
<tr>
<td>2X</td>
<td>2.57 ± 0.30a</td>
<td>2.67 ± 0.3bc</td>
<td>18.5 ± 2.8ab</td>
<td>0.76 ± 0.10bc</td>
<td>93.5 ± 1.7ab</td>
<td>146 ± 34a</td>
<td>257 ± 46a</td>
<td>2.1 ± 0.2c</td>
<td>234 ± 9a</td>
</tr>
</tbody>
</table>

HOMA, homeostatic model assessment; HOMA, homeostatic model assessment; IR, insulin resistance as [blood glucose (mmol/L)*serum insulin (mU/L))/22.5]; %β, percent beta cell function as [20* serum insulin (mU/L))/blood glucose (mmol/L) – 3.5]*100%; DPP-IV, dipeptidyl peptidase IV; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase. GGT, gamma-glutamyl transferase, was not detected in any of the groups (except 0.1X, 0.1 ± 0.1 U/L) and total bilirubin was not different between the groups (p > 0.05) and ranged from 0.1 ± 0.01 mg/dL for 2X to 0.2 ± 0.03 mg/dL for all other groups. All values are mean ± standard error of the mean. Different letters in each column indicate significant differences between groups for each column (p < 0.05).
Figure 10.7. Representative images hematoxylin and eosin stained tissue indicating average diameter (µm) of A) pancreatic islets (40x zoom), and B) islets stained for glucagon (-a) and insulin (-b) as an example of staining in order to do calculations of β-cell functionality (40x zoom); and C) diameter of adipocytes in epididymal fat pads (10x zoom); where numbered subscripts indicate treatment group: 1 - water, 2 - Sitagliptin, 3 - AFFB, 4 - 0.1X PAE, 5 - 1X PAE, 6 - 2X PAE. Different letters indicate significant differences (p < 0.05) for that tissue.
**Histomorphologic features of adipose.** The macrophage infiltration in the adipose was not markedly different between treatments (Figure 10.7 C1-C6), however the adipocyte size (diameter) was significantly smaller for both the 1X and 2X mice compared to the water group that had the largest adipocyte diameters (94.8 ± 2.6 μm). There was no difference seen by the sitagliptin nor the 0.1X dose of PAE compared to water; but the AFFB was also able to reduce the adipocyte diameter compared to water (p < 0.05). The results for the adipocyte diameter mimic the results for the total visceral adipose weight (Table 10.2).

**Histomorphologic features of the liver.** The weights of the livers in the mice receiving the PAE were significantly higher than all the other groups of mice (Table 10.3); therefore, the livers were analyzed by histopathological assessments. The livers in these groups appeared pink and smooth during necropsy, with no obvious indications of fat accumulation or damage. Staining of livers with H&E indicated no clear inflammation, necrosis or apoptosis (Figure 10.8 A1-A6); however, we detected an unexpected lipidosis present in the PAE groups (Figure 10.8 A5 and 10.8 A6). These lipids were both macro- as well as microvesicular and located focally only in the periportal region compared to the lipids in the HFD-fed water mice that were located in zone 2 (Figure 10.8 A1). Using a standard NAFLD scoring tool to assess the degree of lipidosis from 1-5 where 5 indicates the most severe lipidosis present throughout the whole liver, there were lipids present in the HFD-water control mice that was at a similar level to those present in the sitagliptin group (p > 0.05). There was no lipidosis present in any of the AFFB mice, however the PAE extract exhibited an increasing amount of lipids and distribution with the higher doses indicated in Figure 10.8A. The liver lipids in the 1X and 2X groups indicated an adverse effect due to dose, rather than a response to the high-fat feeding as the lipid distribution and intensity differs from the HFD-induced lipids in the water control mice.
A)

Figure 10.8. Representative images of livers stained for A) Hematoxylin and eosin (20x zoom) with the subjective NAFLD score; B) malondialdehyde to determine lipid peroxidation present with percentage of livers that stained moderately, as represented, or strongly in that group (20x zoom). Subscripts for A) and B) indicate group: 1 - water, 2 - Sitagliptin, 3 - AFFB, 4 - 0.1X PAE, 5 - 1X PAE, 6 - 2X PAE; and C) proliferating cell nuclear antigen to determine cell proliferation between the water mice (C1) and 2X (C2) at 20x zoom. B) and C) have a background stain of Hematoxylin. Different letters indicate significant differences (p < 0.05) for that tissue.
To further assess the changes observed in the liver due to PAE, malondialdehyde (MDA) staining was obtained to determine the degree of fatty acid peroxidation, as well as proliferating cell nuclear antigen (PCNA) to determine the effect on proliferation. Figure 10.8B illustrates the representative oxidation seen by malondialdehyde present in the livers; while most groups indicated MDA staining that was moderate or strong, there was no increase in the proportion in the 1X or the 2X group compared to the water-control group; the toxicity by these high doses was apparently not due to lipid peroxidation. There was a difference seen in the amount of proliferating hepatocytes present in the livers of the 1X and 2X PAE fed mice and water mice; there were many more proliferating hepatocytes focally distributed in the 2X mice (Figure 10.8C). Plasma was analyzed for liver function by screening for ALT, AST, GGT, ALP and total bilirubin (Table 10.3). There was no difference in aspartate transaminase (AST) but there was a 2.1-fold increase in the alanine transaminase (ALT) levels seen by the mice receiving the highest dose of the PAE. An increase in ALT but not AST can be due to excess alanine amino-group transfer to form glutamate and pyruvate. The AST to ALT ratio is also helpful in differentiating causes of liver damage; in this study the ratios for all groups were above 2, and were actually lowest for the 2X group. Alkaline phosphatase (ALP) was the only liver enzyme with a significant increase for both the 1X and 2X PAE groups. Total bilirubin was not increased (p > 0.05) in any of the groups (average of 0.16 ± 0.01 mg/dL for all mice).

**Gene expression analysis.** To determine what mechanisms may have contributed to the lowering of HFD-driven hyperglycemia in the mice receiving PAE, gene expression changes in the pancreas were evaluated. Figure 10.9 shows the heat map generated for the Illumina array; there was a trend to shift the expression profile with the water and sitagliptin groups having a similar pattern, but different from both the AFFB and 0.1X, and the 1X and 2X.
10.9. Heat map illustrating the trend of up-regulated (red) or down-regulated (blue) genes in pancreatic RNA of the mice extracted from RNAlater-stabilized frozen pancreatic tissue (n = 4/group) and gene expression analyzed using an Illumina MouseWG-6 v2.0 Expression BeadChip.
Within the full list of the represented genes in the microarray compared to the control of the water group, 1, 29, 202 and 123 were found to be differentially expressed for AFFB, 0.1X, 1X and 2X respectively (Figure 10.10). No genes were significantly modified compared to water for the sitagliptin group. With FDR < 0.3 (30% chance of false discovery rate, based on the fact that the most highly expressed genes within the pancreas will be secretory or enzyme-related due to the highly specialize pancreas function), 1X and 2X treatments shared 66 differentially expressed genes (DEGs). Interestingly, 0.1 X, 1X and 2X treatments shared 7 DEGs and only one Kdsr (3-ketodihydrosphingosine reductase), which encodes proteins involved in the biosynthesis of sphingolipids, was shared by the four treatments including AFFB.

10.10. Venn diagram of shared differentially expressed genes (DEGs) between the treatments, false discovery rate < 0.3.

Analysis of this set of genes using DAVID showed that using functional annotation clustering, 8, 52 and 26 were generated for 0.1X, 1X and 2X respectively. However, only 6 and 1
clusters had an enrichment score $> 1.3$ (-log p-value) equivalent to p=0.05) for 1X and 2X respectively (Tables 10.4 and 10.5). The highly-enriched clusters were associated mainly to cellular localization of the molecules, as there were no associations found with canonical pathways. The main clusters affected by 1X were related to ribosomal function, Ubl conjugation, oxidation reduction, mitochondrial structure, NAD(P) binding, and antioxidant activity. There was only one cluster with significant enrichment scores for the 2X treatment that was related to ribosomal structure and function.

Table 10.4. Functional annotation clustering with significant enrichment scores for 1X PAE treatment.

<table>
<thead>
<tr>
<th>Annotation cluster 1</th>
<th>Enrichment Score: 4.93</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosome</td>
<td>11</td>
<td>2.3E-8</td>
<td></td>
</tr>
<tr>
<td>Translation</td>
<td>16</td>
<td>4.7E-7</td>
<td></td>
</tr>
<tr>
<td>Structural constituent of ribosome</td>
<td>12</td>
<td>4.7E-7</td>
<td></td>
</tr>
<tr>
<td>Intracellular non-membrane-bounded organelle</td>
<td>29</td>
<td>3.7E-2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Annotation cluster 2</th>
<th>Enrichment Score: 2.19</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubl conjugation</td>
<td>14</td>
<td>1.6E-3</td>
<td></td>
</tr>
<tr>
<td>Cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)</td>
<td>7</td>
<td>9.3E-3</td>
<td></td>
</tr>
<tr>
<td>Isopeptide bond</td>
<td>8</td>
<td>1.8E-2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Annotation cluster 3</th>
<th>Enrichment Score: 2.07</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation reduction</td>
<td>15</td>
<td>5.7E-3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Annotation cluster 4</th>
<th>Enrichment Score: 1.51</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrion</td>
<td>25</td>
<td>4.6E-3</td>
<td></td>
</tr>
<tr>
<td>Respiratory chain</td>
<td>5</td>
<td>4.6E-3</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>4</td>
<td>2.4E-1</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial lumen</td>
<td>4</td>
<td>2.4E-1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Annotation cluster 5</th>
<th>Enrichment Score: 1.42</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide phosphate-binding region:NADP</td>
<td>4</td>
<td>2.7E-2</td>
<td></td>
</tr>
<tr>
<td>NAD(P)-binding domain</td>
<td>4</td>
<td>1.7E-1</td>
<td></td>
</tr>
</tbody>
</table>
Genes were analyzed using DAVID functional annotation clustering; 52 total clusters were generated for 1X PAE. Enrichment scores are considered significant if > 1.3 [-log(p-value) equivalent to p=0.05].

Table 10.5. Significant enrichment scores generated by functional annotation clustering for 2X PAE treatment

<table>
<thead>
<tr>
<th>Annotation cluster 6</th>
<th>Enrichment Score: 1.38</th>
<th>Count</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase activity</td>
<td>3</td>
<td>3.4E-2</td>
<td></td>
</tr>
<tr>
<td>Oxidoreductase activity, acting on peroxide as acceptor</td>
<td>3</td>
<td>3.4E-2</td>
<td></td>
</tr>
<tr>
<td>Antioxidant activity</td>
<td>3</td>
<td>6.5E-2</td>
<td></td>
</tr>
</tbody>
</table>

Genes were analyzed using DAVID functional annotation clustering; 26 total clusters were generated for 2X PAE. Enrichment scores are considered significant if > 1.3 [-log(p-value) equivalent to p=0.05].

An alternative approach used to identify biological processes affected by the treatments was Ingenuity® Pathway Analysis (IPA). Further enrichment analysis on canonical pathways of IPA Knowledge Base provided significant overlaid pathways through the list of DEGs, finding potential activation of amino acid metabolism pathways by the treatments (Table 10.6). Highly correlated gene network among DEGs in pancreatic tissue was highest for cell cycle and cellular development (S=41), carbohydrate metabolism, molecular transport and small molecule biochemistry (S=38), and metabolic disease, lipid metabolism and molecular transport (S=25) for 0.1X, 1X and 2X treatments respectively.
Table 10.6. Comparison analysis among PAE treatments of differentially expressed genes by top canonical pathway, predicted downstream function and network.

<table>
<thead>
<tr>
<th>Samples Comparison</th>
<th>0.1X</th>
<th>1X</th>
<th>2X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top canonical pathways</td>
<td>Glycine biosynthesis I (p=0.00209, ratio=1/2)</td>
<td>Methionine degradation I (p-value = 0.00356, ratio= 2/17)</td>
<td>Methionine degradation I (p-value=0.00153, ratio=2/17)</td>
</tr>
<tr>
<td></td>
<td>dTMP de novo biosynthesis (p-value=0.00522, ratio=1/5)</td>
<td>Cysteine biosynthesis III (p-value = 0.00445, ratio= 2/17)</td>
<td>Cysteine biosynthesis III (p-value=0.00191, ratio=2/19)</td>
</tr>
<tr>
<td></td>
<td>Folate polyglutamylation (p-value=0.00522, ratio=1/5)</td>
<td>Alanine degradation III (p-value = 0.0105, ratio= 1/2)</td>
<td>Superpathway of methionine degradation (p-value=0.00539, ratio=2/32)</td>
</tr>
<tr>
<td>Top biological functions</td>
<td>Cell cycle (p-value=0.036, M=4)</td>
<td>Carbohydrate metabolism (p-value = 0.000142, M=15)</td>
<td>Amino acid metabolism (p-value=0.0238, M=6)</td>
</tr>
<tr>
<td></td>
<td>Cellular development (p-value=0.047, M=8)</td>
<td>Molecular transport (p-value = 0.000142, M=16)</td>
<td>Molecular transport (p-value=0.0271, M=12)</td>
</tr>
<tr>
<td></td>
<td>Lipid metabolism (p-value=0.049, M=6)</td>
<td>Small molecule biochemistry (p-value = 0.000142, M=24)</td>
<td>Small molecule biochemistry (p-value=0.0371, M=17)</td>
</tr>
<tr>
<td>Top toxicological functions</td>
<td>Increased levels of bilirubin (p-value=0.00626, M=1)</td>
<td>Pulmonary hypertension (p-value=0.000410, M=4)</td>
<td>Liver cholestasis (p-value=0.00686, M=1)</td>
</tr>
<tr>
<td></td>
<td>Cardiac arrhythmia (p-value=0.00209, M=1)</td>
<td>Glutathione depletion in liver (p-value = 0.00528, M=2)</td>
<td>Liver steatosis (p-value=0.00754, M=4)</td>
</tr>
<tr>
<td></td>
<td>Liver cholestasis (p-value=0.0417, M=1)</td>
<td>Glomerular injury (p-value = 0.00528, M=2)</td>
<td>Cardiac fibrosis (p-value=0.0103, M=1)</td>
</tr>
<tr>
<td>Top networks</td>
<td>Cell cycle, cellular development (S=41)</td>
<td>Carbohydrate metabolism, molecular transport and small molecule biochemistry (S=38)</td>
<td>Metabolic disease, lipid metabolism and molecular transport (S=25)</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular disease, hematological disease, hereditary disorder (S=3)</td>
<td>Cell cycle, cellular development and cellular growth and proliferation (S=24)</td>
<td>Protein synthesis, cellular assembly and organization, cellular compromise (S=25)</td>
</tr>
<tr>
<td></td>
<td>Embryonic development, infectious disease (S=2)</td>
<td>Connective tissue disorders, dermatological diseases (S=24)</td>
<td>DNA replication, recombination, cell cycle (S=23)</td>
</tr>
</tbody>
</table>
Table 10.6 (contd.)

<table>
<thead>
<tr>
<th>Top molecules</th>
<th>Fold change up-regulated</th>
<th>Fold change down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecules</td>
<td>Fold change</td>
<td>Molecules</td>
</tr>
<tr>
<td>Acot1</td>
<td>↑7.42</td>
<td>GDF15</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>↑3.94</td>
<td>REEP</td>
</tr>
<tr>
<td>NR1H4</td>
<td>↑2.57</td>
<td>SLAMF9</td>
</tr>
<tr>
<td>SHMT2</td>
<td>↑1.81</td>
<td>AZGP1</td>
</tr>
<tr>
<td>RANGFR</td>
<td>↑1.74</td>
<td>SLC7A3</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>↑3.84</td>
<td>REEP</td>
</tr>
<tr>
<td>NR1H4</td>
<td>↑3.88</td>
<td>SLC7A3</td>
</tr>
<tr>
<td>SHMT2</td>
<td>↑2.46</td>
<td>NDRG2</td>
</tr>
<tr>
<td>RANGFR</td>
<td>↑2.05</td>
<td>SLAMF9</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>↑2.43</td>
<td>NDRG2</td>
</tr>
<tr>
<td>NR1H4</td>
<td>↑2.09</td>
<td>SLAMF9</td>
</tr>
<tr>
<td>SHMT2</td>
<td>↑2.08</td>
<td>NDRG2</td>
</tr>
<tr>
<td>RANGFR</td>
<td>↑2.08</td>
<td>SLAMF9</td>
</tr>
</tbody>
</table>

Results were interpreted using Ingenuity® Pathway Analysis at FDR <0.3, significance indicates the probability of association of molecules from the dataset with the canonical pathway by random chance alone. M: number of molecules; S: Score = -log(Fisher’s Exact test result) = 6.

Additionally, a Downstream Effect Analysis was performed using IPA, which identified biological functions that were expected to be increased or decreased given the observed gene expression changed in a data-set uploaded to the software. The differential gene expression of the treatments resulted in a high (absolute z-score of ≥ 2) predicted decrease of concentration of glucose for 0.1X (z-score = -2.12), 1X (z-score = -2.12) and 2X (z-score = -2.71) (Figure 10.11).

![Prediction Legend](image)

Figure 10.11. Predicted downstream function to inhibit D-glucose concentration by all three PAE treatments 0.1X (A), 1X (B) and 2X (C). RNA extracted from RNAlater-stabilized frozen pancreatic tissue (n = 4/group) and gene expression analyzed using an Illumina MouseWG-6 v2.0 Expression BeadChip. Results were interpreted using Ingenuity® Pathway Analysis at FDR <0.3.
Discussion

We found that C57BL/6j mice in the groups consuming the most anthocyanins had the lowest body weight gain due to an attenuated gain in fat mass induced by the HFD, while absolute lean mass did not differ. Mice in the 2X group did consume less of the HFD than the mice getting only water, but it is not clear whether the reduction of diet intake was the cause or effect of reduction in body weight. The fact that a dose-response was not found between the two highest doses of the PAE agree with previous work showing that lower (1 mg/mL) doses of ANC
may be effective to reduce obesity markers but higher doses may not [15]. Another study indicated a dose of 2 mg ANC/mouse/d may be close to the upper limit that is effective for ANC as they tested 1.8 mg ANC/mouse/day and it was not effective, while the upper limit of purified ANC effectiveness was determined to be 6 mg/mouse/day [20]. Additionally, the fact that our study utilized a combination of ANC with other phenolic compounds may affect the results in comparison with purified ANC in these previous studies. It has been previously seen that purified ANC from blueberries and strawberries can prevent HFD-induced dyslipidemia and obesity in mice, but whole blueberries do not, and that the sugars and lipid components may be masking the benefits of the ANC and other polyphenols [15, 19, 43]. Prevention of diet-induced obesity of C57BL/6 mice with ANC in the diet has been done at 50, 100, or 200 mg/kg food [18], which is equivalent to 0.2 and 0.6 mg ANC/day; this is the level of ANC in the AFFB and 1X groups and the 2X groups, respectively. More studies are needed to determine which compound present in the mixture of phenolic compounds extracted from the alcohol-free fermented blueberry-blackberry beverage was most effective to blunt the diet-induced obesity.

After 3 weeks of treatment, all groups were hyperglycemic, however, at the end of the study mice consuming 1X and 2X groups had significantly lower fasting blood glucose than the water and the sitagliptin group. The values of fasted insulin were in range of that seen by other studies of HFD in C57BL/6J mice as it is expected for this strain of mice to become hyperinsulinemic, and supports no differences seen among the groups [15-20, 29]. Improved fasting blood glucose and glucose tolerance was seen previously at 12.5 mg ANC/mouse/d [43] however these were extracted ANC that were added to the diets at a dose much higher than those used in this study. An intervention trial in humans with the addition of blueberry powder equivalent to about 2 cups of fresh whole blueberries (668 mg of anthocyanins, or 6.7 mg
ANC/kg BW/d) reported that these compounds in blueberries improved insulin sensitivity in obese, insulin-resistant men and women [10]. Translating to an effective dose in mice, this is equivalent to an intervention of 0.16 mg ANC per mouse per day. So, this does fall between the dose for the AFFB or 1X PAE and the 2X PAE and indicates the levels of ANC used in our study can be consumed by humans and would lead to a positive effect on insulin sensitivity.

As no significant reduction in DPP-IV activity by the mice receiving the highest doses of the phenolic compounds was observed, the reduction of hyperglycemia by these two groups cannot be attributed to an action on DPP-IV. The reduction seen by AFFB but not the 1X and 2X PAE suggests there may have been another compound present in the extract which could have interfered with the inhibition of DPP-IV. We have previously established ANC, as well as other berry phenolic compounds, as effective DPP-IV inhibitors using enzymatic and computational modeling, so likely these are the compounds present in AFFB that are effective at reducing the activity of DPP-IV in the plasma of the mice, but may also be due to the metabolites produced from these compounds [22, 33].

The effect on plasma, tissue morphological changes, and gene expression were evaluated to understand the reduction in fat mass and fasting blood glucose in the mice consuming AFFB or the three doses of PAE. All of the groups were in the range of islet sizes known to have a proper insulin secretory response (100-150 µm) [44]. Previous studies have found that there was improved β-cell function with purified ANC, primarily malvidin-3-glucoside and –galactoside as well as delphinidin-3-glucoside, provided in the water at levels of 0.2 mg/mL [15]; which is equivalent to 0.49 mg/mouse/d. In our study the 2X mice were consuming ANC close to this dose (0.47 mg C3G/mouse/d) indicating that there may have been an effect on the improvement
of β-cell function to secrete insulin in response to glucose. The results we found for adipocyte diameter agrees with the literature in that less obese mice have smaller adipocytes [45].

The beneficial results seen by the highest doses need to be taken into consideration with the adverse effects seen for increased liver weights. The unexpected periportal liver lipids observed in the highest doses of the PAE that were not seen in mice receiving the AFFB indicated that there are some compounds in the extract that may have potential toxic effects at the provided doses. It was found that the AFFB protected against the HFD-induced lipid deposition which may suggest there is a compound in the AFFB blunting the negative effects of the extract, or during the extraction process some compounds were increased beyond their beneficial effect. As the anthocyanin content was not different between the AFFB and the 1X group, these adverse effects seem to be independent of the anthocyanin concentration. Further, previous studies conducted using C57BL/6j mice fed blueberries or purified anthocyanins were found to have no adverse effects or an improvement in hepatic steatosis caused by high-fat feeding [16, 46-49]. Previous studies that found polyphenolic compounds from berry fruit prevented inflammation in diet-induced obese mice were an equivalent dose of 120 mg per day in humans [46]; this dose is just above that seen for the 0.1X PAE group. Additionally, higher levels than those used in this study in db/db mice (equivalent to consuming 160-300 mg C3G per kg BW per day of ANC) have been reported to attenuate obesity-associated hepatic steatosis without any noticeable detrimental effects on behavior or appetite, and the mice tolerated the high doses well [48]. This previous study indicated the kidneys, liver, and heart all showed improved or unchanged histopathology as indicated by less heavy livers than the HFD control, reduced microvesicular steatosis, and significant reductions in ALT levels. PACs have also been previously reported to reduce HFD-induced steatosis at a dose of 100 mg/kg BW/d [50]. The
effect of polyphenols from blueberry to inhibit hepatic steatosis in HepG2 cells in vitro was previously seen to be primarily due to the phenolic acids, specifically the caffeic acid present [51]. These phenolic acids would have been removed during the phenolic extraction process with the Amberlite, therefore the PAE may have been less effective than the AFFB, which still contained phenolic acids, to inhibit the high-fat diet-induced hepatic lipids.

Of the liver functional panel enzymes tested in plasma, ALP was the only enzyme with a significant increase for both the highest PAE doses, and increases in this enzyme can occur due to peroxisome proliferation or glycogen accumulation rather than lipidosis [52]. Additionally, the detected bilirubin levels for all mice fall towards the low end of the reference range (between 0.1 -1.0 mg/dL) indicating that there was no severe liver failure or hepatitis present in any of the groups [53]. Considering the transaminases ALT and AST are markers of liver damage resulting in more permeability of hepatocytes, most liver cell injuries result in ASL levels that are lower than ALT levels, however this is not what was observed. As GGT was not detected in any of the mice, the potential for cholestatic damage or alcoholic liver disease can be eliminated. It is well established that rodents are not a good model for studying cholesterol metabolism; as we saw a dramatic increase in ALP, it is possible there could have been an alteration on the cholesterol metabolism in the livers of the mice causing the lipids to become accumulated rather than directed toward circulation and eventual storage in adipose. The histological characterization of the increase in ALP and microvesicular steatosis resembles Reye’s syndrome, but additional studies will be needed to evaluate any mitochondrial toxicity in the liver. Similar results to reduce HFD-induced obesity were seen with purified ANC from mulberry at up to 0.68 mg C3G per day, which is higher than the 2X dose of ANC, without any reported toxicity and an attenuation of the HFD-induced increases in ALT and AST [16]. A polyphenol-rich extract from
lotus has also been used at a dose of 166 mg/kg/d to reduce the ASL, ALT, and ALP levels in ethanol-fed mice [54]. The increases in liver enzymes appear to not be due to the dose of the ANC but other phenolic compounds present in the PAE.

Gene expression changes, while measured in the pancreatic tissue, indicated that an up-regulation of methionine degradation and cysteine biosynthesis which could have affected the liver cholesterol concentrations as methionine is known to induce hypercholesterolemia through increased expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase levels [55]. Also, highly expressed changes in the cysteine biosynthetic pathway were observed, which occurs due to the alternative pathway of methionine breakdown via the reverse-transulfuration pathway. LPL was significantly reduced by both the 1X and 2X groups, indicating these groups may not have been able to hydrolyze the triglycerides in lipoproteins such as VLDL. This could also explain the focal lipid deposition in the periportal region indicating triglycerides coming from the intestine in chylomicrons may not have been hydrolyzed. Taken together, it appears that pathways in the liver to maintain normal plasma cholesterol and triglycerides were affected by the highest doses of the PAE resulting in lipid accumulation in the periportal region of livers in mice consuming 1X and 2X.

In the present study, gene-expression profiling in the pancreatic tissue revealed that the treatments had effects on amino acid metabolism in addition to its effect on genes that are directly involved in concentration of glucose. Although the underlying mechanisms are not well understood, glucagon is known to play an important role in amino acid metabolism [56, 57]. Hypoaminoacidemia, weight loss, and muscle wasting are key features of glucagonoma [58]; conversely, lean body mass and circulating amino acids are increased in mice with targeted deletion of the glucagon receptors [59]. Interestingly, the profile of differential gene expression
did not overlap with specific canonical pathways; however, the predicted downstream effect for
the three treatments 0.1X, 1X and 2X pointed to a decrease of glucose concentration. As part of
these set of genes that predicted a downstream decrease of glucose, the three treatments up-
regulated zinc-a2-glycoprotein 1(AZGP1) gene. In a previous study done by Wargent et al. [60],
the authors reported that AZGP1 reduced body fat content and improved glucose homeostasis
and the plasma lipid profile in obese mice, and was suggested that this might be mediated via
physiological agonism of β3- and possibly β2-adrenoceptors. Moreover, 1X and 2X treatments
down-regulated LPL. It has been reported that islet LPL contributes to glucose homeostasis by
acting as an insulin secretion regulator [61], and furthermore, stimulation of LPL activity
suggests that it may contribute to increasing delivery of lipids to the islets. Acute exposure of
islets to fatty acids potentiates glucose-stimulated insulin secretion [62], but chronic exposure
causes impaired insulin responses and beta cell death [63]. On the other hand, an important
marker down-regulated by 0.1X treatment was FOXO1. Although FOXO1 expression is
considered as a double-edged sword in the pancreas [64], it has been reported that its inhibition
protects pancreatic islet β-cells against fatty acid and endoplasmic reticulum stress–induced
apoptosis [65]. Similarly high doses of ANC from purple corn (10 mg/kg body weight/d) have
been seen to be protective against pancreatic beta-cell death in db/db mice, with no reported
adverse effects [66]. From the microarray evaluation of pancreatic tissue of mice, it can be
inferred that the treatments 0.1X, 1X and 2X, caused differential gene expression of networks
related to cell proliferation, carbohydrate and protein metabolism. Additionally, the treatments
exerted their contributing-hypoglycemic effect in pancreas mainly through the modulation of
genes that have been strongly related to phenotypic changes for improving glucose homeostasis.
While not necessarily an indication of severe liver damage, the unexpected adverse effects to increase liver weight and some of the liver enzymes indicated that there is a potentially beneficial dose that must be optimized. The amount of blueberry polyphenolics seen previously to have a benefit in reducing hyperglycemia, weight gain, and cholesterol in mice was 13.9 mg/d, which is half as much as the total polyphenolic concentration of the 2X dose (6.4 mg gallic acid equivalents/mouse/d) [19]. Toxicological evaluation of a procyanidin-rich extract from red grape skins and seeds determined an LD$_{50}$ of 5000 mg/kg [67]. The 2X dose was 250 mg phenolics/kg BW/d, and because the adverse effects seen have not been reported for ANC, it is important to identify which other non-anthocyanin phenolic compound may have been increased to cause the adverse effects seen. Compounds that contain a gallic acid moiety or a propyl gallate appear to be more cytotoxic to hepatocytes through collapsing the mitochondrial membrane potential and forming reactive oxygen species to deplete glutathione levels [68]. It will also be important to consider the formation of compounds that may form similar cytotoxic structures during absorption and metabolism.

Most studies that have reported adverse effects on liver function are due to extremely high doses of phenolics at levels that could usually only be consumed in supplement form. For example, the toxic dose for epigallocatechin-3-gallate (EGCG) appears to be between daily intakes of 500-700 mg/kg for mice, which translates to 30-90 mg/kg for a human, or between 10-32 cups of green tea [69]. One recent review on the role of isoquercetin is an example of what needs to be done to establish a recommended dose of phenolic compounds and that likely there may be a U-shaped curve of effect; there are protective or benefits at a middle dose below which no benefit is seen, but above which may exhibit adverse events [70]. At higher doses, the very same mechanisms that allow the antioxidant and anti-carcinogenic response may cause
flavonoids to act as mutagens, pro-oxidants, and inhibitors of key enzymes involved in hormone metabolism [71-73]. More studies and controlled treatment trials are needed to add to the literature on incidence of adverse effects of polyphenolic compounds at higher doses, especially when consumed as a whole extract.

In summary, it was found that fasting blood glucose for diet-induced obese mice consuming either 9 or 19 mg C3G/kg BW/d was significantly lower than both the water and the sitagliptin groups, indicating there were effects beyond DPP-IV inhibition. Additionally, mice in the group consuming the most anthocyanins had the lowest body weight gain through reduced fat mass that was correlated with visceral fat and plasma triglycerides, while absolute lean mass was not affected. There was an unexpected finding of enlarged livers by the two highest doses indicating some of the adverse effects that can be seen due to excess consumption of compounds that are beneficially effective at lower doses, which requires additional studies to optimize benefits with no adverse effects. Overall, phenolic compounds extracted and concentrated from the fermented blueberry-blackberry beverage reduced weight gain, fat mass accumulation, plasma triglycerides, and fasting blood glucose in diet-induced obese mice. Considering the efficacy and the unexpected enlarged livers, the dose at which the combination of compounds present in the PAE will be most beneficial is between 1.1 mg C3G/kg/d and 9 mg C3G/kg/d.

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Authors' disclosure.

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References


CHAPTER 11

CONCLUSIONS

• Wines produced from blueberries and blackberries have high total polyphenolics and total anthocyanin contents which correlate with their antioxidant capacity.

• Blueberries and their fermented products are good natural sources of polyphenols, antioxidants, and *in vitro* inhibition of α-amylase and α-glucosidase; fermentation retained the *in vitro* starch degrading enzyme inhibitory capacity of Highbush blueberries (*Vaccinium corombosum*).

• ANC and PAC at 100 µM C3G or epicatechin equivalents obtained from blueberry-blackberry blends reduced the phosphorylation of the p65-subunit NFκB, decreased iNOS and COX-2 expression, and decreased NO production in RAW 264.7 mouse macrophages. Along with the high antioxidant capacity and phenolic content and ability to decrease enzyme activity related to improved glucose utilization, bioactive ANC and PAC compounds present in fermented berry beverages may act synergistically to reduce risk factors for chronic inflammatory diseases.

• ANC isolated from blueberry-blackberry wine blends and a variety of other phenolic compounds commonly present in citrus, berry, soy, and other plants could strongly inhibit DPP-IV activity, supporting the role of flavonoids to act as naturally occurring DPP-IV inhibitors.

• Blueberry-Blackberry blend ANCIs are potential inhibitors of inflammation-related adiposity response and sensitizers of insulin signaling in adipocytes through their action to blunt adiponectin gene expression induced by TNF-α treatment (18.2%) and reduce glycerol release in adipocytes. (15.9%) induced by LPS-induced macrophage conditioned media.

• Bioactive ANC and PAC from fermented berry beverages reduced incretin-cleaving hormone DPP-IV protein activity and protein expression in Caco-2 cells, increased insulin secretion of iNS-1E pancreatic β-cells both directly and following a dual-cell layered model of simulated absorption, up-regulated genes of insulin-receptor associated proteins and GLP-1, and down-regulated PTP1B in iNS-1E cells, and enhanced insulin receptor signaling and up-regulation of proteins IGFBP-2 and -3, IGF-II, and VEGF.

• Diet-induced obese mice consuming either 9 mg C3G/kg BW/d had significantly lowered fasting blood glucose, the lowest body weight gain via reduced fat mass gain, and reduced visceral fat and plasma triglycerides.
INTEGRATION AND FUTURE WORK

There are increasing numbers of teens and adults that have pre-diabetes in the United States and worldwide, the number of people with diabetes is projected to increase from over 360 million in 2014 to over 550 million by 2030. Diabetes causes severe complications if glucose is not monitored and is a major contributing factor of rising health costs. In general, diets that are high in fruit and vegetable intake have been linked to a decreased risk of chronic diseases; specifically, increased berry consumption has been linked to improved cognitive function and decreased incidence of inflammatory diseases such as cardiovascular disease and cancer. There is a need to develop strategies for diabetes management in order to decrease the incidence and deaths from diabetes-related complications. It is important to first understand the chemistry and investigate inflammatory pathways leading to increased chronic diseases in order to suggest ways to prevent or manage them.

My research presented in this dissertation was designed to investigate the overall understanding of berry fruit compounds as they relate to inflammatory diseases focusing on type-2 diabetes. This work focused on the evaluation of blueberry (Vaccinium corymbosum) and blackberry (Rubus spp.) fermented beverages and their bioactive components to reduce progression and onset of type-2 diabetes. Blueberries and blackberries are a rich source of anthocyanins (ANC), water-soluble pigments that give berries their red, blue, and purple color. Increased consumption of ANC and ANC-containing foods results in decreased risk for type-2 diabetes. Berries also contain proanthocyanidins (PAC), polymerized forms of ANC. Berry fermentation increases extraction from fruit skins and is a viable way to increase the phenolic content of berry juice products, thus increasing the potential for health benefits. Current incretin-based therapies have potential for treatment of type-2 diabetes as the incretin hormone glucagon-
like peptide-1 (GLP-1) is able to stimulate insulin release; however, it is rapidly degraded to inactive metabolites by the enzyme dipeptidyl peptidase-IV (DPP-IV). Our results using computational and enzymatic studies indicated that ANC are strong inhibitors of DPP-IV. We have also shown that fermented blueberry-blackberry beverages have a high antioxidant capacity, total phenolics, and have identified these compounds using HPLC-MS/MS analysis of anthocyanin and proanthocyanidin extracts. We showed in vitro inhibition of markers of inflammation and the enzyme DPP-IV as well as the ability of these compounds to increase insulin secretion from pancreatic β-cells in vitro. Finally, we showed the efficacy of these compounds animal study investigating their potential to reduce obesity and hyperglycemia in C57BL/6j mice fed a high-fat diet. The primary findings from each of the five aims presented in this dissertation are included below, with a brief discussion of their relevance.

**Aim 1 Conclusions:** Blackberry wines (n = 6) had an average total phenolic content of 2212.5 ± 1090.3 mg ellagic acid equivalents (EAE) per liter, total anthocyanin content of 75.56 ± 70.44 mg/L, and antioxidant capacity (AC) of 26.39 ± 17.95 mmol trolox equivalents (TEs) per liter. Blueberry wines (n = 4) had an average total phenolic content of 1623.3 ± 645.5 mg EAE per liter, total anthocyanin content of 20.82 ± 12.14 mg/L, and AC of 21.21 ± 7.71 mmol TE per liter. Strong positive correlations were found between total phenolic compounds and AC (r = 0.88). Fruit wines made from blueberries and blackberries may have potential health applications and therefore could contribute to the economy of the wine industry, especially in the state of Illinois. The fermentation process may create more stable polymers of the beneficial pigment compounds as well as new compounds that may also have health benefits.

![Graph](image-url)
**Aim 2 Conclusions:** The wines maintained similar action to inhibit α-amylase and α-glucosidase as the berries, relative to the positive control acarbose, a known anti-diabetic drug (from 91.8 to 103.3% for α-amylase and from 103.2% to 190.8% for α-glucosidase). Cold temperature fermentation had a larger increase in antioxidant capacity of blueberry wine than room temperature fermentation.

The overall impact of these initial studies provides valuable information that blueberries grown in southern Illinois are a good source of polyphenols and antioxidant capacity, and a natural source of starch degrading enzyme inhibition *in vitro*. Wine made from blueberries and blackberries, especially when fermented at cold temperatures, have increased antioxidant capacity. The commercial availability of wines containing high amounts of beneficial anthocyanins and antioxidants provides grounds to optimize high quality characteristics with potential health beneficial effects. These results suggest that fruit wines made from blueberries and blackberries may have potential health applications based on their high polyphenolic concentration.

**Specific Objective 3.1 Conclusions:** Total ANCs of the wine were strongly correlated to total phenolics \((r = 0.99, p < 0.05)\) and to antioxidant capacity \((r = 0.77, p < 0.05)\). Both ANC- and PAC-enriched fractions inhibited starch-degrading enzyme α-glucosidase and dipeptidyl peptidase-IV activity. ANC and PAC (100 µM C3G and epicatechin equivalents, respectively) from blueberry–blackberry blends reduced *in vitro* LPS-induced inflammatory response in mouse macrophages via the nuclear factor kappa B-mediated pathway through decreased iNOS and COX-2 expression, and decreased NO production. Computational modeling interaction energy of D3A when bound to DPP-IV was -3227.7 kcal / mol, similar to known inhibitor diprotin A of -3208.3 kcal / mol. Bioactive ANC and PAC compounds present in fermented berry beverages may act synergistically to reduce risk factors for chronic inflammatory diseases.

![Figure 11.2: Effect of anthocyanin (ANC) and proanthocyanidin (PAC) on NFκB expression. Values are means ± SEM with significantly different letters (n=4, p < 0.05).](image1)

**Figure 11.3:** Surface representation of delphindin-3-arabinoside, the predominant anthocyanin present in blackberry, bound to DPP-IV, thus inhibiting its action to degrade GLP-1.
**Specific Objective 3.2 Conclusions:** Computational docking analyses showed for the first time that natural phenolics could inhibit DPP-IV activity by binding tightly into the active sites of the enzyme. DPP-IV has three binding pockets/active sites (S1, S2 and S3). ANC from blueberry-blackberry wine blends strongly inhibited DPP-IV activity (IC\textsubscript{50}, 0.07 ± 0.02 to > 300 \(\mu\text{M}\)) by binding to the catalytic site. Compared to other anthocyanins found in berries, (cyanidin and pelargonidin), delphinidin had the most potent DPP-IV inhibitory effect, suggesting the importance of interactions of the hydroxyl groups on the B ring of anthocyanins.

**Specific Objective 3.3 Conclusions:** Anthocyanin blends affected lipolysis by preventing LPS-induced glycerol release into culture media, inducing intracellular fat accumulation (28.2% max.) when applied during differentiation, and inhibiting isoproterenol-induced lipolysis (18.6% max.) of mature 3T3-L1 cells. ANC treatment slightly restored the insulin-induced glucose uptake of adipocytes that was blunted by conditioned medium treatment. Anthocyanins from blueberry and blackberry fermented beverages are potential inhibitors of inflammation and sensitizers of insulin-signaling, possibly through inhibition of lipolysis in adipocytes.

The overall impact of the findings from aim 3 is that the chemical composition of blended blueberry and blackberry fermented beverages provide valuable information as to which compounds are able to have potential for improving the management of diabetes. Studying the bioactive compounds using \textit{in vitro} models allows the mechanism of action to be better understood in preventing chronic inflammatory diseases. This is important in order to promote future studies \textit{in vivo} in order to fully understand the bioactivity and potential mechanism of action of these compounds to reduce inflammatory diseases. Overall, these results suggest that due to the presence of phenolic compounds, specifically anthocyanins, blueberry and blackberry fermented beverages are good natural sources of antioxidants, are starch-degrading enzyme inhibitors, can bind strongly to the active site of DPP-IV to inhibit its activity, and are able to reduce markers of adipogenesis. These findings give light to specific roles of these compounds to

![Figure 11.4](image-url): Modulation of lipolysis measured by glycerol release from fully differentiated adipocytes activated by macrophage conditioned media (CM-LPS) in presence or absence of each blend ANC (100 \(\mu\text{M}\) C3G equivalents).
reduce inflammation and inhibit enzymes that are current targets of pharmaceutical therapy, and thus highlight their potential to impact type-2 diabetes.

**Aim 4 Conclusions:** Anthocyanins (ANC) from 50% blueberry: 50% blackberry and 100% blackberry fermented beverages at 50 µM cyanidin-3-glucoside equivalents were able to increase glucose-stimulated insulin secretion from iNS-1E cells by 233 and 100 µIU insulin/mL directly and after Caco-2 transport, respectively. ANCs from both 50% and 100% blackberry increased the relative protein expression of IGF-II, IGFBP-2 and 3, and VEGF in iNS-1E cells. ANC also up-regulated the gene expression of the incretin hormone GLP-1 (fold-change 3.0 ± 1.4 and 2.0 ± 0.3) and of genes in the insulin secretory pathway, including iGF1R (2.3 ± 0.6 and 1.6 ± 0.3) for ANC50% and ANC100%, respectively. Also, PTP1B gene expression was down-regulated by ANC 100% (-1.9 ± 0.1), a potential therapeutic target of type-2 diabetes.

**Aim 5 Conclusions:** Daily consumption of anthocyanins was 8.4, 1.1, 9.0, and 19.0 mg C3G/kg BW/d for AFFB, 0.1X, 1X, and 2X, respectively, with anthocyanins present predominantly C3G (65.8%). The body weight/food intake ratio and sucrose consumption were similar (p > 0.05). Mice in the 3x group had the lowest body weight gain (10.3 ± 0.3 g) and the water group the most weight gain (16.7 ± 1.2 g, p < 0.05). The weight increase was attributed to a gain in fat mass (18.0 ± 0.8 % BW for 2X vs. 31.3 ± 1.1 % BW for water) which was correlated with visceral fat (0.8 ± 0.1 g for 2X vs. 2.4 ± 0.2 g for water), while absolute lean mass did not differ (p > 0.05). After 3 weeks of treatment, all groups had fasting blood glucose (FBG) above 126 mg/dL. At the end of the study (week 12), FBG for 1X and 2X groups was significantly lower (168 and 184 mg/dL, respectively) than the water (222 mg/dL), and the sitagliptin group (217 mg/dL, p < 0.05). These two highest doses, however, had an increase in liver weights and liver lipids, indicating that adverse effects need to be further evaluated. Overall, phenolic compounds, mainly anthocyanins, from a fermented blueberry-blackberry beverage reduced BW gain, fat mass accumulation, and FBG in diet-induced obese mice. Future studies to evaluate the mechanistic targets will add to the understanding of the reduction in fat mass and fasting blood glucose in the mice consuming anthocyanins. At the completion of this research we expect to understand the effect and mechanism of action of berry phenolic ANC and
PAC to reduce hyperglycemia, and therefore reduce the progression and onset of type-2 diabetes, through the mechanisms as indicated in the diagram below.

![Diagram of research findings integration pathway]

**Figure 11.5: Research findings integration pathway.** Blue arrows indicate an increase/up-regulation or a decrease/reduction of specific measurements evaluated.

The results of this research will allow better understanding of the anti-diabetic mechanisms of dietary bioactive compounds found in fermented berry beverages to prevent chronic inflammatory diseases such as type-2 diabetes using animal, cellular and molecular approaches, and establish physiological relevance by determining a dose that is translatable to human consumption. Further, the role of phenolic compounds to increase proliferation and response to glucose of pancreatic β-cells could reduce the rate of progression of patients with type-2 diabetes to become dependent on insulin therapy. Translation of our results to future clinical trials will be needed to attribute any reduction of onset of type-2 diabetes to specific compounds in berries. Potential synergism of compounds may provide additional evidence.
behind reduced hyperglycemia following consumption of berries seen in future and previous epidemiological and clinical trials. It is important to determine the effective dose of phenolics needed to reduce blood glucose in humans in order to reduce the onset and progression of obesity or hyperglycemia. The animal study suggests consumption of the 1x dose of anthocyanins from fermented berry beverages per day (9 mg/kg BW) would be able to have an effect to reduce the onset of obesity and hyperglycemia, even when consuming a high-fat diet, however at the doses provided there were some adverse effects in the liver that warrant further investigation. Importantly, this amount of anthocyanins is feasibly achieved by humans consuming 2 cups of blueberries per day (8 mg/kg BW) and is associated with no known toxicity, but the concentration of other phenolic compounds present may be reaching a dose at which they exert adverse effects. Further investigation into the optimum dose and toxicity observed will help to add to the discussion of a recommended dose of phenolic compounds and dietary flavonoids that will be beneficial without safety concerns. With the knowledge gained from this research, the role of bioactive compounds from dealcoholized fermented berry beverages to reduce type-2 diabetes will be expanded, thus contributing to the field of research on flavonoids and nutrition to improve human health.

Future directions of this research will provide mechanistic evidence, effective dose, and potential new targets of DPP-IV inhibition and incretin action for future animal and clinical trials or drug discovery. This research could impact both current practices and dietary recommendations, and suggest future targets of type-2 diabetes therapy, and will provide understanding of the role of phenolic compounds to affect insulin secretion. This study will add to the growing field of research investigating health effects of flavonoids by understanding of the mechanisms of dietary bioactives found in fermented berry beverages to increase insulin secretion and reduce chronic diseases such as type-2 diabetes using both in vitro and in vivo models. Future studies are needed to address the diverse roles of the complex mixtures of chemical components in blueberries and blackberries and phenolic-rich products produced from these fruits and their potential to reduce obesity-associated inflammation and type-2 diabetes.

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