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The Ability of Berry Extracts to Inhibit Alpha-Glucosidase In Vitro

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THE ABILITY OF BERRY EXTRACTS TO INHIBIT ALPHA-GLUCOSIDASE IN VITRO

by

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Abstract

Type 2 diabetes is a growing public health concern. Drugs, such as acarbose, are used to slow the rise in blood glucose levels after meals by inhibiting the enzyme, alpha-glucosidase, which is responsible for digesting complex carbohydrates. The objective of this study is to compare the alpha-glucosidase inhibitory activity of extracts from Maine blueberries and blackberries to acarbose because these plants are very anthocyanin-rich. Varietal differences in highbush blueberries (*Vaccinium corymbosum*) and agricultural practices for growing wild or lowbush blueberries (*Vaccinium angustifolium* Ait.) are also experimental variables. The twelve berry samples were obtained from farms around the state of Maine in August of 2013. Results were obtained through four assays, including alpha-glucosidase inhibition, total free phenolic content, total anthocyanin content, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA). Analysis of Variance (ANOVA), Tukey's Honestly Significant Difference (HSD) test, and Pearson Correlations were used to compare means. The alpha-glucosidase inhibition assay showed that blueberries and blackberries can inhibit at least 50% of alpha-glucosidase activity under these experimental conditions and within sixty minutes. None of the four assays yielded data that strongly correlated with one another, and the mean organic wild versus high-input wild data did not show any significant differences except for radical scavenging activity where the high-input wild mean was significantly higher.

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1 Background & Introduction

Type 2 diabetes is increasing in prevalence worldwide, and especially in Maine. Worldwide, 347 million people have diabetes (World Health Organization, 2013). According to the United States Centers for Disease Control and Prevention, 25.8 million children and adults in the United States have diabetes (2011). Between 1995 and 2010, the diabetes prevalence rate in Maine increased from 3.5% to 8.7%, which means it more than doubled in only five years (Maine DHHS, 2012), so it is clear that diabetes is a very serious concern.

Type 2 diabetes occurs when the body's cells have become insulin-resistant or when the pancreas no longer makes adequate insulin. This condition prevents blood glucose from being taken into cells, causing high blood glucose and cell starvation. Over time, type 2 diabetes can lead to many serious problems, such as heart disease, stroke, high blood pressure, blindness, kidney disease, nervous system disease (neuropathy), and amputation (American Diabetes Association, 2014). Treatment of type 2 diabetes often requires multiple approaches for reducing blood glucose levels.

Diet modification is one strategy to prevent and treat type 2 diabetes. By decreasing the consumption of simple sugars and increasing intake of dietary fiber and healthy foods, such as fruits, vegetables, whole grains, and lean protein, blood sugar levels can be improved. Medical nutrition therapy goals are to provide the patient with tools to achieve the best glycemic control possible as well as cardiac health maintenance to prevent complications. The total amount of digestible carbohydrates eaten per meal, regardless of the source, is the primary determinant of postprandial glucose (Mahan et al., 2012). Therefore, monitoring total grams of carbohydrate remains the key strategy and in

patients, day-to-day consistency in amount of carbohydrate eaten and meal timing improves glycemic control.

Currently, the most common recommendations for type 2 diabetes meal planning options are either carbohydrate (carb) counting or the diabetic exchange lists. With carb counting, all food portions consisting of 15 grams of carbohydrate (regardless of the source) are considered to be one carb serving (Mahan et al., 2012). These servings are spread out throughout the day with a consistent number of servings divided amongst meals and snacks. The diabetic exchange list groups food into categories that include starches, fruits, milk, sweets, other carbs, nonstarchy vegetables, meat and meat substitutes, fats, and free foods, with each food group having approximately the same nutritive value (American Diabetes Association, 2014). The carb counting or the exchange list method is selected depending on what works best for the patient.

The diabetic diet is adapted to meet the needs of each specific patient, so calorie needs are calculated first, with consideration for current weight, height, age, gender, and activity level. Then, according to the patient's calorie needs, the diet is broken down into macronutrient categories. A standard diabetic diet will usually consist of 50% of kilocalories coming from carbohydrates, 30% of kilocalories coming from fat, and 20% of kilocalories coming from protein (American Diabetes Association, 2014). Other recommendations for diabetes treatment can include strategic meal composition, which means that fiber and lean protein sources are important to include in meals. This is because fiber and protein are complex molecules (compared to simple sugars) that slow the process of digestion and, therefore, can decrease how quickly sugar enters the blood stream and aid in glycemic control.

Physical activity is also recommended to help with diabetes management because exercise improves insulin sensitivity, reduces cardiovascular disease risk, and helps control weight (Mayo Foundation, 2013). The Mayo Foundation for Medical Education and Research (2013) recommends 150 minutes per week of moderate intensity aerobic exercise or for 90 minutes per week of vigorous aerobic exercise at least three times per week, plus resistance training at least three times per week.

Even with dietary and physical activity modifications, most patients with type 2 diabetes will also require medications to treat their condition. There are many forms of diabetes medications that act in different ways. Some commonly prescribed medications are shown in Table 1.

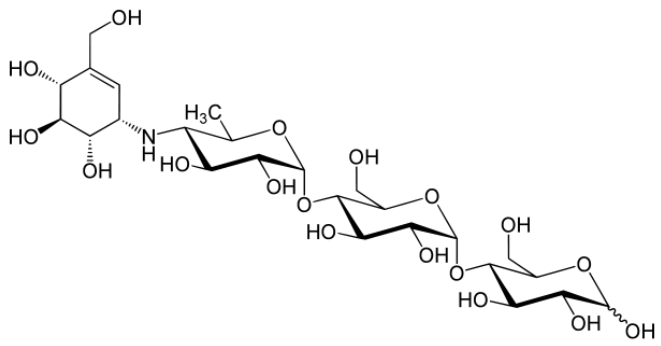
Table 1. Type 2 Diabetes Medications and Mechanisms of Action[a]

Medication	Mechanism of Action	Brand / Generic Names
Biguanide	Decreases hepatic glucose production	Glucophage, Metformin
Sulfonylureas	Stimulate insulin secretion from pancreatic beta-cells	Amaryl, Glimepiride, Glucotrol, Glipizide, Glyburide
Thiazolidinediones	Improve peripheral insulin sensitivity	Actos, Pioglitazone
Glucagon-like peptide-1 (GLP-1) Agonists	Increase glucose-dependent insulin secretion, suppress postprandial glucagon secretion	Byetta, Exenatide, Victoza
Alpha-Glucosidase Inhibitors	Delay carbohydrate absorption	Precose, Glyset, Acarbose, Miglitol, Voglibose
Glinines	Stimulate insulin secretion from pancreatic beta-cells	Prandin, Starlix
Amylin Agonists	Decrease glucagon production	Symlin
Dipeptidyl Peptidase 4 (DPP-4) Inhibitors	Prevent degradation of insulin stimulants	Januvia, Onglyza
Insulin	Supplements endogenous insulin	Humalog, Insulin Lispro, Novalog, Levemir

[a] Adapted from Mahan et al. (2012).

One of the categories of medication is alpha-glucosidase inhibitors, which help reduce blood glucose spikes by slowing the digestion of carbohydrates by the enzyme, alpha-glucosidase, which hydrolyzes alpha (1→4) bonds in carbohydrate compounds (Wang et al., 2013). Alpha-glucosidase binds to its substrate mainly through hydrogen bonds (Wang et al., 2013). Therefore, alpha-glucosidase inhibitors (termed "glycomimetics") have structural similarity to alpha-glucosidase substrates (maltose and oligosaccharides), but bear a positive charge near the glycosidic bond, which acts as a competitive inhibitor (Wang et al., 2013). The glycomimetic molecule binds to the enzyme active center through charge interactions with the catalytic group in addition to hydrogen bonds (Wang et al., 2013). Alpha-glucosidase glycomimetics include some well-known and commercialized molecules such as acarbose, miglitol, and voglibose (Etxeberria et al., 2012).

Figure 1. Acarbose Chemical Structure[a]



[a] Adapted from Wehmeier et al. (2004).

Acarbose (O-{4,6-dideoxy-4[1S-(1,4,6/5)-4,5,6-trihydroxy-3-hydroxymeth-yl-2-cyclohexen-1-yl]-amino-α-D-glucopyranosyl}-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose) is an alpha-glucosidase inhibitor (Figure 1) that can be prescribed for persons with type 2 diabetes, and has been used since 1990 (Wehmeier et al., 2004).

However, as with all medications, acarbose has side effects that can include: shakiness, dizziness, sweating, nervousness, numbness or tingling around the mouth, weakness, and clumsy or jerky movements (National Institutes of Health, 2014).

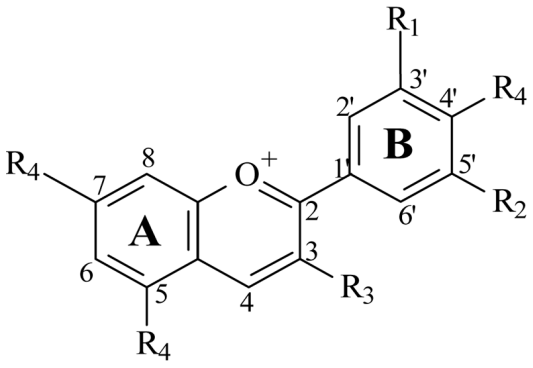
Certain plant compounds can have a similar inhibitory effect on alpha-glucosidase in vitro. For example, Kwon et al. (2008) studied four sources of red and white wines as well as four types of teas to determine the alpha-glucosidase inhibitory potential. They found that black tea and red wine had the highest inhibitory activity and that the inhibitory activity correlated with the phenolic content and antioxidant activity of the extracts. Similarly, Misbah et al. (2013) found that *Ficus deltoidea* fruit extracts also inhibited alpha-glucosidase and concluded that the study of fruit compounds having both antihyperglycemic and antioxidant activities may provide a new approach in the treatment of diabetes.

One of the major beneficial phytochemical groups is polyphenols. They are the most abundant antioxidants in the human diet and are the most common and widespread component in plants (Cohen et al., 2010; Coté et al., 2010; van Dorsten et al., 2010). Polyphenols contain at least one aromatic ring with at least one hydroxyl group in addition to other substituents, and they can be divided into fifteen major classes according to their chemical structures (Xiao et al., 2012). Polyphenol-rich food sources include blackberry, raspberry, black currant, blueberry, artichoke, potato, coffee, red cabbage, red grape, curly kale, and rhubarb, among others (Manach et al., 2004). Flavonoids are a subgroup and are the major polyphenols present in wide variety of plant sources (Blade et al., 2010). Over 10,000 flavonoids have been separated and identified

from plants and are divided into subclasses, including anthocyanidins, flavanones, flavonols, flavones, and isoflavones (Brand et al., 2010).

In the large group of polyphenolics and within the subgroup of flavonoids, anthocyanins consist of more than five hundred compounds (McGhie et al., 2007). Anthocyanins make up the most numerous group of water-soluble pigments found in plants, and they give various flowers, fruits, and leaves their colors (Fernandes et al., 2012), so most people come into contact with anthocyanins every day. To understand how anthocyanins may impact human health, the first point to consider is the chemical structure. Anthocyanins are derived from anthocyanidins, so their structure is based on the aglycone, or sugar-free, anthocyanidin structure. When an anthocyanidin becomes glycosylated, it is then classified as an anthocyanin (Glover and Martin, 2012). There are six anthocyanidins most commonly found in nature: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Lohachoompol et al., 2008).

Figure 2. The Flavylium Cation: Basic Anthocyanin Structure. R_1, R_2 =H, OH, or OCH_3; R_3 = OH or glycosyl; R_4= OH or glycosyl[a]



[a] Adapted from McGhie et al. (2007).

Anthocyanins are molecules that have three defining characteristics (as shown in Figure 2). First, anthocyanins are glycosylated, containing one or more sugar molecules.

Second, anthocyanins are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium (McGhie et al., 2007), which means that they contain multiple hydroxyl or methoxy groups. The third and final defining characteristic of an anthocyanin molecule is that they contain two benzoyl rings separated by a heterocyclic ring (McGhie et al., 2007). There are hundreds of different anthocyanin structures that differ based on the number of hydroxyl groups the molecule contains, the degree of methylation, the number and type of sugar molecules that are attached, and the number of aliphatic or aromatic acids attached to the sugars (McGhie et al., 2007). Basically, all anthocyanins have the same general structure but vary in the particular side groups that are attached. These attached groups are what define and differentiate each specific anthocyanin.

Anthocyanins are primarily known for contributing color to plants, so another important aspect of the biochemistry of anthocyanins is color. Anthocyanins give plants very distinct colors, ranging from red in grapes, pink in flowers, purple in eggplants, to blue in blueberries (Glover et al., 2012). Anthocyanins confer colors because their light absorption falls in the visible range between 465 and 550 nanometers (Glover et al., 2012), which humans see as red, pink, purple, or blue. The color conveyed by anthocyanins is pH-dependent and they are most intensely colored at a low pH. At an acidic pH below 2, the red flavylium cation is the most abundant. When the pH becomes more basic, different anthocyanin structures (hemiketal, chalcones, and quinonoidal bases) are present in equilibrium (Teixeira et al., 2013). The alterations in pH cause changes in the molecular structure because protons are lost rapidly (McGhie et al., 2007), and as structure changes, color also changes.

According to the USDA Database for the Flavonoid Content of Selected Foods (Bhagwat et al., 2011), the mean anthocyanin profiles for blackberries, cultivated blueberries, and wild blueberries are shown in Table 2.

Table 2. Mean Anthocyanin Content by Berry Variety[a]

Anthocyanin / Anthocyanidin Category	Mean Blackberry Content (mg/100g)	Mean Highbush Blueberry Content (mg/100g)	Mean Lowbush Blueberry Content (mg/100g)
Cyanidin	90.49	7.10	17.92
Delphinidin	0.00	30.91	34.00
Malvidin	0.00	59.64	54.00
Pelargonidin	0.15	0.00	2.65
Peonidin	0.00	15.36	9.11
Petunidin	0.00	28.02	15.43
Total	90.64	141.03	133.11

[a] Adapted from Bhagwat et al. (2011).

Not only do the polyphenol and anthocyanin compounds contribute to alpha-glucosidase inhibition and, subsequently, to diabetes management, but there may also be many other benefits involved. Granfeldt et al. (2011) demonstrated that the effect of polyphenol-rich fruits is sometimes more evident in the insulin response than on glycemia because a fermented oatmeal drink enriched with 47% bilberries was shown to reduce the insulin index significantly. This research suggests that the presence of polyphenols, such as anthocyanins, may also have induced an effect on an enhanced insulin-independent pathway for glucose uptake similar to the one reported for cinnamon and berries (Thondre, 2013). Additionally, Johnston et al. (2002) reported that fruit and berries may delay the digestion and absorption of carbohydrates and suppress postprandial glycemia, oxidative stress, and low-grade inflammation. These beneficial effects of fruit and berries on glucose metabolism may be due to the presence of dietary fiber and polyphenols (Torronen et al., 2012; Hanhineva et al., 2010).

Mursu et al. (2014) studied the relationship between the intake of fruit, berries, and vegetables, and the risk of type 2 diabetes in Finnish men and concluded that fruit and vegetable consumption, particularly berries, may reduce the risk of type 2 diabetes. Since it is often assumed that type 2 diabetes simply comes from eating too much sugar, people with diabetes may believe that they need to avoid all carbohydrate sources equally, including fruits. Due to this misconception, many people with diabetes are not consuming the recommended amounts of fruit, which negatively impacts their overall health and diabetes management. For example, a study performed in Queensland, Australia found that only 32% of individuals with diabetes reported eating enough fruit, according to National Health and Medical Research Council criteria (McCulloch et al., 2003). With all of the proven health benefits from fruit compounds, adequate fruit consumption should be a priority, especially for people with diabetes.

In the United States, Chun et al. (2005) studied the daily intake of total phenolics, total flavonoids, and antioxidants in the American diet from fruit and vegetable consumption. The research showed that orange contributed the highest amount of total phenolics [117.1 mg gallic acid equivalent (GAE) person^{-1} day^{-1}] and antioxidants [146.6 mg vitamin C equivalents (VCE) person^{-1} day^{-1}] because of its high consumption by Americans. Apples' phenolics and antioxidants contribution is the second highest. Even though potatoes had lower levels of phenolics and antioxidant capacity, they were third due to the fact that their consumption is the highest (137.9 lb person^{-1} year^{-1}) in the American diet. The daily intake by Americans of phenolics, flavonoids and antioxidants from fruits and vegetables was estimated to be 450 mg GAE, 103 mg catechin equivalents, and 591 mg VCE, respectively.

The objective of this study is to compare the alpha-glucosidase inhibitory activity of extracts from Maine blueberries and blackberries to acarbose because these plants are very anthocyanin-rich. The inhibition results will also be correlated with phenolic content, anthocyanin content, and antioxidant activity. Varietal differences in cultivated (highbush) blueberries (*Vaccinium corymbosum*) and agricultural practices for growing wild (lowbush) blueberries (*Vaccinium angustifolium* Ait.) are also experimental variables. I hypothesize that phenolic content, anthocyanin content, and antioxidant activity will positively correlate with alpha-glucosidase inhibition and that the wild varieties will produce the greatest inhibition because cultivated berries can exhibit a reduced phytochemical profile for the sake of achieving a larger berry.

2 Materials

Berry samples were obtained from farms around the state of Maine in August of 2013. The specific blackberry species cultivar is unknown. The cultivated blueberry varieties include Patriot, Blue Ray, and Blue Crop. Wild blueberries were collected as part of a National Institute of Food and Agriculture research project that compared the effects of various levels of agricultural inputs on fruit quality. Two blocks within each of four fields were sampled for each level of agricultural input. For this project, organically-managed fields were compared to fields receiving high levels of agricultural inputs such as pesticides, chemical fertilizers, and irrigation. Blocks within fields were composited. The organic wild sites were (O2, O3), (O6, O7), (O5, O8), and (O1, O2). The high-input wild sites were (H7, H8), (H2, H4), (H5, H6), and (H1, H2). All samples were sorted and cleaned to remove leaves, debris, and damaged berries. Samples were then stored frozen in Ziploc® brand containers at -18° Celsius. For analysis, berries were ground with mortar and pestle.

Reagents purchased from Sigma-Aldrich (St. Louis, MO) included acarbose, pepsin from porcine gastric mucosa, alpha-glucosidase, sodium phosphate monobasic anhydrous, sodium phosphate dibasic anhydrous, 4-nitrophenyl alpha-D-glucopyranoside, chlorogenic acid, Folin-Ciocalteau (F-C) reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®). Reagents purchased from Fisher Scientific (Fair Lawn, NJ) included sodium hydroxide pellets, acetone, hydrochloric acid, sodium bicarbonate, potassium chloride, sodium acetate trihydrate, and methanol. Reagents purchased from Acros Organics (Geel, Belgium) included potassium phosphate monobasic and potassium phosphate dibasic.

Instruments used were a Julabo SW22 incubator (Seelbach, Germany), Fisher Flexa-Mix stir plate and 350uL flat-bottom well plates (Fair Lawn, NJ), Sartorius Analytic A200S scale (Gottingen, Germany), Corning pH Meter 240 (Pittsburgh, PA), FLUOstar Omega BMG Labtech platereader (Weymouth, MA), Thomas Scientific Labquake Barnstead Thermolyne rocker table (Swedesboro, NJ), Beckman Centrifuge Model TJ-6 (Brea, CA), and Spectronic 20D+ Spectrophotometer (Bedfordshire, UK).

3 Methods

3.1 In Vitro Digestion & Alpha-Glucosidase Inhibition

Before determining alpha-glucosidase inhibition, samples were subjected to in vitro digestion as adapted from Rom et al. (1992), Mertz et al. (1984), and Nunes et al. (2004). To 4.0 g fresh sample, reagent blank, and positive control (1 mM acarbose), 35 mL pepsin solution (1.5 g of pepsin/L of 0.1 M KH_2PO_4 buffer, pH 2) was added. Samples were ground using mortar and pestle, then incubated at 37°C for 1 hour. Approximately 750 uL of 4 M NaOH was added to increase to pH 6.3.

Alpha-glucosidase inhibition was measured using adaptations of the procedures of Johnson et al. (2013) and Kwon et al. (2008). To a 96-well plate in a random order, 50 uL of digested berry samples, 50 uL positive control, or 50 uL reagent blank were added according to the plate layout diagram, which specifies which well receives which sample. (The order of analysis on a plate is randomized to decrease the error that can occur due to the samples' location on the plate.) Then, 100 uL of a 1.0 U/mL a-glucosidase solution (in 0.1 M sodium phosphate buffer, pH 6.9) was added. The plate was incubated at 25 °C for 10 min. The plate reader injected 50 uL of a 5 mM *p*-nitrophenyl-a-D-glucopyranoside solution (in 0.1 M sodium phosphate buffer, pH 6.9) to each well. The reaction mixtures were incubated at 25°C for 5 min. Before and after incubation, the absorbance was recorded at 405 nm. Percent inhibition was calculated relative to the diabetes drug, acarbose, as the positive control, and to the negative control, which had 50 uL of buffer solution in place of the extract.

$$\%\text{Inhibition} = [(\Delta\text{absControl}_{405\text{nm}} - \Delta\text{absExtract}_{405\text{nm}}) / \Delta\text{absControl}_{405\text{nm}}] * 100$$

3.2 Extraction

The extraction procedure was adapted from Wrolstad et al. (2005). One L of solvent contained 70% acetone, 30% reverse osmosis (RO) water, acidified with 1 mL HCl. Acetone was used in place of methanol because it is safer for the environment and evaporates more quickly, so less time in the turbovap is needed.

Five g of sample was weighed into 50 mL conical tubes (USA Scientific, Ocala, FL) and then extracted with 20 mL of extraction solvent. The sample was combined with the solvent and then ground with mortar and pestle until the skins were broken down. The tubes were rocked for 20 minutes on the Barnstead Shaker and then centrifuged (uncapped to allow for airflow and to facilitate acetone evaporation) for 15 minutes at 4800 rpm. The supernatant was decanted and then the pellet was re-extracted with 20 mL of extraction solvent until no pink color was visible in the extract (5 extractions in total were performed). The solvent was evaporated using a turbovap at 40°C. The aqueous extract was brought to a final volume of 100 mL with RO water and stored frozen for two weeks at -18°C.

3.3 Total Free Phenolic Content

To determine the total free phenolic content, methods were adapted from Velioglu et al. (1998). Twenty uL of blank (RO water), standards, and extracted samples were dispensed into the appropriate well according to the plate layout diagram. Then, 150 uL of F-C reagent was added to each well (Folin-Ciocalteau reagent diluted 1:10 with RO water) and left at 22°C for 5 minutes. Next, 150 uL of sodium bicarbonate was added to each well (6 g/100 mL) and left at 22°C for 90 minutes. The absorbance was read on the

FLUOstar Omega plate reader at 725 nm. A regression equation was determined using Excel where absorbance was the dependent variable (y) and concentration was the independent variable (x).

ug Chlorogenic Acid Equivalents/100 g = ((ug/mL * extraction volume(mL)) / sample wt (g)) *100 *2(dilution factor)

Chlorogenic acid standards were prepared as follows. Stock: 0.1 g chlorogenic acid brought up to 100 mL with extraction solvent (70% acetone, 30% RO, 0.01% HCl).

Table 3. Chlorogenic Acid Standards

Standard Level (ug)	Stock Solution Volume Used (mL)	Final Volume Achieved By Adding Extraction Solvent (mL)
50	0.5	10
100	1.0	10
150	1.5	10
200	2.0	10
250	2.5	10
300	3.0	10

3.4 pH Differential Total Anthocyanin Content

To determine anthocyanin content, the pH differential method was adapted from Giusti et al. (2001), Lee et al. (2005), and Wrolstad (2005). Aqueous sample extracts were diluted with pH 1.0 buffer to obtain an absorbance between 0.2 and 0.9 at 520 nm. A 3.5 mL cuvette and the Spectronic 20D+ was used to determine the proper dilution. The dilution determined for the pH 1 buffer was used with the pH 4.5 buffer. To make potassium chloride buffer (0.025 M, pH 1.0), 1.86 g KCl and ~980 mL RO water were mixed in a beaker. The pH was measured and adjusted to 1.0 with concentrated HCl. The

solution was transferred to a 1 L volumetric flask and brought up to volume with RO water. To make sodium acetate buffer (0.4 M, pH 4.5), 54.43 g sodium acetate trihydrate and ~960 mL distilled water were mixed in a beaker. The pH was measured and adjusted to 4.5 with concentrated HCl. The solution was transfer to a 1 L volumetric flask and brought up to volume with RO water.

Once the dilution was determined, 300 uL (150 uL diluted sample + 150 uL buffer) of buffered sample was added to the appropriate well according to the plate layout diagram. Using the BMG Labtech Fluostar Omega Platereader, a table was generated that contained the sample ID and raw data for the absorbance at 520 and 700 nm.

Absorbance = ($A_{520\ nm}$ pH 1.0 – $A_{700\ nm}$ pH 1.0) – ($A_{520\ nm}$ pH 4.5 – $A_{700\ nm}$ pH 4.5)

Concentration mg/L = (Absorbance * molecular wt * dilution * 1000) / molar extinction coefficient

Mg extracted = ((concentration mg/L)/1000)* extraction volume

Mg/100g = (mg extracted / sample wt) * 100

Notes: The dilution factor used was 4 and was made by adding (1 mL full-strength extract + 1 mL RO water) + 1 mL pH 1 buffer where DF 2 multiplied by DF 2 equals DF 4. The anthocyanin, malvidin-3-glucoside, was used for calculations. The molecular weight is 493.3 g/mol and the molar extinction coefficient is 28,000 L/(cm)(mol) (Wrolstad, 2005).

3.5 DPPH Antioxidant Activity

Radical scavenging activity was determined by methods adapted from Musa et al. (2013) and Abderrahim et al. (2013). To prepare DPPH dry reagent array (a method where DPPH is dried into the well plate instead of in liquid form), a stock solution of 1 mg DPPH in 1 mL methanol was prepared. Thirty uL stock solution was added to each of 96 wells using a 12-channel pipettor. The plates were evaporated under nitrogen gas to form dry reagent (30 ug DPPH per well). Plates were sealed with aluminum foil after preparation to avoid DPPH photodecomposition and then stored in the fridge.

To prepare Trolox standards, 0.005 g Trolox® was weighed and filled to 100 mLs with extraction solvent (70% acetone, 30% RO water, 0.01% HCl). It was then filled to 200 mLs with extraction solvent to achieve 100 umol/L (stock solution). All standards were stored in the fridge.

Table 4. Trolox Standards

Standard Level (umol/L)	Stock Solution Volume Used (mL)	Final Volume Achieved by Adding Extraction Solvent (mL)
80	8	10
60	6	10
40	4	10
20	2	10

To determine antioxidant activity, the plate seal was removed. To each well, 200 uL methanol was added to reconstitute the dried DPPH using a 12-channel pipettor. According to the plate layout diagram, 100 uL blank (methanol), Trolox® standard, or extracted sample (DF 10) were added. Readings were taken in the Omega plate-reader at times 0, 15, 30, 45, and 60 minutes at 25°C. The plate was sealed between readings to

prevent evaporation and photodecomposition. Results were calculated as percent radical scavenging activity:

% RSA = [absorbance$_{517\ nm}$ (t=0) – absorbance$_{517\ nm}$ (t=t) x 100 / absorbance$_{517\ nm}$ (t=0)], where (t=0) is the absorbance at time 0 and (t=t) is the absorbance at 15, 30, 45, or 60 mins.

3.6 Statistical Analysis

The statistical significance comparing data between groups was assessed by one-way analysis of variance (ANOVA), Tukey's Honestly Significant Difference Test, and Pearson Correlations. Statistical analyses were performed using SYSTAT (Version 12. Systat Software, Inc. San Jose, CA). Statistical significance was declared when $p \leq 0.05$.

4 Results

4.1 Alpha-Glucosidase Inhibition

The positive control, 1mM acarbose, inhibited a mean of 92.3% of the enzyme activity. Acarbose inhibition results did stay consistent during runs. Published data using a similar method and percent inhibition calculation yielded 100% inhibition by acarbose (Johnson et al., 2013), so the results from this project are similar.

Under these experimental conditions, the berry extracts all inhibited more than half of the alpha-glucosidase activity. Mean percent inhibition varied from 60.4% for cultivated, 54.5% for organic wild, to 54.3% for high-input wild. Patriot was significantly the lowest inhibitor amongst the cultivated berries, while (O2, O3) and (O6, O7) were significantly lowest amongst the wild berries. There was not a significant difference in the mean alpha-glucosidase inhibition between the organic wild blueberries versus the high-input wild blueberries. Alpha-glucosidase inhibition did not strongly correlate with any of the other assays as its correlation with total free phenolics was 0.239, with total anthocyanins was 0.134, and with % RSA was -0.026.

Hogan et al. (2010) reported that red grape extract inhibited alpha-glucosidase enzyme activity by 47%, and the inhibition potency was significantly higher than that of the white grape extract they tested, which only inhibited alpha-glucosidase activity by 39%. Although grape extracts do not have a phytochemical profile identical to blackberries or blueberries, this research shows that an extract with more phenolics and anthocyanins is more inhibitory.

Table 5. Alpha-glucosidase Inhibition by Cultivated Berries[a]

Sample	% a-Glucosidase Inhibition
Blackberry	62.9 ± 5.9 a
Patriot	55.4 ± 4.6 b
Blue Ray	62.6 ± 2.4 a
Blue Crop	60.6 ± 2.3 a

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 6. Alpha-glucosidase Inhibition by Wild Blueberries[a]

Agricultural Input Level	Harvest Site	% a-Glucosidase Inhibition
Organic	(O2, O3)	51.5 ± 6.4 b
	(O1, O2)	60.4 ± 3.8 a
	(O5, O8)	55.2 ± 3.6 a
	(O6, O7)	50.9 ± 3.9 b
High	(H2, H4)	54.5 ± 2.6 a
	(H7, H8)	55.8 ± 4.9 a
	(H1, H2)	53.2 ± 3.3 a
	(H5, H6)	53.9 ± 2.5 a

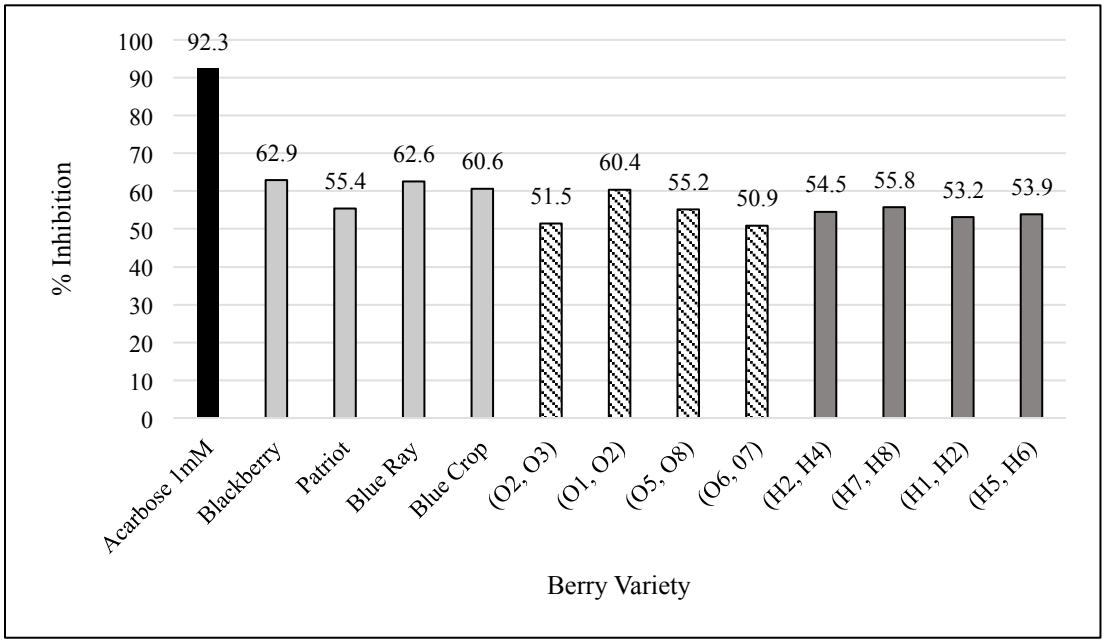
[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 7. Alpha-glucosidase Inhibition by Wild Blueberries by Input Level[a]

Agricultural Input Level	% a-Glucosidase Inhibition
Organic	54.5 ± 5.8 a
High	54.3 ± 3.4 a

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Figure 3. Alpha-glucosidase Percent Inhibition



4.2 Total Phenolic Content

The total free phenolics procedure measures the total amount of phenol compounds the berry samples contain and the results are expressed as chlorogenic acid equivalents in mg/100 g. Amongst the cultivated berries, blackberry was significantly the highest, and amongst the wild berries, (O1, O2) and (H7, H8) were significantly the highest in phenolic content. However, there was not a significant difference between the mean phenolic content of the organic wild berries and the high-input wild berries. There were no strong correlations evident between phenolic content and the other assays as the correlation with alpha-glucosidase inhibition was 0.239, with total anthocyanins was 0.643, and with % RSA was 0.578.

Published data reports significantly lower phenolic content results. For example, Prior et al. (1998) reported a mean total phenolic content of 260.9 mg/100 g for cultivated blueberries (*Vaccinium corymbosum* L. / Northern Highbush) and 299.0 mg/100 g for wild blueberries (lowbush / *Vaccinum angustifolium*). The differences from published data could be caused by such factors as variations in berry storage time or conditions, variations in extraction procedure and standard used, or variations in assay execution.

Table 8. Total Free Phenolic Content of Cultivated Berries[a]

Sample	Total Free Phenolics (mg/100 g)
Blackberry	896.6 ± 15.8 a
Patriot	545.9 ± 79.1 b
Blue Ray	689.9 ± 100.0 b
Blue Crop	565.6 ± 21.3 b

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 9. Total Free Phenolic Content of Wild Blueberries[a]

Agricultural Input Level	Harvest Site	Total Free Phenolics (mg/100 g)
Organic	(O2, O3)	699.2 ± 61.4 b
	(O1, O2)	850.0 ± 33.9 a
	(O5, O8)	664.0 ± 43.1 b
	(O6, O7)	664.1 ± 17.1 b
High	(H2, H4)	660.1 ± 39.9 b
	(H7, H8)	763.8 ± 62.3 a
	(H1, H2)	642.7 ± 68.4 b
	(H5, H6)	747.1 ± 67.7 b

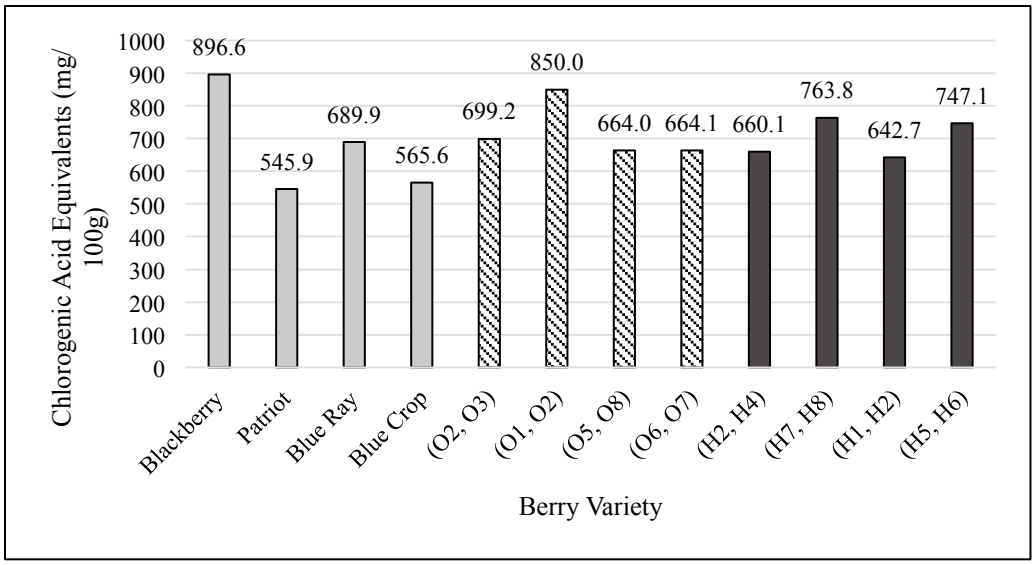
[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 10. Total Free Phenolic Content of Wild Blueberries by Input Level[a]

Agricultural Input Level	Total Free Phenolics (mg/100 g)
Organic	719.3 ± 87.7 a
High	703.4 ± 73.5 a

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Figure 4. Total Free Phenolic Content (mg/100 g)



4.3 Anthocyanin Content

The pH differential method is used to measure the anthocyanin content of a sample and the results are reported as malvidin-3-glucoside equivalents in mg extracted/100 g. Since anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5) (Lee et al., 2005) the pH differential procedure measures the difference in absorbance between these two forms to determine anthocyanin content.

Amongst the cultivated berries, Blue Ray was significantly highest in anthocyanin content. Amongst the wild blueberries, (O1, O2) was significantly highest. When the anthocyanin content is averaged by variety, organic wild had an average of 97.5 mg/100 g. High-input wild had an average of 92.3 mg/100 g. Finally, the cultivated variety had an average anthocyanin content at 84.2 mg/100 g. However, the mean anthocyanin content of the organic wild blueberries was not significantly different from the high-input wild

blueberries. Anthocyanin content did not strongly correlate with the other assays as its correlation with alpha-glucosidase inhibition was 0.134, with total free phenolics was 0.643, and with % RSA was 0.356. In published data, Prior et al. (1998) reported a mean total anthocyanin content of 129.2 mg/100 g for cultivated blueberries and 95.4 mg/100 g for wild blueberries, so the wild blueberry results in this project were similar.

Table 11. Anthocyanin Content of Cultivated Berries[a]

Sample	Anthocyanin Content (mg/100 g)
Blackberry	84.9 ± 5.8 b
Patriot	72.6 ± 15.3 b
Blue Ray	117.9 ± 11.0 a
Blue Crop	61.6 ± 1.3 b

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 12. Anthocyanin Content of Wild Blueberries[a]

Agricultural Input Level	Site	Anthocyanin Content (mg/100 g)
Organic	(O2, O3)	85.9 ± 11.1 b
	(O1, O2)	120.8 ± 7.3 a
	(O5, O8)	98.5 ± 2.9 b
	(O6, O7)	84.6 ± 6.3 b
High	(H2, H4)	77.8 ± 4.5 b
	(H7, H8)	107.1 ± 6.1 b
	(H1, H2)	84.5 ± 5.7 b
	(H5, H6)	99.7 ± 7.9 b

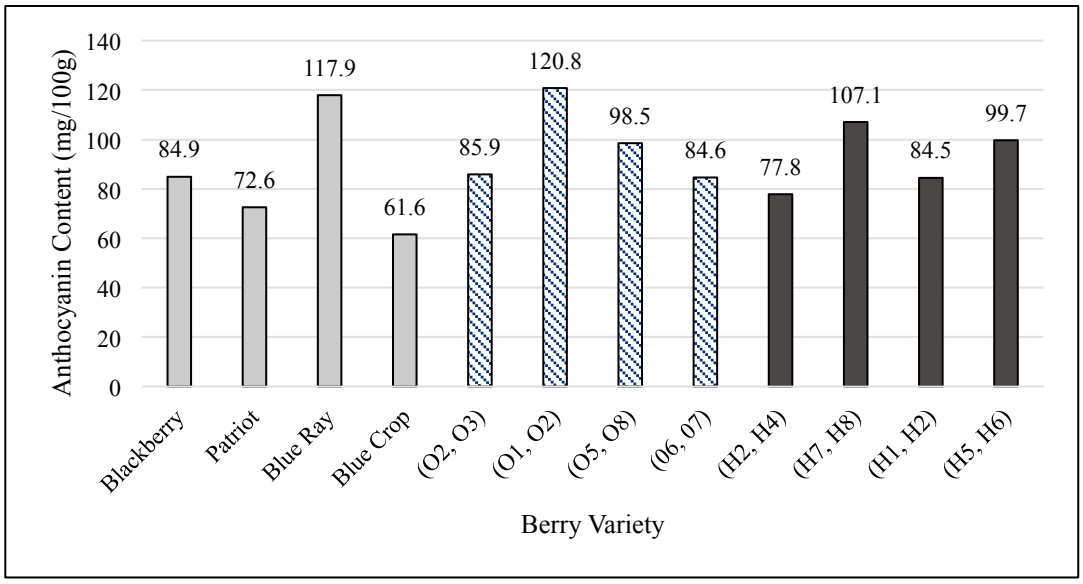
[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 13. Anthocyanin Content of Wild Blueberries by Input Level[a]

Agricultural Input Level	Anthocyanin Content (mg/100 g)
Organic	97.5 ± 16.4 a
High	92.3 ± 13.2 a

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Figure 5. Anthocyanin Content (mg/100 g)



4.4 Antioxidant Activity

The antioxidant activity was measured using the dry DPPH reagent array method and results are expressed as percent RSA at t=60 mins with a berry extract concentration of 5.0 mg/mL. Percent RSA measures the ability of the berries to scavenge free radicals, or in other words, their ability to act as antioxidants.

There were trends evident in the % RSA data. Amongst the cultivated berries, blackberry was significantly the highest. Amongst the wild blueberries, (O1, O2), (O6, O7), (H7, H8), and (H5, H6) were significantly the highest. Altogether, the data shows that cultivated blueberries have a mean RSA of 14.3%, high-input wild have a mean RSA of 18.4%, and organic wild have a mean RSA of 16.7%. The radical scavenging activity of the high-input wild blueberries was significantly higher than the organic wild blueberries. However, there was no strong correlation evident between % RSA and the

other assays because its correlation with alpha-glucosidase inhibition was -0.026, with total free phenolics was 0.578, and with total anthocyanins was 0.356.

Published data does vary from these results. Huang et al. (2012) reported that, at 2.0 mg/mL, blueberry and blackberry extracts could scavenge nearly all DPPH radicals (96.96% and 95.37%, respectively). At 5.0 mg/mL, this project's RSA data is much lower than that. The variations from published data could be due to such factors as berry storage time and condition differences, antioxidant degradation due to freezing, extraction differences, and assay execution differences.

Table 14. Radical Scavenging Activity of Cultivated Berries[a]

Sample	% RSA at t=60 mins
Blackberry	18.9 ± 1.8 a
Patriot	11.0 ± 2.5 b
Blue Ray	13.3 ± 2.6 b
Blue Crop	14.1 ± 4.1 b

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 15. Radical Scavenging Activity of Wild Blueberries[a]

Agricultural Input Level	Sample	% RSA at t=60 mins
Organic	(O2, O3)	14.4 ± 2.0 b
	(O1, O2)	19.3 ± 1.4 a
	(O5, O8)	15.6 ± 2.2 b
	(O6, O7)	17.7 ± 4.7 a
High	(H2, H4)	16.7 ± 1.3 b
	(H7, H8)	18.8 ± 1.6 a
	(H1, H2)	16.9 ± 1.0 b
	(H5, H6)	21.3 ± 1.7 a

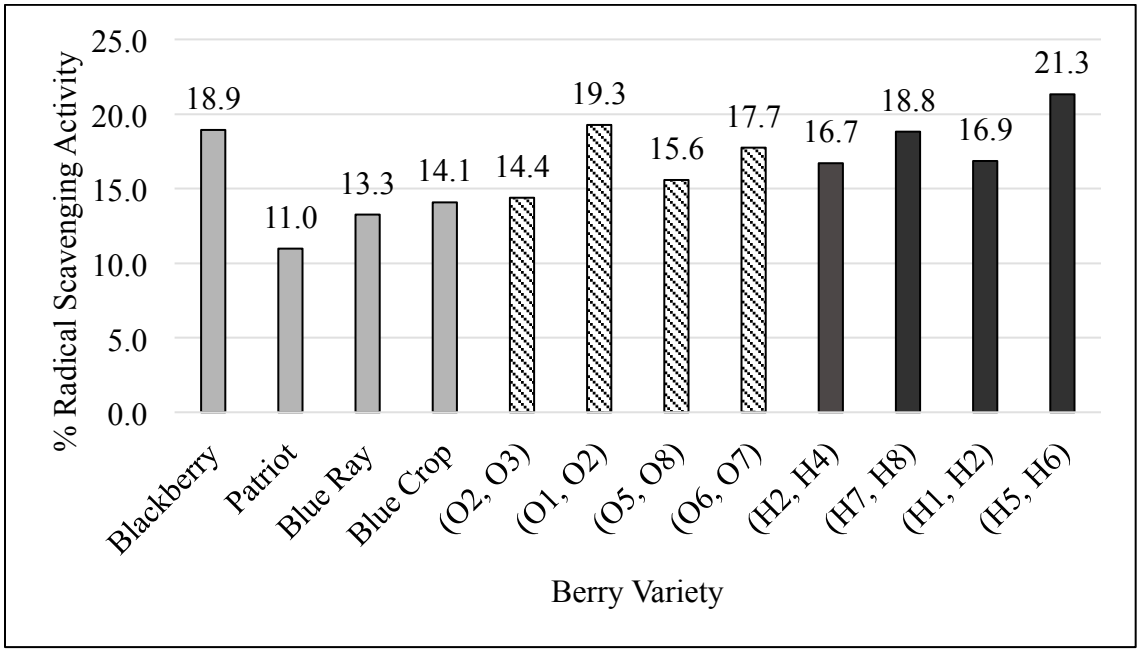
[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Table 16. Radical Scavenging Activity of Wild Blueberries by Input Level[a]

Agricultural Input Level	% RSA at t=60 mins
Organic	16.7 ± 3.3 b
High	18.4 ± 2.3 a

[a] Means sharing a letter are not statistically significant from each other (Tukey's HSD, $p \leq 0.05$).

Figure 6. Percent Radical Scavenging Activity (t = 60 mins)



5 Discussion

Overall, the assay results for alpha-glucosidase inhibition, total free phenolics, total anthocyanins, and percent radical scavenging activity demonstrate some of the health-beneficial qualities of blackberries and blueberries with some variations seen between varieties and agricultural input practices. The alpha-glucosidase inhibition assay shows that blueberries and blackberries can inhibit at least 50% of alpha-glucosidase activity under these experimental conditions and within sixty minutes. Under different experimental conditions (such as with a stronger berry concentration or longer run time), alpha-glucosidase inhibition would be even higher.

Therefore, people with diabetes should be educated and encouraged to consume berries (and other fruits and vegetables) because they contain phytochemicals that can act in a similar way to diabetes medications, like acarbose, and other alpha-glucosidase inhibitors. Grace et al. (2009) performed a study that demonstrated that anthocyanins from blueberry have the potency to alleviate symptoms of hyperglycemia in diabetic C57b1/6J mice. Additionally, Abidov et al. (2006) performed a study where adults with type 2 diabetes consumed blueberry extract; the results showed that the supplement reduced plasma glucose and it possessed pharmacologically relevant anti-inflammatory properties. Due to these properties, berries can be incorporated into meals to help decrease postprandial glucose rises from consumption of starchy foods. While inhibitory medications can have side effects and drug interactions, consumption of fruit is a natural and easy way for people to improve glycemic control and overall health.

None of the four assays yielded data that strongly correlated with one another, and the mean organic wild versus high-input wild data did not show any significant

differences except for radical scavenging activity where high-input wild was significantly higher. More data would be needed to support or refute the hypothesis that cultivated berries have a worse phytochemical profile than wild types.

Published data has presented conflicting results as to whether organic versus conventionally grown produce yields better phytochemical profiles. For example, Wang et al. (2008) found that organically grown blueberries were higher in total free phenolics, anthocyanin content, and antioxidant capacity when compared with non-organically grown berries. However, You et al. (2011) found no significant difference in anthocyanin content between organic and non-organic cultivation of blueberries and inconsistent results for total free phenolics and antioxidant capacity. Thus, research on the impact of agricultural practices on blueberry phytochemical content is ongoing.

In conclusion, it is clear that phytochemical research is a promising field that continues to grow and expand. Further research into this subject could include acquiring more data by assay repetition, using a wider range of samples and input levels, determining the anthocyanin profiles by chromatography, or testing the extracts for even more enzyme inhibitory activity (such as alpha-amylase), as this can also influence type 2 diabetes management.

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