PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT, ANTI-HYPERGLYCEMIC, AND ANTI-HYPERTENSIVE PROPERTIES OF SERVICEBERRY AND BLACKBERRY

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MASTER OF SCIENCE

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ABSTRACT

Production and consumption of edible berries are increasing rapidly in the United States, mostly due to their superior flavor profile, and popular diet-related value with their human health relevant bioactives and nutritional benefits. However, bioactive and nutritional qualities, especially human health protective phenolic antioxidants and associated non-communicable chronic disease (NCD) relevant health benefits of berries vary widely among accessions/cultivars and due to different production practices (organic vs conventional). Therefore, the aim of this thesis was to screen and select high phenolic and high antioxidant serviceberry and blackberry accessions/cultivars and to investigate the effect of different weed management and fertilization (organic vs. conventional) practices on phenolic bioactive linked antioxidant and anti-diabetic properties of blackberry using *in vitro* assay models. Overall, high phenolic-bioactive linked antioxidant and anti-hyperglycemic properties were observed in both serviceberry and blackberry accessions/cultivars and further for blackberry it was significantly higher under organic weed management and fertilization practices.

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Finally, I would like to express my profound gratitude to my family and girlfriend for providing me with their unfailing love and support through my graduate career. My sincerest thanks.

DEDICATION

To everyone who suffers from a metabolic syndrome-related disease, may you find hope and healing through this work and to God, through whom all things are possible.

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LIST OF ABBREVIATIONS

T2D	Type 2 Diabetes Mellitus
NCD	Non-Communicable Chronic Diseases
ROS	Reactive Oxygen Species
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ABTS	2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic)acid

CHAPTER 1. INTRODUCTION

1.1. Background to Introduction

As non-communicable chronic disease (NCD) burden increases globally from poor diets that are rich in macronutrients largely from soluble carbohydrate dense foods and poor intake of fruit and vegetables, there is increase need to identify diverse range of fruits and vegetables as antidotes to NCD through our diets. Among the NCD challenges, oxidative stress-linked early stages of type 2 diabetes, such as hyperglycemia and its complications towards hypertension can be countered by improved diets rich in fruits and vegetables. Therefore, the focus of this thesis is on screening different cultivars/genotypes and targeted organic production strategies towards understanding the potential of select berries such as antioxidant rich serviceberry (*Amelanchier* spp.) and blackberry (*Rubus* spp.) as potential antidotes to counter hyperglycemia and hypertension using *in vitro* assay models. This study will then provide a foundation to improve the diversity of fruits and vegetables available to counter NCD related challenges.

1.2. Non-communicable Chronic Disease Epidemic

Non-communicable chronic diseases (NCDs) are the leading causes of death worldwide, accounting for 71% of all deaths and share a common pathogenesis linked to oxidative stress-induced metabolic breakdowns and associated cellular dysfunctions (Ceriello and Motz 2004; Lin and Beal 2006; WHO 2017). Such oxidative stress-induced common pathogenesis involved in NCD incidence and development can lead to chronic inflammatory state which is associated with several macro- and micro-vascular complications in humans (Ceriello and Motz 2004; Lin and Beal 2006). In general, NCDs encompass a wide range of oxidative stress-linked non-infectious diseases such as diabetes mellitus (DM), cardiovascular diseases (CVDs), cancers, respiratory disease, musculo-skeletal disorders, and dyslipidemia (Unwin et al. 2006). With regard to these common NCDs, the most prevalent with greater morbidity and mortality are CVDs, cancers, respiratory disease (COPD), and type 2 diabetes (T2D) (WHO, 2016). Several of these NCDs can be prevented and it has been estimated that up to 80% of T2D and CVDs, and 40% of all cases of cancer could be prevented with better diets and healthy lifestyle choices (Ley et al. 2016; Unwin and Alberti 2006). In this context of healthy diet and lifestyle choices, higher consumption of nutritionally balanced foods, avoiding smoking, exercising at least 30 minutes per day, moderate consumption of alcohol, and having a BMI of less than or equal to 25 kg/m^2 are widely recommended in order to reduce the risks of NCDs (Piernas et al. 2016). Furthermore, higher intake of nutritionally balanced diet is especially most critical in overall prevention and management of diet and lifestyle-linked NCDs, such as T2D, and CVDs. Diet related factors that have been associated with increased risk of T2D and CVDs include; higher intake of hyper-processed and calorie dense foods; higher daily consumption of refined carbohydrates; higher intake of trans fatty acids and sugar-sweetened beverages; low intake of vegetables and fruits; and higher consumption of alcohol (Ley et al. 2016). On the contrary, diet rich in dietary fiber, minerals, and phytochemicals such as different plant-based whole foods provide protective functions against diet and lifestyle-linked NCDs and therefore can be targeted in preventative dietary support strategies to counter the rapidly emerging epidemic of NCDs, especially higher prevalence of T2D and associated CVDs (Ley et al. 2016). Based on these dietary benefits and potential NCD protective functions of plant-based whole foods, the aim of this thesis was to investigate the anti-hyperglycemic, antioxidant and anti-hypertensive properties of berries, including some cold hardy accessions such as serviceberry and blackberry using rapid *in vitro* screening strategies with goal to advance their dietary support strategies or to develop health targeted edible ingredients for overall prevention and management of early stages of T2D and associated chronic inflammation and hypertension.

Type 2 diabetes is linked to chronic metabolic disorders and the pathogenesis of T2D involves insulin resistance, insulin insensitivity, impaired insulin production, beta-cell dysfunction, chronic hyperglycemia, and chronic oxidative stress (Cheung et al. 2012; Egan and Dinneen 2019; Khangura et al. 2000; Odegaard et al. 2016; Shanmugam et al. 2016; Vijan 2016). Several of these metabolic disorders are interconnected and widely characterized as "Metabolic Syndrome" or "Syndrome X". In the context of "Metabolic Syndrome" and associated T2D, there are different oxidative-stress induced metabolic and physiological breakdowns which can lead to several micro-vascular (retinopathy, nephropathy, and neuropathy), and macro-vascular complications (heart attacks, stroke, and peripheral vascular disease) (Cerf 2013; Cheplick et al. 2015; Dal et al. 2016). Due to such metabolically-linked interrelated and common pathogenesis involving vascular complications, T2D has been shown to increase the risk of other NCDs, especially 2-3 folds higher risks of CVDs which include stroke and myocardial infarction, (Abdul-Ghani et al. 2017; Almdal et al. 2004). Among all pathogenesis, chronic hyperglycemia due to improper balance of glucose homeostasis is a major cause of T2D development. Therefore, managing chronic hyperglycemia and countering associated vascular complications is most critical to prevent and reduce the risks of T2D associated morbidity and mortality globally.

1.3. Type 2 Diabetes and Chronic Hyperglycemia

Development of T2D is a multistage process and involves insulin resistance, beta-cell dysfunction, and associated chronic hyperglycemia (Egan and Dinneen 2019). The progression from pre-diabetic stage to actual development of T2D is directly linked to increasing fasting and postprandial glucose level in the blood due to chronic hyperglycemia (Fonseca 2009).

Additionally, onset of hyperglycemia can trigger both insulin resistance and beta cell dysfunction and therefore can escalate the progression of T2D development in pre-diabetic individuals (Cerf 2013). Other risk factors and indicators associated with progression and development of chronic hyperglycemia-linked T2D are higher body mass index (BMI), chronic hypertension, high triglycerides, and lower level of HDL cholesterol (Cerf 2013). Furthermore, chronic hyperglycemia may also lead to or cause several additional diabetes-associated vascular complications including chronic oxidative stress-linked inflammation and chronic hypertension (Dal et al. 2016). Therefore, managing chronic hyperglycemia is not only just important for countering T2D, but also essential reduce the risks of chronic hypertension and inflammation commonly associated with T2D.

1.4. Type 2 Diabetes, Oxidative Stress, and Inflammation

Chronic oxidative stress and associated inflammation are closely linked to the development of T2D and other NCDs (Odegaard et al. 2016). Chronic oxidative stress is mostly caused by an imbalance between reactive oxygen species (ROS) and antioxidant defense systems which increases the production of ROS in cells and leads to chronic inflammation as part of the temporary response of the innate immune system towards redox imbalance (Betteridge 2000; Velasquez 2014). Higher concentration of ROS and subsequent breakdown of redox homeostasis are believed to be responsible for the development of several NCDs, such as T2D, CVDs, and hypertension (Yuliandra et al. 2017). This oxidative stress-induced common pathogenesis could explain how both hypertension and CVDs are prevalent among diabetic individuals and up as 80% of diabetic patients die mostly from CVDs and associated vascular complications (Dal and Sigrist 2016; Mohan et al. 2010; Vijan 2016).

The pathology of chronic oxidative stress and inflammation in individuals with T2D is commonly attributed to T2D-associated chronic hyperglycemia (Dal et al. 2016). Hyperglycemia causes oxidative stress through the production of free radicals and the impairment of antioxidant defense systems (Maritim et al. 2002). Additionally, chronic hyperglycemia also causes excess cellular energy (ATP) to build up in the body and the process is directly linked to the oxygen malfunction in the mitochondria. This excess of ATP most often results in the incomplete reduction of oxygen to occur in the mitochondria, ultimately leading to the generation of ROS and subsequent development of chronic oxidative stress (Ceriello and Motz 2004; Lin and Beal 2006).

Chronic hyperglycemia subsequently causes both localized and systemic inflammation, through raising the level of pro-inflammatory proteins and causing macrophages to expel inflammatory cytokines (Dal et al. 2016). Furthermore, chronic oxidative stress has been shown to cause inflammation and such higher inflammatory states can also lead to aggravated oxidative stress and reduce overall cellular antioxidant capacity (Joseph et al. 2014; Khansari et al. 2009; Reuter et al. 2010; Tarique et al. 2016) Therefore, countering chronic oxidative stress and associated inflammation to prevent and manage T2D and associated vascular complications such as chronic hypertension is essential (Akash et al. 2013; Dembinska et al. 2008; Maritim et al. 2002).

1.5. Type 2 Diabetes and Hypertension

Among several type 2 diabetes associated macro-vascular complications, hypertension, also known as “high blood pressure”, is the most prevalent NCDs in the United States and the most attributable risk factor for death globally (Hewett 2010). Chronic hypertension resulting in elevated blood pressure and blood glucose that occurs due to several metabolic breakdowns including chronic oxidative stress, and chronic hyperglycemia both commonly associated with T2D (Hewett 2010). Additionally, inflammation associated with oxidative stress-linked T2D could potentially explain higher occurrence of hypertension in diabetic patients (Cheung et al. 2012; Dembinska et al. 2008; Maritim et al. 2002). Furthermore, hypertension associated inflammation could also contribute to the pathology of T2D as inflammation has been shown to be involved in diabetes pathogenesis and is highly common in individuals with T2D (Cheung et al. 2012; Dembinska et al. 2008; Maritim et al. 2002; Savoia et al. 2006; Vijan 2016). Due to such common metabolically linked pathogenesis, hypertension has been estimated to be 1.5 to 3 times more common in individuals with T2D than in non-diabetic population (Vijan 2016). In the United States, 50% - 80% of individuals with T2D have been diagnosed with hypertension (Cheung et al. 2012; Khangura et al. 2000). In addition, the coexistence of T2D and hypertension has been shown to play a crucial role in the progression and initiation of other macro-vascular diseases (ie: peripheral vascular disease, stroke, ischemic heart disease) and individuals with both T2D and hypertension have a 4-fold higher risk of developing such CVDs (Khangura et al. 2000; Khangura et al. 2018). Therefore, managing hypertension is essential for prevention of both T2D and CVDs and to address the NCD challenges globally. Due to such complex mechanism in pathogenesis of these development and their interconnections, it is important to address these NCD-linked health challenges using holistic integrated strategies. Plant-based foods rich in health promoting compounds can be targeted as part of such holistic disease preventative strategies, and can be advanced complementarily with other therapeutic strategies to address the global epidemic of T2D and associated CVDs.

1.6. Plant-Based Foods as a Dietary Antidote against Type 2 Diabetes

Plant-based foods, especially fresh and whole foods are rich in health protective bioactives such as phenolic antioxidants with diverse human health relevant functionalities (Attele et al 2002; DeFuria et al. 2009; Hanhineva et al. 2010; Kowalska and Olejnik 2016; Lehtonen et al 2010; Shama et al. 2014; Ştefănuţ et al. 2013; Zhang et al. 2012). Therefore, higher intake of such bioactive enriched plant-based foods as part of daily diet is highly recommended for prevention and management of oxidative stress-linked NCDs, especially early stages of T2D and associated health complications such as chronic inflammation and hypertension (Hanhineva et al. 2010; Shama et al. 2014). In general, exogenous supply of antioxidants is important to maintain cellular redox homeostasis specially to counter NCD-induced chronic oxidative stress. In this context, higher consumption of plant based foods rich in antioxidants such as phenolic bioactives with higher antioxidant potentials is the safe and most inexpensive strategy to counter T2D and other NCD-induced chronic oxidative stress and associated metabolic breakdowns (Hanhineva et al. 2010). Additionally, plant-based foods rich in phenolic antioxidants have also been shown to have anti-hyperglycemia, anti-inflammatory, and anti-hypertension functionalities (Barbosa et al. 2011; Cheplick et al. 2015). Therefore, plant-based foods rich in such phenolic antioxidants such as berries are important dietary sources which can be targeted as part of safe and inexpensive dietary intervention strategies for the prevention and management of T2D and associated health complications (Carlsen et al. 2010).

In general, berries are rich source of phenolic bioactives, and have shown high *in vitro* anti-hyperglycemic and anti-inflammatory properties linked to early stages of T2D (Cheplick et al.2007; 2010; 2015; Pinto et al. 2008; 2010a; 2010b; 2010c; Sarkar et al. 2016; 2017). Additionally, epidemiological studies have also suggested an association between higher consumption of berries and reduced risk of T2D and associated macro and micro-vascular complications (Castro-Acosta 2016). Therefore, berries can be targeted as dietary antidote to manage chronic hyperglycemia, and chronic oxidative stress commonly associated with early stages of type 2 diabetes and associated health complications. Based on this rationale of T2D linked health benefits of berries, we have hypothesized that berries such as serviceberry and blackberry rich in phenolic bioactives would also have high anti-hyperglycemic and antioxidant functionalities relevant for dietary prevention and management of early stages T2D and associated chronic inflammation and chronic hypertension.

CHAPTER 2. REVIEW OF LITERATURE

2.1. Berries as a Healthy Food Choice

Consumers across different geographic locations, ethnic origins, and economic backgrounds are becoming increasingly aware about fresh and healthy food choices and therefore driving the rising demand for fresh fruit produce with value-added nutritional profiles. In this context, fresh fruits such as berries are highly sought agricultural commodities and demand for these healthy fresh foods is increasing both in the United States and in the global market (Lucier et al. 2006; Monson 2009). The per capita consumption of berries has risen by 55% from 1990 to 2004 in the United States alone (Monson 2009). According to the current market estimate, fresh berries are available in the retail market throughout the year in the United States and berry producers can earn higher economic returns per unit of land than any other agricultural commodity produce (Sobekova et al. 2013).

Berries are high-value specialty crops with very high dietary antioxidants and other health promoting phenolic bioactives among commonly consumed fresh fruits and vegetables (Carlsen et al. 2010; Lucier et al. 2006; Monson 2009; Pérez-Jiménez et al. 2010). The growing interest and demand for fresh berries among consumers is mainly due to their high antioxidant profiles with diverse human health benefits. Both serviceberry and blackberry contains significant amounts of human health relevant phenolics (anthocyanins, flavols and ellagitannins) with high antioxidant activity (Jurikova et al. 2013; Kaume et al. 2011; Lavola et al. 2012; Moyer et al. 2002; Szajdek and Borowska 2008). Such high phenolic-linked antioxidant activity of serviceberry and blackberry is also associated with different health relevant functionalities and can be targeted in value-added dietary application as fresh fruits and as functional food ingredients to counter non-communicable chronic diseases (NCDs) such as T2D and CVDs.

Previous research has indicated that berries have anti-inflammatory activities (Srivastava et al. 2010), higher antioxidant activities (Kähkönen et al. 2001; Srivastava et al. 2010; Wang and Lin 2000), and glucose metabolism enzyme inhibitory activities (Cheplick et al. 2007; McDougall et al. 2005; Sarkar et al. 2016; Tundis et al. 2010; Zhang 2012). Therefore, berries in general are ideal targets to be utilized in value-added dietary strategies to prevent and manage NCDs including T2D and associated health risks.

However, the future domestic and export market of berries will largely depend on understanding and improving the different value-added quality parameters such as critical human health relevant bioactive profiles, sensory qualities, and other post-harvest preservation qualities such as shelf-life of berries. These value-added qualities of berries, including human health relevant nutrient profiles, of serviceberry and blackberry varies widely among cultivars, different cultivation practices, growing conditions, time of harvest, and storage conditions (Cheplick et al. 2016; Sarkar et al. 2016; Talcott 2007; Wang and Lin 2000). Therefore, based on our previous findings and consistent body of preliminary evidence from published literature, the major aim of this thesis was to investigate human health relevant phenolic bioactive profiles and associated antioxidant, anti-hyperglycemic and anti-hypertensive properties in cultivars and accessions of serviceberry and blackberry, including select winter hardy accessions under both organic and conventional production practices using rapid *in vitro* screening strategies.

2.2. Phenolic Bioactives of Berries

Phenolic compounds are plant secondary metabolites mostly produced in response to abiotic and biotic stresses. Such stress-induced phenolic compounds are widely present across plant-based foods and can be found as simple phenolic acids, stilbenoids, lignans, or flavonoids (Anhe et al. 2013). When consumed as part of a diet, phenolic bioactives from plant-based food sources can provide diverse T2D relevant protective functions such as anti-hyperglycemia countering benefits through their inhibition of key glucose metabolism relevant enzymes such as α-amylase, and intestinal lumen α-glucosidase, and through improving insulin function, sensitivity, and the uptake of glucose in adipocytes and muscle cells (Anhe et al. 2013; Hanhivena et al. 2011). Additionally, phenolic bioactives also serve as powerful dietary antioxidants for defense against oxidative stress due to their structural composition and associated functions; containing at least one aromatic carbon ring and one or more hydroxyl groups (Rice –Evans et al. 1997). Such structure-function relationship gives phenolic compounds the ability to act as hydrogen donors, to delocalize or stabilize unpaired electrons, and to chelate transition metal ions and overall ability to scavenge oxidative stress-induced ROS (Clark 2002; Hanhivena et al. 2011). Additionally, phenolic bioactives can act as antioxidants by modulating enzymatic reactions and cellular metabolic pathways that are involved in ROS generation and/or inhibiting enzymes responsible for ROS formation (Song et al. 2005). In recent times, dietary antioxidants and the antioxidant functionalities of phenolic bioactives of plant-based foods are gaining increasing attention, especially for their potential incorporation in dietary intervention strategies as humans largely depend on external plant-derived antioxidants to combat NCD-induced chronic oxidative stress and to manage its associated inflammatory complications (Hanhineva et al. 2010).

2.3. Berry Phenolics: Antioxidant and Anti-inflammatory Properties

Several previous *in vivo* studies with human health-related models have concluded that berries are beneficial in T2D due to their capacity to reduce oxidative stress (Kowalska and Olejnik 2016). In humans, berries reduce oxidative stress by modulating both lipid and protein oxidation and the improvement of total antioxidant status (Kowalska and Olejnik 2016). The most effective antioxidants found among berry phenolic compounds are anthocyanins and therefore targeting anthocyanin-rich berries has significant relevance in dietary support strategies to counter T2D associated chronic oxidative stress (Yang et al. 2018). Both the anthocyanin components of berries and the total berry phenolics have been shown to reduce inflammation in numerous *in vivo* human studies through favorably impacting both the inflammatory and immune processes contributing to chronic inflammation (Joseph et al. 2014). This suggests that anthocyanins and other phenolic acids are not only an important bioactives for contributing to antioxidant capacity of berries, but also its potential anti-inflammatory properties. Additionally, phenolic compounds have been shown to cause this anti-inflammatory effect through quenching of free radicals, by directly blocking inflammatory cytokine expression, NFKB activity, and MAPK pathways (Anhe et al. 2013). The treatment of chronic inflammation with plant-based foods including berries and other plant-based herbal medicines have been practiced for centuries in different parts of the world, including in the United States. Specifically, in the United States, American Indians traditionally used plant sources, including berries, for treating several types of health issues associated with metabolic disorders and breakdown in body functions. Therefore, such traditional therapeutic approach has significant relevance to investigating the potential to manage oxidative stress and chronic inflammation associated with common NCDs, such as T2D, and to improve overall well-being in contemporary life. Therefore, berries, especially phenolic bioactives of these berries including winter-hardy cultivars and accessions, can be targeted for evaluation to potentially manage chronic oxidative stress-induced inflammation commonly associated with T2D and other common NCDs.

2.4. Berry Phenolics: Anti-Hyperglycemic Properties

One of the strategies to manage T2D is to suppress postprandial hyperglycemia via lessening the amount of absorbed glucose in the bloodstream through the inhibition of α-amylase and/or α-glucosidase enzymes (Barbosa et al. 2011; Cheplick et al. 2015). α -Amylase is an enzyme responsible for catalyzing the hydrolysis of starch alpha-1, 4-glucosidic linkages and α-glucosidase is an enzyme responsible for catalyzing the last step in the digestion of carbohydrates in the small intestine and absorption of glucose into the bloodstream (Barbosa et al. 2011; Wang et al. 2012). Blackberry (*Rubus* spp.), raspberry (*Rubus idaeus*), blueberry (*Vaccinium* spp.), strawberry (*Fragaria x ananassa* Duch.), black currant (*Ribes nigrum* L.), red currant (*Ribes rubrum* L.), gooseberry (*Ribes uva-crispa*), and serviceberry (*Amelanchier alnifolia* Nutt.) all exhibited significant α-amylase and/or α-glucosidase enzyme inhibitory activities in *in vitro* studies (Cheplick et al. 2007; 2010; 2015; Pinto et al. 2008; 2010b; 2010c; Sarkar et al. 2016; Wang et al. 2012; Zhang et al. 2012). Similarly, several berries have demonstrated *in vivo* anti-hyperglycemic functionalities in both animal and human models (Bispo et al 2015; Hanhineva et al. 2010). Blueberries (*Vaccinium ashei* and *Vaccinium corymbosum*) were reported to attenuate insulin resistance *in vivo* in mice; Panax ginseng (*Panax ginseng*) berries were reported to significantly improve glucose tolerance, blood glucose levels, reduce in serum insulin levels *in vivo* in mice; serviceberries (*Amelanchier alnifolia* Nutt.) were reported to lower post-prandial blood glucose concentrations, inhibit intestinal a-glucosidase, and delay the absorption of carbohydrate *in vivo* in mice; blackberries (*Rubus fruticosus*) and mulberries (*Morus nigra* L.) were reported to decrease glucose levels *in vivo* in mice; sea buckthorn (*Hippophae rhamnoides* ssp. turkestanica) berries were reported to help stabilize postprandial hyperglycemia *in vivo* in humans (Attele et al 2002; DeFuria et al. 2009; Lehtonen et al 2010; Ştefănuţ et al. 2013; Zhang et al. 2012) .

Synthetic pharmaceutical drugs, such as Acarabose, that target inhibition of these key enzymes (α-amylase and α-glucosidase), have been reported to cause adverse side effects including: meteorism, flatulence, abdominal distention, and possibly diarrhea (Barbosa et al. 2011; Kwon et al. 2006). Current research suggest that fresh fruits and vegetables rich in phenolic bioactives with high anti-hyperglycemic functionalities could be targeted in safe dietary intervention strategies and could be used complementarily with synthetic drugs to inhibit α-amylase and α-glucosidase enzymes in order to control postprandial hyperglycemia without any adverse side effects (Barbosa et al. 2011). Therefore, berries such as serviceberry and blackberry rich in phenolic antioxidants are ideal dietary targets to counter both chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of T2D. Additionally, finding safe dietary sources to manage chronic hypertension commonly associated with T2D and CVDs is also of great interest.

2.5. Anti-hypertensive Properties of Berries

Inhibition of angiotensin-I-converting enzyme (ACE) is a common therapeutic target for hypertension management, especially in patients with weak kidney functions (Anderson et al. 2010). ACE is an enzyme that catalyzes the conversion of angiotensin I to angiotensin II, which acts as an effective vasoconstrictor. Inhibition of ACE is the current pharmaceutical method for managing hypertension (Wagner et al. 1991). However, many synthetic ACE inhibitors have shown significant side effects including hypotension, diarrhea, cough and rash (Townsend et al. 2018). Therefore, finding safe dietary sources with ACE inhibitory potential is important, especially to control chronic hypertension associated with T2D (Forbes et al. 2002).

Several berries, such as raspberries (*Rubus* spp.), cranberries (*Vaccinium* spp.), and strawberries (*Fragaria* X a*nanassa* Duch.) exhibited *in vitro* ACE inhibition in previous studies (Cheplick et al. 2007; 2010; Pinto et al. 2010a). Additionally, several berries and berry-derived products [ie: grapes (*Vitis* spp.), cherries (*Prunus* spp.), chokeberries (*Aronia* spp.), bilberries (*Vaccinium* spp.), blueberries (*Vaccinium* spp.), and strawberries (*Fragaria* spp.)] have demonstrated anti-hypertensive functionalities, *in vivo,* in human studies including reduction of systolic blood pressure, diastolic blood pressure, augmentation index, central pulse wave velocity, and improving arterial stiffness (Kowalska and Olejnik 2016). However, the anti-hypertensive potential of serviceberry and blackberry especially ACE inhibitory properties of these berries were not extensively investigated. Therefore, rapid *in vitro* screening strategies for determining anti-hypertensive and anti-diabetic properties of different cold hardy cultivars of these berries are needed prior to targeting them in dietary intervention strategies to prevent and manage early stages of T2D and associated complications.

2.6. Serviceberry as a Model Crop

Serviceberry was used in traditional medicinal practices of Native Americans, including for management of what was suspected to be T2D type "sugar disease" for centuries (Zhang et al. 2012). Recent *in vitro* and *in vivo* studies have also found several anti-diabetic functionalities in serviceberries (Burns et al 2008; Zhang et al. 2012). In previous *in vitro* studies, serviceberries have shown to have anti-hyperglycemic (improvement of glucose uptake, inhibition of aldose reductase, a-glucosidase inhibition), high antioxidant [2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)], and anti-inflammatory properties (nitric oxide, lipid peroxidation inhibition, COX-2, TNF-induced expression) (Burns et al. 2008; Juríková et al. 2013; Zhang et al. 2012). Similarly, previous *in vivo* study also found anti-hyperglycemic effect and α-glucosidase enzyme inhibitory functions of serviceberry in diet-induced obese rats (Zhange et al. 2012). In general, serviceberries are rich in human health relevant bioactive profiles (Jennings et al. 1988). Some of these bioactives include: quercetin, rutin, cyanidin-3-galactoside, and cyanidin-3-glucoside and have shown to impart two or more of the following anti-diabetic relevant functionalities of a-amylase inhibition, a-glucosidase inhibition, or angiotensin-converting enzyme (ACE) inhibition activity using *in vitro* and/or *in vivo* models (Adefegha et al. 2018; Adisakwattana et al. 2004; Akkarachiyasit et al. 2011; Kaume 2011; Oboh 2014; Ożarowski et al. 2018; Sarinya Akarachiyasit 2010). However, phenolic bioactives and associated T2D relevant health benefits of serviceberry vary widely among cultivars and further due to different growing conditions and cultivation practices. Therefore, it is important to screen existing cultivars of serviceberry for their potential antioxidant, anti-hyperglycemic, and anti-hypertensive properties using rapid *in vitro* screening strategy prior to incorporating them in health targeted dietary solutions against early stages T2D and associated health risks.

2.7. Blackberry as a Model Crop

Similar to serviceberry, blackberry is also rich source of phenolic bioactives with high antioxidant potential. In addition, previous studies have reported several anti-diabetic functionalities in blackberry in both *in vitro* and *in vivo* models (Azofeifa et al. 2016; Cuevas-Rodríguez et al. 2010; Sarkar et al. 2016; Srivastava et al 2010; Ştefănuţ et al. 2013; Verma et al. 2014). Previous *in vitro* studies have found high anti-hyperglycemic (α-amylase and α-glucosidase enzyme inhibitory activities), antioxidant, and anti-inflammatory properties in different blackberry cultivars (Cuevas-Rodríguez et al. 2010; Sarkar et al. 2016; Srivastava et al 2010). Furthermore, *in vivo* studies with diabetic rats also reported both antioxidant and anti-hyperglycemic properties in blackberry and blackberry derived products (Azofeifa et al. 2016; Ştefănuţ et al. 2013). Interestingly, the anti-hyperglycemic activity of whole blackberry fruits was present even after 5-6weeks of administration of whole blackberry fruits to the rats (Azofeifa et al. 2016; Ştefănuţ et al. 2013). These previous studies suggest that blackberry with rich phenolic bioactive profile have potentials to manage chronic hyperglycemia and chronic oxidative stress commonly associated with early stages T2D and other NCDs (Jennings et al. 1988). Common phenolic bioactives present in blackberry are ellagic acid, rutin, catechin, protocatechuic acid, gallic acid (Lugasi et al. 2011). However, the phenolic acid content and phenolic profile of blackberry vary widely among cultivars, and due to different cultivation practices and growing environment. Therefore, optimization of these different variables (cultivars and cultivation practices) is essential in order to produce blackberry with rich phenolic bioactive profiles and to target them in health-focused food solutions.

2.8. Impact of Cultivar Differences, Growing Condition, Growth Stages, and Cultivation Practices on Berry Bioactives and Associated Health Benefits

The content and profile of phenolic bioactives of berries vary widely between cultivation practices, cultivars/genotypes, different maturation stages, different cultivation practices and due to growing conditions (Kaume et al. 2011; Zia-Ul-Iaq et al. 2014). Additionally, phenolic bioactive-linked functionalities, such as antioxidant, anti-hyperglycemic (α-amylase and α-glucosidase enzyme inhibitory activities), and antihypertensive properties vary between cultivars, growing conditions and due to different cultivation practices (organic vs conventional) (Aneta et al. 2013, Conti et al. 2014, Mishra et al. 2017; Sarkar et al. 2016; 2017). Plants grown under high abiotic stress environments, such as higher latitude, cold and temperate climate, and with higher biotic stress pressure (common under organic production system) produce higher amount of stress inducible secondary metabolites such as phenolics as part of their natural stress adaptive responses (Ankolekar et al. 2012; Zhang et al. 2015; Lavola et al. 2012; Ribas-Agusti 2017; Schulz et. al. 2016; Wang et. al. 2013; Zhang et al. 2015). Based on this rationale, we hypothesize that berries grown under the high latitude, cold temperate conditions of the Northern Plains have higher concentration of stress-induced phenolic metabolites and therefore can be targeted for diet-based strategies to prevent and manage NCDs such as early stages of T2D and associated complications. However, the commercial production of blackberry and serviceberry is limited in North Dakota. Like many leading states with berry production such as Maine, New York, and Vermont, North Dakota also has similar climatic conditions and agricultural infrastructure with good potential for production of high-value serviceberry and blackberry in this region. However, lack of sufficient winter-hardy cultivars and the sufficient know-how on production practices restrict the expansion of berry production in North Dakota and other parts of the Northern Plains. Furthermore, the current productivity of serviceberry and blackberry, both floricane and primocane cultivars, is significantly low (especially after overwintering) and not economically viable for commercial production. Therefore, it is essential to screen winter-hardy cultivars of serviceberry and blackberry with superior health targeted nutritional profiles prior to expanding their commercial production in the Northern Plains. Based on these needs of better defining the value added health relevant functionalities of winter hardy cultivars of serviceberry and blackberry, the primary goal of this thesis was to screen existing serviceberry and blackberry cultivars for their phenolic bioactive-linked antioxidant, anti-hyperglycemic and anti-hypertensive properties using rapid *in vitro* assay models. Furthermore, the effect of different environmental conditions (crop year differences in serviceberry) and cultivation practices (organic vs. conventional in blackberry) on berry phenolics and associated T2D relevant health benefits were also determined using same rapid *in vitro* screening strategy.

CHAPTER 3. OBJECTIVES

The major objective of this thesis was to screen health relevant phenolic bioactive profile and associated T2D-linked health benefits of serviceberry and blackberry, including winter-hardy cultivars in order to target them in long-term diet based interventions against early stageT2D and associated health risks. Furthermore, the goal was also to support growing consumer demands of locally grown value-added fresh berries by better defining and optimizing their T2D-linked health benefits using rapid *in vitro* screening strategies. With this broad aim, the specific objectives of this thesis were:

- To screen serviceberry cultivars, including those that are winter-hardy from two different crop years for phenolic bioactive–linked antioxidant, anti-hyperglycemic, and anti-hypertensive properties using rapid *in vitro* assay models.
- To analyze and compare phenolic-linked antioxidant and anti-hyperglycemic functionalities of two blackberry cultivars grown under organic and conventional cultivation practices.

CHAPTER 4. SCREENING OF SERVICEBERRY ACCESSIONS/CULTIVARS FOR PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT, ANTI-HYPERGLYCEMIC, AND ANTI-HYPERTENSIVE FUNCTIONALITIES USING *IN VITRO* ASSAY MODELS

4.1. Abstract

Serviceberry (*Amelanchier* spp.), a cold-tolerant native berry commonly found in temperate regions of the Northern Hemisphere and also known as Saskatoon and Juneberry is widely used in ethnic foods and traditional medicines of many Native American tribes. Recent human health-focused *in vitro* and *in vivo* studies have also found high antioxidant and high anti-diabetic properties (α-glucosidase enzyme inhibitory activity) in leaf, twigs, and fruits of Serviceberry. However, such health relevant phenolic bioactive profile and associated antioxidant and anti-diabetic properties of serviceberry potentially vary among different accessions or cultivars and under different environmental (under cold and other abiotic stress pressure) conditions. Therefore, it is essential to screen existing cold-hardy accessions and commercial cultivars of serviceberry for their phenolic antioxidant-linked anti-diabetic properties prior to incorporating them in health-targeted dietary interventions for potential management of chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of type 2 diabetes (T2D). Based on this rationale, the aim of this study was to screen 12 serviceberry accessions (North Dakota biotypes) and 8 cultivars from two different crop years (2016 and 2017) for their total soluble phenolic content, phenolic acid profiles, antioxidant activity, anti-hyperglycemia relevant α-amylase, α-glucosidase enzyme inhibitory and anti-

hypertensive relevant angiotensin-I-converting enzyme (ACE) inhibitory activities using rapid *in vitro* assay models. Overall, very high phenolic content (2.1-3.0 mg/g F.W.), high antioxidant activity (79-92% DPPH inhibition), high T2D management relevant α-amylase (63-100%) and high α-glucosidase (80-93%) enzyme inhibitory activity was observed in all serviceberry accessions and cultivars. Significant differences ($p<0.05$) in phenolic antioxidant-linked anti-hyperglycemic properties were also observed between accessions/cultivars and between different crop years. Additionally, moderate to high anti-hypertensive relevant ACE enzyme inhibitory activity (44-85%) was found in all serviceberry accessions/cultivars evaluated in this study. Therefore, this *in vitro* study provides biochemical rationale to screen and select high phenolic, high antioxidant serviceberry accessions and cultivars (ND 12-1, ND 41-1, Parkhill, Smoky, Buffalo) to incorporate them in potential dietary intervention strategies especially for healthy ethnic food design to counter oxidative stress-linked T2D epidemic in contemporary indigenous and non-indigenous communities of North America and globally.

4.2. Introduction

Serviceberry, Saskatoon berry or Juneberry (*Amelanchier alnifolia* Nutt.) is a temperate edible berry native to and grown widely in the Northern Plains region of the United States and in Canada. The name Saskatoon was derived from the Cree name "*misaskwatomin*" and this native berry is widely used in ethnic foods and traditional medicines including for the ailment of several chronic diseases by many Native American tribes. Serviceberry grows well in semi-shaded or sunny environments, is extremely tolerant to several abiotic stresses (salt, drought, extreme soil pH, ranging from around 5.5 to 7.0, and cold tolerant, even up to -60^0C), and is grown for both for commercial and non-commercial purposes in North America, Europe, and temperate regions of the Asia (Donno et al. 2016; UMN Extension). However, the commercial production and its economic importance is mostly restricted in North America, especially in Canada, where the current demand is greater than the actual supply (Jurikova et al. 2013). Most of the commercial cultivars were developed in Canada and the common cultivars of serviceberry are Martin, Regent, Northline, Thiessen, Honeywood, and Smoky. In general, serviceberry and other common small fruits grown under cold temperate climate of the North America are regarded as one of the best sources of dietary antioxidants and other health promoting bioactives such as phenolic compounds (Heinonen 2007). Additionally, serviceberry has been shown to have higher antioxidant capacity than other native berries, including blueberry (Guerrero et al. 2010; Li et al. 2009).

Berries rich in such phenolic antioxidants and other human health promoting bioactives have shown diverse health benefits including anti-inflammatory, anti-diabetic, anti-hypertensive, vascular health protective, and gut health relevant functions (Beattie et al. 2005; Yang and Kortesniemi 2015). Due to such diverse health protective functions, berries are gaining increasing interest among consumers to integrate them as part of healthy dietary choices and therefore can be targeted in dietary interventions to manage oxidative stress-linked common non-communicable chronic diseases (NCDs) such as T2D and associated cardiovascular complications. In this context of NCD-linked health benefits of berries, several *in vitro, in vivo* and epidemiological studies have suggested an association between higher consumption of berries and reduced risk of T2D and associated macro and micro-vascular complications (Castro-Acosta 2016; Nile and Park 2014; Yang and Kortesniemi 2015). Previously, blackberry (*Rubus* spp.), raspberry (*Rubus* spp.), blueberry (*Vaccinium* spp.), strawberry (*Ribes uva-crispa*), black currant (*Ribus rubrum* L.), and serviceberry all exhibited significant anti-hyperglycemia relevant α-amylase and/or α-glucosidase enzyme inhibitory activities both in *in vitro* and *in vivo* assay models-based studies and these studies indicated strong and positive correlation between phenolic content and antioxidant and anti-hyperglycemic properties in these berries (Cheplick et al. 2007; 2010; 2015; Pinto et al. 2008; 2010b; Sarkar et al. 2016; 2017; Wang et al. 2012; Zhang et al. 2012). The diverse group of phenolic compounds and other bioactives widely distributed in these berries, especially in serviceberry also have other human health relevant protective functions such as anti-inflammatory and anti-hypertensive properties and therefore can be targeted in dietary support strategies to manage chronic oxidative stress, hyperglycemia and hypertension commonly associated with early stages of T2D and other NCDs (Donno et al. 2016; Seeram 2008).

Furthermore, serviceberry is traditionally consumed fresh, in processed foods, and in the preparation of Native American meats has significant dietary relevance for improving overall health outcomes of contemporary Native American communities facing higher NCD-linked health disparities including T2D epidemic. On average, Native American mortality attributed to diabetes is three times greater than all races in the United States combined (Indian Health Service, 2015). Additionally, Traditional Native American medicine uses native plants, such as serviceberry, for healing and the promotion of health opposed to conventional medicine which Native American’s tend to reject (Koithan and Farrell 2010; Zhang et al. 2012).

The major human health relevant bioactives of serviceberry include anthocyanins, specifically cyanidin-based anthocyanins that comprise ~63% of the phenols, flavanes, flavonols, and hydroxycinnamic acids. Among these bioactives, quercetin, rutin, cyanidin-3-galactoside, and cyanidin-3-glucoside commonly found in serviceberry, have shown to impart diverse human health benefits including anti-inflammatory, anti-diabetic, and anti-hypertensive functionalities (Fontana Pereira 2011; Jurikova 2013; Lavola 2012; Li 2009; Oboh 2014; Ożarowski 2018;

Sarinya Akarachiyasit 2010; Wagner et al. 1991). Though these previously published studies have validated the antioxidant and anti-diabetic potential of the serviceberry using *in vitro* and *in vivo* models, there is need for further research on serviceberry accession/cultivar screening. Such screening strategies are especially required to obtain winter-hardy accessions and cultivars for the Northern Plains to advance their use for wider value-added applications in contemporary health-focused dietary strategies to counter T2D and associated health risks as part of overall preventive health management approaches through better diets. Further, similar to other berries, phenolic bioactive content and associated health benefits such as T2D relevant benefits of serviceberry potentially vary among different accessions and cultivars (differences in genetic make-up), due to different growing conditions (soil and cultivation practices), and with environmental variations (abiotic and biotic stress) (Green and Mazza 1986; Sarkar et al. 2017). Therefore, prior to targeting serviceberry in contemporary ethnic and non-ethnic healthy food design, it is important to screen existing winter–hardy accessions and cultivars for phenolic bioactive-linked health benefits using rapid and inexpensive screening strategies.

Based on this rationale, the aim of this study was to screen different accessions and cultivars of serviceberry grown under cold temperate climate of the Northern Plains for phenolic bioactive-linked antioxidant, anti-hyperglycemic, and anti-hypertensive properties using rapid *in vitro* assay models. Furthermore, the screening of serviceberry accessions/cultivars was conducted for two years, to determine any potential impact of crop years (environmental variations) on phenolic bioactive associated health benefits of serviceberry accessions/cultivars (Lavola et al. 2011). We hypothesized that winter-hardy serviceberry accessions and cultivars grown under cold temperate climate of the Northern Plains would have higher stress inducible phenolic bioactives and proportionately higher human health relevant functionalities when compared to low abiotic stress tolerant accessions/cultivars of serviceberry (Lavola et al. 2012; Schulz et. al. 2016; Zhang et. al. 2015). Therefore, advancing the screening of winter-hardy accessions and cultivars of serviceberry for phenolic bioactive linked anti-diabetic and anti-hypertensive properties using rapid *in vitro* assay models has significant relevance, especially for selecting high phenolic and high antioxidant serviceberry accessions and cultivars for incorporating in dietary support strategies or for future clinical studies targeting T2D-linked health benefits.

4.3. Materials and Methods

4.3.1. Serviceberry accessions/cultivars and growing conditions**:** Twelve North Dakota (ND) serviceberry accessions (ND 1-2, 1-4, 1-5, 1-6, 1-7, 12-1, 14-2, 16-1, 17-1, 18-1, 41-1, and 48-2) and eight commercial cultivars (Buffalo, Honeywood, Martin, Parkhill, Pearson, Regent, Smoky, and Thiessen) from two crop years (2016 and 2017) were collected from North Dakota State University Horticulture Research Farm and Arboretum located near Absaraka, North Dakota (46°58′41″N 97°23′40″W.). All serviceberry plants were propagated in tissue culture in 2012 and planted in the field in 2012, according to a randomized complete block design (RCBD). In 2016 and 2017, serviceberries were grown under conventional production systems. All berries were harvested at full maturation according to visual and brix data and frozen at -20^{o}C immediately after harvest. Other than specifically mentioned, all chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Company (St Louis. MO, USA).

4.3.2. Preparation of berry extracts: Total weight of 20 g of whole berry fruits were added to 50 mL of distilled water and homogenized using a Waring laboratory blender (Winsted, CT) set on high for 5 min. The remaining homogenate was then centrifuged at 10,000g for 20 min. The supernatant was then removed and again centrifuged at 10,000g for additional 15 min. After two consecutive centrifugations, berry supernatant was then removed and transferred into 1.5 mL Eppendorf tubes and stored in -20^{o}C for less than 2 weeks until all *in vitro* assays were conducted. For each cultivar or accession, twelve total replications were used and the *in vitro* assays were repeated two times from each crop year.

4.3.3. Total soluble phenolics assay: Total soluble phenolics were determined by the Folin-Ciocalteu method based on the modifications by Shetty et al. (1995). Briefly, 0.5 mL of distilled water and 0.5 mL of serviceberry sample extract were combined and transferred to a 10 mL test tube. After initial dilution with distilled water, 1 mL of 95% ethanol and 5 mL of distilled water was added to this mixture. Then 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and the mixture was vortexed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and was incubated in the dark at room temperature for 60 min. After the incubation period, the absorbance was read at 725 nm. A standard curve was created using various concentrations of gallic acid in distilled water and the results were represented as mg of gallic acid per gram of sample fresh weight (FW).

4.3.4. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) inhibition antioxidant assay: Antioxidant activity of serviceberry cultivars was determined by using DPPH radical cation decolorization assay method modified by Kwon et al. (2006). Briefly, 1.25 mL of 60 μM DPPH stock solution prepared in 95% ethanol was added to 250 μL of berry sample extract (initially 12.5 μL of each serviceberry extract was diluted in 237.5 μL of distilled water for 1:20 dilution, prior to conducting the assay). The decrease in absorbance was monitored after 5 min at 517 nm. The absorbance of a control, using distilled water instead of sample extract, was also recorded after 5 min at the same wavelength for comparison. The percentage of inhibition was then calculated by the following equation:

$$DPPH\ Inhibition\ (\%) = \frac{(Abs\ control - Abs\ sample)}{Abs\ control} \times 100$$

4.3.5. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant assay: Additionally, antioxidant activity of serviceberry cultivars was determined by using ABTS radical cation decolorization assay (Re et al. 1999). The ABTS radical cation was created by combining 5 mL of 7-mM ABTS solution with 88 mL of 140-mM $K_2S_2O_4$ solution. The solution was then kept at room temperature in the dark for 12-16 hours before use. Before the assay, the solution was combined with 95% ethanol to create a 1:88 dilution (ABTS solution: 95% ethanol) and the absorbance was adjusted accordingly at 734 nm to 0.70. Then1 mL of the adjusted ABTS solution was added to 50 µL of sample extract (initially 2.5 µL of serviceberry extract was diluted with 47.5 µL of distilled water) and the solution was then vortexed for 30 seconds. After vortexing, solution was then incubated at room temperature for 2.5 minutes and then absorbance of the incubated mixture was recorded at 734 nm using UV-VIS spectrophotometer. The antioxidant activity of the sample extracts was then represented as percent inhibition of ABTS radical formation and calculated using the formula:

$$ABTS\ Inhibition\ (\%) = \frac{(Abs\ control - Abs\ sample)}{Abs\ control} \times 100$$

4.3.6.α-Amylase enzyme inhibition assay: The α-Amylase inhibitory activity of serviceberry accessions/cultivars was measured by using an *in vitro* assay method modified from Worthington Enzyme Manual (Worthington Biochemical Corp. 1993a). Briefly, a solution of 500 µL of serviceberry sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α−amylase solution (0.5 mg/mL) was incubated at 25°C for

10 min. After incubation, 500 μL of 1% starch solution, diluted in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl), was added and then the test tubes were incubated again at 25°C for 10 min. Afterwards, 1.0 mL of dinitrosalicylic acid was added and the solution mixtures were incubated in boiling water for 10 min. After taking out from the water bath, solution mixtures were allowed to cool at room temperature. A volume of 10 mL distilled water was then added to the solution to adjust the baseline reading of the control at 1.0 ±0.2 and the absorbance was measured at 540 nm.

The absorbance of sample blank (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The final absorbance (A540 extract) of the extract was obtained by subtracting its corresponding sample blank reading. Additionally, 1:2 and 1:5 dilution of the sample extract (250 μL sample + 250 μL of distilled water and 100 μL sample + 400 μL of distilled water respectively) were performed to investigate potential dose dependence. The a-amylase inhibitory activity was calculated according to the equation below:

$$\alpha - Amylase\ Inhibition\ (\%) = (\frac{Abs\ control - (Abs\ sample - Abs\ Sample\ Blank)}{Abs\ Control} \times 100$$

4.3.7. α-Glucosidase enzyme inhibition assay: The *in vitro* assay method in this study was modified from the Worthington Enzyme Manual for *a*−glucosidase inhibition (Worthington Biochemical Corp. 1993b, McCue et al. 2005). A volume of 50 μL of sample extract diluted with 50 μL of 0.1 M potassium phosphate buffer (pH 6.9) and 100 μL of 0.1 M potassium phosphate buffer (pH 6.9) containing glucosidase solution (1.0 U/mL) was incubated in 96-well plates at 25 °C for 10 min. After pre-incubation, 50 μL of 5 mM *p*-nitrophenyl- a-d-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader

(Thermomax; Molecular Devices Co., Sunnyvale, CA) and compared to a control which had 50 μL of buffer solution in place of the extract (A405 control). The *a*-glucosidase inhibitory activity was expressed as a percentage of inhibition and calculated as follows:

$$\alpha - Glucosidase\ Inhibition\ (\%) = \frac{(\Delta\ Abs\ control - \Delta\ Abs\ sample)}{\Delta\ Abs\ control} \times 100$$

4.3.8. Angiotensin-I-converting enzyme (ACE) inhibition assay: Anti-hypertensive function relevant ACE inhibitory activity of serviceberry accessions/cultivars was determined using an *in vitro* assay method modified by Kwon et al. (2006). A volume of 50 µL of sample extract was incubated with 200 µL of 0.1 M NaCl-borate buffer (0.3 M NaCl, pH 8.3) containing 2 mU of ACE enzyme solution at 25°C for 10 min. After pre-incubation, 100 µL of a 5.0 mM substrate (hippuryl-histidyl-leucine) solution was added to the reaction mixture. Test solutions were then incubated in water bath at 37°C for 1 h. Sample blanks (buffer in place of enzyme and substrate), a control (distilled water instead of sample extract) and a blank (buffer instead of sample extract and enzyme) were also included. The reaction was then stopped by adding 150 µL of 0.5 N HCl to all reaction mixtures. The hippuric acid formed was detected by using High Performance Liquid Chromatography (HPLC) based quantification protocol. For HPLC analysis, a volume of 5 µL of sample was injected using an Agilent ALS 1200 auto-sampler into an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for the gradient were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was Agilent Zorbax SB-C18, 250 − 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 228 nm and the chromatogram was integrated using the

Agilent Chemstation enhanced integrator for detection of liberated hippuric acid. Pure hippuric acid was used to identify the spectra and retention time. The percentage of inhibition was calculated considering the area of the hippuric acid peak according to the equation below:

$$ACE\ Inhibition\ (\%) = \frac{((Abs\ control - Abs\ blank) - Abs\ sample)}{(\ Abs\ control - Abs\ blank)} \times 100$$

4.3.9. High performance liquid chromatography (HPLC) for phenolic acid characterization: The serviceberry sample extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample was injected using an Agilent ALS 1200 auto-sampler into an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a D1100 CE diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 − 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 0.7 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of chlorogenic acid, gallic acid, ellagic acid, catechin, rutin, benzoic acid, and *p*-coumaric acid in 100% methanol were used to calibrate the standard curves and retention times.

4.3.10. Data analysis: Two extractions were performed for each serviceberry sample, and all *in vitro* assays were replicated six times for each extraction (n = 12). Means, standard errors, and standard deviations were calculated from replicates using MS-Excel. All data was subjected to a two-way ANOVA using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC), and the least mean square differences for cultivar, crop years, and accessions/cultivar × crop year interactions were compared using Tukey's test ($p < 0.05$).

4.4. Results and Discussions

4.4.1. Total soluble phenolic content and phenolic acid profile of serviceberry cultivars:

The total soluble phenolic (TSP) content of serviceberry extracts was determined using the Folin-Ciocalteu based spectrophotometric assay. Overall, all 12 serviceberry accessions and 8 cultivars had very high TSP content (2.1-3.3 mg gallic acid equivalent (GAE) /g F.W.) (Fig. 4.1). Previously, Donno et al. (2016) reported around 5.3 mg GAE/g F.W. of total phenolic content in a cultivated genotype of *Amelanchier canadensis* (L.) Medik, which is a similar serviceberry species as in this study. In this current study, significant differences (*p <0.05*) in TSP content was observed among different serviceberry accessions/cultivars, due to different crop years (2016 vs. 2017), and further due to accessions/cultivars × crop year interactions (Fig 4.1.) (Appendix II).

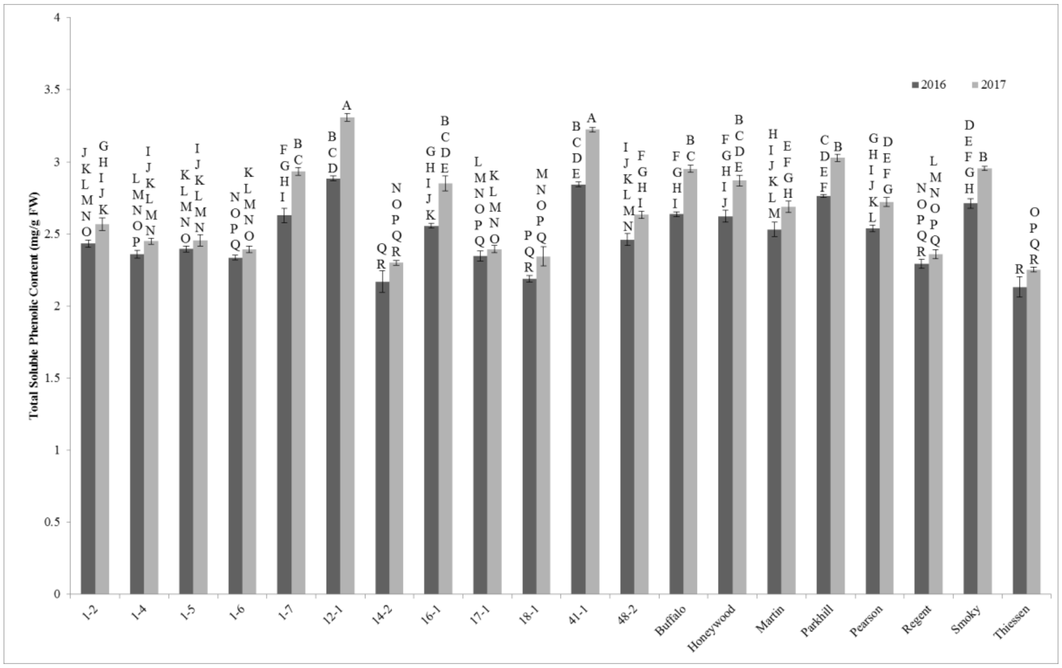

Figure 4.1. Total soluble phenolic (TSP) content (mg/g F.W.) of 20 serviceberry accessions/cultivars from two different crop years (2016 & 2017). Different capital letters for each bar represent significant differences in TSP content between accessions/cultivar × crop year interactions at the *p*<0.05 level.

Overall, serviceberry accessions ND 12-1 and ND 41-1 had significantly ($p < 0.05$) higher TSP content (≥3.0.) followed by Parkhill, Smoky, and Buffalo serviceberry cultivars. Among all serviceberry accessions and cultivars investigated in this study, accessions ND 18-1 and ND 14-2, and cultivars Regent and Thiessen had comparatively lower TSP content. Overall, serviceberry fruits from 2017 crop year had significantly ($p < 0.05$) higher TSP content when compared to berry fruits from 2016 crop year (Figure 4.1). Variations in environmental conditions (temperature and rainfall) between these two crop years may have contributed to these significant differences in stress inducible TSP content of serviceberry accessions and cultivars (Appendix I). Overall, 2017 had lower mean temperature than 2016, and potentially cold stress may have resulted in significantly higher TSP content in serviceberry fruits harvested in 2017 crop year. Therefore, this current study indicated that both accessions/cultivars and environmental conditions (crop year) had significant impact on TSP content of serviceberry. Previously, Green and Mazza (1986) reported significant variations of anthocyanin, total phenolic content, and soluble solid content in 8 different serviceberry cultivars. Similar to the results of the current study, Sarkar et al. (2017) found significant effect ($p < 0.05$) of genotype × environment (crop years and locations) interactions on phenolic content and associated health benefits of rabbit-eye blueberry (*Vaccinium virgatum*). Not only just TSP content but individual phenolic acid content of berry fruits also varies significantly among cultivars and due to variations in environmental conditions.

In this current study, characterization and determination of individual phenolic acids content of the serviceberry accessions/ cultivars were carried out using HPLC-based chromatographic analysis. Overall, major phenolics found in this current study were rutin, caffeic acid, catechin, chlorogenic acid, gallic acid, and benzoic acid (Table 4.1.). Previously, Donno et al. (2016) reported caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, quercetin, rutin, ellagic acid, and epicatechin as major phenolics in serviceberry genotype.

Table 4.1. Individual phenolic content (µg/g F.W.) of serviceberry accessions/cultivars from two different crop years.

Accessions/ Cultivars	Benzoic Acid		Caffeic Acid		Catechin		Chlorogenic Acid		Gallic Acid		Rutin	
	µg/g F.W.											
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
1-2	0.8±0	0.9±0	0.9±0.1	6.5±0	0.5±0.1	2.1±0	0.3±0	0.9±0	0.4±0	0.6±0	4.5±0	6.8±0
1-4	0.8±0.1	1±0	0.4±0	5.6±0	0.4±0.2	2.5±0	N.D.	0.7±0	0.6±0	0.5±0	4.2±0	6.6±0
1-5	0.6±0	0.9±0	0.3±0	4.4±0	0.3±0.2	1.6±0	N.D.	0.7±0	0.3±0	0.5±0	2.5±0	6.7±0
1-6	0.6±0	1.2±0	3.9±0	5.4±0	2.8±0	2.8±0	0.8±0	0.9±0	0.7±0	0.6±0	7.5±0	6.4±0
1-7	0.7±0	0.9±0	4.2±0	6.8±0	1.4±0	3±0	0.8±0	0.8±0	0.4±0	0.4±0	6.6±0	7.4±0
12-1	0.7±0	1±0	6.5±0	5.3±0	1.7±0	4.5±0	2±0	2.2±0	0.7±0	0.6±0	16.8±0	27±0
14-2	0.8±0	1.2±0	19.6±0	4.6±0	5.6±0	5.9±0	1.8±0	0.9±0	0.4±0	0.6±0	12.4±0	15.4±0
16-1	0.8±0	1.1±0.1	0.5±0	8.9±0	0.3±0	1.7±0	N.D.	1.6±0	0.2±0	0.3±0	6.8±0	15.6±0
17-1	0.7±0	1±0	7.8±0	4.7±0	2.7±0	2.8±0	1.2±0	0.7±0	0.6±0	0.5±0	11±0	6±0
18-1	0.7±0	1.1±0	4.7±0	16.2±0	1±0	4.1±0	1±0	3.2±0	0.2±0	0.7±0	4.6±0	24±0.1
41-1	0.9±0	1±0.2	0.7±0	12.6±0	0.2±0.1	4.2±0	0.3±0	2.8±0	0.5±0	0.4±0	12.5±0	16.3±0.1
48-2	0.9±0	0.9±0	1.1±0	4.1±0	0.3±0	1.7±0	0.2±0	0.6±0	0.2±0	0.5±0	3.6±0	5.7±0
Buffalo	0.6±0	1.1±0	1.1±0.1	9.2±0.1	0.3±0.1	2.8±0	0.4±0	1.4±0	0.6±0	0.7±0	12.2±0	17.5±0
Honeywood	0.4±0.1	1±0	0.4±0	5±0	0.2±0	3.4±0	N.D.	2.2±0	0.3±0	0.8±0	6.9±0	23.5±0
Martin	0.4±0.2	1±0.1	4.2±0	5.3±0	2±0	2.1±0	0.8±0	0.7±0	0.2±0	0.4±0	16.2±0	18.4±0
Parkhill	0.4±0	0.9±0	2.7±0	6.2±0	0.6±0	3.5±0	0.8±0	0.8±0	0.1±0	0.6±0	3.7±0	7.4±0
Pearson	0.3±0	0.9±0	6.7±0	4.3±0	2.5±0	1.3±0	0.6±0	0.5±0	0.4±0	0.4±0	10.7±0	9.3±0
Regent	0.2±0	1±0	5.2±0	3.1±0	2±0	1.6±0	0.9±0	0.7±0	0.5±0	0.5±0	5.8±0	6.2±0
Smoky	0.3±0	1.1±0	11.5±0	5.5±0	3.3±0	8.4±0	1.6±0.8	1±0	0.6±0	0.5±0	17.2±0.2	15.6±0
Thiessen	0.5±0	1±0.2	5.8±0	3.9±0	3.2±0	1.5±0	1±0	0.6±0	0.4±0	0.3±0	20.3±0	14.8±0

± Standard Error

N.D. Not Detected

Furthermore, mean content of individual phenolic acids of serviceberry accessions and cultivars were significantly higher in 2017 crop year when compared to fruits of same accessions/cultivars from 2016 crop year. Therefore, these results on individual phenolic acid, suggested that both differences in accessions/cultivars and impact of crop years (environmental variations) had significant effect on phenolic acid composition and their concentration in serviceberry fruits. Such variations in composition and concentration of individual phenolic acids of serviceberry accessions/cultivars may also have relevance in the associated health benefits such as antioxidant, anti-hyperglycemic and anti-hypertensive properties.

4.4.2 Antioxidant activity of serviceberry cultivars: Total antioxidant activity of serviceberry fruit extracts was quantified using the DPPH and ABTS-based radical scavenging activity assays. We hypothesized that serviceberry accessions/cultivars with high TSP content would also have higher total antioxidant activity, as many previous studies reported strong correlation between TSP content and antioxidant activity of berry fruits (Cheplick et al. 2010; Henonen et al. 1998; Sarkar et al. 2016; 2017; Sariburun et al. 2010; Sellappan et al. 2002). In this study, very high antioxidant activity (both ABTS and DPPH based assays) was observed in all serviceberry accessions/cultivars (Figure 4.3 A & B).

Due to such high antioxidant potentials (close to 100 % DPPH and ABTS free radical ion inhibition in undiluted sample), serviceberry fruit extracts were further diluted to 1:5 for DPPH based antioxidant assay and 1:20 for ABTS based antioxidant assay. Overall, 51-98 % ABTS free radical inhibition (1:20 diluted samples) and 62-100% DPPH free radical inhibition (1:5 diluted samples) were observed in serviceberry accessions/cultivars investigated in this study.

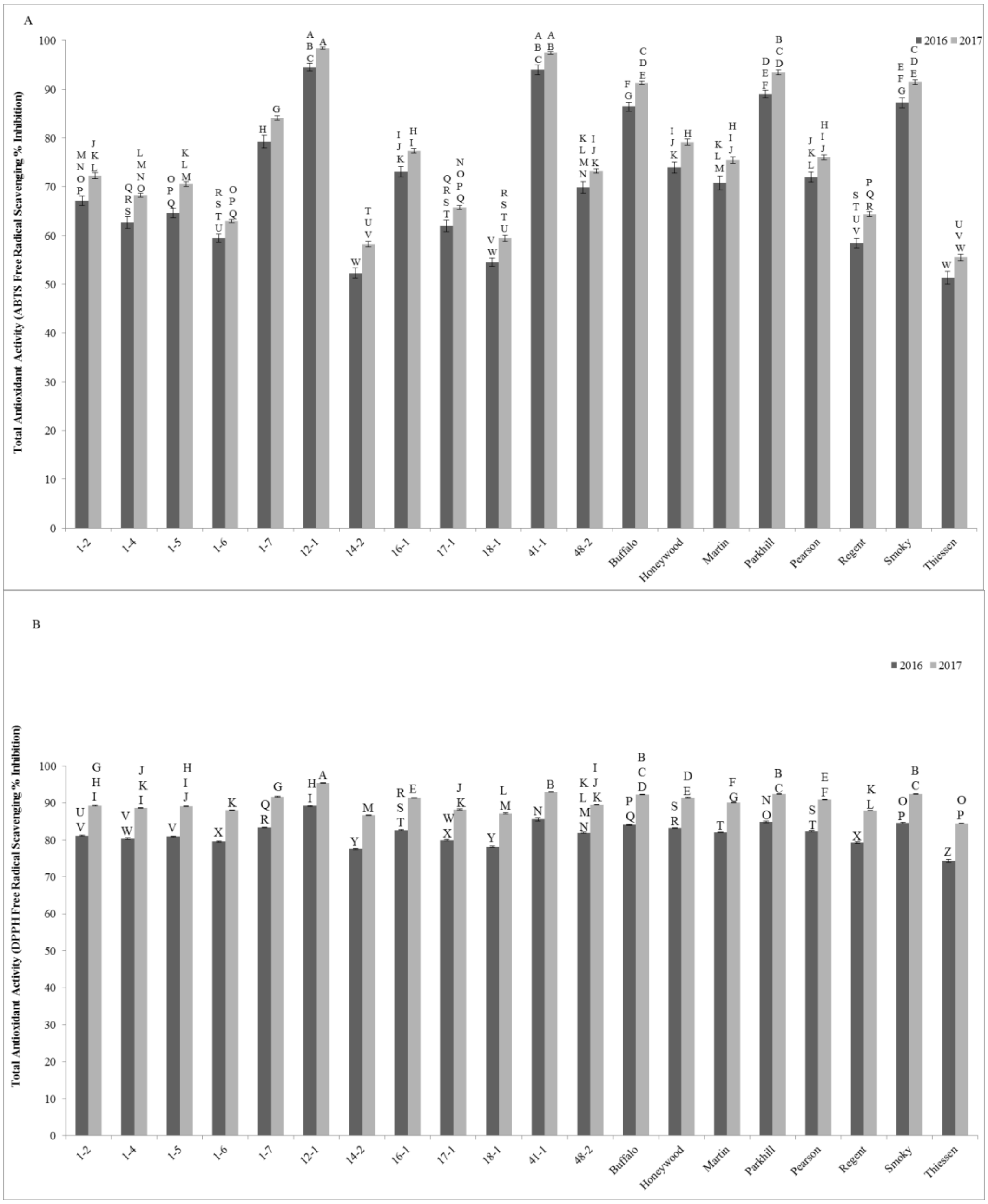

Figure 4.2. Total antioxidant activities (A- ABTS free radical scavenging % inhibition & B- DPPH free radical scavenging % inhibition) of serviceberry accessions/cultivars from two different crop years (2016 &2017). Different capital letters for each bar represent significant differences in total antioxidant activity between accessions/cultivar × crop year interactions at the *p*<0.05 level.

The positive control resveratrol, in 1 mg/ mL had 55-60% DPPH based inhibition which was lower than the DPPH based inhibition (62-100) value of all serviceberry extracts (0.2 mg/

mL). Previous studies also reported very high antioxidant activity similar to the result of the current study, in fresh, stored and processed serviceberry fruits (Cazares-Franco et al. 2014; Donno et al. 2016; Michalczyk and Macura 2010).Significant differences ($p < 0.05$) in antioxidant activity (in both ABTS and DPPH-based assays) were observed among serviceberry accessions/cultivars, due to different crop years (2016 vs. 2017), and also between accessions/cultivars × crop year interactions. Furthermore, similar to TSP content, significantly high antioxidant activity (in both ABTS and DPPH-based assays) was observed in serviceberry accessions ND 12-1, ND 41-1, and cultivars Parkhill, Smoky, and Buffalo, while comparatively low antioxidant activity was found in ND18-1, ND 14-2 serviceberry accessions and in Regent and Thiessen cultivars. Therefore, this result validated our hypothesis that antioxidant activity of serviceberry accessions/cultivars is associated with their TSP content. Furthermore, serviceberry fruits from 2017 crop year had significantly higher ($p < 0.05$) antioxidant activity (in both ABTS and DPPH-based assays) when compared to fruits from 2016 crop year. Therefore, both accessions/cultivars differences and environmental variations (crop years) had significant impact on phenolic-linked antioxidant activity of serviceberry. Previously, similar effect of genotypes and crop years on differences in antioxidant activity of serviceberry was reported by Lachowicz et al. (2017). Overall, results of the current study indicated that serviceberry accessions ND 12-1, ND 41-1, and cultivars Parkhill, Smoky, and Buffalo with high TSP content and high antioxidant activity can be targeted towards strategies for dietary interventions to counter chronic oxidative stress commonly associated with diet and lifestyle-linked NCDs such as T2D and associated complications. It is also possible that these high phenolic and high antioxidant serviceberry accessions and cultivars screened in this study may have other human health benefits relevant functions such as anti-hyperglycemic and anti-hypertensive properties and therefore such analysis were undertaken.

4.4.3 Anti-hyperglycemic property relevant α-amylase and α-glucosidase enzyme inhibitory activity of serviceberry cultivars: To determine the anti-hyperglycemic potentials of serviceberry, key glucose metabolism relevant α-amylase and α-glucosidase enzyme inhibitory activities of serviceberry accessions/cultivars from two different crop years (2016 & 2017) were analyzed using rapid *in vitro* assays. Overall, all serviceberry accessions/cultivars had high α-amylase enzyme inhibitory activity (62-100%) in undiluted sample (Table 4.2). Even after half (1:2) and one-fifth (1:5) dilution, all serviceberry accessions/cultivars had significant *in vitro* α-amylase enzyme inhibitory activity (31-80 % in half dilution and 10-32% in one-fifth dilution) (Table 4.2). Previously, α-amylase enzyme inhibitory activity was observed in *A. parviflora* var. *dentata* (Zengin et al. 2018). In the current study, among serviceberry accessions and cultivars, accessions ND 1-5, ND 14-2 and cultivars Regent and Buffalo had significantly ($p < 0.05$) higher α-amylase enzyme inhibitory activity. Overall, similar to TSP content and antioxidant activity, significant differences ($p < 0.05$) in α-amylase enzyme inhibitory activity was observed among serviceberry accessions/cultivars, between two crop years (2016 & 2017), and accessions/cultivars × crop year interactions. However, in contrast to the TSP content and total antioxidant activity, mean α-amylase enzyme inhibitory activity of serviceberry accessions/cultivars was significantly higher in 2016 crop year when compared to the mean α-amylase enzyme inhibitory activity of 2017 serviceberry samples.

Similarly, serviceberry accessions such as ND 1-5, ND 14-2 with higher α-amylase enzyme inhibitory activity had comparatively low TSP content and total antioxidant activity. However, serviceberry cultivar Buffalo had both high TSP content and high α-amylase enzyme inhibitory activity. Therefore, this result indicated that α--amylase enzyme inhibitory activity of all serviceberry accessions/cultivars may not be directly related to TSP content alone.

Table 4.2. α-Amylase enzyme inhibitory activity of serviceberry accessions/cultivars from two different crop years (2016 &2017).

Accessions/Cultivars	Undiluted (1:1)		Half-Diluted (1:2)		One-Fifth Diluted (1:5)	
	% Inhibition					
	2016	2017	2016	2017	2016	2017
1-2	100±0A	94.6±0.5BCD	51.7±1.2HIJ	46.2±0.8LMNOP	20.6±1GHIJK	16.7±0.9JKLMNOP
1-4	71.6±0.9M	65.6±0.8N	33.4±1WX	30.8±0.8X	17±1.1JKLMNOP	10.8±0.9SR
1-5	100±0A	95.7±0.7B	80.1±0.8A	77.5±0.8A	32.4±0.8A	27.7±0.7ABCD
1-6	93.9±0.9BCD	89.1±0.7EFG	46.1±0.9MNOP	42.3±0.9OPQRST	19.1±0.9HIJKLM	13.1±0.8OPQSR
1-7	63.8±0.9N	62.2±0.9N	35.3±1.4VWX	33.2±0.9WX	12.9±0.9OPQRS	10.2±0.9S
12-1	100±0A	97.8±0.9AB	57.7±0.8FG	51.4±0.9HIJKL	24.7±0.8CDEFG	22±0.9EFGHI
14-2	100±0A	100±0A	70.3±1BC	65.9±0.9CD	28.7±0.8ABC	24.2±0.9CDEFG
16-1	81±0.9JK	78.8±0.9KL	41.2±1PQRSTU	37.1±0.9UVW	16.4±0.9JKLMNOP	12.6±1PQROS
17-1	100±0A	95.2±0.7BC	53.3±1.2GHI	55.4±0.9FGH	23.1±1DEFGH	19.9±1GHIJKL
18-1	89.2±1.1EFG	91.1±1DE	45.7±1MNOPQ	40.8±1.1QRSTU	15.8±0.9KLMNOPQR	11.2±1QRS
41-1	86.6±0.8FGH	84±0.9HIJ	42.9±0.9NOPQRS	38.9±0.9STUV	17.5±0.9IJKLMNO	12.8±0.8POQRS
48-2	100±0A	100±0A	59.9±0.8EF	53.3±1GHI	25.6±0.9BCDEF	20.8±1FGHIJ
Buffalo	100±0A	100±0A	65.2±0.9CD	63±1DE	27.6±0.8ABCD	22.5±1EFGH
Honeywood	94.9±0.8BCD	89.4±0.8EF	47.8±1.1JKLMN	42.9±0.8NOPQRS	18.2±0.8HIJKLMN	15.2±0.8LMNOPQRS
Martin	89.7±0.8EF	85.5±0.9GHI	45.3±0.9MNOPQ	41.2±0.9PQRSTU	18.4±0.8HIJKLM	14.5±1MNOPQRS
Parkhill	84.3±0.8HIJ	82.6±0.9IJ	43.1±0.8NOPQRS	38.3±1STUVW	17.5±0.8IJKLMNO	13.4±0.9NOPQRS
Pearson	95.8±0.9B	91.5±1CDE	48.3±0.9IJKLM	44.8±0.9MNOPQR	18.8±0.9HIJKLM	15.1±0.8LMNOPQRS
Regent	100±0A	100±0A	75±1AB	72.1±0.9B	30.4±0.9AB	26.4±1BCDE
Smoky	100±0A	100±0A	51.5±0.6HIJK	46.4±0.8KLMNO	22.2±1EFGHI	17.2±0.8IJKLMNO
Thiessen	81.1±1JK	76.2±0.9L	39.9±0.8RSTUV	37.3±0.8TUVW	16.1±0.9JKLMNOPQ	12.3±0.9PQRS

± Standard Error

*Different capital letters for each dilution represent significant differences in α-amylase enzyme inhibitory activity between accessions/cultivar × crop year interactions at the *p*<0.05 level.

Serviceberry is rich source of anthocyanin and previous studies have shown high α-amylase inhibitory activities in anthocyanin rich berries (Grussu et al. 2011; McDougall et al. 2005).Therefore, other bioactive compounds of serviceberry beyond just phenolic acids may have significant role in dictating their α-amylase enzyme inhibitory activity. Not only just high α-amylase enzyme inhibitory activity, but very high α-glucosidase enzyme inhibitory activity was observed in all serviceberry accessions and cultivars investigated in this study. The inhibitory activity of serviceberry accessions/cultivars against α-glucosidase enzyme was quantified to determine the potential of serviceberry fruit to lower the rate of digestion of carbohydrates in the small intestine and absorption of glucose in the bloodstream (McDougall and Stewart 2005). Overall, all serviceberry accessions/ cultivars had very high α-glucosidase inhibitory activity in undiluted samples (80-93%) (Table 4.3). A significant dose dependent response in α-glucosidase enzyme inhibitory activity was also observed in all serviceberry accessions and cultivars. Even after one-fifth (1:5) dilution, 45-67 % α-glucosidase enzyme inhibitory activity was observed in serviceberry fruit extracts. Previously, Zhang et al. (2012) reported α-glucosidase enzyme inhibitory activity in serviceberry leaves, twigs, and leaves with berry extracts. Overall significant differences ($p < 0.05$) in α-glucosidase enzyme inhibitory activity was observed among serviceberry accessions/cultivars, between two crop years (2016 & 2017), and accessions/cultivars × crop year interactions (Table 4.3). In this current study, serviceberry accessions ND 1-5, and ND 12-1 and serviceberry cultivar Parkhill had high α-glucosidase enzyme inhibitory activity when compared to other serviceberry accessions and cultivars. Both ND 12-1 and Parkhill also had high TSP content and high antioxidant activity. Therefore, high α-glucosidase enzyme inhibitory activity of some serviceberry accessions and cultivars may be linked to the TSP content and composition of phenolics.

Table 4.3. α-Glucosidase enzyme inhibitory activity of serviceberry accessions/cultivars from two different crop years (2016 &2017).

Accessions/Cultivars	Undiluted (1:1)		Half-Diluted (1:2)		One-Fifth Diluted (1:5)	
	% Inhibition					
	2016	2017	2016	2017	2016	2017
1-2	89.5±1CDEFGHI	89.9±0.4BCDEFG	80±0.4BCD	76.6±0.4DEFGHI	62.8±1ABCD	52.8±1HIJKLM
1-4	90.5±0.6ABCDE	92±0.4ABC	81.9±0.3AB	75.4±0.5GHIJKLM	63.4±0.8AB	53.6±0.7GHIJKL
1-5	91.3±1ABCDE	90.3±0.5ABCDEF	84±0.7A	74.1±0.6HIJKLMN	66±0.6A	51±1.4KLMNO
1-6	90.7±0.7ABCDE	91±0.3ABCDE	81.3±0.5ABC	75.8±0.6EFGHIJKL	60.7±0.7BCDE	48.9±0.8LMNOPQ
1-7	88.5±0.5DEFGHIJ	86.1±0.3JKLM	72.6±0.8JKLMNO	72±0.4LMNOP	51.7±1.2JKLMNO	55.3±0.6FGHIJK
12-1	93±0.2A	92.3±0.4ABC	79.4±0.3BCDEF	75.5±0.5FGHIJKL	56.1±1.1EFGHIJ	52±0.8IJKLMN
14-2	84±0.6ML	83.8±0.5MN	68.7±0.6PQR	67.9±0.8QR	42±1.3RS	46±0.8PQR
16-1	89.2±0.8CDEFGHI	86.7±0.3HIJKLM	76.2±1.3DEFGHIJK	69.3±0.3OPQR	57.8±1.1DEFG	47.1±0.7NOPQ
17-1	87±0.4GHIJKL	86.1±0.7IJKLM	73.7±0.6IJKLMN	71.6±0.7MNOPQ	51.9±0.8IJKLMNO	45.2±0.7QR
18-1	84.9±0.3KLM	85.6±0.5JKLM	70.7±0.6NOPQR	70.6±1NOPQR	48.4±1.3MNOPQ	47±0.8OPQ
41-1	92.7±0.3AB	93±0.4AB	79.5±0.7BCDE	78.1±0.6BCDEFG	59.6±1.1BCDEF	47.3±0.7NOPQ
48-2	92±0.5ABC	92.6±0.3AB	79.8±0.5BCD	79.6±0.5BCD	60.8±0.7BCDE	49.4±0.7LMNOPQ
Buffalo	88.4±1DEFGHIJ	92±0.2ABC	73.3±1.3IJKLMN	76.5±0.9DEFGHIJ	50.6±1.1KLMNOP	56.7±0.5EFGHI
Honeywood	87.4±0.3FGHIJ	80.7±0.3N	70.4±1.2NOPQR	61.1±0.6S	52.2±0.3IJKLM	37.7±0.9S
Martin	92.2±0.6ABC	91.9±0.2ABC	78.8±1.2BCDEFG	76.9±0.9DEFGHI	62.4±0.4ABCD	53.7±0.9GHIJKL
Parkhill	93.3±1A	92±0.6ABC	84.8±0.6A	76.8±0.8DEFGHI	67.2±0.8A	51±0.9KLMNO
Pearson	90.9±0.5ABCDE	91.2±0.7ABCDE	75.7±1.3EFGHIJKL	77.6±0.5CDEFGH	62.9±0.7ABC	58±1.3CDEFG
Regent	90.7±1ABCDE	91.4±0.4ABCD	81.2±0.4ABC	75.7±0.4EFGHIJKL	62.9±0.6ABC	53.4±0.8GHIJKL
Smoky	88.3±0.5EFGHIJ	91±0.4ABCDE	72.6±0.7KLMNOP	79.4±0.8BCDE	49.2±1.2LMNOPQ	57.3±1.4EFGH
Thiessen	85.8±0.9JKLM	84.8±0.5KLM	54.5±0.6KLMNOP	67.1±0.8R	54.5±0.6GHIJK	44.9±0.7QR

± Standard Error

Different capital letters for dilution represent significant differences in α-glucosidase enzyme inhibitory activity between accessions/cultivar × crop year interactions at the *p*<0.05 level.

Previously, high *in vitro* and *in viv*o α-glucosidase enzyme inhibitory activity was observed with quercetin (rutin) and hydroxycinnamic acid (caffeic) derivatives (Adisakawattana et al. 2009; Li et al. 2009; Pereira et al. 2011).Therefore, high rutin and high caffeic acid content found in all serviceberry accessions/cultivars may have relevance for high α-glucosidase enzyme inhibitory activity of serviceberry that was observed in this current study. Overall, this current study provides biochemical rationale to select serviceberry accessions/ cultivars such as ND 1-5, ND 12-1, Parkhill for future animal model and possibly clinical studies to validate the anti-hyperglycemic functionalities of serviceberry and for their potential integration in health-focused dietary solutions to counter T2D and associated complications such as chronic hypertension.

4.4.4. Anti-hypertensive property relevant angiotensin-I-converting enzyme (ACE) inhibitory activity of serviceberry cultivars: Chronic hypertension is most common health complication directly associated with T2D and other common NCDs (Lukic et al. 2014). The higher rate of T2D associated mortality is mostly attributed to higher prevalence of chronic hypertension and CVDs in diabetic patients (Hu et al. 2005). Therefore, managing chronic hypertension is most critical to counter T2D associated mortality and morbidity. Though there are several pharmaceutical drugs currently available such as synthetic ACE inhibitors to manage chronic hypertension, but many plant-based foods with ACE inhibitory potentials are safer and inexpensive choices to prevent and manage T2D associated chronic hypertension (Guang and Philips 2009). Previously, anti-hypertensive relevant ACE inhibitory activity was observed in berries such as raspberry (*Rubus* spp.), red currant (*Ribes rubrum*), blueberry (*Vaccinium* spp.), and chokeberry (*Aronia* spp.) (Cheplick et al. 2007; Hellström et al. 2010; Pinto et al. 2010b; Wiseman et al. 2010). In this current study, moderate to high ACE inhibitory activity (44-85%) was observed in fruit extracts of all serviceberry accessions/cultivars in *in vitro* assay (Fig 4.4).

Previously, Wagner et al. (1991) reported ACE inhibitory activity in serviceberry leaf extract. Overall, serviceberry accessions ND 1-2, and ND 41-1, and serviceberry cultivar Parkhill and Smoky had high ACE inhibitory activity when compared to other serviceberry accessions and cultivars investigated in this study.

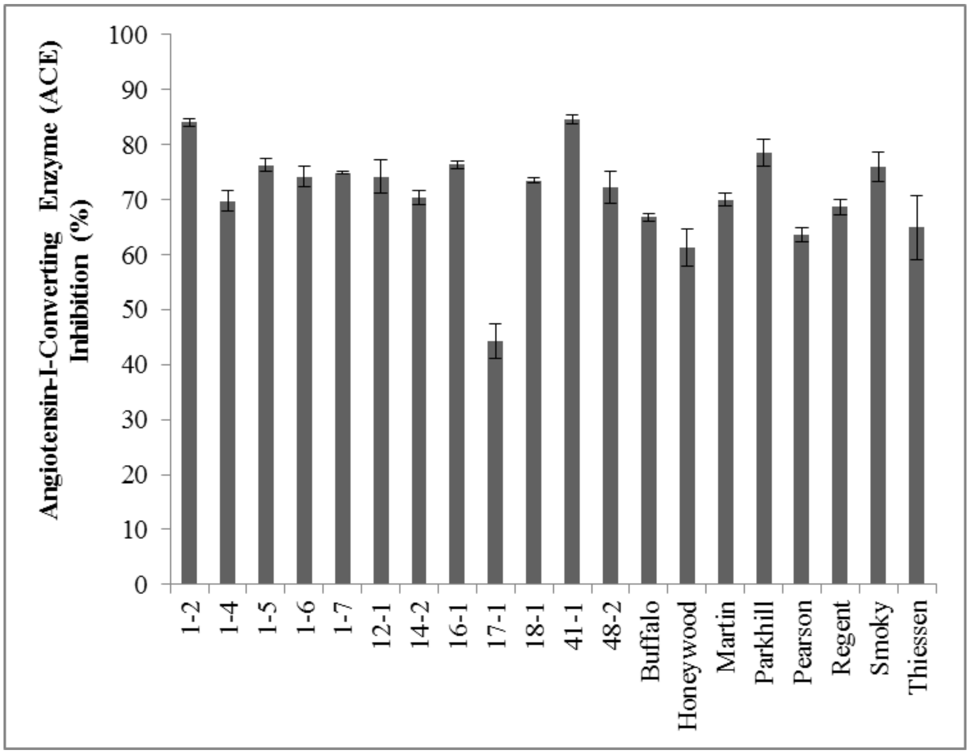

Figure 4.3. Angiotensin-I-Converting Enzyme (ACE) inhibitory activity of serviceberry accessions/cultivars from 2016 crop year.

Interestingly, serviceberry accession ND 41-1, and serviceberry cultivar Parkhill and Smoky also had high TSP content and high antioxidant activity. Therefore, these serviceberry accessions/cultivars can be targeted for countering chronic hypertension and chronic oxidative stress commonly associated with T2D and other NCDs. However, animal model-based *in vivo* study and clinical studies will be needed to validate the anti-hypertensive and anti-hyperglycemic properties of serviceberry accessions/cultivars which were observed in current *in vitro* study.

4.5. Conclusions

Overall, berry fruits are considered as one of the best dietary source of phenolic antioxidants and due to such high bioactive-linked health benefits, fresh and processed berries are becoming more popular food choice among consumers. Therefore, berries rich in phenolic bioactive profiles can be targeted in contemporary health-focused dietary intervention strategies to counter diet and lifestyle-linked NCDs, such as early stages of T2D and associated health risks. In this context of health benefits of berries, wild and native berries of North America with cold-hardy trait such as in serviceberry are rich source of stress inducible phenolic bioactives with high antioxidant and anti-hyperglycemic potentials. However, such stress-inducible phenolic bioactive profile and associated health benefits of serviceberry potentially vary among accessions/cultivars (genotypic), and due to environmental variations (phenotypic). Therefore, this study screened and selected high phenolic, high antioxidant serviceberry accessions/cultivars suitable to be grown in cold temperate climate of North Dakota and had health relevant phenolic-linked anti-hyperglycemic and anti-hypertensive functionalities. Therefore, such superior accessions/cultivars can be targeted in further dietary support strategies to potentially manage chronic hyperglycemia and chronic hypertension commonly associated with early stages of T2D and other NCDs. Overall, in this *in vitro* assay model based rapid screening study, high phenolic-linked antioxidant, anti-hyperglycemic, and moderate to high anti-hypertensive properties were observed in 12 North Dakota (ND) successions and 8 commercial cultivars of serviceberry. Furthermore, significant differences in phenolic-linked antioxidant, anti-hyperglycemic (α-amylase and α-glucosidase enzyme inhibitory activities), and anti-hypertensive (ACE inhibitory activity) properties were observed among different accessions/cultivars and due to environmental variations (two different crop years). However, it is important to further investigate the anti-diabetic and anti-hypertensive properties of these serviceberry accessions and cultivars using animal model-based *in vivo* and clinical studies, prior to incorporating them in dietary and therapeutic strategies for T2D-linked health benefits.

CHAPTER 5. PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT AND ANTI-HYPERGLYCEMIC FUNCTIONALITIES OF BLACKBERRY CULTIVARS GROWN UNDER ORGANIC AND CONVENTIONAL PRODUCTION PRACTICES

5.1. Abstract

Blackberries are a rich source of dietary phenolic bioactives with potential positive human metabolism modulating health relevant functionalities. Therefore, blackberries can be targeted in dietary support strategies to potentially manage diet and chronic oxidative stress-linked non-communicable chronic diseases (NCDs) such as early stages of type 2 diabetes (T2D). In previous *in vitro* and *in vivo* studies high phenolic antioxidant-linked anti-hyperglycemic functionalities (α-amylase and α-glucosidase inhibitory activity) were observed in blackberries. However, such phenolic bioactive-linked antioxidant and anti-diabetic functionalities potentially vary widely among cultivars and due to different production practices such as organic vs. conventional production systems. Therefore, the aim of this study was to screen and compare two blackberry cultivars grown under organic and conventional production systems for their phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities using *in vitro* assay models. Food grade relevant cold water extracts of two blackberry cultivars (Prime-Ark 45 & Prime-Ark Freedom) grown under 6 different cultivation practices, 4 organic (mulch-based weed management + organic fertilization) and 2 conventional cultivation practices (herbicide + chemical fertilizer) were used to determine total soluble phenolic content, phenolic acid profile, antioxidant activity, α-amylase, and α-glucosidase enzyme inhibitory activities using *in vitro* assay models. Overall, high soluble phenolic content, high antioxidant activity and

high α-amylase and α-glucosidase enzyme inhibitory activities were observed in both blackberry cultivars. However, blackberry grown under organic weed management and with organic fertilization had significantly higher phenolic-linked antioxidant and anti-hyperglycemic properties. Further, Prime-Ark 45 grown under black fabric and black plastic mulch with feather meal had significantly high phenolic-linked antioxidant and anti-hyperglycemic properties and therefore such organic production strategy can be advanced to potentially improve phenolic content and associated T2D-linked health benefits in blackberry and can be extended to other berries.

5.2. Introduction

Blackberry (*Rubus* spp.) is an edible berry with high human health relevant bioactive profiles and very high antioxidant potentials among common fruits and vegetables (Jennings et al. 1988). Overall, production, acreage, productivity, and consumption of blackberry are increasing rapidly in the United States and around the world. Currently, North America is the leading producer of blackberry and its production is expected to increase significantly in the future to satisfy the growing consumer demand both as fresh fruit and also as processed fruit (Kaume et al. 2011). However, the future improvement of domestic market and potential export value of blackberry and blackberry-based processed food products will largely depend on understanding and enhancing the different value-added quality parameters such as critical human health relevant nutritional qualities, sensory qualities, and other post-harvest preservation qualities such as shelf-life of berries. In general, the growing interest and demand of fresh berries among consumers is mainly due to their diverse human health relevant benefits.

When comparing the health benefits of berries, blackberry has significant amount of human health relevant phenolic bioactives (anthocyanains, flavols and ellagitannins) with high antioxidant activity (Borowska 2008; Kaume et al. 2011; Moyer et al. 2002; Sarkar et al. 2016). This high phenolic-linked antioxidant activity of blackberry is also associated with several other human health relevant functionalities including anti-diabetic properties and has potential to advance value-added dietary application as fresh fruits and as functional food ingredients to counter oxidative stress-linked non-communicable chronic diseases (NCDs), such as type 2 diabetes (T2D), cardiovascular diseases (CVDs), and obesity. Therefore, blackberry is an ideal fruit choice to be utilized in value-added dietary strategies to counter and manage NCDs including T2D and associated health risks. Previous research findings have indicated that blackberry has anti-inflammatory activities (Srivastava et al. 2010), higher antioxidant activities (Kähkönen et al. 2001; Srivastava et al. 2010; Wang and Lin 2000), and glucose metabolism relevant enzyme inhibitory activities (McDougall et al. 2005; Sarkar et al. 2016; Tundis et al. 2010). However phenolic bioactive profile, antioxidant activity, and anti-hyperglycemic properties of blackberry vary widely among cultivars and types (Sarkar et al. 2016). Furthermore, these value-added qualities of berries including human health relevant nutrient profiles of berries also varies widely due to different cultivation practices, growing conditions, time of harvest, and storage conditions (Cheplick et al. 2015; Kaume et al. 2011; Sarkar et al. 2017; Talcott 2007; Wang and Lin 2000; Zia-Ul-Iaq et al. 2014).

Therefore, optimizing production practices, especially organic production of blackberry to improve human health relevant phenolic bioactive profile has significant merit. The perception of higher nutritional and sensory quality, especially human health relevant bioactive profile of the organic berries is one major driving factor for its higher demand among consumers. Better nutritional quality also could potentially provide significant marketing advantage to organic produce and ensure higher economic return to organic berry growers. However, many previous studies with berries were inconclusive and did not find any significant differences in phenolic bioactives and associated nutritional qualities of berries between organic vs. conventional production systems (Vian et al. 2006; You et al. 2011). Therefore, determination of optimal organic or conventional agricultural practices and selecting appropriate cultivar of blackberry are required for improving phenolic bioactives and associated health benefits prior to incorporating them in dietary strategies to counter chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of T2D.

Furthermore, the commercial production of blackberry both under organic and conventional production is limited in North Dakota and other states of the Northern Plains due to lack of sufficient winter-hardy cultivars and lack of sufficient knowledge on production practices. However, current acreage and production of winter-hardy blackberry in the North Dakota can be expanded with innovative organic production strategies for improving nutritional and human health relevant functional qualities during production. This approach through new innovations, especially organic production of blackberry will help to advance the major challenge to have economically viable yield and quality of existing winter-hardy blackberry cultivars suitable to grow in the climate of North Dakota and other parts of the Northern Plains. In order to address this blackberry production challenges, the aim of the current study was to compare the impact of organic and conventional weed control and fertilization management practices on phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities of two primocane-fruiting blackberry cultivars grown in North Dakota. In this study, rapid *in vitro* screening strategy was used to determine phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities of blackberry relevant for targeting them in dietary support strategies to prevent and manage early stages of T2D and associated health risks.

5.3. Materials and Methods

5.3.1. Blackberry cultivars and treatments: Two blackberry cultivars, Prime-Ark 45 and Prime-Ark Freedom (Initially released by University of Arkansas primocane breeding program) were grown in North Dakota State University Horticulture Research Farm (NDSU HRF) near Absaraka, ND (46°58′41″N 97°23′40″W.) under the following organic or conventional weed management and fertilization practices (4 organic cultivation practices, Treatment 1: black landscape fabric with feather meal (224 kg/ha N; nitrogen: phosphorus: potassium-12.8:0:0); Treatment 2: black landscape fabric with fish emulsion (224 kg/ha N; nitrogen: phosphorus: potassium-9.6:3:0); Treatment 3: black plastic mulch with feather meal (224 kg/ha N; nitrogen: phosphorus: potassium-12.8:0:0); Treatment 4: black plastic mulch with fish emulsion (224 kg/ha N; nitrogen: phosphorus: potassium-12.8:0:0); and 2 conventional production practices, Treatment 5: application of herbicide + urea (224 kg/ha N; nitrogen: phosphorus: potassium-46:0:0); Treatment 6: application of herbicide + Environmentally Smart Nitrogen (ESN) (224 kg/ha N nitrogen: phosphorus: potassium-44:0:0).For herbicide treatments under conventional weed management, Rely280 (Glufosinate) @4.12 lt. /ha + Spartan (Sulfentrazone) @0.72 lt./ha were applied using a CO_2 pressurized backpack sprayer (20GPA, 8002 FF nozzles) surrounding blackberry plants (blackberry plants were covered with cardboard to protect from herbicide drift) on 26th June 2018. The blackberry plants were planted (1st June 2017) from 1 year old root stocks and harvested on the second year of growth. For overwintering, the blackberries were covered with a 16 cm layer of straw followed by a thermal blanket. The berries were harvested at full maturation and immediately frozen in -20°C freezer.

5.3.2. Chemicals: Other chemicals used in in vitro biochemical assays such as 3,5-Dinitrosalicyclic acid (DNS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azinobis(3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS), Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), were purchased from Sigma Chemical Co. (St. Louis, MO).

5.3.3. Preparation of blackberry extracts: Total 40 g of whole blackberry fruits were added to 100 mL of distilled water and homogenized in a Waring laboratory blender (Winsted, CT) set on high for 5 min. The remaining homogenate was then centrifuged at 10,000g for 20 min. The supernatant was then removed and again centrifuged at 10,000g for 15 min. The supernatant was then removed and transferred into 1.5 mL Eppendorf tubes and stored in -20^{o}C for less than 2 weeks until all *in vitro* assays were conducted. For each cultivar or accession extract, six replications were used and the *in vitro* assays were conducted two times for each year.

5.3.4. Total soluble phenolics assay: Total soluble phenolics were determined by the modified method developed by Shetty et al. (1995) based on previous Folin-Ciocalteu method, Distilled water of 0.5 mL and 0.5 mL of blackberry sample extract were combined and transferred to a 10 mL test tube. After initial dilution with distilled water, 1 mL of 95% ethanol and 5 mL of distilled water was added to this mixture. Following this 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and the mixture was vortexed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and was incubated in the dark at room temperature for 60 min. After the incubation period, the absorbance was read at 725 nm. A standard curve was created using various concentrations of gallic acid in distilled water and the results were represented as mg of gallic acid per gram of sample fresh weight (FW).

5.3.5. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) inhibition antioxidant assay: Antioxidant activity of blackberry cultivars grown under organic and conventional production systems was determined by using DPPH radical cation decolorization assay method using the modified method developed by Kwon et al. (2006). In this assay 1.25 mL of 60 µM DPPH stock solution prepared in 95% ethanol was added to 250 µL of blackberry sample extract. The decrease in absorbance was monitored after 5 min at 517 nm. The absorbance of a control, using distilled water instead of sample extract, was also recorded after 5 min at the same wavelength for comparison. The percentage of inhibition was then calculated by the following equation:

$$DPPH\ Inhibition\ (\%) = \frac{(Abs\ control - Abs\ sample)}{Abs\ control} \times 100$$

5.3.6. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant assay: Antioxidant activity of blackberry was also determined by using a second ABTS radical cation decolorization assay (Re et al. 1999). ABTS radical cation was created by combining 5 mL of 7-mM ABTS solution with 88 mL of 140-mM $K_2S_2O_4$ solution. The solution was then kept at room temperature in the dark for 12-16 hours before use. Before the assay, the solution was combined with 95% ethanol to create a 1:88 dilution (ABTS solution: 95% ethanol) and the absorbance was adjusted accordingly at 734 nm to 0.70. Then1 mL of the adjusted ABTS solution was added to 50 µL of blackberry sample extract. After vortexing, solution was then incubated at room temperature for 2.5 minutes and then absorbance of the incubated mixture was recorded at 734 nm using UV-VIS spectrophotometer. The antioxidant activity of the blackberry was then represented as percent inhibition of ABTS radical formation and calculated using the formula:

$$ABTS\ Inhibition\ (\%) = \frac{(Abs\ control - Abs\ sample)}{Abs\ control} \times 100$$

5.3.7.α-Amylase enzyme inhibition assay: α-Amylase inhibitory activity of blackberry was measured by using an *in vitro* assay method modified from Worthington Enzyme Manual

(Worthington Biochemical Corp., 1993a). Briefly, a solution of 500 μL of blackberry sample extract and 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α−amylase solution (0.5 mg/mL) was incubated at 25°C for 10 min. After incubation, 500 μL of 1% starch solution, diluted in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl), was added and then the test tubes were incubated again at 25°C for 10 min. Afterwards, 1.0 mL of dinitrosalicylic acid was added and the solution mixtures were incubated in boiling water for 10 min. After removing from the water bath, solution mixtures were allowed to cool at room temperature. A volume of 10 mL distilled water was then added to the solution to adjust the baseline reading of the control at 1.0 ±0.2 and the absorbance was measured at 540 nm.

The absorbance of sample blank (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The final absorbance (A540 extract) of the extract was obtained by subtracting its corresponding sample blank reading. Additionally, 1:2 and 1:5 dilution of the sample extract (250 μL sample + 250 μL of distilled water and 100 μL sample + 400 μL of distilled water respectively) were performed to determine potential dose dependence. The a-amylase inhibitory activity was calculated according to the equation below:

$$\alpha - Amylase\ Inhibition\ (\%) = (\frac{Abs\ control - (Abs\ sample - Abs\ Sample\ Blank)}{Abs\ Control} \times 100$$

5.3.8. α-Glucosidase enzyme inhibition assay: The *in vitro* assay method in this study was modified from the Worthington Enzyme Manual for *a*−glucosidase inhibition (Worthington Biochemical Corp. 1993b, McCue et al. 2005). A volume of 50 μL of blackberry sample extract diluted with 50 μL of 0.1 M potassium phosphate buffer (pH 6.9) and 100 μL of 0.1 M potassium phosphate buffer (pH 6.9) containing glucosidase solution (1.0 U/mL) was incubated in 96-well plates at 25 °C for 10 min. After pre-incubation, 50 μL of 5 mM *p*-nitrophenyl- a-d-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (Thermomax; Molecular Devices Co., Sunnyvale, CA) and compared to a control which had 50 µL of buffer solution in place of the extract (A405 control). The *a*-glucosidase inhibitory activity was expressed as a percentage of inhibition and calculated as follows:

$$\alpha - Glucosidase\ Inhibition\ (\%) = \frac{(\Delta\ Abs\ control - \Delta\ Abs\ sample)}{\Delta\ Abs\ control} \times 100$$

5.3.9. Angiotensin-I-converting enzyme (ACE) inhibition assay: Anti-hypertensive function relevant ACE inhibitory activity of blackberry was determined using an *in vitro* assay method modified by Kwon et al. (2006). A volume of 50 µL of sample extract was incubated with 200 µL of 0.1 M NaCl-borate buffer (0.3 M NaCl, pH 8.3) containing 2 mU of ACE enzyme solution at 25°C for 10 min. After pre-incubation, 100 µL of a 5.0 mM substrate (hippuryl-histidyl-leucine) solution was added to the reaction mixture. Test solutions were then incubated in water bath at 37°C for 1 h. Sample blanks (buffer in place of enzyme and substrate), a control (distilled water instead of sample extract) and a blank (buffer instead of sample extract and enzyme) were also included. The reaction was then stopped by adding 150 µL of 0.5 N HCl to all reaction mixtures. The hippuric acid formed was detected by using High Performance Liquid Chromatography (HPLC) based quantification protocol. For HPLC analysis, a volume of 5 µL of sample was injected using an Agilent ALS 1200 autosampler into an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for the gradient were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was Agilent Zorbax SB-C18, 250 − 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 228 nm and the chromatogram was integrated using the Agilent Chemstation enhanced integrator for detection of liberated hippuric acid. Pure hippuric acid was used to identify the spectra and retention time. The percentage of inhibition was calculated considering the area of the hippuric acid peak according to the equation below:

$$ACE\ Inhibition\ (\%) = \frac{((Abs\ control - Abs\ blank) - Abs\ sample)}{(\ Abs\ control - Abs\ blank)} \times 100$$

However, ACE inhibition was not observed in this study and therefore not presented in results and discussion section.

5.3.10. High performance liquid chromatography (HPLC) for phenolic acid characterization: The blackberry sample extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample was injected using an Agilent ALS 1200 autosampler into an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a D1100 CE diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 − 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 0.7 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of chlorogenic acid, gallic acid, ellagic acid, catechin, rutin, benzoic acid, and *p*-coumaric acid in 100% methanol were used to calibrate the standard curves and retention times.

5.3.11. Data analysis: Two extractions were performed for each blackberry sample, and all *in vitro* assays were replicated six times (n = 6). Means, standard errors, and standard deviations were calculated from replicates using MS-Excel. All data was subjected to a two-way ANOVA using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC), and the least mean square differences for cultivar, treatments, and cultivar × treatments interactions were compared using Tukey's test ($p < 0.05$).

5.4. Results and Discussions

5.4.1. Total soluble phenolic (TSP) content and phenolic characterization of blackberry grown under organic and conventional production systems: Total soluble phenolic (TSP) content was determined in order to understand any potential impact of different production practices (organic vs. conventional) and cultivars on phenolic content of blackberry. Overall, significant differences ($p<0.05$) in TSP content was observed between two cultivars and also due to different weed and fertilization management practices (Fig 5.1) (Appendix III). Both blackberry cultivars had high TSP content, however cultivar Prime-Ark 45 (mean-2.12 mg GAE/g F.W.) had significantly high ($p<0.05$) TSP content when compared to Prime-Ark Freedom (mean- 1.71 mg GAE/g F.W.). Previously, Sarkar et al. (2016) reported 1.1-2.1 mg GAE/g F.W. of TSP content in 13 blackberry cultivars grown in Alabama. Similarly, Wang and Lin (2000) also found 2.0-2.4 mg /g F.W. TSP content in mature blackberry fruits. Among different management practices, both blackberry cultivars with feather meal (organic) treatment had significantly ($p<0.05$) high TSP content when compared to fish emulsion, urea (conventional), and ESN (conventional). Similarly, blackberry grown under organic weed management (black landscape fabric and black plastic mulch) had significantly ($p<0.05$) high TSP content when compared to blackberry grown under conventional weed management practice (herbicide). Therefore, the result of this study suggested that organic fertilization and organic weed management practices may have positive impact on TSP content of two blackberry cultivars. Previously, Cavender et al. (2014) observed high total phenolic content in blackberry with hand weeding treatment when compared to no weed management and weed mat treatments. In another study, Crecente-Campo et al. (2012) found high anthocyanin content and high ascorbic acid content in organically grown strawberry (*Fragaria* spp.) (cow manure +organic pest management) when compared to strawberry grown under conventional production practices. However, they did not observe any significant differences in total phenolic content of strawberry from two different production systems (organic vs. conventional). Therefore, it is important to conduct multi-year and multi-location studies to validate the findings of the current study. Furthermore it is also important to understand the overall impact of organic vs. conventional production practices and cultivars on phenolic acid profile of blackberry.

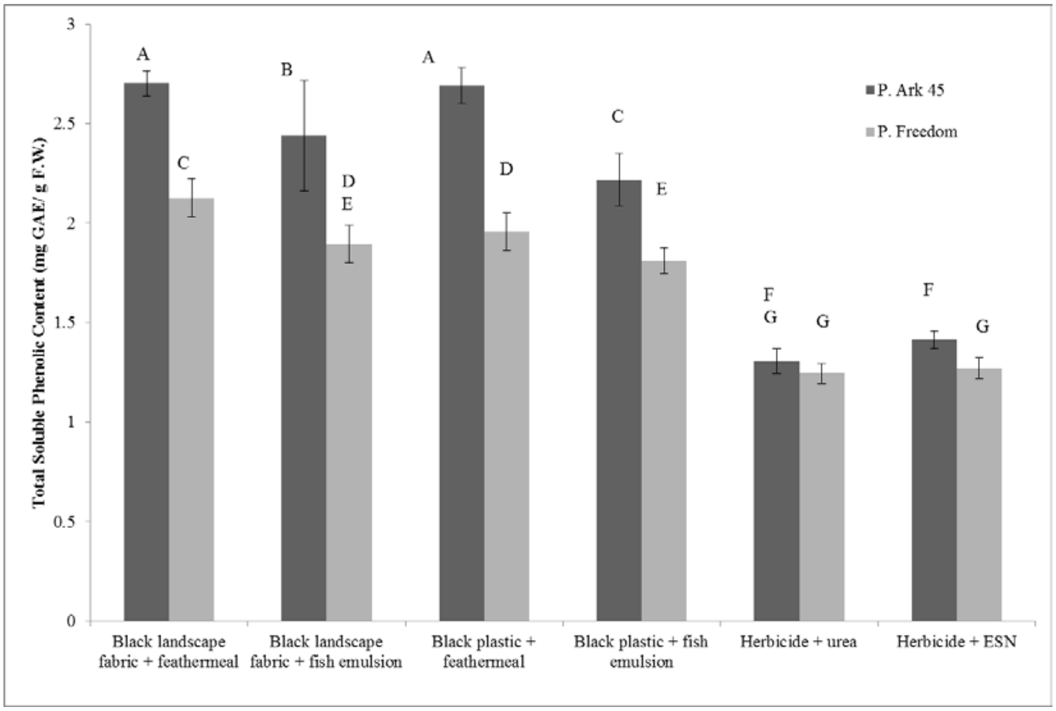

Figure 5.1. Total soluble phenolic content (mg GAE/ g F.W.) of two blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) grown under organic and conventional weed and fertilization management practices. Different capital letters represent significant differences in TSP content due to different cultivar × treatment interactions at *p*<0.05.

Table 5.1. Individual phenolic acid content (µg / g F.W.) of two blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) grown under organic and conventional weed and fertilization management practices.

Treatments	Catechin		Gallic Acid		Ellagic Acid		Protocatechuic acid		Rutin	
	µg / g F.W.									
	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom
Black landscape fabric + feather meal	9.8±0.1	3.4±0.1	0.3±0.1	0.5±0.1	1.3±0.3	1.1±0.1	0.3±0.1	0.2±0.1	7.4±0.6	5.5±0.1
Black landscape fabric + fish emulsion	14.1±0.2	1.3±0.1	0.02±0.1	0.6±0.1	1.5±0.1	0.9±0.1	0.2±0.1	0.3±0.1	3.8±2.9	3.3±0.1
Black plastic + feather meal	16.8±0.1	1.1±0.1	0.1±0.1	0.6±0.1	1.4±0.1	0.9±0.1	0.3±0.1	0.1±0.1	6.9±0.1	1.8±0.1
Black plastic + fish emulsion	28.4±0.1	3.1±0.1	0.1±0.1	0.4±0.1	1.8±0.1	1.4±0.1	0.3±0.1	0.2±0.1	8.2±0.1	7.1±0.1
Herbicide + urea	13.1±0.1	1.3±0.1	0.3±0.1	0.6±0.1	1.6±0.1	1.2±0.1	0.2±0.1	0.2±0.1	7.3±0.1	6.5±0.1
Herbicide + ESN	12±0.1	4.6±0.1	0.2±0.1	1±0.1	1.6±0.1	2±0.1	0.2±0.1	0.3±0.1	7.7±0.2	6.8±0.1

± Standard Error

In this study, major phenolic compounds found in blackberry were catechin, gallic acid, ellagic acid, protocatechuic acid, and rutin (Table 5.1.). Among all phenolic compounds, both blackberry cultivars had higher concentration of catechin followed by rutin and ellagic acid. Previously, Sellappan et al. (2002) reported high catechin and ellagic acid content in blackberry. Similarly, Sarkar et al. (2016) also found high catechin and rutin content in 13 blackberry cultivars. In this current study, similar to the result of the TSP content, high catechin, ellagic acid, and rutin were observed in cultivar Prime-Ark 45 when compared to Prime-Ark Freedom. Among different weed management and fertilization treatments, black plastic mulch + fish emulsion treatment resulted in high catechin and rutin in both blackberry cultivars. Therefore, these results indicated that different cultivation practices not only have impact on TSP content of blackberry, but also have significant effect on phenolic acid profile of blackberry cultivars. Furthermore, differences in TSP content and phenolic profile of blackberry cultivars may also have relevance in other human health relevant functions such as antioxidant and anti-hyperglycemic properties.

5.4.2. Total antioxidant activity of blackberry grown under organic and conventional production systems: Blackberry fruits are rich in dietary antioxidants, and gaining interest in health-focused food market, especially to counter chronic oxidative stress commonly associated with major NCDs, such as T2D and CVDs (Huang et al., 2012). In this study, two different free radical scavenging (DPPH & ABTS) assay methods were used to determine total antioxidant activity of blackberry grown under organic and conventional production systems (Fig. 5.2 A&B). Due to very high (100% inhibition) antioxidant activity observed in ABTS-based free radical scavenging assay, blackberry extracts were further diluted to 1:10 using distilled water, and results of diluted samples is presented (Fig 5.2 B). Overall, very high antioxidant activity (78-95% DPPH-based free radical inhibition in undiluted sample and 49-94% ABTS-based free radical inhibition in 1:10 diluted sample) was observed in both blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) and from all different cultivation practices. Similar high antioxidant activity of blackberry cultivars was reported by previous *in vitro* studies (Sariburun et al., 2010; Sarkar et al. 2016; Siriwoharn et al., 2004). In this current study, similar to TSP content, significantly high ($p<0.05$) antioxidant activity (both DPPH & ABTS based assays) was observed in Prime-Ark 45 when compared to Prime–Ark Freedom. Furthermore, organic fertilization (feather meal and fish emulsion) and organic weed management (black landscape fabric and black plastic mulch) resulted in significantly high ($p<0.05$) antioxidant activity in both blackberry cultivars.

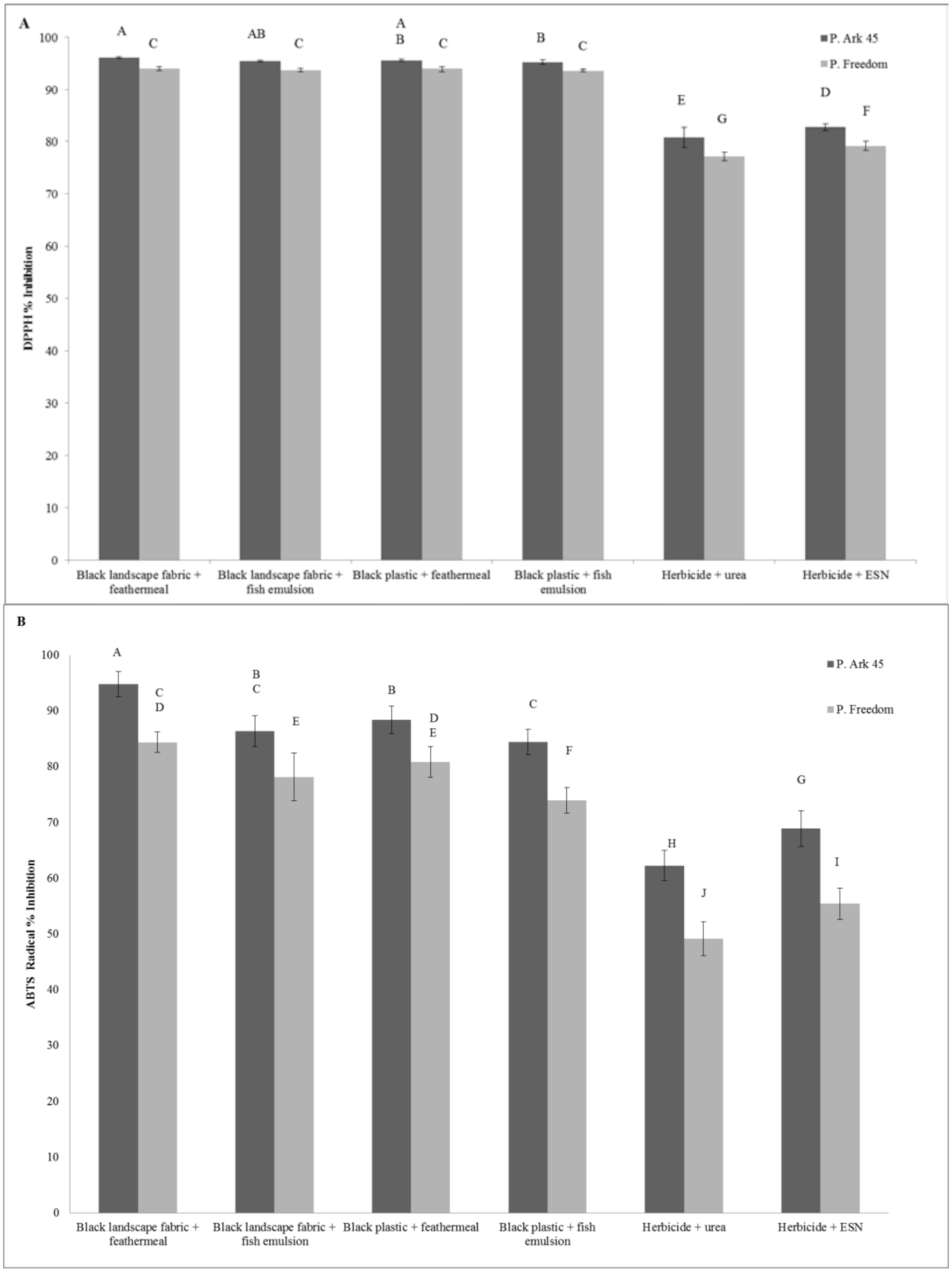

Figure 5.2. Total antioxidant activity (A- DPPH free radical scavenging % inhibition B-ABTS free radical scavenging % inhibition with 1:10 dilution) of two blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) grown under organic and conventional weed and fertilization management practices. Different capital letters represent significant differences in TSP content due to different cultivar × treatment interactions at *p*<0.05.

Overall, Prime-Ark 45 grown under organic production system had significantly high TSP content and total antioxidant activity. In this study positive and strong correlation between TSP content and antioxidant activity was observed in blackberry. Previously, Wang et al. (2008) reported higher correlation between phenolic content and antioxidant activity (ORAC) in organically grown blueberries. Similarly, Mikami-Konishide et al. (2013) found significant effect of growing condition and cultivation practices on antioxidant potential of fruit crops including berries in Japan. Overall, results of the current study suggested that organic fertilization and weed management strategy can be rationally advanced to improve phenolic-linked antioxidant activity and related human health benefits in blackberry. Such organic production innovation will help to advance value-added production of high-quality organic berries in North Dakota and other regions of the Northern Plains, and will ensure better economic returns to organic growers.

5.4.3. Anti-hyperglycemia relevant α-amylase and α-glucosidase enzyme inhibitory activities of blackberry grown under organic and conventional production systems: In this study, potential anti-hyperglycemic functions of blackberry were also determined using *in vitro* assay methods and based on potential inhibition of two key glucose metabolism relevant enzymes α-amylase and α-glucosidase. Inhibition of these enzymes help to slow down the breakdown of complex carbohydrate into glucose and reduce the immediate absorption of glucose in the bloodstream, which is essential to manage postprandial glucose spike in the bloodstream and related chronic hyperglycemia (Tundis et al. 2010). Previously, extracts of berry fruits have shown high α-amylase and α-glucosidase enzyme inhibitory activities (Cheplick et al. 2007; 2010; 2015; Pinto et al. 2008; 2010b; Sarkar et al. 2016; 2017; Wang et al. 2012; Zhang et al. 2012).

Similar to the findings of these previous studies, the current study also observed very high α-amylase (84-100 % inhibition in undiluted sample) and α-glucosidase (82-99% inhibition in undiluted sample) enzyme inhibitory activities in two blackberry cultivars grown under organic and conventional production systems (Table 5.2 & 5.3). Significant dose dependent responses in α-amylase and α-glucosidase enzyme inhibitory activities were also observed in both blackberry cultivars. Overall, Prime-Ark freedom had high α-amylase inhibitory activity when compared to Prime-Ark 45. On the contrary Prime-Ark 45 had high and α-glucosidase enzyme inhibitory activity in this study, especially in half and one-fifth diluted sample.

Table 5.2. α-Amylase enzyme inhibitory activity of two blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) grown under organic and conventional weed and fertilization management practices.

Accessions/ Cultivars	Undiluted (1:1)		Half-Diluted (1:2)		One-Fifth Diluted (1:5)	
	% Inhibition					
	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom
Black landscape fabric + feather meal	96.4±0.7B*	100±0A	48.6±0.8CDE	50±1CD	19.6±1BCD	21±0.7BC
Black landscape fabric + fish emulsion	100±0A	100±0A	61.3±0.9B	65.6±0.9A	22.7±1AB	26±0.6A
Black plastic + feather meal	96.9±0.6B	94.5±1B	51.1±0.9C	46.4±0.9DEFG	19.6±0.9BCD	18±0.7CD
Black plastic + fish emulsion	88.7±0.9CD	96.6±0.8B	45.5±0.7EFGH	47.6±0.8CDEF	18.8±0.8CD	19.3±0.6BCD
Herbicide + urea	87.5±0.7D	84.6±0.6E	43.1±0.9GH	42.6±1.2GH	16.5±0.9D	17.1±0.8D
Herbicide + ESN	85.9±0.6DE	91.4±0.8C	42.4±0.8H	44.3±0.8FGH	18.3±0.8CD	17.5±0.8CD

± Standard Error

*Different capital letters for each dilution represent significant differences in α-amylase enzyme inhibitory activity between cultivar × treatment interactions at the $p<0.05$ level.

Similar to TSP content and antioxidant activity, organic weed management and fertilization treatments also resulted in significantly ($p<0.05$) high α-amylase and α-glucosidase enzyme inhibitory activities in blackberry fruits. The significant differences in α-amylase and α-glucosidase enzyme inhibitory activities of blackberry due to different cultivation practices (organic vs. conventional) were more prominent in one-fifth diluted sample. Therefore, results of the current study suggested that organic production practices may also have role in improving anti-hyperglycemic functions in blackberry cultivars. However, future studies with different cultivars, multiple years, and multi-locations are required to confirm the findings of this current study. Furthermore, organic blackberry can be targeted as dietary antidote against chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of T2D and other NCDs.

Table 5.3. α-Glucosidase enzyme inhibitory activity of two blackberry cultivars (Prime-Ark 45 & Prime–Ark Freedom) grown under organic and conventional weed and fertilization management practices.

Accessions/Cultivars	Undiluted (1:1)		Half-Diluted (1:2)		One-Fifth Diluted (1:5)	
	% Inhibition					
	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom	P. Ark 45	P. Freedom
Black landscape fabric + feather meal	99.6±0.3A*	98.3±0.6AB	94.4±0.3A	92.3±0.5BCDE	83.1±0.7B	74.9±1.2DEF
Black landscape fabric + fish emulsion	97.8±0.3AB	96.6±0.5B	94±0.3BC	91.8±0.5CDE	83.3±1.5B	79±1.1BCD
Black plastic + feather meal	99.1±0.6A	97.5±0.4AB	97.8±0.3A	89.8±1EF	94.1±0.7A	76.2±0.9CDE
Black plastic + fish emulsion	97.9±0.3AB	97.3±0.5AB	90.9±0.4DEF	92.6±0.6BCD	71.8±1.2EF	80.7±1.4BC
Herbicide + urea	98.1±0.3AB	82±0.6D	88.9±0.5F	62.8±1.1H	70.3±1.3F	37.6±1H
Herbicide + ESN	98.1±0.4AB	89.7±1.1C	89.9±0.6EF	78±0.8G	73.7±1.4EF	55.6±0.9G

± Standard Error

*Different capital letters for each dilution represent significant differences in α-glucosidase enzyme inhibitory activity between cultivar × treatment interactions at the $p<0.05$ level.

5.5. Conclusions

North Dakota and other regions of the Northern Plains have significant potential to become leaders in organic berry production in the United States. However, it is important to develop and optimize new organic production strategies to expand and strengthen organic berry production, such as production of blackberry in this region. Furthermore, it is also important to understand the effect of different organic production practices and different cultivars on value added human health relevant bioactive profiles and associated health benefits of berries, including blackberry. Based on these needs, the goal of this study was to determine the impact of different organic and conventional weed management and fertilization practices on phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities of two blackberry cultivars using rapid *in vitro* screening strategy. Overall, very high phenolic-bioactive linked antioxidant and anti-hyperglycemic properties were observed in both blackberry cultivars (Prime-Ark 45 & Prime-Ark Freedom). Furthermore, high TSP content, high antioxidant activity and high anti-hyperglycemic (α-amylase and α-glucosidase enzyme inhibitory activities) properties were observed in blackberry grown under organic weed management and organic fertilization treatments when compared to same blackberry cultivars grown under conventional weed management and fertilization practices. However, more practical evidences based on future field-based studies will be needed to confirm the above findings and to advance organic production practices for production of high-value berries with superior human health relevant nutritional qualities, especially for application as dietary antidote against T2D and other diet-linked NCDs.

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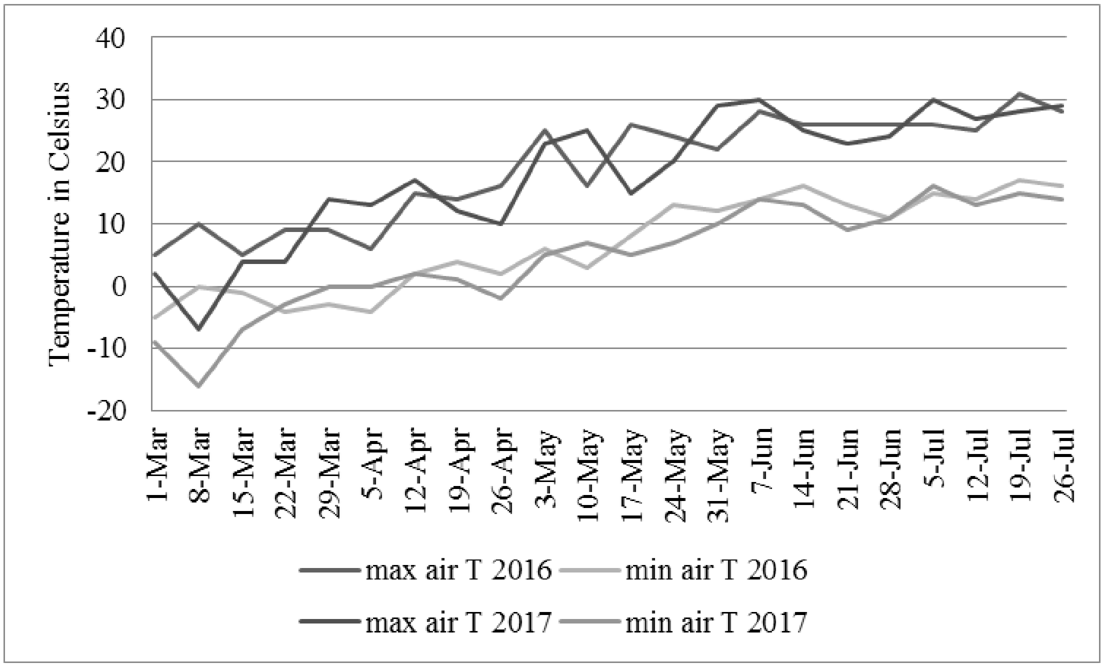
APPENDIX A. TEMPERATURE OF 2016 AND 2017 SERVICEBERRY CROP YEARS



APPENDIX B. ANALYSIS OF VARIANCE (ANOVA) TABLE FOR SERVICEBERRY

Variables	DF	TSP	DPPH	ABTS	α-amylase Inhibitory Activity			α-glucosidase Inhibitory Activity		
					Undiluted	1:2 dilution	1:5 dilution	Undiluted	1:2 dilution	1:5 dilution
		MS	MS	MS	MS	MS	MS	MS	MS	MS
Accessions/cultivars	19	1.7***	203.8***	4322.48***	2855.3***	3966.25***	698.8***	214.7***	434.4***	544.66***
Crop Years	1	4.4***	8147.8***	2559.8***	830.5***	1683.56***	2134.5***	NS [a]	1070.8***	5465.7***
Accessions/cultivars × Crop Years	19	0.07***	3.48***	3.35*	33.8***	21.92*	5.25*	26.71***	110.39***	315.2***

MS-Mean Sum of Square Value

*$P<0.05$

** $p<0.01$

*** $p<0.001$

[a] Not Significant

APPENDIX C. ANALYSIS OF VARIANCE (ANOVA) TABLE FOR BLACKBERRY

Variables	DF	TSP	DPPH	ABTS	α-amylase Inhibitory Activity			α-glucosidase Inhibitory Activity		
					Undiluted	1:2 dilution	1:5 dilution	Undiluted	1:2 dilution	1:5 dilution
		MS	MS	MS	MS	MS	MS	MS	MS	MS
Cultivars	1	6.07***	206.8***	3992.7***	138.8***	NS [a]	NS	824.7***	2635.6***	5215.1***
Cultivation Practices	5	5.95***	1397.6***	4369.7***	718.0***	1371.3***	168.1***	270.3***	1256.1***	3326.4***
Cultivars × Cultivation Practices	5	0.4***	5.5***	35.4**	115.3***	57.2***	17.2*	233.1***	606.5***	1212.1***

MS-Mean Sum of Square Value

*$P < 0.05$

** p<0.01

*** p<0.001

[a] Not Significant