PHENOLIC-LINKED ANTIOXIDANT AND ANTI-HYPERGLYCEMIC PROPERTIES OF SELECTED CEREAL, PSEUDO-CEREAL, AND MILLET USING IN VITRO SCREENING METHODS

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ABSTRACT

Improving diversity of food systems by targeting whole grain cereals, pseudo-cereals, and millets is essential to enhance nutritional qualities beyond macro and micronutrient balance and to address emerging global food and nutritional security-linked public health challenges. However, human health relevant nutritional parameters of whole grains vary widely among species, genotypes, growing conditions, and further due to different processing methods. Therefore, it is important to screen human health relevant nutritional parameters of these whole grains prior to targeting them for wider public health solutions linked to non-communicable chronic diseases (NCD). Based on this rationale, oats from different processing stages and from different production systems, buckwheat, teff, pearl millet, and different genotypes of sorghum were analyzed for health relevant phenolic bioactive linked antioxidant and anti-hyperglycemic properties using *in vitro* assay models. Overall, high phenolic-linked antioxidant and anti-diabetic properties were observed in whole grain oats, rolled oat, buckwheat, teff, and select sorghum genotypes.

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"Whoever teaches me a letter, I will be his/her servant."….Ancient Arabic Wisdom

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DEDICATION

To those who were and remain my steadfast supporters, to my wife, my children, and my parents

I dedicate this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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. Global Food and Nutritional Security-Linked Public Health Challenges

Global food and nutritional security solutions must be based on diversity of food systems to ensure higher human health relevant nutritional quality to meet the needs of emerging global public health challenges. Due to lack of diversity of foods and associated nutritional imbalance, global food security challenges have become more complex as a result of the rapid upsurge in the incidence and prevalence of diet-linked non-communicable chronic diseases (NCDs) such as type 2 diabetes, cardiovascular disease, obesity, dyslipidemia, and cancer (Kimokoti and Millen 2011). The global NCD epidemic is largely associated with higher consumption of macronutrient-dense hyper-processed foods, especially from refined carbohydrates and saturated fat, along with rapid urbanization, and lifestyle changes (sedentary) (Rosegrant and Cline, 2003). Therefore, food security is no longer just an issue of supplying adequate macronutrients and micronutrients to the global population, but rather ensuring nutritionally balanced diversity of foods to address global food security-coupled public health challenges linked to NCDs. Furthermore, even with the increase of global population in the next few decades reaching a maximum of 10-11 billion (by 2100), the present food production based on macronutrient levels will have sufficient stock and surplus, with high proportion of cereal grain production (Hübert et al. 2011). Though 1 billion people globally are still fighting hunger from deficient macronutrients, this food security challenge is due to the lack of proper distribution, difficulties with fresh food accessibility, lack of long term storage facilities, and increasing price of foods (Godfray et al. 2010; Hübert et al. 2011). In this above context, it is not just meeting the calorie and macronutrient demand of the increasing population, but to provide healthy and nutritious diversity of food to combat global epidemic of preventable diet-linked diseases, such as NCDs.

As a result, in the next few decades there will be higher global mortality due to the prevalence of NCDs than malnutrition and infectious disease both in developed and in developing countries (WHO 2016). It is therefore very clear that to sustain and improve global food security and concurrently to address present and emerging nutritional and related health security challenges of both hunger and NCDs co-existing in the same population it is necessary to improve the diversity of plant-based foods, especially whole foods with higher NCD relevant nutritional quality beyond current macronutrient and micronutrient models. This will require diversity of food choices with balanced nutrition to counter NCDs. In this context, diversity of whole grains such as underutilized cereals, pseudo-cereals, and millets is essential as it will ensure balanced supply of essential nutrients in daily diets and will also provide diverse group of health relevant bioactive compounds with health promoting and cellular protective functions to prevent and manage common NCDs such as type 2 diabetes.

1.2. Changes in Dietary Pattern and Rise of Non-Communicable Chronic Diseases (NCDs)

In general, rapid rise and prevalence of NCDs is directly associated with rapid changes in dietary pattern across the world in the last century. Changes in dietary pattern and in nutritional composition have had significant evolutionary impact in the past and they now affect the health of the contemporary human populations (Heber and Bowerman 2001). The evolution of human dietary pattern has been mostly driven by the necessity, availability, taste, ecological and socio-cultural interactions and cost of the diet. With the development and advancement of industrialization of agriculture in recent times, humans have gradually shifted from diverse plant-based diets that provided essential vitamins, minerals, and over 25,000 phytochemicals to a narrow diet based on refined grains, added oils, sugar and salt (Eaton and Eaton 2003). During the last century, after addressing the major macronutrient and calorie needs to counter hunger

through improved breeding technologies and food processing advancements, carefully designed foods on the basis of taste, cost and convenience was promoted in the human diet worldwide. The development of such modern packaged and hyper-processed fast foods often disregarded or neglected a wider and more comprehensive nutritional requirements and overall health value of our food system (Schlosser et al. 2001). The marketing of popular and profitable large-scale manufactured foods along with globalization of economies and mostly advancement of mono-cropping agriculture threatens to homogenize diet and also reduce the diversity of food choices. Such changes in human diet and sedentary lifestyle changes have significantly impacted in the rapid global emergence of major NCDs, such as type 2 diabetes, cardiovascular diseases, and cancer (Zimmet et al. 2001).

To address this urgent global diet-linked public health challenges, attention have been diverted to develop dietary guidelines to counter cellular metabolic breakdowns associated with chronic diseases such as obesity-linked diabetes, cardiovascular disease, and cancer. Over the past 40 years international studies comprising of controlled trials of risk factors and prospective cohort study of disease end points have documented the lack of diversity of human dietary pattern and its association with increased risk of chronic diseases (Mann 2002). Therefore, to improve the diversity of food choices and to enhance nutritional qualities of diet, fruits, vegetables, whole grains, and nuts have been recommended and advanced to lower the risk of NCDs. However, the overall impact of food and dietary pattern on human health largely depends on the complex, synergistic contributions and interactions among food structure, preparation methods, fatty acid profiles, carbohydrate quality (glycemic index, fiber content), protein type, micronutrients and phytochemicals (Mozaffarian and Ludwig 2010). The diet relevant to manage NCDs should be low in salt content, *trans* fat, saturated fat, refined carbohydrates, and added

sugar. Furthermore, such diet must be balanced with unsaturated fats, fiber, antioxidants, minerals and phytochemicals. In this context, whole grains are considered as healthy food choices as they have range of health promoting nutritional components and when consumed as part of diet whole grains can have diverse cellular protective functions against chronic diseases such as type 2 diabetes.

1.3. Type 2 Diabetes Epidemic

Type 2 diabetes is one of the most common NCDs and prevalence of this major chronic disease is rising rapidly worldwide (WHO 2015). According to International Diabetes Federation (IDF, 2017), currently 425 million adults have diabetes with 50% still remaining undiagnosed. Furthermore, 1 in 6 births is affected by hyperglycemia during pregnancy, which also increases the risk of type 2 diabetes development as adults (IDF 2017). Diabetes patients in the United States is around 30.8 million (2017) currently and type 2 diabetes has been considered as a new epidemic in the American pediatric population (Kaufman 2002). The trend of total mortality associated with T2D continues to steadily rise, with the World Health Organization projecting that diabetes will be the 7th leading cause of death in 2030 (WHO 2017). Population growth, ageing of population and urbanizations are key factors which will lead to a 54% increase in numbers with diabetes by 2030 (Shaw et al. 2010). The global emergence of obesity and diabetes is also an economic issue as it is a health issue and type 2 diabetes along with cardiovascular disease has a significant socio-economic impact on individuals, families, health system and countries (Yach et al. 2006).

Overweight and obesity is one major contributor in the onset of type 2 diabetes especially, mother's body mass index and child's birth weight, growth trajectories of children in the earliest years of life, subsequent eating and activity patterns, and behavior in childhood are

strongly related to the onset of type 2 diabetes (Bloomgarden 2004). In this context, all factors which promote obesity like consumption of excess energy, particularly increases in the intake of saturated fatty acids, sugar-sweetened beverages and hyper-processed and refine foods, and the consumption of less fiber with sedentary lifestyle are responsible for the global outbreak of type 2 diabetes (Kolb and Mandrup-Poulsen 2010). Type 2 diabetes and cardiovascular disease have many common risk factors and they are highly associated based on common pathogenesis and cellular metabolic breakdowns. In this context, "Metabolic Syndrome" which is a common link between type 2 diabetes and cardiovascular disease also includes other chronic metabolic disorders such as hypertension, dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance and obesity (particularly central obesity) (Gluckman and Hanson 2004).

In general, type 2 diabetes mellitus is a chronic metabolic disorder characterized by relative insulin deficiency due to impaired insulin production from defective pancreatic β-cells combined with peripheral insulin resistance (Green et al. 2003). The primary cause of fasting hyperglycemia is due to an elevated rate of basal hepatic glucose production in the presence of hyperinsulinemia; whereas postprandial (after a meal) hyperglycemia is due to the impaired suppression of hepatic glucose production by insulin and decreased insulin-mediated glucose uptake by muscle (De Fronzo 1999). Furthermore, different morbidity and mortality from microvascular (retinopathy, nephropathy, and neuropathy) and macro-vascular (heart attacks, stroke, and peripheral vascular disease) complications arise from type 2 diabetes (Dandona et al. 2004). Major risk factors of type 2 diabetes are generally associated with local or systemic low-grade inflammation and such chronic inflammation eventually leads to on-set of diabetes if counter-regulation of inflammation and metabolic stress are compromised because of a genetic or epigenetic predisposition (Dandona et al. 2004). Therefore, cellular oxidation and production

of free radicals as part of chronic oxidative stress is common risk factor of type 2 diabetes and associated complications (Grattagliano et al. 2008).

1.4. Type 2 Diabetes: Chronic Oxidative Stress and Chronic Hyperglycemia

In general, as an aerobic Eukaryotic organism, human cells are commonly impacted by redox imbalances from metabolic and environmental fluctuations, where reactive oxygen species (ROS) interact with major macromolecules which inevitably lead to cellular damages and dysfunction (Aruoma 1998; Wilson et al. 2005). Demand of excessive cellular energy (ATP) usually results in an incomplete reduction of oxygen in the mitochondria and leading to the subsequent generation of excess ROS. While oxidizing environment favors cell death, reducing environments favor cell proliferation. Current animal models based *in vivo* and clinical studies suggest that while most NCDs are symptomatically diverse, a shared pathogenesis for development and incidence exists: chronic inflammation induced from oxidative stresses and related chronic hyperglycemia (Ceriello and Motz 2004; Lin and Beal 2006). Pathogenesis of vascular degeneration involves oxidative stress either by triggering or exacerbating the biochemical processes accompanying metabolic syndrome (Hanhineva et al. 2010; Wilson et al. 2005). Chronic antioxidant deficiency may favor the propagation of oxidative alterations from intra- to extra-cellular spaces that induces a systemic state of oxidative stress. Reactive oxygen species (ROS) not only damage cellular structures, but also contribute to cellular aging, mutagenesis, carcinogenesis, coronary heart disease and apoptosis (Kaneto et al. 2007).

In addition it is suggested that malnutrition or nutrient deficiency in the early stage of life is also associated with the later more rapid development of glucose impairment and impact on subsequent cardiovascular disease under high soluble carbohydrate nutrient intake (Anderson et al. 2009). Many studies have shown that early exposure to famine or lower birth weight can lead

to higher rate of impaired glucose tolerance and type 2 diabetes (Inoguchi et al. 2003). Generation of ROS under stress mediated environment may result from the reduced activity of electron transport complexes in the mitochondria. Altered mitochondrial function under limited energy environment can have deleterious effect on cells like β-cell that have higher energy requirement. The damage of β-cell can have long term implications and can lead to insulin resistance and type 2 diabetes, respectively (Anderson et al. 2009).

Dysfunction of β-cells either due to the pre-exposure to nutrient deficiency or due to excessive nutrition from refined carbohydrate and fat intake in later stages of life is strongly associated with the oxidative stress and ROS formation. In diabetic state such oxidative stress decrease insulin biosynthesis and secretion, which lead to the aggravation of type 2 diabetes. Oxidation of low density lipoprotein (LDL) is another complication associated with hyperglycemia. Higher blood glucose level cause glycation or glycoloxidation of LDL, which increase serum oxidative stress and lower the capability of high density lipoprotein (HDL) to protect against oxidation of LDL (Kaplan and Aviram 2005). Cholesterol accumulation in diabetic patients may result from the arterial cell oxidative stress, caused by hyperglycemia (Cerielo 2003). Insulin can act as vasodilator and therefore anti-inflammatory and balanced insulin supply and signaling can reduce vascular complications. Hyperinsulinemia induced atherosclerosis increase ROS production and reduce insulin sensitivity (Albacker et al. 2008). Furthermore, continuous exposure to oxidative stress creates glucose toxicity and potentially can cause different organ failure through tissue damage in diabetic patients (Cerielo 2003). Development of type 2 diabetes is a multistage process and thus requires systematic preventative measures like balanced diet, exercise and healthy lifestyle for overall prevention and management of this chronic diseases. Therefore, improved protection against chronic oxidative

stress and chronic hyperglycemia is an integral part for the prevention and management of type 2 diabetes in humans.

1.5. Gut Health and Type 2 Diabetes

Beyond the protection against chronic oxidative stress and chronic hyperglycemia, improvement of gut health is also closely associated with lower risk of type 2 diabetes and other NCDs (Larsen et al. 2010). Changes in composition and function of intestinal microbiota is associated with type 2 diabetes (Forslund et al. 2015; Larsen et al. 2010). The relationship between gut microbiota and type 2 diabetes especially for obesity-induced type 2 diabetes is based on glucose tolerance of the host microbiota. Qin et al (2012) reported that depletion in butyrate-producing bacteria and increase in some pathogenic bacteria is associated with type 2 diabetes prevalence. Relative increase in *Escherichia coli* population was also observed in type 2 diabetes patients even with Metformin treatment (Forslund et al. 2015). The function of gut microbiota is related to fat distribution, insulin sensitivity, lipid and glucose metabolism and therefore contribute to differences in body weight and subsequently impact type 2 diabetes (Diamant et al. 2010). Another important role of gut microbiota is related to host immune system and are critical for type 2 diabetes patients. Dietary intervention can change composition and function of gut microbiota and thus contribute to the management of type 2 diabetes and associated complications (De Filippo et al. 2010). Therefore, foods such as whole foods that favor composition of beneficial bacteria and can inhibit pathogenic bacteria is important dietary strategies to improve gut health in diabetic patients. In this context foods rich in dietary fibers such as whole grains when fermented by gut bacteria release short chain fatty acids such as butyrate, and subsequently create favorable environment for beneficial microbial composition commonly associated with non-diabetic patients (Hartstra et al. 2015). Furthermore, plant-based

foods with rich phenolic bioactive profiles can also inhibit pathogenic bacteria while at the same time supporting and maintaining favorable and beneficial bacterial composition in the gut. Managing gut infection by inhibiting pathogenic bacteria is important for type 2 diabetes patients, as they are more susceptible to bacterial infections (Gentile et al. 1998). Therefore, plant-based foods such as whole grains with balanced profile of dietary fiber, micronutrients, and health relevant bioactive compounds can be targeted to improve favorable composition of gut microbiota and associated human health promoting functions, which is critical in the prevention and management of type 2 diabetes. Furthermore, such dietary strategy can also help to counter chronic infection of gut or other organs and can improve overall health of diabetic patients.

1.6. Importance of Plant-Based Foods to Manage Type 2 Diabetes and Associated Complications

Plant-based foods have diverse cellular protective functions against several risks factors of type 2 diabetes including antioxidant, anti-hyperglycemic and gut health relevant benefits. Calorie restricted diets with balanced micronutrients and phytochemical-enriched foods have the potential to mitigate oxidative stress and maintain cellular redox balance with reduction in degenerative diseases. Diet is the most critical preventative measure that can effectively reduce cellular ROS load, and eventual oxidation of cellular organelles. Dietary antioxidants, particularly from plant-based foods are most important component for the early management of type 2 diabetes (Hanhineva et al. 2010). Animals including humans largely depend on external plant-based antioxidants to counter oxidative stress and its associated complications. Healthy diet with proper nutritional compositions, particularly from plant-based foods is important for better regulation of chronic oxidative stress and additionally critical stages of glucose uptake, vascular distribution and metabolism. Many *in vitro* and *in vivo* studies have shown that dietary

antioxidants, taken either as extracts or part of whole food itself have beneficial effects on overall glucose metabolism (Hanhineva et al. 2010). Some of these dietary antioxidants also mimic low to moderate stress induced protective mechanism and thus stimulate cellular antioxidant responses linked to enzymatic-mediated metabolic defense systems by triggering necessary signals (Dembinska-Kiec et al. 2008). Different cellular metabolic pathways and enzymatic reactions are involved in the cellular ROS generation and dietary antioxidants can even reduce ROS formation by inhibiting enzymes responsible for its synthesis (Song et al. 2005). The interaction of dietary antioxidants with beneficial microorganisms can also potentially suppress oxidative reactions in cellular and extracellular compartments. Dietary antioxidants from plant-based food sources does not act alone and generally participate in a complex and dynamic system of human cellular metabolism and associated beneficial microbiome. Furthermore, plant-based foods can also improve glucose metabolism through different functions including improving insulin sensitivity, insulin function, and by inhibiting key digestive enzymes associated with carbohydrate metabolism. Therefore, as part of strategies to counter different cellular metabolic breakdown associated with chronic oxidative stress and glucose imbalance, plant-based foods rich in micronutrients, fiber, and diverse health promoting bioactives are important dietary sources and can be targeted to prevent and manage type 2 diabetes. Furthermore, the bioactive compounds of plant-based foods can also be targeted as dietary antidote against diverse chronic diseases and therefore has relevance to address both food security challenges and nutritionally-linked public health challenges worldwide. Many of the human health-relevant attributes and benefits of plant-based foods are due to their high bioactive content, specifically diverse phenolic compounds, which are widely distributed across different plant systems for ecological adaptations including whole grains. Therefore dietary intervention

strategies can target plant-based foods such as whole grains with stress adaptive high bioactive compounds to provide protection against chronic oxidative stress, chronic hyperglycemia, and host immune breakdown and associated infection commonly associated with type 2 diabetes.

CHAPTER 2. REVIEW OF LITERATURE

2.1. Role of Phenolic Bioactives of Plant-Based Foods to Prevent and Manage Type 2 Diabetes

In general, phenolic compounds or phenolic phytochemicals are secondary metabolites synthesized by plants to counter biotic and abiotic stresses. This diverse group of phenolic compounds that contain at least one aromatic ring and usually one or more hydroxyl substitutes that have evolved in different plant lineages to address specific needs, mostly as defense, and signal compounds for stress and reproductive adaptations (Rice-Evans et al. 1997). Phenolic compounds help in overall adaptive strategy for natural selection in plants in diverse niche environments, and thus plants accumulate a vast number of these compounds. Due to the free radical scavenging properties, bioactive phenolics can work as powerful cellular antioxidants and have potential in therapeutic measures against oxidative stress related diseases. The structural chemistry of phenolics suggests different antioxidant functions such as; i) high reactivity as hydrogen or electron donor, ii) ability to stabilize or delocalize unpaired electron, iii) and ability to chelate transition metal ions (Hanhineva et al. 2010). These phenolic antioxidants are virtually present in all plant-based foods. However their levels significantly vary among diets depending on the type and quantity of plant-based food sources. Due to their high antioxidant potentials, phenolic compounds can scavenge the harmful free radicals and inhibit the oxidative reactions or can protect cells from oxidative breakdown through stimulation of cellular antioxidant enzyme responses. Therefore phenolic compounds form plant-based food sources possesses cellular protective functions against specific diseases, like early stages of disease development when oxidative stress condition is in part involved in initiation and progression (Pandey and Rizvi 2009). Diverse health relevant cellular bioactive functions of phenolics are

anti-inflammatory, antioxidative, chemopreventive and neuroprotective activities and thus associated with lowered risk of major chronic diseases like diabetes, cardiovascular diseases and cancer (Hanhineva et al. 2010; Song et al. 2005). Furthermore, bioactive phenolics from different plant-based foods also influence glucose metabolism in several ways, such as inhibition of carbohydrate digestive breakdown and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva et al. 2010; Johnston et al. 2005; Lin et al. 2016).

Carbohydrate digestion (starch breakdown) and glucose absorption are obvious targets for better glycemic control after high carbohydrate meals, and α-amylase and α-glucosidases are key enzymes responsible for the digestion of dietary starch to uptake of glucose. Dietary soluble starches are generally hydrolyzed by pancreatic α-amylase with absorption aided by α-glucosidase in order to absorb by the small intestine (Caspary 1992). Important therapeutic approaches available for managing early stages of type 2 diabetes is by controlling the absorption of glucose through the reduction of starch hydrolysis by inhibiting pancreatic α-amylase and limiting the absorption of glucose by inhibiting intestinal α-glucosidase (Tundis et al. 2010). Many *in vitro* and *in vivo* studies have been reported that phenolics including flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tanins (proanthocyanidinins and ellagitannins) inhibit α-amylase and α-glucosidase enzyme activity (Choi et al. 2008; Hanhineva et al. 2010; Kwon et al. 2006; McCue et al. 2005; Nair et al. 2013).

Furthermore, cellular bioactive phenolics also influence glucose transporters and thus mediate intestinal absorption of glucose. Studies with animal models have shown that flavonoids

like chlorogenic, ferulic, caffeic, tannic acids, quercetin monoglucosides, tea catechins and naringenin could inhibit Na^+-dependent SGLT1-mediated glucose transport and alter postprandial blood glucose response (Hanamura et al. 2005). Choi et al. (2008) found that isoflavonoids like genistein and daidzein preserved insulin production by β-cells in mice. Dietary polyphenols may also influence glucose metabolism through stimulation of peripheral glucose uptake in both insulin-sensitive and insulin-nonsensitive tissues. Cells typically respond to phenolic phytochemicals mainly through direct interactions with receptors or enzymes involved in metabolic processes of digestion, signal transduction, or through modifying gene expressions which may result in the modification of the redox status of the cellular systems which may trigger a series of redox-dependent reactions (Shalaby and Horwitz 2014; Tsao 2010). In addition, phenolic bioactives of plant-based foods also influence gut health benefits by supporting favorable and beneficial microbial composition and associated functions, which is critical for improving glucose metabolism (Cardona et al. 2013; Qin et al. 2012). Although human cells cannot produce bioactive phenolic antioxidants themselves, however dietary consumption of such plant-based secondary metabolites can help to counter chronic oxidative stress, chronic hyperglycemia, and other benefits essential in prevention and management of type 2 diabetes and associated complications (Kumar et al. 2011; Pandey and Rizvi 2009; Nair et al. 2013). In this context, among different plant-based foods, whole grains contain diverse group of phenolic compounds with several human health related protective functions and has significant relevance in advancing dietary support strategies to prevent and manage NCDs, such as type 2 diabetes.

2.2. Human Health Relevant Functionalities of Whole Grains: Focus on Lowering Risks of Type 2 Diabetes

The primary group of phenolic compounds present in whole grain cereals, pseudo-cereals and millets are flavonoids, phenolic acids and tannins (Dykes and Rooney 2007). The bran and germ fractions derived from conventional milling process are rich source of biologically active compounds found in grain (Slavin 2004). Major nutrients in grains are vitamin B (thianin, niacin, riboflavin and pantothenic acid), minerals (calcium, magnesium, potassium, phosphorus, sodium and iron), basic amino acids (arginine and lysine) and other bioactives including tocols and lipids (Slavin 2004). If all components of the kernel such as bran, germ and endosperm are present in natural proportion then it is considered as whole grains. Many previous studies reported a substantial inverse association between whole grain consumption and risk of chronic diseases including type 2 diabetes. Sun et al. (2010) reported that substitution of whole grains including brown rice for white rice lowered the risk of type 2 diabetes. Similarly, Aune et al. (2013) conducted a meta-analysis and confirmed that higher intake of whole grains is associated with lower risk of type 2 diabetes, and opposite trend was observed with higher intake of refined grain such as white rice. In another meta-analysis study, Ye et al. (2012) emphasized that the benefits of whole grain consumption related to diabetes management was associated with higher intake of dietary fibers from whole grains. Cho et al. (2013) reported that higher consumption of whole grains is also associated with lower risk of type 2 diabetes-linked cardiovascular diseases. Parker et al. (2013) suggested that type 2 diabetes relevant benefits of whole grains are associated with low energy intake, prevention of weight gain, and direct influence on insulin resistance. Not only just dietary fibers of whole grains, but also other bioactive compounds such as phenolics of whole grain also have protective functions against type 2 diabetes. Belobrajdik and Bird (2013)

highlighted that species and variety of whole grains with high phenolic bioactive content exhibited high anti-inflammatory properties and relevant in the management of chronic oxidative stress commonly associated with type 2 diabetes. The unique composition of whole grain phenolics and their interactions with other bioactive compounds are linked to potential health benefits of whole grains, including type 2 diabetes relevant health benefits (Okarter and Liu 2species, cultivars, growing conditions, due to variation in environment, and also with different food processing methods. .

2.3. Phenolic Bioactives and Human Health Benefits of Oats

Oats (*Avena sativa* L.) are commonly consumed as whole-grain cereals and a significant dietary source for diverse human health relevant bioactive compounds. The major phenolic acids present in oats are ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, vanillic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, sinapic acid, tricin, apigenin, luteolin, kaempferol, and quercetin (Peterson et al. 2001). Bioactivity of oat syringic acid and avenanthramides in plasma and protection against LDL oxidation was observed previously (Chen et al. 2004). In another study Ji et al. (2003) observed *in vivo* antioxidant effect of synthetic avenanthramides in rats. High *in vitro* antioxidant activity of whole grain oat when compared to wheat and rye was also found (Zielinski and Kozlowska 2000). Furthermore, many studies reported type 2 diabetes relevant benefits of oats. Low post-prandial glycemic response was observed with bran flour of oats (Tapola et al. 2005). Similarly, reduction of glycemic index with β-glucan enriched breakfast cereal was also reported (Jenkins et al. 2002). Lammert et al. (2008) reported 40% reduction in insulin doses with oatmeal-based dietary intervention. Improvement in insulin resistance and reduction of LDL-cholesterol was found in type 2 diabetes patients after β-glucan-enriched bread consumption. Similarly, improvement in glycemic control

and reduction in hyperlipidemia among patients with type 2 diabetes was also observed with oat based diet rich in soluble fiber (Donahue et al. 2009). Furthermore, reduction of fasting blood glucose and glycated hemoglobin (HbA1c) was observed with high fiber diet including oat cereals (Post et al. 2012). All these previous studies suggested that oats and oat-based foods have significant antioxidant and anti-hyperglycemic functionalities and therefore have relevance in the prevention and management of type 2 diabetes.

2.4. Phenolic Bioactives and Human Health Benefits of Buckwheat and Teff

Buckwheat (*Fagopyrum esculentum*) is a pseudo-cereal belonging to family *Polygonaceae* and is becoming increasingly popular as part of healthy foods due to its diverse nutritional qualities and human health benefits, and further for gluten-free diet. Buckwheat is good source of dietary fibers, minerals such as manganese and magnesium and other cellular bioactive compounds including phenolics (Wijngaard and Arendt 2006). Gallic acid, p-coumaric acid, epicatechin, caffeic acid, rutin, hiperin, and quercetin are major phenolics commonly found in buckwheat flour (Inglett et al. 2011). Furthermore whole grain buckwheat contain 2-5 times more phenolic content than oats, and bran and hulls of buckwheat are rich source of diverse phenolic compounds (Holasova et al. 2002). Antioxidant function relevant to protection against lipid peroxidation was previously observed in tartary buckwheat bran extract (Wang et al. 2009). Kim et al. (2008) reported that antioxidant potential of buckwheat might be based on its phenolic acid composition. Previously, Sharma et al. (2012) reported high antioxidant and α-glucosidase enzyme inhibitory activity from leaves of tartary buckwheat. In another study, Stringer et al. (2013) observed that consumption of buckwheat was associated with increase in gastrointestinal satiety hormones in type 2 diabetes individuals. Furthermore, reduction of serum glucose in Streptozotocin-diabetic rats was also observed with buckwheat concentrate (Kawa et al. 2003).

Improvement of lipid metabolism in diabetic rat was found with buckwheat sprouts (Watanabe and Ayugase 2010). However, studies investigating anti-diabetic properties of buckwheat grain and flour are very limited.

Another underutilized cereal grain with high nutritional qualities is teff (*Eragrostis tef*) commonly grown under tropical climate. Teff is a good source of iron and calcium, as well as protein and other cellular bioactive compounds. The major phenolic acids of teff are protocatechuic, benzoic, vanillic, caffeic, syringic, coumaric, ferulic, and cinnamic acid (Abebe et al. 2007; McDonough et al. 2000). High antioxidant activity linked to phenolic content was observed in teff (Kotaskova et al. 2016). Due to balanced nutritional profiles and rich source of phenolic bioactives, teff has been considered as healthy food source. However investigation related to anti-hyperglycemic property of teff is limited. Therefore, analysis of phenolic bioactive-linked antioxidant and anti-hyperglycemic properties of buckwheat and teff has significant relevance for its value added utilization.

2.5. Phenolic Bioactives and Human Health Benefits of Sorghum and Pearl Millet

Millets are also ancient grains and yet mostly underutilized in modern food systems. The importance of millets as food crops is mostly restricted in arid and semi-arid regions of the world. However, due to diverse health benefits, interest in millets as value-added food crop, especially as source of functional food ingredients is increasing rapidly. In general, millets are good source of protein (essential amino acids), fatty acids, minerals, vitamins, dietary fibers, and phenolics (Amadou et al. 2013). Furthermore, millets also have good prebiotic potentials and used as fermented foods in Africa. Phenolics are mostly present in bound forms in millet and major groups of phenolics are hydroxybenzoic acid, hydroxycinnamic acid, and flavonoids (Chandrasekara and Shahidi 2011). Major phenolic acids present in sorghum and other millet

grains are gallic, protocatechuic, p-hydroxybenzoic, gentisic, vanillic, syringic, ferulic, caffeic, *p*-coumaric, cinnamic, and sinapic acid (Dykes and Rooney 2006). High antioxidant and anti-hyperglycemic properties were reported previously in different millets. In this context, high α-amylase and α-glucosidase enzyme inhibitory activity was observed in sorghum extracts (Kim et al. 2011). Similarly, high antioxidant, low α-amylase and high and α-glucosidase enzyme inhibitory activity was observed in finger millet (*Elusine coracana*) (Kunyanga et al. 2012). In another study Shobana et al. (2010) reported hypoglycaemic, hypochloresteroleimic, nephroprotective properties in diabetic rat fed with finger millet seed coat extracts. Decrease in blood glucose level was also found in diabetic individuals after replacing regular 100% wheat flat bread (chapatti) with 30% millet+ 70% wheat flat bread (Pradhan et al. 2010).

Therefore, all these previous studies suggested that whole grains such as oats, buckwheat, teff and millets have significant antioxidant and anti-hyperglycemic potentials and can be targeted in dietary complimentary strategies to prevent and manage type 2 diabetes and associated complications. However the phenolic bioactive profiles and associated health benefits of these grains vary widely between species, genotypes, growing conditions, environment, and further due to different food processing methods. Therefore metabolically targeted rapid *in vitro* screening of these whole grains for their potential phenolic-linked antioxidant and anti-hyperglycemic functionalities has significant merit. Furthermore, it is also important to understand the effect of different food processing methods, growing conditions, and differences in genotypes on such health relevant functionalities of these whole grains in order to optimize and select for more expensive animal model based *in vivo* or clinical studies.

CHAPTER 3. OBJECTIVES

Based on the above scientific rationale the broad objective of this study was to analyze the phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities of specialty cereals such as oat, select pseudo-cereals, pearl millet, and sorghum in order to target them in improved dietary support strategies in the management of early stages of type 2 diabetes and associated complications. The specific objectives are:

1. To determine phenolic bioactive-linked antioxidant, anti-hyperglycemic, and gut health benefits of different oats, buckwheat, teff, and pearl millet using rapid *in vitro* assay models.
2. To compare phenolic bioactive–linked type 2 diabetes relevant benefits of oat groats and rolled oat from conventional and organic production systems.
3. To analyze phenolic bioactive-linked antioxidant and anti-hyperglycemic functionalities of different sorghum genotypes using rapid *in vitro* assay models.

CHAPTER 4. PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT, ANTI-HYPERGLYCEMIC, AND GUT HEALTH RELEVANT BENEFITS OF OAT, BUCKWHEAT, TEFF, AND PEARL MILLET

4.1. Abstract

Whole grain cereals, pseudo-cereals, and millets are rich source of human health promoting nutrients and can be targeted as part of balanced nutrition-based dietary support strategies to manage diet-linked non-communicable chronic diseases (NCDs) such as type 2 diabetes and its complications. However, human health relevant bioactive profiles such as phenolics and associated nutritional qualities of whole grains vary widely between species, cultivars, growing conditions, and further due to different post-harvest processing. Therefore, the objective of this study was to compare the phenolic-linked antioxidant, anti-hyperglycemic, and gut health relevant pathogenic bacterial inhibiting properties of different oats (whole grain organic, organic rolled, whole grain conventional, whole grain Quaker, and instant oatmeal), teff, buckwheat, and pearl millet using *in vitro* assay models. Cold water extracts of all grain samples were evaluated for total soluble phenolic (TSP) content, phenolic acid profiles, antioxidant activity, α-amylase and α-glucosidase enzyme inhibitory activities, and anti-bacterial activity against *Helicobacter pylori* using *in vitro* assay models. Among all samples, high TSP content, high antioxidant activity, and moderate type 2 diabetes relevant α-amylase and α-glucosidase enzyme inhibitory activities were observed in buckwheat, whole grain organic oat, organic rolled oat, and teff. Furthermore, anti-bacterial activity against *Helicobacter pylori* was also observed

in whole grain organic oat. Therefore, based on these *in vitro* rapid screening tools whole grain organic oat, organic rolled oat, buckwheat, and teff can be further targeted and evaluated as functional food ingredients or as part of dietary support strategies to manage chronic hyperglycemia and oxidative stress which are commonly associated with early stages of type 2 diabetes and its complications.

4.2. Introduction

Cereals, pseudo cereals, and millets are important grains that are integral part of human diet meeting the calorie and overall macro- and micronutrients needs for 70% of the global population (Fisher et al. 2014). Beyond the basic macro-nutrients, cereals, pseudo cereals, and millets are also rich source of human health promoting nutrients such as vitamins (thianin, niacin, riboflavin and pantothenic acid), minerals (calcium, magnesium, potassium, phosphorus, sodium and iron), amino acids (arginine and lysine), dietary fibers, and bioactive compounds (Slavin 2004). Furthermore, diverse groups of phenolic bioactives such as flavonoids, phenolic acids, and tannins are widely distributed in different grain components of cereal and also in pseudo-cereals and millets (Borneo and Leon 2012; Liu 2007). Therefore, improving access to such whole grains for their macro- and micronutrient balance and also for other health promoting bioactive compounds is essential to address needs of emerging global food and nutritional security coupled public health challenges (Godfray et al. 2010). In this context, health relevant and targeted bioactive profiles of whole grains can help to develop improved food quality and dietary support strategies for countering rapid rise in diet- and lifestyle-linked non-communicable chronic diseases (NCDs) which is a major global public health challenge in the 21st century (Gani et al. 2012).

Based on these above needs and scope, there are renewed interest in whole foods including whole grain cereals, pseudo-cereals, and millets to support balanced nutritional needs and for improved health relevant bioactive rich diets to counter and manage NCDs, such as type 2 diabetes, cardiovascular disease, lipid disorder, and obesity. Many *in vitro* and *in vivo* studies reported a substantial inverse association between whole grain consumption and risk of chronic diseases (Aune eta al. 2013; de Munter et al. 2007; Shahidi and Chandrasekara 2013) including type 2 diabetes and cardiovascular diseases. Previously, de Munter et al. (2007) observed that high intake of whole grain particularly bran intake significantly reduced risk of type 2 diabetes in over 150,000 women. Whole grains are known to slow digestion and absorption of carbohydrates and thus regulate postprandial blood glucose and insulin response (Aune et al. 2013). McKeown et al. (2004) observed inverse association of whole-grain intake with body mass index and fasting insulin in Framingham offspring study. Consumption of small grain like finger millet based diet resulted in significantly lower plasma glucose levels in six type 2 diabetes patients (Kumari and Sumathi 2002). The reduction of risk for type 2 diabetes may involve the synergistic effect of several whole grain components such as various phytochemicals, vitamin E, minerals and other physiological relevant health targeted bioactives.

Therefore, whole grains such as cereals, pseudo-cereals, and millets with rich phenolic bioactive profiles, micronutrients, and fiber are gaining increasing interest to develop functional foods and food ingredients and to incorporate in dietary and therapeutic strategies as part of the diet to manage NCDs, such as type 2 diabetes and cardiovascular diseases (Okarter and Liu 2010). In general, diet-based bioactives managing cellular and metabolic targets of chronic hyperglycemia, countering oxidative stress-induced chronic inflammation, improving insulin secretion and function, and enhancing cellular immunity responses are critical for overall

prevention and management of type 2 diabetes (Ley et al. 2014). Furthermore, providing protection against chronic infection is also essential, as type 2 diabetes patients are more susceptible to infectious diseases, and recovery from such infection is also difficult with advanced stages of diabetes (Larsen et al 2010; Naing et al. 2012). In this context, health targeted phenolic bioactives of plant-based foods, including cereals, pseudo-cereals and millets have diverse human health relevant cellular protective functions such as antioxidant, anti-hyperglycemic and antimicrobial properties and can be targeted as part of dietary support strategies to prevent and manage type 2 diabetes and its associated complications including chronic infection (Edreva et al. 2008; Fraga et al. 2010).

Therefore, targeting cereals, pseudo cereals, and millets such as oat (*Avena staiva*), teff (*Eragrostis tef* Trotter), buckwheat (*Fagopyrum esculentum*), and pearl millet (*Pennisetum glaucum*) with balanced nutritional qualities and additional human health relevant bioactive profiles to develop diverse dietary support strategies for prevention and management of NCDs such as type 2 diabetes has merit. Whole grain oat, teff, buckwheat, and pearl millet potentially have significantly higher total phenolic, flavonoid, and phenolic acid content, and associated antioxidant activity spread across all their grain components (Fardet 2010; Schaffer-Lequart et al. 2017). Previously, high antioxidant potential of oat, teff, buckwheat, and pearl millet have been reported which has indicated potential relevance against chronic oxidative stress linked metabolic breakdown, commonly associated with type 2 diabetes and other NCDs (Gimenez-Bastida et al. 2016; Hitayezu et al. 2015; Kotaskova et al. 2016; Kim et al., 2011; Li et al. 2016; Pushparaj and Urooj, 2014). Furthermore, phenolic bioactives of these whole grains have also shown potential to inhibit carbohydrate digestive enzymes such as α-amylase and glucose uptake modulating enzyme such as α-glucosidase activity (Liu et al. 2011) which in turn helps to

maintain post-prandial glucose homeostasis (Baron 1998). Therefore, targeting whole grain cereals, pseudo cereals, millets rich in health relevant phenolic bioactives and associated antioxidant, anti-hyperglycemic, and antimicrobial functionalities for dietary and therapeutic uses against early stages of type 2 diabetes and its associated complications has significant relevance. However, such health relevant phenolic bioactive profile and associated health benefits varies between species, cultivars, due to growing conditions (organic vs conventional) and due to other post-harvest storage and food processing variables (Brennan et al. 2011, Chnadrasekara et al. 2012). Therefore, metabolically targeted rapid screening strategy using *in vitro* assay models is important for wide biochemical analysis of health relevant bioactive profiles and understanding health benefits of whole grains or foods derived from such grains prior to incorporating in animal model-based study or clinically relevant analysis and for dietary interventions or to design functional foods and ingredients. Based on this rationale, the hypothesis of this proposed study was that cultivar or species of cereal, pseudo-cereals, and pearl millet with higher phenolic bioactive content would have high antioxidant, anti-hyperglycemic, and antimicrobial properties with gut health relevant benefits, and can be targeted as dietary support for managing early stages of type 2 diabetes and associated risks. In this proposed study, four grain species and foods derived from whole grains such as oat (whole grain organic and conventional, rolled oat, whole grain and oatmeal from commercial sources), commercial teff flour, organically grown buckwheat, and one pearl millet accession were targeted and screened for total soluble phenolic (TSP) content, characterization of phenolic acids (high performance liquid chromatography-HPLC), total antioxidant activity (DPPH and ABTS based), type 2 diabetes relevant α-amylase and α-glucosidase enzyme inhibitory activities, and antibacterial activity against ulcer causing bacteria *Helicobacter pylori* using rapid *in-vitro* assay models.

4.3. Materials and Methods

4.3.1. Materials: Whole grain organic oat (Doubting Thomas Farm, Minnesota), Grains' Miller oat (conventional from local Minnesota farm), organic rolled oat (Doubting Thomas Farm, Minnesota), whole grain oat from commercial source (Quaker, Ohio, USA), and instant oatmeal from commercial source (Family Gourmet Quick Oats, Bob's Red Mill, Oregon, USA), organic teff flour from commercial source (Bob's Red Mill, Oregon, USA), and organic buckwheat (Doubting Thomas Farm, Minnesota) and one pearl millet accession (Gem-X from North Dakota State University Millet Variety Trial) were collected from 2016 crop year and screened for health relevant phenolic bioactive-linked antioxidant, anti-hyperglycemic, and antibacterial (*Helicobacter pylori*) properties using rapid *in vitro* assay models.

4.3.2. Analytical Material: Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 3, 5-Dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and of analytical grade.

4.3.3. Extraction: Except teff flour and instant oatmeal, all other grain samples were milled using WonderMill Grain Mill (Wonder-Mill, Pocatello, ID). After milling all flour samples were extracted using cold water extraction procedure. For all cold water extractions, 10 g of each grain sample weighed and then homogenized with 50 mL of distilled water for 5 min using a Warring blender set at Low. All extracted samples were then centrifuged at 8,500 rpm for 20 minutes for two times. The sample extracts were then stored at 4ºC during the period of the biochemical analysis.

4.3.4. Total Soluble Phenolic Content: The total soluble phenolic content of all cold water extracted grain samples was analyzed by the Folin-Ciocalteu method (Shetty et al. 1995).

In a glass test tube, 0.5 mL of supernatant was transferred and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and vortexed and kept in dark incubation to stand for 60 min. After 60 min the absorbance of the mixture was read at 725 nm. The absorbance values were then expressed in milligram equivalents of gallic acid per gram dry weight (DW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

4.3.5. High Performance Liquid Chromatography (HPLC): The sample extracts of oat, buckwheat, teff, and pearl millet (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample from 100 µL of transferred supernatant was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 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 from 0% 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, 333, 540, 580 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of gallic acid, benzoic acid, ferulic acid, p-coumaric acid, o-coumaric acid, cinnamic acid, protocatechuic acid, catechin, caffeic acid, rutin, and quercetin in 100% methanol were used to calibrate the standard curves and retention time and individual phenolic acids were expressed as µg per gram dry weight.

4.3.6. Antioxidant Activity by 2, 2-diphenyl-1-picrylhydrazyl Free Radical (DPPH) Scavenging Assay:

The DPPH free radical scavenging activity of all grain samples was determined by an assay method modified by Kwon et al. (2006). At first, 1.25 mL of 60 µM DPPH in 95% ethanol was added to 250 µL of each sample extract, with the decrease in the absorbance monitored after 5 min at 517 nm (A517 extract). The absorbance of a control (distilled water instead of sample extract) was also recorded after 5 min at the same wavelength (A517 control). The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{A517(\text{control}) - A517(\text{extract})}{A517(\text{control})} \right) \text{x } 100$$

4.3.7. Antioxidant Activity by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) Free Radical Scavenging Assay:

The total antioxidant activity of all grain samples was also measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation as described by Re et al. (1999). $ABTS^+$ radical cation was prepared by mixing five mL of 7 mM ABTS stock solution with 88 µL of 140 mM potassium persulphate, and the mixture was allowed to stand in the dark at room temperature for 12 to 16 h to mature before use. Then $ABTS^+$ stock solution was then diluted with 95% ethanol (ratio 1:88) to give an absorbance of 0.70 ± 0.02 at 734 nm and was equilibrated. One milliliter ABTS was then added to Eppendorf tubes containing 50 µL of each sample extract and was mixed by a vortex mixer for 3-5sec and subsequently centrifuged for 1min at 7558*g*. After 2 min 30 sec incubation in the dark, absorbance of the mixtures was read at 734 nm. The readings were compared with the control, which contained 50 µL of 95% ethanol instead of the sample extract. The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}} \right) \text{x } 100$$

4.3.8. α-Amylase Enzyme Inhibition Assay: The *in vitro* α-amylase enzyme inhibitory activity of all grain samples was determined using an assay method modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A total of 500 µL of each 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) were incubated at room temperature for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube and then incubated at 25 ℃ for 10 min. Next, 1.0 mL of 3, 5 dinitrosalicylic acid color reagent was added. The test tubes were then incubated in a boiling water bath for 10 min and cooled to room temperature to stop the reaction. The reaction mixture was then diluted after adding 10 mL of distilled water, and 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 extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. The *a*-amylase enzyme inhibitory activity of all undiluted grain extracts was then calculated according to the equation below:

$$\% \text{ Inhibition} = \left(\frac{\text{A540(Control)} - [\text{A540(Extract)} - \text{A540(Sample blank)}]}{\text{A540(Control)}} \right) \text{x } 100$$

4.3.9. α-Glucosidase Enzyme Inhibition Assay: The α-glucosidase enzyme inhibition assay method was modified from the Worthington Enzyme Manual (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 ℃ for 10 min. After pre-incubation, 50 µL of 5mM 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 ℃ for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (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 enzyme inhibitory activity of all grain samples was expressed as a percentage of inhibition and calculated as follows:

$$\%\ \text{Inhibition} = \left(\frac{\Delta\ \text{A405(control)} - \Delta\ \text{A405(extract)}}{\Delta\ \text{A405(control)}} \right) \text{x } 100$$

4.3.10. Helicobacter pylori Inhibition Disc Assay: Isolate of *H. pylori* (strain ATCC 43579) were obtained from the American Type Culture Collection (Rockville, MD, USA). Standard plating medium was prepared according to Stevenson et al. (2000) using 10 g of special peptone, 15 g of granulated agar, 5 g of sodium chloride, 5 g of yeast extract and 5 g of beef extract per liter of distilled water. Antimicrobial activity of all grain sample extracts against *H. pylori* was tested by the standard agar diffusion method. One-hundred (100) mL of stock *H. pylori* was added to test tubes containing 10 mL of broth media and incubated at 37°C for 48 h. One- hundred (100) mL (1×10^8 cfu/mL) was seeded on the surface of the agar–starch plates. Sterile paper disks (0.67 cm diameter) containing 100 µL of the sample extracts were placed onto the surface of the seeded agar plate. Plates were incubated at 37°C for 48 h in Gas Pack jars (BBL Microbiology Systems, Cockeysville, MD) with BBL microaerophilic envelopes. The diameter of the inhibition zone (no growth) surrounding each disk was measured and reported as the zone of inhibition in millimeters.

4.3.11. Lactic Acid Bacterial Proliferation Assay: Initially, 100 mL of frozen stock from the lactic acid bacterial strain *Bifidobacterium longum* was inoculated into 10 mL of MRS broth (Difco) and incubated for 24 h at 37C. Then, 100 mL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37C. Sample volumes of 1 mL were filter sterilized using sterile filters Millex GP 0.22 mm (Millipore Corp., Bedford, MA). Filter sterilized oat sample extracts (1 mL) and 100 mL of the 48-h grown strain (diluted 100 times in sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37C for 24 h. Control with 1 mL of sterile distilled water instead of sample extract was also included. One hundred microliters of the serially diluted samples were plated in triplicates every 0, 6, 9, 12 and 24 h on MRS agar (Difco) plates and incubated in anaerobic BBL GasPak jars (Becton, Dickinson & Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson & Co.) at 37C for 48 h to determine the cfu/mL. This assay were undertaken to confirm that extracts inhibiting pathogenic bacteria did not inhibit beneficial bacteria which do not depend on oxygen metabolism for energy generation.

4.3.12. Statistical Analysis: Two extractions were performed for each sample, and all *in vitro* assays were replicated six times for each extraction (n = 12). Experiments for *H. pylori* inhibition assay were run in triplicates and the entire assay was run two times (n = 6). Means, standard errors, and standard deviations were calculated from replicates using MS-Excel. All data was subjected to a one- way ANOVA using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC), and the least mean square differences were compared using Tukey's test ($p < 0.05$).

4.4. Results and Discussion

4.4.1. Total Soluble Phenolic Content and Phenolic Acids Characterization: Phenolic acid content and composition of whole grains vary widely among different species, among different cultivars within same species, due to different growing conditions, and with different post-harvest storage conditions and food processing methods (Belobrajdic and Bird 2013). Therefore, screening and optimization of health relevant bioactive phenolic compounds of whole grains is essential prior to incorporating them in human health relevant dietary applications. Total soluble phenolic (TSP) content of different oat samples (whole grain organic, organic rolled oat, whole grain conventional, whole grain Quaker, and instant oatmeal), organic buckwheat, teff, and pearl millet was determined using Folin-Ciocalteu method (Shetty et al. 1995). In the current study, significant statistical differences ($p<0.05$) in TSP content was observed between different oat samples, buckwheat, teff, and pearl millet (Fig. 4.1).

Among all grain samples, buckwheat (2.8 mg GAE/ g DW) had highest TSP content followed by whole grain organic oat (2.4 mg GAE/ g DW), and the values were statistically significant when compared to all other oat samples, teff, and pearl millet. Similar range of phenolic content was previously reported in buckwheat and whole grain oat extracts (Alvarez-Jubete et al. 2010; Cai et al. 2011; Hodzic et al. 2009). In this study, the lowest TSP content was observed in instant oatmeal (1.2 mg GAE/ g DW) and it was significantly lower than whole grain organic and organic rolled oat. Loss of phenolic content of oat was observed previously due to commercial processing (drum drying of steamrolled oat) (Bryngelsson et al. 2002). This previous study also reported that reduction of phenolic content was mostly due to the decrease in concentration of specific phenolics such as cinnamic acid and avenanthramides (Bryngelsson et al. 2002).

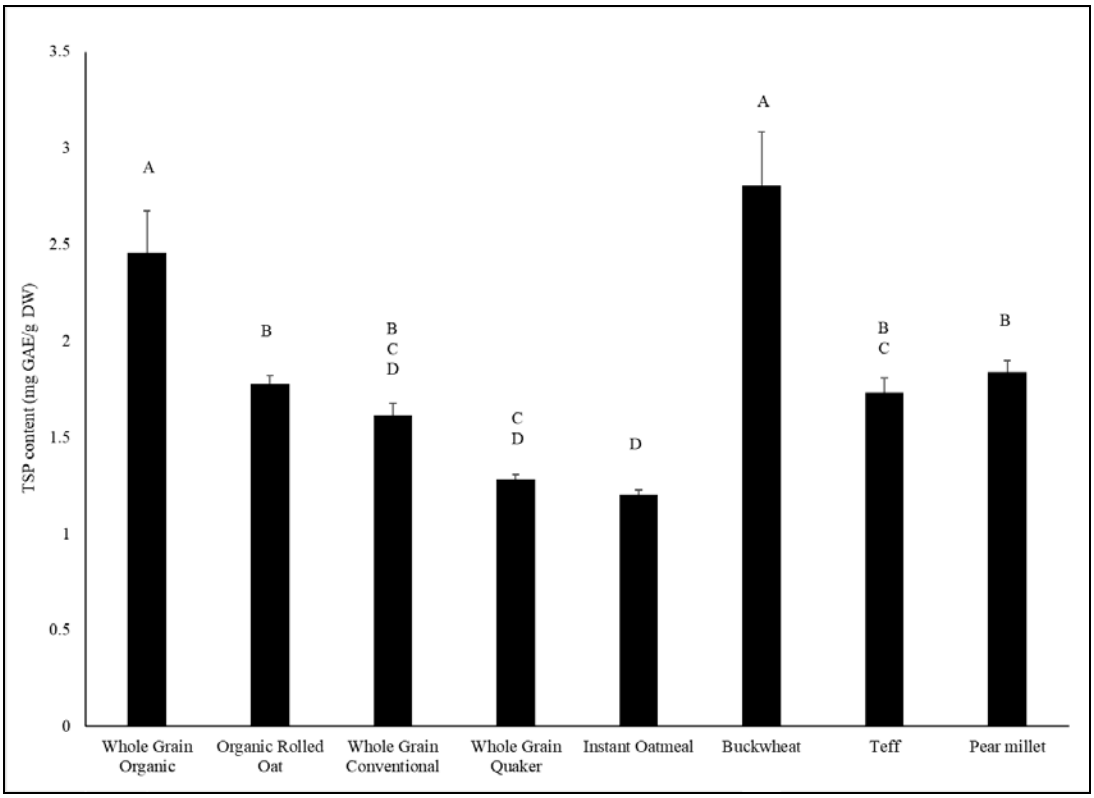

Figure 4.1. Total soluble phenolic (TSP) content (mg GAE/ g D.W.) of different oats (whole grain organic, organic rolled oat, whole grain conventional, whole grain Quaker, and instant oatmeal), buckwheat, teff and pearl millet. Different letters indicate significant differences in TSP content between all grain samples at the $p<0.05$ level.

In another study, Xu et al (2009) observed changes in phenolic content and associated antioxidant activity in oats with steeping treatments. Therefore, lower TSP content found in whole grain Quaker oat and instant oatmeal in this study was probably due to the loss of phenolic compounds during commercial processing. However, the TSP content of whole grain oat from conventional production system was at par with whole grain oat from commercial source (Quaker). Therefore, not only just post-harvest processing, but also the growing conditions, environment, and cultivar might have significant impact on TSP content of oats. Other minor grains such as teff and different millets are also gaining increasing interest especially for healthy

food design due to their balanced nutritional profile and associated health benefits (Dykes and Rooney 2007).

Table 4.1. Individual phenolic acid concentration in different oats (whole grain organic, organic rolled oat, whole grain conventional, whole grain Quaker, and instant oatmeal), buckwheat, teff and pearl millet sample.

Sample	Gallic acid[a]	Catechin	Ferulic acid	*p*-coumaric acid	Protocatechuic acid	Benzoic acid	Quercetin	Rutin	Cinnamic Acid
Whole Grain Organic Oat	0.7e	1.4e	4.4b	ND	ND	0.2c	ND	ND	ND
Organic Rolled Oat	0.6e	7.7b	3.6c	ND	ND	0.4c	ND	ND	ND
Whole Grain Conventional Oat	0.6e	1.6d	ND	0.6c	ND	2.9a	ND	ND	ND
Whole Grain Quaker	10.1a	1.9c	ND	ND	ND	1.4b	ND	ND	ND
Instant Oatmeal	0.7e	1.4e	ND	ND	ND	1.1b	ND	ND	ND
Buckwheat	6.4b	20.4a	19.7a	5.1a	1.8b	ND	4.2	ND	ND
Teff	5.5c	1.3e	ND	2.5b	4.8a	ND	ND	1.8	ND
Pearl millet	4.8d	ND	0.9d	0.1c	1.5b	0.3c	ND	ND	0.7

[a] Expressed as microgram per gram dry weight (µg/g DW)
Different lowercase letters in a column indicate significant differences ($p< 0.05$) in individual phenolic acid among all grain samples.
ND- not detected

In this study, Pearl millet (1.8 mg GAE/ g DW) and teff (1.7 mg GAE/ g DW) also had high TSP content and it was at par with organic rolled oat and whole grain oat from conventional production system. Dykes and Rooney (2006) previously reported similar baseline value of phenolic content in pearl millet and teff. Overall, the current study indicated that TSP content

vary significantly among different cereals, pseudo-cereal, and millet, and also due to different growing conditions (organic vs conventional) and different processing methods (whole grain, rolled and commercial processing). However, more than total soluble phenolic content, individual phenolic acid concentration varies significantly among species, cultivars, growing conditions, due to different abiotic and biotic stress pressure and due to different post-harvest food processing methods (Emmons et al. 1999). Based on the above rationale, phenolic acids of all grain samples were characterized using high performance liquid chromatography (HPLC) (Table 4.1).

Similar to the TSP content, higher concentration of individual phenolic acids was also observed in buckwheat. Major phenolics found in buckwheat sample were catechin, ferulic acid, gallic acid, *p*-coumaric acid, protocatechuic acid, and quercetin. Among all grain samples, significantly higher ($p< 0.05$) concentration of catechin and ferulic acid were observed in buckwheat. Furthermore, quercetin was only detected in buckwheat. Previously Guo et al. (2011) and Gallardo et al. (2006) reported ferulic, caffeic, gallic, p-coumaric, hydroxybenzoic, sinapic, and vanillic acid as major phenolic compounds in buckwheat.

In whole grain organic oat and organic rolled oat, ferulic acid, catechin, gallic acid, and benzoic acid were found. Similar phenolic acid profile found in these two oat samples might be due to their same growing condition, location, and from the same crop year. However, ferulic acid content slightly decreased after partial steaming and rolling in organic rolled oat, while as catechin and benzoic acid concentration slightly increased with this processing step. Among different oat samples, significantly higher concentration of ferulic acid was observed in whole grain organic oat, followed by organic rolled oat. Interestingly, ferulic acid was not detected in other oat samples such as whole grain oat from conventional production system, whole grain oat

from Quaker and instant oatmeal. Previously Mishra et al. (2017) reported higher ferulic acid concentration in different rye cultivars from organic production system when compared to same rye cultivars from conventional production system. However, *p*-coumaric acid was only detected in whole grain oat from conventional production system and same oat sample also had significantly higher ($p< 0.05$) benzoic acid content. However, significantly high ($p< 0.05$) gallic acid content was observed in whole grain Quaker oat. Previously, *p*-coumaric acid, sinapic acid, syringic acid, *p*-hydroxybenzoic acid, ferulic acid, caffeic acid, vanillic acid, Avenathramide A, Avenathramide B were the major phenolic acids reported in oat (Chen et al. 2004; Emmons et al. 1999). The reason for not finding some of these major phenolics of oat was probably due to the food grade (cold water) extraction and HPLC protocol used in the current study.

In commercial teff flour, gallic acid, catechin, *p*-coumaric acid, protocatechuic acid, and rutin were found as major phenolic compounds. Significantly high ($p< 0.05$) protocatechuic acid was observed in teff, and rutin was only detected in this grain sample. Ferulic acid, *p*-coumaric acid, gallic acid, catechin, vanillic acid, syringic acid, *p*-hydroxybenzoic acid, rutin, and quercetin were previously reported in whole grain extract of brown and white teff flour (Kotaskova et al. 2016; Salawu et al. 2014). Free and conjugated forms of phenolic acids such as derivatives of hydroxybenzoic and hydroxycinnamic acids are most common in millets (Shahidi and Chandrasekara 2013). In the current study, gallic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, benzoic acid, and cinnamic acid were found in the pearl millet (cv. Gem X) sample. Among all phenolic acids, high gallic and protocatechuic acid were observed in the pearl millet. Furthermore, cinnamic acid was only detected in the pearl millet and was not found in other grain sample. Phenolic acid results of the current study suggested that more than total phenolic content, phenolic acid profile and individual phenolic acid content vary significantly among

different whole grains. Such wide variation in phenolic composition of different whole grains also dictates their cellular and metabolic bioactive functionalities and associated human health benefits. Therefore, not only just phenolic content and composition, but screening of different whole grains for their phenolic bioactive-linked functionalities such as antioxidant, anti-hyperglycemic, and antimicrobial properties is also essential, especially prior to targeting in different dietary and therapeutic applications.

4.4.2. Total Antioxidant Activity (DPPH & ABTS Free Radical Scavenging) Assay: The renewed interest in phenolics of plant based food is mostly due to its high antioxidant potential, which has significant relevance both in improving post-harvest preservation of foods and for human health related applications (Lobo et al. 2010). Phenolics are potent antioxidants, and can directly quench free radicals or can upregulate endogenous cellular defense responses to mitigate overall oxidative stress in cells (Rice-Evans et al. 1997). Countering oxidative stress and maintaining cellular redox homeostasis is critical for managing chronic inflammation and other metabolic breakdowns commonly associated with NCDs such as type 2 diabetes, cardiovascular diseases, and cancer (Fernanadez-Sanchez et al. 2011; Visioli et al. 2011). Therefore, plant-based foods such as whole grains with high phenolic content and high antioxidant potentials are gaining increasing interest for designing human health relevant functional foods and food ingredients. In the current study, antioxidant activity of all grain samples was determined using two different free radical scavenging assays (DPPH and ABTS). Significantly high antioxidant activity based on both ABTS and DPPH free radical scavenging assay was observed in buckwheat, teff, and pearl millet (Fig 4.2 & 4.3). Interestingly, significantly high ($p<0.05$) antioxidant activity (80-88% inhibition) was observed in whole grain organic oat and organic rolled oat sample and it was at par with buckwheat, teff, and pearl millet when measured using

ABTS free radical scavenging assay. The differences in antioxidant activity of grain samples, especially for oats between two assays might be due to the higher affinity of ABTS free radicals towards hydrophilic antioxidants when compared to the DPPH free radicals (Re et al. 1999). Higher total soluble phenolic content of whole grain organic oat, and organic rolled oat was positively correlated with antioxidant activity based on ABTS free radical scavenging assay.

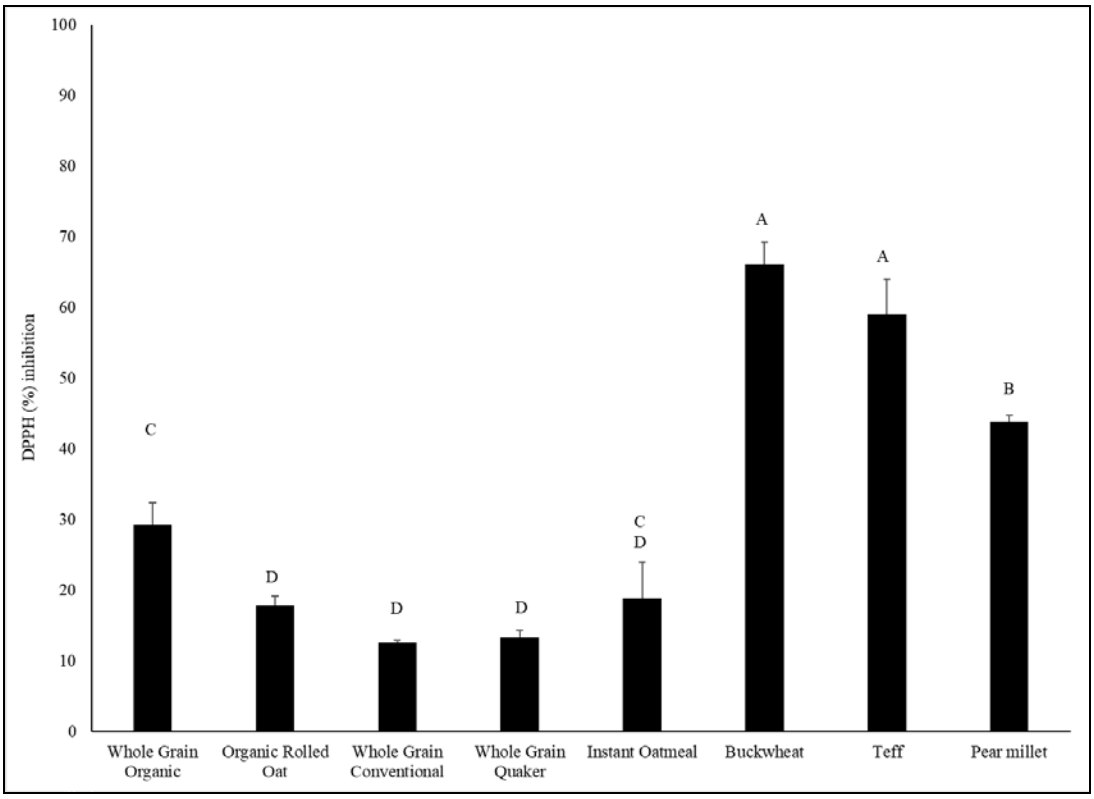

Figure 4.2. Total antioxidant activity (DPPH % inhibition) of different oats (whole grain organic, organic rolled oat, whole grain conventional, whole grain Quaker, and instant oatmeal), buckwheat, teff and pearl millet. Different letters indicate significant differences between grain samples at the $p<0.05$ level.

Similar to the TSP content, low antioxidant activity (based on both DPPH and ABTS) was observed in whole grain conventional oat, whole grain Quaker oat, and in instant oatmeal. Cai et al. (2011) reported similar DPPH assay based antioxidant activity in ethyl acetate, *n*-butanol and water fraction of ethanol crude extract from oats. Other previous studies also

reported high antioxidant activity in buckwheat, oat, teff, and pearl millet (Chandrasekara and Shahidi 2011; Chu et al. 2013; Inglett et al. 2011; Kotaskova et al. 2016). Increased antioxidant activity with roasting was previously observed in oat (Gujral et al. 2011).

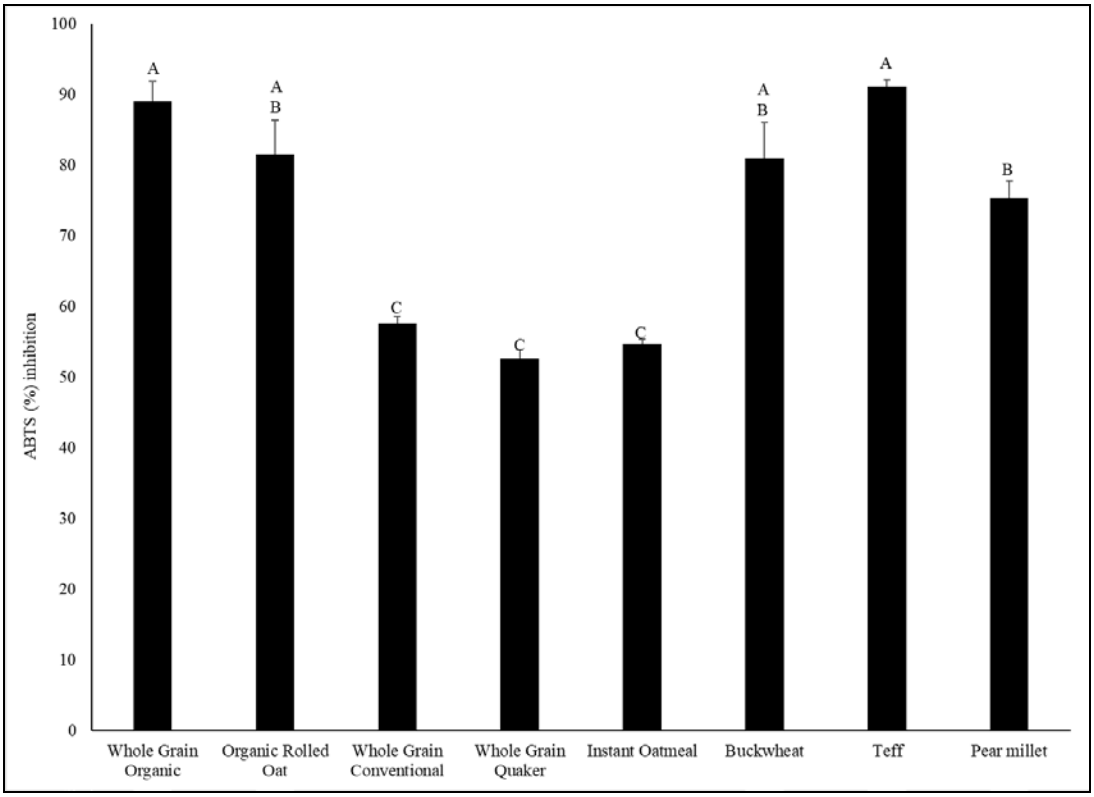

Figure 4.3. Total antioxidant activity (ABTS % inhibition) of different oats (whole grain organic, organic rolled oat, whole grain conventional, whole grain Quaker, and instant oatmeal), buckwheat, teff and pearl millet. Different letters indicate significant differences between grain samples at the $p<0.05$ level.

However, in the current study the antioxidant activity of instant oatmeal based on DPPH based assay was at par with whole grain organic oat, however the antioxidant activity of same sample was significantly lower when determined using ABTS based assay. Overall, high antioxidant activity was observed in buckwheat, teff, and pearl millet and this was probably due to their phenolic acid composition and presence of other metabolically relevant bioactives. High antioxidant activity of teff was probably due to the higher protocatechuic acid content and

presence of rutin (Li et al. 2011). Similarly, high ferulic acid, gallic acid, and catechin in buckwheat and gallic and protocatechuic acid in pearl millet might be contributed to high antioxidant activity of these grains (Kerio et al. 2013; Urbaniak et al. 2013). The results of this study suggested that buckwheat, teff , pearl millet, and whole grain organic oat have high antioxidant potentials, and these whole grains can be targeted in dietary intervention strategies to counter chronic oxidative stress commonly associated with type 2 diabetes and other NCDs. These whole grains with high antioxidant function can be especially targeted to replace or complement with calorie dense and hyper-processed cereals as part of overall dietary interventions to prevent and manage type 2 diabetes and cardiovascular diseases. Furthermore, beyond antioxidant property, these whole grains and their phenolic bioactives also have other human health relevant functionalities and can provide protection against other relevant cellular and metabolic breakdowns commonly involved in the pathogenesis of chronic diseases such as type 2 diabetes.

4.4.3. Anti-hyperglycemic Properties (α-Amylase and α-Glucosidase Enzyme Inhibitory Activities): Managing chronic hyperglycemia and maintaining glucose homeostasis is the major focus in the management of type 2 diabetes (Inzucchi et al. 2015). Slow breakdown of complex carbohydrate in the intestine and subsequent low uptake of glucose in blood is desirable for management of post-prandial hyperglycemia (Khan et al. 2014). Therefore, inhibiting key enzymes such as α-amylase and α-glucosidase involved in carbohydrate metabolism are common therapeutic approach to manage chronic hyperglycemia. However, major synthetic drugs such as Acarbose, Miglitol, Voglibose used as α-amylase and α-glucosidase inhibitors to manage chronic hyperglycemia have harmful side effects such as diarrhea, flatulence, and other digestive complications (Rosak and Mertes 2012). Therefore, finding α-amylase and α-glucosidase

enzyme inhibitors from natural plant-based food sources have significant merit, especially for developing dietary support strategies to counter chronic hyperglycemia commonly associated with type 2 diabetes. Moderate α-glucosidase enzyme inhibitory activity was previously observed in tartary buckwheat (Qin et al. 2013). Similarly, high anti-diabetic activity of oat β-D-glucan was also observed in mice study (Liu et al. 2016). Based on the above rationale and previous findings, α-amylase and α-glucosidase enzyme inhibitory activities of all grain samples were determined using *in vitro* assays.

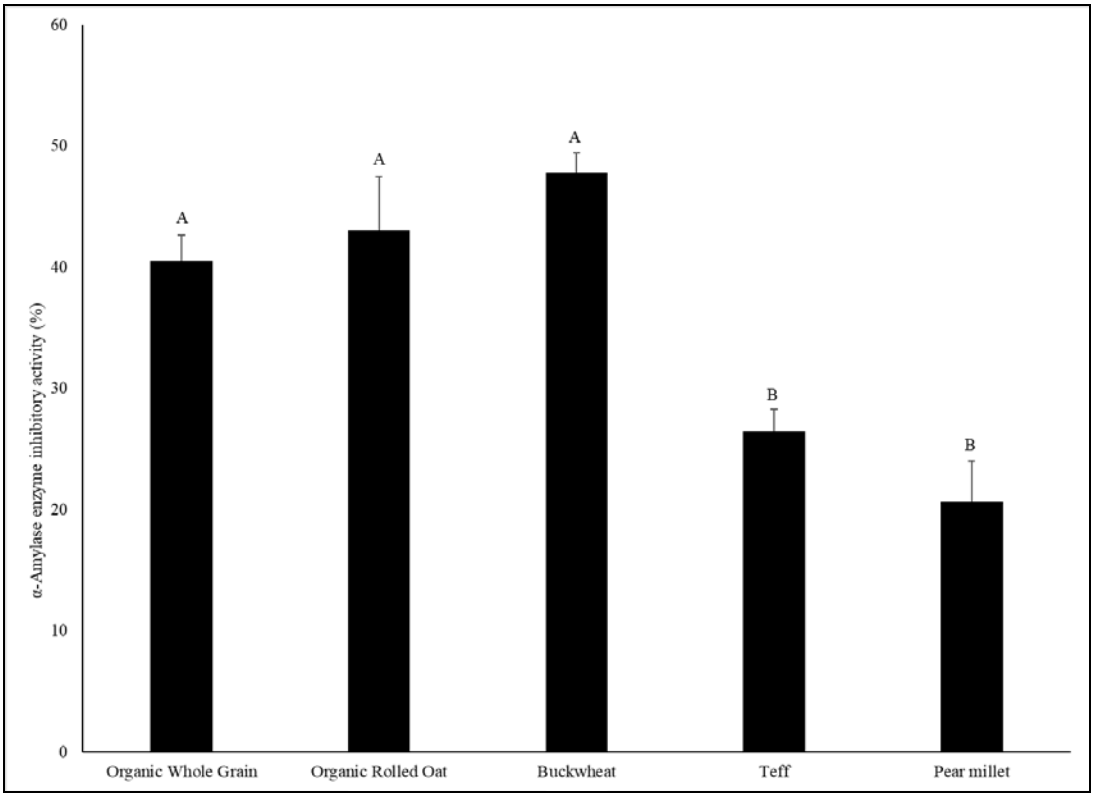

Figure 4.4. Alpha-amylase inhibitory activity (%) of undiluted sample of different oats (whole grain organic, organic rolled oat), buckwheat, teff and pearl millet. Different letters indicate significant differences between grain samples at the $p<0.05$ level.

In the current study, moderate α-amylase enzyme inhibitory activity (40-47%) was observed in buckwheat, organic rolled oat, and whole grain organic oat while low α-amylase enzyme inhibitory activity (20-26%) was observed in teff and pearl millet (Fig 4.4). The α-

amylase enzyme inhibitory activity of buckwheat, organic rolled oat, and whole grain organic oat was significantly high (p<0.05) when compared to teff and pearl millet.

Table 4.2. α-Glucosidase enzyme inhibitory activity (%) of different oats (whole grain organic, organic rolled oat), buckwheat, teff and pearl millet.

Sample	Undiluted sample[a]	Half diluted sample	One-fifth diluted sample
Whole Grain Organic	38.3 ± 2.9a	38.2 ± 3.8a	33.1 ± 5.4a
Organic Rolled oats	22.6 ± 1.7bcd	17.6 ± 1.9bc	9.7 ± 1.1b
Whole Grain Conventional	17.2 ± 1.3def	14.4 ± 2.6bcd	NA
Whole Grain Quaker	13.7 ± 0.9ef	11.2 ± 1.7cd	NA
Instant Oatmeal	18.7 ± 1.3cde	16.5 ± 2.0bcd	NA
Buckwheat	25.8 ± 1.3bc	19.3 ± 1.4bc	13.8 ± 1.1b
Teff	29.9 ± 1.5b	21.3 ± 1.2b	16.2 ± 2.2b
Pearl millet	9.9 ± 3.5f	7.8 ± 2.8d	NA

[a] Mean ± standard error
Different lowercase letters indicate significant differences (p< 0.05)
NA- no activity

No α-amylase enzyme inhibitory activity was observed in whole grain oat from conventional production system, whole grain Quaker oat, and in instant oatmeal. The α-amylase enzyme inhibitory activity of grain samples was positively correlated with TSP content. Therefore, the results indicated that the α-amylase enzyme inhibitory activity might be related to TSP content or phenolic acid compositions of these grain samples. In this study, all grain samples had low to moderate (9-38% in undiluted sample) *in vitro* α-glucosidase enzyme

inhibitory activity (Table 4.2). Among all grain sample whole grain organic oat had significantly higher α-glucosidase enzyme inhibitory activity, followed by teff, buckwheat, and organic rolled oat.

Dose dependent response in α-glucosidase enzyme inhibitory activity was also observed in this study. Interestingly, pearl millet had lowest α-glucosidase enzyme inhibitory activity (9.9 % in undiluted sample). Overall, buckwheat, whole grain organic oat, organic rolled oat, and teff had both α-amylase and α-glucosidase enzyme inhibitory activities and therefore these whole grains can be targeted in dietary strategies to manage chronic hyperglycemia commonly associated with type 2 diabetes and other NCDs. However, further animal model based *in vivo* and clinical studies are required to validate this finding and to determine other anti-diabetic properties of these whole grains.

4.4.4. Gut Health Relevant Benefits (Helicobacter pylori Inhibition and Bifidobacterium longum Proliferation): In general, increased rate of infection and severity is most common in patients with type 1 and type 2 diabetes (Muller et al. 2005). The lack of metabolically controlled hyperglycemia in diabetic patients makes them more susceptible to chronic pathogenic infection, including colonization of *Helicobacter pylori* in the gastric antrum (Bener et al. 2007; Jeon et al. 2012; Ko et al. 2001). Overall, *H. pylori* infection-induced gastric ulcer is one of the most common infectious disease worldwide (McColl 2010). Therefore, finding antimicrobials with potential to reduce *H. pylori* infection in natural plant-based food sources is extremely important both for diabetic and non-diabetic patients. In this study, among all samples only whole grain organic oat showed inhibitory activity (> 2 mm) against *H. pylori* in disc assay (Fig 4.5).

Figure 4.5. *Helicobacter pylori* inhibitory activity (zone inhibition) of whole grain organic oat sample (2) compared to whole grain conventional oat (1) and organic rolled oat sample (3).

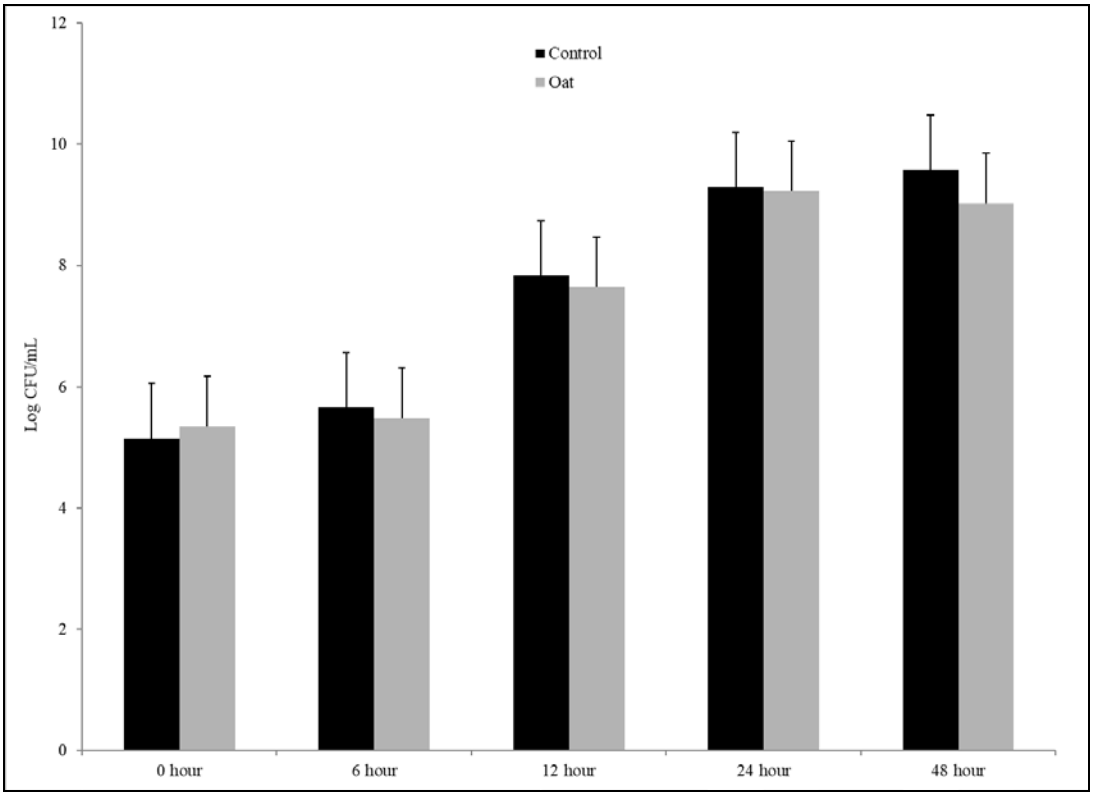

Figure 4.6. *Bifidobacterium longum* proliferation (Log CFU/mL) with whole grain organic oat sample at different time points (0, 6, 12, 24, and 48 hrs.) compared to the control.

This result indicated that whole grain organic oat not only just have antioxidant and anti-hyperglycemic properties, but also have potential anti-bacterial activity and can be targeted to reduce *H. pylori*-induced gastric infection. However, one of the major limitations of most synthetic antibiotics is its detrimental effect on beneficial gut microbiome. Therefore, it is also important to evaluate the impact of the whole grain organic oat against beneficial gut bacteria such probiotic *Bifidobacterium longum*. In this study, no significant inhibition of *Bifidobacterium longum* (still 24 h) was observed in whole grain organic oat (Fig 4.6). However, proliferation of *B. longum* was also not observed and the growth slightly reduced after 48 h when compared to the control.

Previously, increased adhesion of *Bifidobacterium* strains to colonic tissue was observed after exposure of bacteria to liquid oat-based media (Laine et al. 2003). Therefore, the positive effect of oat and oat bioactives on beneficial gut bacteria might be based on other mechanisms such inhibition of cellular energy metabolism targets that depend on oxygen in bacterial pathogens, and not just based on proliferation. However, inhibitory effect of the whole grain organic oat against microaerobic *H. pylori* found in this study has significant relevance in developing oat-based dietary strategies to target countering of gastric ulcer both in diabetic and non-diabetic patients complimenting with drug targets. Furthermore, same whole grain organic oat with high antioxidant and moderate anti-hyperglycemic functions can provide multiple benefits and can be targeted in dietary support strategies to manage type 2 diabetes and associated health risks.

4.5. Conclusions

Maintaining balanced nutrition in daily diet and making sound lifestyle choices are most critical to address emerging epidemic of NCDs, such as type 2 diabetes, cardiovascular disease,

obesity, and cancer worldwide. In this context, whole grains which are rich in human health promoting nutrients can be targeted to develop dietary support strategies to manage type 2 diabetes and other NCDs. Based on this rationale, whole grain oat from different sources, rolled oat, instant oatmeal, buckwheat, teff, and pearl millet were screened for health relevant phenolic bioactive-linked antioxidant, anti-hyperglycemic, and *H. pylori* infection relevant anti-bacterial properties using rapid *in vitro* assay models. High phenolic bioactive-linked antioxidant activity was observed in buckwheat, teff, pearl millet, whole grain organic oat, and in organic rolled oat. Similarly, moderate anti-hyperglycemic property was also observed in buckwheat, teff, whole grain organic oat, and in organic rolled oat. Furthermore anti-bacterial property against *H. pylori* was found in whole grain organic oat. The results of the current study suggested that phenolic bioactive-linked human health relevant functionalities of whole grains vary significantly among species, due to growing condition (organic vs conventional), and due to processing. However, overall whole grain organic oat, rolled oat, buckwheat, teff, and pearl millet can be targeted in dietary support-based interventions or to design functional foods complimentary pharmacological targets to counter chronic inflammation and chronic hyperglycemia commonly associated with type 2 diabetes and other NCDs.

CHAPTER 5. PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT AND ANTI-HYPERGLYCEMIC PROPERTIES OF OAT GROATS AND ROLLED OAT GROWN UNDER ORGANIC AND CONVENTIONAL PRODUCTION SYSTEMS

5.1. Abstract

The perception of higher nutritional and sensory quality, especially human health relevant bioactive profile of the organic produce is one major driving factor for its higher demand among consumers. In this context, stress modulated secondary metabolites such as health relevant phenolic bioactives from organic plant-based foods such as whole grains can be important targets for health benefits such as antioxidant, anti-diabetic, and anti-hypertensive properties. However the evidence regarding benefits of organic food production systems related to any human health relevant nutritional qualities of food crops such grains and legumes are limited. Therefore, the aim of this study was to compare phenolic bioactive-linked antioxidant and anti-hyperglycemic properties of oat groats and rolled oats from organic and conventional production systems using *in vitro* assay models. Based on this objective, total soluble phenolic (TSP) content, phenolic acid profile, antioxidant activity (based on DPPH & ABST assays), type 2 diabetes relevant α-amylase, and α-glucosidase enzyme inhibitory activities of oat groats (from two crop years) and rolled oat (single crop year) from conventional and organic production systems were investigated using targeted and relevant *in vitro* assays. In this study, significantly high TSP content and α-glucosidase enzyme inhibitory activity was observed in groats when compared to rolled oats. Furthermore, the statistically significant effect of crop year on phenolic bioactives, antioxidant

and anti-hyperglycemic properties of groats were observed, while the effect of organic vs conventional production systems on such human health relevant properties of oats were not conclusive in this study. However, significantly high catechin concentration was observed in organic groats across two crop years and in organic rolled oats when compared to groats and rolled oats from conventional production systems. The results of this study indicated that effect of environment (varied between crop years) and further food processing method (groats vs rolled oats) had more significant effect on phenolic bioactive-linked antioxidant and anti-hyperglycemic properties of oats over the differences in agricultural production systems (organic vs. conventional).

5.2. Introduction

Oat (*Avena sativa* L.) is widely considered as a healthy food source among major cereal grains due to its rich human health relevant nutritional profiles and associated benefits. Increasing consumer demand for whole grain oat, rolled oat, oatmeal, and oat flakes is driving its market share worldwide, and major food industries are also investing substantially to develop value-added oat-based food products and functional food ingredients from oats (Sterna et al. 2016). The nutritional benefits of oats are attributed to its high dietary fibers (β-glucans and other dietary fiber components), minerals (manganese, phosphorus, copper, iron, and magnesium), well-balanced protein, several vitamins (vitamin B and E), lipids (linoleic acid), and other phytochemicals (Singh et al. 2013). Furthermore, oat is also good source of natural antioxidants such as tocopherols, alk(en)ylresorcinols, sterols, phytic acids, phenolic acids and their derivatives, especially unique class of bioactive compounds known as “avenanthramides” (Dykes and Rooney 2007; Peterson et al. 2002). Major phenolic acids present in oats are caffeic, ferulic, vanillic, sinapic, *p*-coumaric, protocatechuic, and gallic acid (Cai et al. 2012). Due to the

presence of such diverse groups of bioactive compounds across different grain components and their higher bioavailability when consumed as part of a diet, oat-derived foods are considered as functional foods and can be targeted as dietary complimentary strategies to prevent and manage non-communicable chronic diseases (NCDs), such as cardiovascular diseases, obesity, dyslipidemia, cancer, and type 2 diabetes.

In this context, recent clinical and epidemiological studies suggested diverse dietary and health benefits of oat such as reduction of serum cholesterol level, lower risk of cardiovascular diseases, reduction of systolic and diastolic blood pressure, prevention of gastrointestinal cancer, immune-response enhancing activities, and prevention of type 2 diabetes (Evans et al. 2015; Martinez-Villaluenga and Penas 2017; Tosh 2013). Based on the outcomes of various such clinical and epidemiological studies, U.S. Food and Drug Administration and European Food and Safety Agency approved low cholesterol and cardiovascular disease protective health claims for oat-derived food products. However, majority of oat-based health research have focused on oat β-D-glucan or avenanthramides and their antioxidant, anti-diabetic, anti-cholesterol, and anticancer relevant properties (Blaszczyk et al. 2015; Hussain et al. 2018; Liu et al. 2016; Peterson et al. 2002). However, the health benefits of oat and oat-derived foods are beyond just the β-D-glucan and avenanthramides and few studies suggested that other health relevant bioactives compounds of oats, such as phenolic acids, protein, resistant starch and their synergistic effects when consumed as part of a diet might be relevant in managing chronic diseases such as type 2 diabetes, obesity, and cardiovascular diseases (Xu et al. 2017). The lipid and glucose metabolism relevant benefits of oat β-D-glucan is due to its function in increasing viscosity in the upper digestive tracts and by increasing the expression of plasma peptide Y-Y and intestine peptide Y-Y (Huang et al. 2011; Wood 2007). Such physiological and health

protective functions of β-D-glucan help to slow down carbohydrate breakdown and glucose absorption in the blood and also to lower appetite, both are critical for prevention and management of type 2 diabetes, especially to counter obesity associated diabetes (Liu et al. 2016). However, bioactive phenolics of whole grains such as oats also have carbohydrate metabolism relevant physiological functions and can help to improve glucose homeostasis in both obese and non-obese individuals (Hanhineva et al. 2010; Sarkar and Shetty 2014). Therefore, investigating health relevant bioactives of oats such as phenolics for targeting their human health relevant functionalities such as antioxidant and anti-hyperglycemic properties has significant merit, especially to build improved dietary support strategies in the management of NCDs such as early stages of type 2 diabetes and its associated complications.

However, the phenolic bioactive profiles and their associated health benefits of cereals such as oats vary widely among cultivars, growing conditions, due to various abiotic and biotic stress pressure, and further due to different post-harvest processing. One of the major driving factor for increasing whole food market is the perception of consumers that whole foods such as whole grains, especially from organic production system contain better nutritional profile when compared to whole and hyper-processed foods from conventional production system (Hunter et al. 2011; Kitchen et al. 2003; Strobel et al. 2001). Though the environmental benefits, especially soil health relevant benefits and better flavor profile of organic produce are well accepted, however the empirical evidence supporting superior nutritional quality and human health relevant benefits of organic produce are inconclusive (Lairon and Huber 2014; Mader et al. 2007; Mazzoncini et al. 2015). The hypothesis that organic produce have better bioactive profiles is based on the rationale that the absence of chemical fertilizer and pesticides may provoke higher biosynthesis of disease protective secondary metabolites in food crops (Rembialkowska

2007). Furthermore, diversity and higher population of beneficial microbes in rhizosphere of organic production system might also influence stimulation of health relevant phenolic bioactive profiles of food crops produced under such growing conditions. Previously, higher total phenolic content, and higher concentrations of ferulic and *p*-coumaric acid were observed in organically produce spring and winter wheat (Zuchowski et al. 2011). Similarly, higher ferulic and benzoic acid content along with high *in vitro* α-amylase inhibitory activity relevant to type 2 diabetes benefit were observed in organic rye cultivars when compared to same cultivars from conventional production system (Mishra et al. 2017). However, Dimberg et al. (2005) did not find any significant difference between organic and conventional oats for avenanthramides and hydroxyl-cinnamic acid concentrations over three years of production. They observed significant differences in bioactive profiles of oats between different crop years and cultivars and concluded that genotype × environment interaction is most critical for bioactive profile of oat and other grains. Therefore, evaluation of phenolic bioactive-linked type 2 diabetes relevant functionalities of oat groats and rolled oat from two different production systems (organic and conventional) over two crop years have significant merit. Based on this rationale, the aim of this study was to compare oat groats and rolled oats from organic and conventional production systems for phenolic bioactive-linked antioxidant and anti-hyperglycemic properties using rapid *in vitro* assay models.

5.3. Materials and Methods

5.3.1. Oat Sample: Organic oat groats and organic rolled oat (USDA certified organic) were collected from Doubting Thomas Farm (Moorhead, MN), while groats conventional and rolled oat conventional were collected from Agronomy Seed Farm (Casselton, ND) of North Dakota State University. Organic and conventional oat groats were collected from two different

crop year (2016 & 2018), while organic and conventional rolled oats were from 2017 crop year. All oat samples (organic and conventional; groats and rolled oat) are from single cultivar (spring hulless oat cultivar: Paul) developed at the North Dakota Agricultural Experiment Station and released in 1994.

5.3.2. Analytical Material: Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (30931-67-0), and 3, 5-Dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and of analytical grade.

5.3.3. Extraction: Groats and rolled oats from conventional and organic production systems were milled using WonderMill Grain Mill (Wonder-Mill, Pocatello, ID). After milling all samples were extracted using cold water extraction procedure. For all cold water extractions, 10 g of each grain sample weighed and then homogenized with 50 mL of distilled water for 5 min using a Warring blender set at Low. All extracted samples were then centrifuged at 8,500 rpm for 20 minutes for two times. The sample extracts were then stored at 4ºC during the period of the biochemical analysis.

5.3.4. Total Soluble Phenolic Content: The total soluble phenolic content of all cold water extracted oat samples was analyzed by the Folin-Ciocalteu method (Shetty et al. 1995). In a glass test tube, 0.5 mL of supernatant was transferred and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and vortexed and kept in dark incubation to stand for 60 min. After 60 min the absorbance of the mixture was read at 725 nm. The absorbance values were then expressed in milligram

equivalents of gallic acid per gram dry weight (DW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

5.3.5. High Performance Liquid Chromatography (HPLC): The sample extracts of oat (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample from 100 µL of transferred supernatant was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 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 from 0% 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, 333, 540, 580 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of gallic acid, benzoic acid, ferulic acid, p-coumaric acid, o-coumaric acid, cinnamic acid, protocatechuic acid, catechin, caffeic acid, rutin, and quercetin in 100% methanol were used to calibrate the standard curves and retention time.

5.3.6. Antioxidant Activity by 2, 2-diphenyl-1-picrylhydrazyl Free Radical (DPPH) Scavenging Assay: The DPPH free radical scavenging activity of oat samples was determined by an assay method modified by Kwon et al. (2006). At first, 1.25 mL of 60 µM DPPH in 95% ethanol was added to 250 µL of each sample extract, with the decrease in the absorbance monitored after 5 min at 517 nm (A517 extract). The absorbance of a control (distilled water instead of sample extract) was also recorded after 5 min at the same wavelength (A517 control). The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}} \right) \text{x } 100$$

5.3.7. Antioxidant Activity by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) Free Radical Scavenging Assay: The total antioxidant activity of oat samples was also measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation as described by Re et al. (1999). $ABTS^+$ radical cation was prepared by mixing five mL of 7 mM ABTS stock solution with 88 µL of 140 mM potassium persulphate, and the mixture was allowed to stand in the dark at room temperature for 12 to 16 h to mature before use. Then $ABTS^+$ stock solution was then diluted with 95% ethanol (ratio 1:88) to give an absorbance of 0.70 ± 0.02 at 734 nm and was equilibrated. One milliliter ABTS was then added to Eppendorf tubes containing 50 µL of each sample extract and was mixed by a vortex mixer for 3-5sec and subsequently centrifuged for 1min at 7558*g*. After 2 min 30 sec incubation in the dark, absorbance of the mixtures was read at 734 nm. The readings were compared with the control, which contained 50 µL of 95% ethanol instead of the sample extract. The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}} \right) \text{x } 100$$

5.3.8. α-Amylase Enzyme Inhibition Assay: The *in vitro* α-amylase enzyme inhibitory activity of oat samples was determined using an assay method modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A total of 500 µL of each 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) were incubated at room temperature for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with

0.006 M NaCl) was added to each tube and then incubated at 25 ℃ for 10 min. Next, 1.0 mL of 3, 5 dinitrosalicylic acid color reagent was added. The test tubes were then incubated in a boiling water bath for 10 min and cooled to room temperature to stop the reaction. The reaction mixture was then diluted after adding 10 mL of distilled water, and 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 extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. The *a*-amylase enzyme inhibitory activity of all undiluted oat extracts was then calculated according to the equation below:

$$\% \text{ Inhibition} = \left(\frac{\text{A540(Control)} - [\text{A540(Extract)} - \text{A540(Sample blank)}]}{\text{A540(Control)}} \right) \text{x } 100$$

5.3.9. α-Glucosidase Enzyme Inhibition Assay: The α-glucosidase enzyme inhibition assay method was modified from the Worthington Enzyme Manual (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 ℃ for 10 min. After pre-incubation, 50 µL of 5mM 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 ℃ for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (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 enzyme inhibitory activity of oat samples was expressed as a percentage of inhibition and calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{\Delta \text{ A405(control)} - \Delta \text{ A405(extract)}}{\Delta \text{ A405(control)}} \right) \text{x } 100$$

5.3.10. Statistical analysis: Two extractions were performed for each 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. Oat groats data from two different production systems and from two different crop years was subjected to two –way ANOVA, while rolled oat data from two production systems were analyzed using one- way ANOVA through the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC), and the least mean square differences between treatments and interactions were compared using Tukey's test ($p < 0.05$).

5.4. Results and Discussion

5.4.1. Total Soluble Phenolic Content and Phenolic Acids Characterization: In general, phenolic compounds are secondary metabolites of plants and provide protection against different biotic and abiotic stresses. Diverse group of such protective phenolic compounds and their derivatives are widely distributed in different plants including food crops such as cereals, legumes, fruits, and vegetables and when consumed as part of plant-based diet same phenolic compounds exhibit diverse protective and health promoting functions in humans and animal systems. Oat is considered as a good source of diverse phenolic compounds, and naked or hulless oats (*Avena nuda*) known as "groats" also have significant phenolic content and associated nutritional benefits (Peterson et al. 2001). Furthermore, phenolic compounds and other health relevant bioactives of oats have shown diverse health benefits such as antioxidant, anti-dyslipidemia and anti-diabetic functionalities in various *in vitro* and *in vivo* studies (Bratt et al. 2003; Cai et al. 2012; Handelman et al. 1999; Hitayezu et al. 2015; Martinez-Villaluenga and

Penas 2017; Peterson et al. 2001; Singh et al. 2013; Sterna et al. 2016). However, phenolic content and phenolic acid composition of oat and oat-derived foods may vary among cultivars, soil type, growing conditions, and due to diverse food processing methods. Therefore, evaluating the effect of different agricultural production systems such as organic vs conventional production practices on phenolic content, phenolic acid profiles, and associated antioxidant and anti-hyperglycemic functionalities of oat groats and rolled oat over 2 or more crop years have significant merit. Overall, in this study irrespective of the production systems (organic vs conventional) significantly high total soluble phenolic (TSP) content was observed in groats (1.7-2.4 mg GAE/ g DW) when compared to rolled oats (1.6-1.8 mg GAE/ g DW) (Fig 5.1 A & B). Statistically significant (p<0.05) effect of crop years on TSP content of groats was observed, as groats from 2016 crop year had high TSP content when compared to the oat grots from 2018 crop year. Oat sample from 2018 experienced heat and drought stress, while 2016 oat samples received higher rainfall during the harvest. In general, oat prefers cool and moist condition compared to dry and hot weather and that might resulted in different TSP contents of oat groats from two different crop years. Previously, Dimberg et al. (2005) observed significant differences in avenanthramides, hydroxycinnamic acids and truxinic acid concentrations of oats from different crop years and concluded this difference in weather condition likely had significant effect on these bioactive compounds. Similarly, significant variations in nutritional qualities were observed in wheat due to differences in crop years and crop rotation practices (Mader et al. 2007). In the current study, no significant difference in TSP content of groats from 2016 was observed between organic and conventional production system, while significantly (p<0.05) high TSP content was observed in organic groats from 2018 when compared to groats from conventional production system (Fig 5.1. A).

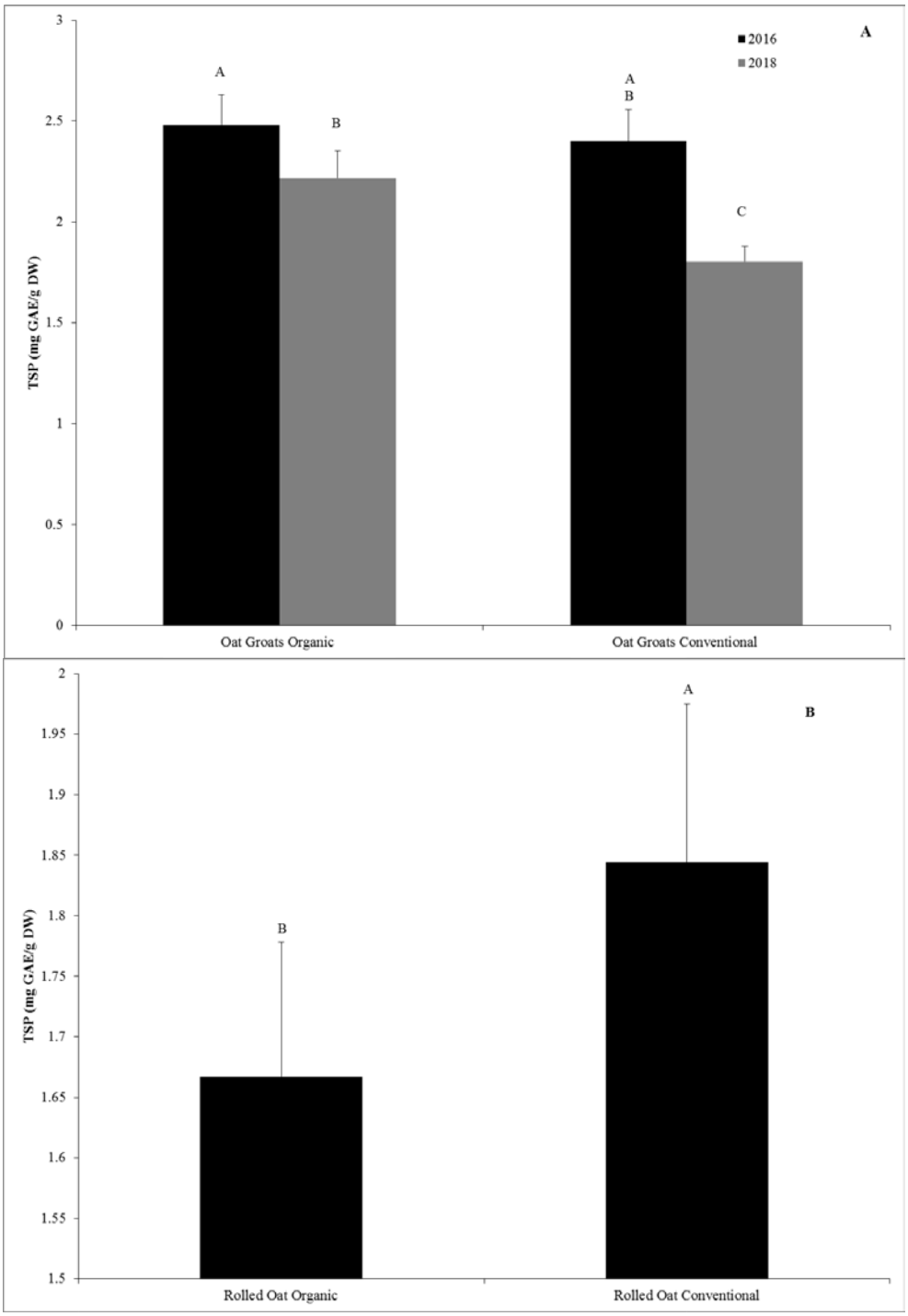

Figure 5.1. Total soluble phenolic (TSP) content (mg GAE/ g D.W.) of oats (A- oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional). Different letters indicate significant differences in TSP content between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at the $p<0.05$ level.

Table 5.1. Individual phenolic acid concentration in different oats (A-oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional).

A	Year	Gallic acid[a]	Catechin	Ferulic acid	Benzoic acid
Oat Groats Organic	2016	0.7c[b]	8.3a	3.9a	0.8c
Oat Groats Conventional		1.3a	1.3d	3.3b	0.8c
Oat Groats Organic	2018	0.9b	5.1b	1.7d	1.5b
Oat Groats Conventional		1.2a	4.2c	2.0c	2.2a

B	Gallic acid[a]	Catechin	Ferulic acid	Benzoic Acid
Rolled Oat Organic	1.0a	6.8a	3.2b	0.27b
Rolled Oat Conventional	1.0a	1.6b	3.8a	0.33 a

[a]Individual phenolic acid concentration was expressed as microgram per gram dry weight (µg/g DW).
[b]Different lower-case letters represent significant differences between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at (p<0.05).

However, rolled oat from conventional production system had significantly (p<0.05) high TSP content when compared to organic rolled oat. Therefore, in this study effect of agricultural production systems (organic vs conventional) on TSP content of groats and rolled oat were inconclusive. Previously, Dimberg et al. (2005) and Zorb et al. (2006) did not observe any significant differences in phenolic bioactives and other metabolites in oats and wheat between organic and conventional production systems. However, Zuchowski et al. (2011) and Mishra et al. (2017) reported significant differences in individual phenolic acid concentrations such as ferulic, *p*-coumaric, and protocatechuic acid in wheat and rye from organic and conventional production systems.

In the current study, gallic acid, catechin, ferulic acid, and benzoic acid were observed both in groats and rolled oats (Table 5.1 A & B). Interestingly, irrespective of the crop years significantly high ($p<0.05$) gallic acid concentration was observed in groats from conventional production system when compared to groats from organic production system. However, no significant differences in gallic acid concentration were observed in rolled oats from organic and conventional production systems. On the contrary, in all crop years significantly high ($p<0.05$) catechin was observed in organic groats and organic rolled oats when compared to groats and rolled oats from conventional production system. Previously, higher concentration of epicatechin gallate and rutin concentration were observed in buckwheat groats from organic production system (Kalinova and Vrchotova 2011). Higher catechin or epigallocatechin concentration in food crops from organic production system might be due to their role in structural supports, which are critical under higher abiotic or biotic stress pressure. However, ferulic and benzoic acid concentrations of groats and rolled oats were significantly high in conventional production system when compared to organic production system, except from 2016 crop year, where no statistically significant differences in ferulic and benzoic acid concentrations of groats were observed between two production systems. The result of this study suggested that phenolic acid profiles of oats might vary between organic and conventional production systems, however studies with multiple years and multiple locations are required to observe clear trends and to find more conclusive evidences.

5.4.2. Total Antioxidant Activity (DPPH & ABTS Free Radical Scavenging) Assay:

Nutritional qualities and health benefits of oats and oat-derived foods are much attributed to its superior antioxidant potentials. Therefore, determining antioxidant activity of groats and rolled oat and comparing between organic and conventional production systems for any potential

differences in antioxidant activities of oats has merit. In this study antioxidant activity of groats and rolled oats were determined using two different free radical scavenging assays (DPPH and ABTS).

Similar to TSP content, high antioxidant activity was observed in groats when compared to rolled oats (Fig 5.2 A&B; Fig 5.3 A&B). However, no significant differences in antioxidant activity of groats based on both DPPH and ABTS based assays were observed between organic and conventional production systems. The antioxidant activity of conventional rolled oat was significantly ($p<0.05$) higher when compared to organic rolled oat based on DPPH based assay. However, no significant differences in antioxidant activity of rolled oats from two different production systems were observed based on ABTS assay. Furthermore, similar to the TSP content, significant effect ($p<0.05$) of crop years on total antioxidant activity of groats was observed based on both DPPH and ABTS assays. However, higher antioxidant activity of groats was observed in 2018 crop year when measured using DPPH based assay, while ABTS based assay resulted in higher antioxidant activity of groats in 2016 crop year. The differences in antioxidant activities between two free radical scavenging assays (DPPH & ABTS) might be due to their different sensitivity and preferences towards hydrophilic and lipophilic antioxidants in oat matrix. Overall, the results of this study indicated that different agricultural production systems such as organic and conventional might not have significant impact on antioxidant activity of oats and might be relevant for other grains too. However, variations in environmental conditions between different crop years might have significant impact on antioxidant profiles and related functionalities of oats.

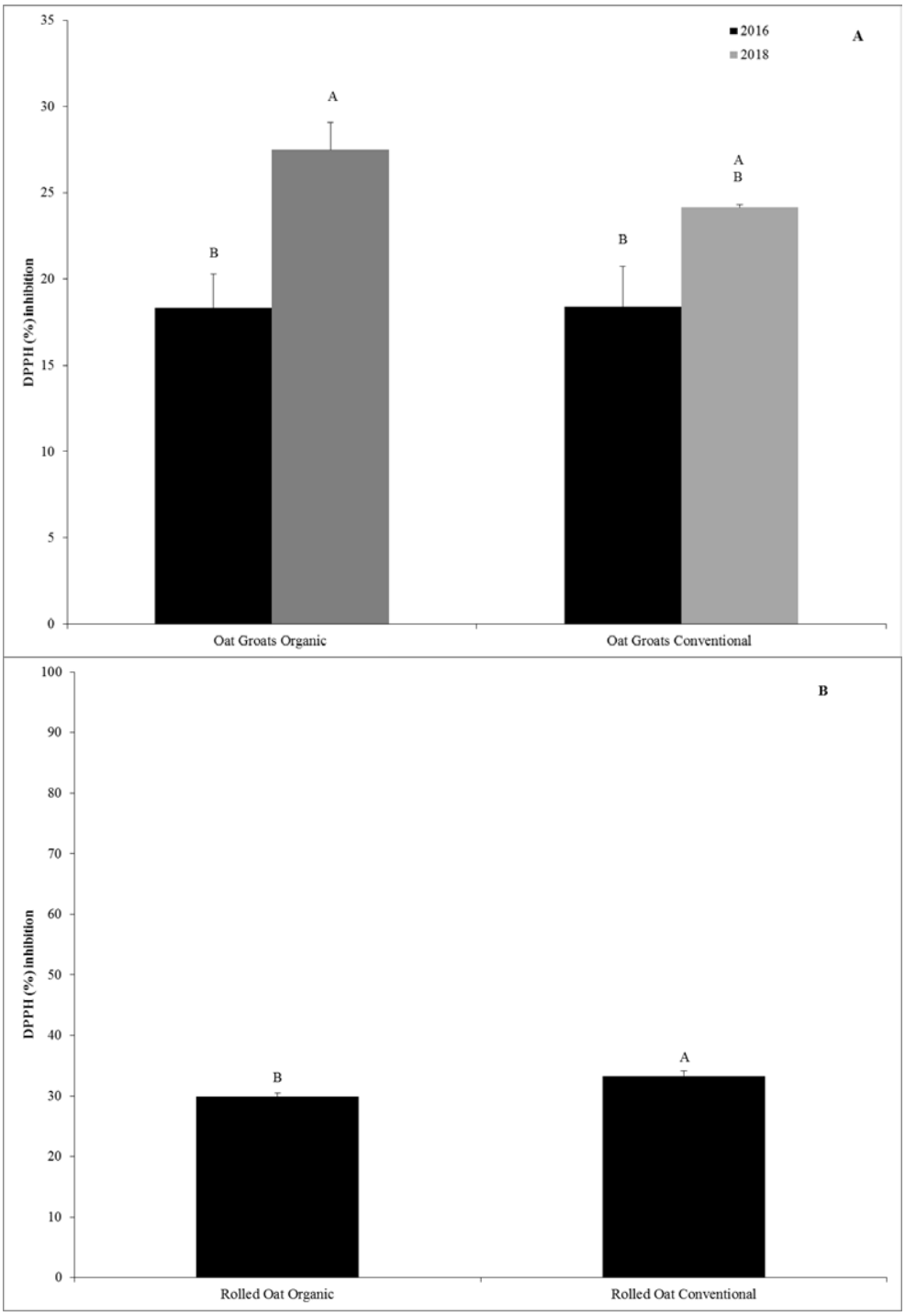

Figure 5.2. Total antioxidant activity (DPPH % inhibition) of oats (A- oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional). Different letters indicate significant differences in TSP content between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at the $p<0.05$ level.

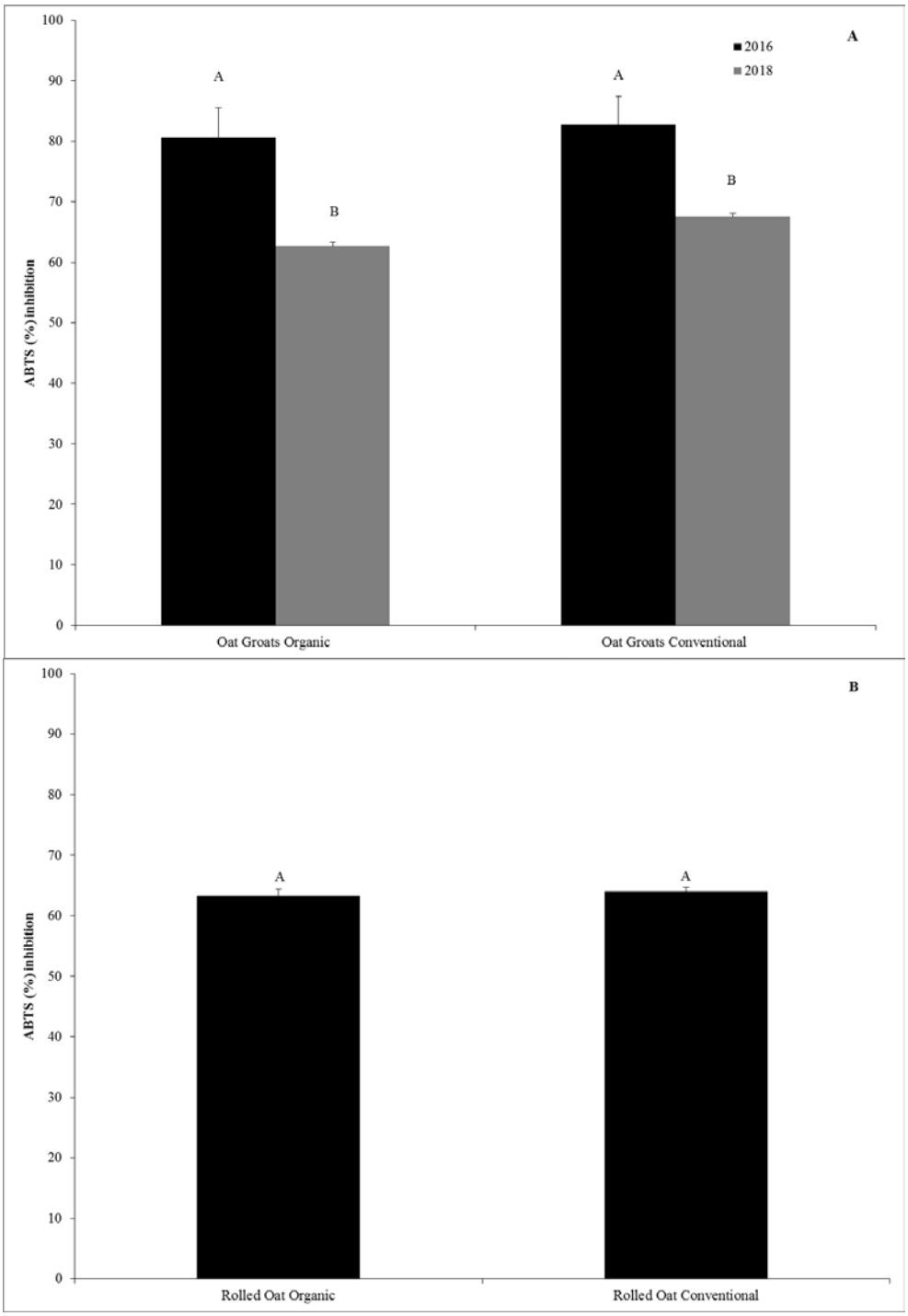

Figure 5.3. Total antioxidant activity (ABTS % inhibition) of oats (A- oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional). Different letters indicate significant differences in TSP content between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at the $p<0.05$ level.

5.4.3. Anti-hyperglycemic Properties (α-Amylase and α-Glucosidase Enzyme Inhibitory Activities): Glucose metabolism relevant key enzyme (α-amylase and α-glucosidase) inhibitory activities of groats and rolled oats from two different production systems (organic and conventional) were measured using *in vitro* assay models in order to determine their type 2 diabetes relevant anti-hyperglycemic functionalities. In this study, significantly high ($p<0.05$) α-amylase enzyme inhibitory activity was observed in groats from 2018 crop year when compared to groats from 2016 crop year (Fig 5.4 A & B). Furthermore, in 2018 crop year groats from conventional production system had significantly high α-amylase enzyme inhibitory activity. However, no significant differences in α-amylase enzyme inhibitory activity of groats between organic and conventional production systems were observed in 2016 crop year. Previously, Mishra et al. (2017) reported higher α-amylase enzyme inhibitory activity in rye cultivars from organic production system when compared to rye from conventional production system. In the current study, significantly high ($p<0.05$) α-amylase enzyme inhibitory activity was observed in organic rolled oat when compared to conventional rolled oat. However, α-amylase enzyme inhibitory activity of groats and rolled oats from two different production systems did not positively correlate with TSP content. Therefore, beyond phenolic compounds, variations in other bioactive components of oat grains such as β-D-glucan or resistant starch might have influenced differences in α-amylase enzyme inhibitory activity of groats and rolled oat. Previously, Hussain et al. (2018) reported significant α-amylase and α-glucosidase enzyme inhibitory activities of oat β-D-glucan. Overall, low to moderate α-glucosidase enzyme inhibitory activity was observed in groats and rolled oat from organic and conventional production systems in this study (Table 5.2 A&B).

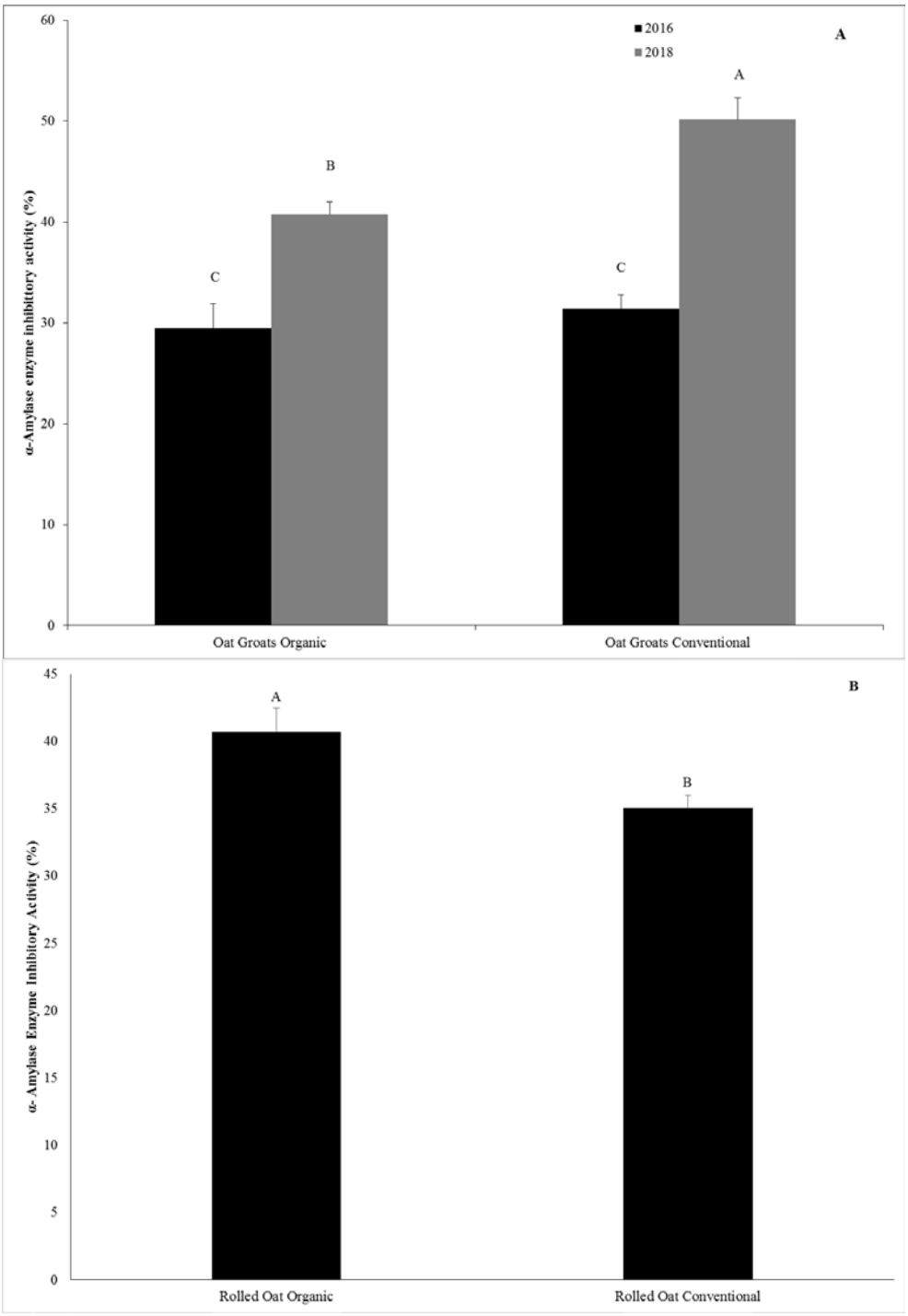

Figure 5.4. Alpha-amylase inhibitory activity (%) of undiluted sample of oats (A- oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional). Different letters indicate significant differences in TSP content between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at the $p<0.05$ level.

Table 5.2. α-Glucosidase enzyme inhibitory activity (%) of different oats (A-oat groats organic and conventional from 2016 and 2018 crop year and B- rolled oat organic and conventional).

A	Year	Undiluted sample	Half diluted sample	One-fifth diluted sample
Oat Groats Organic	2016	34.1 ± 1.9a[b]	26.2 ± 1.4a	28.2 ± 6.5a
Oat Groats Conventional		19.7 ± 2.4b	17 ± 1.9b	11.9 ± 1.4b
Oat Groats Organic	2018	23.2 ± 1b	17.4 ± 0.6b	12.8 ± 0.8b
Oat Groats Conventional		33 ± 2.8a	29.2 ± 2.9a	29.6 ± 3.7a

B	Undiluted sample	Half diluted sample	One-fifth diluted sample
Rolled Oat Organic	12.1 ± 2.0a	7.9 ± 1.5b	5.7 ± 1.4b
Rolled Oat Conventional	15.9 ± 1.1a	12.9 ± 1.9a	11.5 ± 1.6a

[a] Mean ± standard error
[b]Different lower-case letters represent significant differences between growing condition × crop year interaction (A) and between two growing conditions (B) (organic vs. conventional) at (p<0.05).

Similar to TSP content higher α-glucosidase enzyme inhibitory activity was observed in groats when compared to rolled oat. Interestingly, contrasting results regarding α-glucosidase enzyme inhibitory activity of groats and rolled oats were observed between two crop years (2016 & 2018). Organic groats from 2016 crop year had significantly high α-glucosidase enzyme inhibitory activity when compared to the groats from conventional production system. However, opposite trend was observed in 2018 crop year as groats from conventional production system had significantly high (p<0.05) α-glucosidase enzyme inhibitory activity when compared to groats from organic production system. In rolled oats, no significant differences in α-glucosidase

enzyme inhibitory activity between two production systems was observed in undiluted sample, while in half and one-fifth diluted samples significantly high ($p<0.05$) α-glucosidase enzyme inhibitory activity was observed in conventional rolled oat when compared to organic rolled oat. Results of this study suggested that anti-hyperglycemic functionalities of oat might vary due to different growing conditions and production systems, however the effect of environment and further food processing (groats vs rolled oat) methods on different health targeted bioactive components of grain are more critical in determining such anti-diabetic properties of oats. However, further studies with multiple years, different locations, and with different soil types are required to evaluate the specific effect of organic vs conventional production systems on phenolic bioactives and antioxidant and anti-hyperglycemic properties of oats and oat-derived foods.

5.5. Conclusions

Organic food production is gaining increasing interest with rising demand for organic foods among consumers, which is mainly due to the perception about the potential health benefits of organic produce and for ensuring higher environmental sustainability. In this context, organic oats and oat-derived foods are gaining much interest due to their human health relevant nutritional qualities and associated benefits. However there are lack of empirical evidences regarding effect of organic vs conventional production system on human health relevant bioactive profiles of grains such as oat. Based on such needs and scope, the current *in vitro* model-based study compared phenolic bioactive-linked antioxidant and anti-hyperglycemic properties of groats and rolled oat between organic and conventional production systems. No consistent trend on the impact of organic and conventional production system on phenolic content, antioxidant activity and anti-hyperglycemic property was observed in groats and rolled

oats. However, significant effect of crop year on phenolic bioactives and associated antioxidant and anti-hyperglycemic properties of groats were observed. Furthermore, significantly higher catechin concentration in groats and rolled oats from organic production system was observed when compared to groats and rolled oats from conventional production systems. Therefore, further studies are required to improve the understanding on the benefits of organic production system on human health relevant bioactives and associated properties of food crops beyond the advantages of improving soil and water qualities and enhancing flavor profile of fresh produce.

CHAPTER 6. ANALYSIS OF PHENOLIC-LINKED ANTIOXIDANT AND ANTI-HYPERGLYCEMIC FUNCTIONALITIES OF SORGHUM GENOTYPES USING RAPID *IN VITRO* ASSAY MODELS

6.1. Abstract

Millets are widely considered as nutritionally superior food source due to their high dietary fiber, essential amino acids, minerals, and phytochemical content when compared to major cereal grains such as white rice, corn, and wheat. Therefore, with rapid rise in high calorie and hyper-processed diet-linked non-communicable chronic diseases (NCDs) such as type 2 diabetes there is increasing interest towards millets to complement other grains and to improve overall nutritional qualities of cereal-based foods. However, human health relevant bioactive profiles and associated health benefits such as antioxidant and potential anti-hyperglycemic properties of millets such as sorghum vary widely among different genotypes. Therefore, the aim of this study was to screen different sorghum genotypes for their health relevant phenolic bioactive-linked antioxidant and anti-hyperglycemic properties in order to target them in compliementary dietary strategies to prevent and manage chronic diseases such as type 2 diabetes. Based on this objective, cold water extract of sorghum flour from fifty-nine genotypes were evaluated for total soluble phenolic content, phenolic acid profiles, total antioxidant activity (ABTS & DPPH), protein content, and anti-hyperglycemic relevant α-amylase and α-glucosidase enzyme inhibitory activities using *in vitro* assays. Overall, significant differences in TSP content, antioxidant activity, and *in vitro* α-amylase and α-glucosidase enzyme inhibitory activities were

observed in sorghum genotypes. However, high and positive correlation between TSP content and antioxidant activity (DPPH based), and between TSP content and α-glucosidase enzyme inhibitory activity was observed among sorghum genotypes. Furthermore, few sorghum genotypes such as Sweething, M81 E, Special Effort, Trudan Headless were identified with high TSP content, high antioxidant activity, moderate α-amylase, and high α-glucosidase enzyme inhibitory activities, and can be further targeted in animal model based *in vivo* or clinical studies to advance them as part of functional food or nutraceutical design to target management of chronic oxidative stress and chronic hyperglycemia commonly associated with type 2 diabetes.

6.2. Introduction

Sorghum (*Sorghum bicolor* L.) is an annual millet crop used as a staple food in many cultures worldwide especially in Asia and Africa, and is gaining increasing interest in healthy food design due to its rich fiber and bioactive profiles (Tuinstra. 2008). Other than food, sorghum is also used widely as animal feed and bioenergy crop and therefore has significant importance as an industrial crop (Dicko et al. 2006). Furthermore, sorghum is resilient to extreme climate such as drought, salinity, heat stress, and can outperform other cereals under such extreme environmental stresses (Tari et al. 2013). Specific morphological features of sorghum and ability to stimulate endogenous defense responses such as higher biosynthesis of phenolic secondary metabolites under abiotic stresses has the potential to induce higher fitness and resilience against extreme climate and soil variables (Simontacchi et al. 2015). Therefore, due to potential of such high resilience against abiotic stresses and its superior nutritional profiles, sorghum can be advanced as a value-added specialty crop to address global climate change-linked food and nutritional security challenges (Rooney and Awika 2005). Furthermore, due to its rich nutritionally relevant bioactive profiles and potential of associated diverse health

benefits sorghum can be targeted in cellular and biochemically relevant dietary support strategies to address major diet-linked public health challenges, such as epidemic of non-communicable chronic diseases (NCDs) especially against type 2 diabetes and associated macro and micro-vascular complications (Stefoska-Needham et al. 2015). United States is one of the leading producer and exporter of sorghum, however the domestic use of sorghum is mostly as an animal feed and recently it is emerging as a valuable bioenergy crop (Linton et al. 2011). However, with increasing awareness among consumers about healthy food alternatives especially higher demands of gluten-free cereals with rich fiber, high bioactive compounds, and overall balanced nutritional profiles, attention to sorghum for health relevant functional food design is gaining increasing interest.

Sorghum has been traditionally used as a food crop in India, Central America, and some African countries such as Nigeria, Sudan, Ethiopia, Burkina Faso and Egypt and mostly consumed as flat bread, porridge, and fermented condiments (Taylor et al. 2006). All these countries are also burdened with increasing climate change-linked food security and nutritionally-linked public health challenges as part of the population is still facing malnutrition due to lack of access to basic nutrition, while a majority are increasingly suffering with diet-linked chronic diseases like type 2 diabetes from high hyper-processed calorie loads. In this context, millet, such as sorghum provides high energy, high dietary fiber, protein with balanced amino acid profile, minerals, vitamins, and other bioactive compounds and can be targeted to substitute other cereal grains or to complement with cereals and legumes to build economically viable dietary complimentary strategies to prevent and manage both malnutrition and type 2 diabetes challenges that coexist in the same population (Kam et al. 2016). Previously, improved insulin sensitivities, lower blood glucose level, and reduction in triglyceride level was observed

in diabetic mice after administration of millet protein (Nishizawa et al. 2009). In another study, Kim and Park (2012) reported hypoglycemic effect of sorghum extract in diabetic rats and the anti-diabetic mechanism was related to hepatic gluconeogenesis. Similarly, improved insulin sensitivity in mice fed with high fat diet was also observed after incorporation of sorghum extract as part of the diet regime (Park et al. 2012). Furthermore, Chung et al. (2011) reported phenolic-linked antidiabetic effect of sorghum in Streptozotocin-induced diabetic rats. Therefore, targeting sorghum with rich health relevant phenolic bioactives as part of dietary interventions or as functional foods against type 2 diabetes and associated complications has merit.

The health relevant phytochemicals especially major phenolics present in sorghum are phenolic acids, flavonoids, condensed tannins, and deoxyanthocyanidins (Svensson et al. 2010). Sorghum also contains other phytochemicals such as sterols and policosanols (Awika and Rooney 2004). Furthermore, the major phenolic acids of sorghum are benzoic and cinnamic acid derivatives, while different flavonoids are tannins and anthocyanins (Kruger et al. 2003). However, phenolic content and phenolic acid profiles of sorghum vary between genotypes and with the changes in environmental condition (Dykes et al. 2005). Wide variations in phenolic content among sorghum genotypes are related to the color of pericarp of sorghum grain as it varies from white, yellow to red and purple. In general, red and purple color sorghum genotypes have higher phenolic content and high antioxidant potentials (Dykes et al. 2005; 2009). Due to such wide variations in phenolic content, it is important to screen several genotypes of different colored grain for their human health relevant bioactive profiles and associated benefits prior to targeting them in cellular and biochemically relevant dietary intervention strategies to prevent and manage chronic diseases such as type 2 diabetes. Therefore, the objective of this study was to screen several genotype of sorghum for phenolic bioactive-linked antioxidant and anti-

hyperglycemic properties using rapid *in vitro* assay models in order to target them as functional foods or developing functional ingredients to prevent and manage type 2 diabetes and associated complications.

6.3. Materials and Methods

6.3.1. Sorghum Genotype: Fifty-nine genotypes of sorghum (Table 1) were targeted in this study. Grains of sorghum genotype were collected from Sorghum Variety Trial, Department of Plant Sciences, North Dakota State University, Fargo, ND, USA. All sorghum genotype evaluated in this study were from two different locations (Agriculture Experiment Station Research plots) near Fargo, North Dakota in 2017. Sorghum grains were collected after harvest to extract and for use in all biochemical analysis.

6.3.2. Analytical Material: Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (30931-67-0), and 3, 5-Dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and of analytical grade.

6.3.3. Extraction: Sorghum grains were milled using WonderMill Grain Mill (Wonder-Mill, Pocatello, ID). After milling all samples were extracted using cold water extraction procedure. For all cold water extractions, 10 g of sorghum sample weighed and then homogenized with 50 mL of distilled water for 5 min using a Warring blender set at Low. All extracted samples were then centrifuged at 8,500 rpm for 20 minutes for two times. The sample extracts were then stored at 4ºC during the period of the biochemical analysis.

6.3.4. Total Soluble Phenolic Content: The total soluble phenolic content of all cold water extracted sorghum grains was analyzed by the Folin-Ciocalteu method (Shetty et al. 1995).

In a glass test tube, 0.5 mL of supernatant was transferred and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and vortexed and kept in dark incubation to stand for 60 min. After 60 min the absorbance of the mixture was read at 725 nm. The absorbance values were then expressed in milligram equivalents of gallic acid per gram dry weight (DW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

6.3.5. High Performance Liquid Chromatography (HPLC): The sample extracts of sorghum grain (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample from 100 µL of transferred supernatant was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 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 from 0% 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, 333, 540, 580 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of gallic acid, benzoic acid, ferulic acid, p-coumaric acid, o-coumaric acid, cinnamic acid, protocatechuic acid, catechin, caffeic acid, rutin, and quercetin in 100% methanol were used to calibrate the standard curves and retention time.

6.3.6. Antioxidant Activity by 2, 2-diphenyl-1-picrylhydrazyl Free Radical (DPPH) Scavenging Assay: The DPPH free radical scavenging activity of sorghum flour was determined

by an assay method modified by Kwon et al. (2006). At first, 1.25 mL of 60 µM DPPH in 95% ethanol was added to 250 µL of each sample extract, with the decrease in the absorbance monitored after 5 min at 517 nm (A517 extract). The absorbance of a control (distilled water instead of sample extract) was also recorded after 5 min at the same wavelength (A517 control). The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}} \right) \text{x } 100$$

6.3.7. Antioxidant Activity by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) Free Radical Scavenging Assay: The total antioxidant activity of sorghum flour was also measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation as described by Re et al. (1999). $ABTS^+$ radical cation was prepared by mixing five mL of 7 mM ABTS stock solution with 88 µL of 140 mM potassium persulphate, and the mixture was allowed to stand in the dark at room temperature for 12 to 16 h to mature before use. Then $ABTS^+$ stock solution was then diluted with 95% ethanol (ratio 1:88) to give an absorbance of 0.70 ± 0.02 at 734 nm and was equilibrated. One milliliter ABTS was then added to Eppendorf tubes containing 50 µL of each sample extract and was mixed by a vortex mixer for 3-5sec and subsequently centrifuged for 1min at 7558*g*. After 2 min 30 sec incubation in the dark, absorbance of the mixtures was read at 734 nm. The readings were compared with the control, which contained 50 µL of 95% ethanol instead of the sample extract. The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}} \right) \text{x } 100$$

6.3.8. Total Protein Content: Protein content of each sorghum flour sample was determined using combustion method (LECO FP428 Nitrogen analyzer) (AACCI Approved method 46-30.01) (Simsek et al. 2014).

6.3.9. α-Amylase Enzyme Inhibition Assay: The *in vitro* α-amylase enzyme inhibitory activity of sorghum flour was determined using an assay method modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A total of 500 µL of each 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) were incubated at room temperature for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube and then incubated at 25 ℃ for 10 min. Next, 1.0 mL of 3, 5 dinitrosalicylic acid color reagent was added. The test tubes were then incubated in a boiling water bath for 10 min and cooled to room temperature to stop the reaction. The reaction mixture was then diluted after adding 10 mL of distilled water, and 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 extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. The *a*-amylase enzyme inhibitory activity of all undiluted oat extracts was then calculated according to the equation below:

$$\%\ \mathbf{Inhibition} = \left(\frac{\mathbf{A540(Control)} - \mathbf{[A540(Extract)} - \mathbf{A540(Sample\ blank)]}}{\mathbf{A540(Control)}}\right) \mathbf{x\ 100}$$

6.3.10. α-Glucosidase Enzyme Inhibition Assay: The α-glucosidase enzyme inhibition assay method was modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993b; McCue et al., 2005). A volume of 50 µL of sorghum flour 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 ℃ for 10 min. After pre-incubation, 50 µL of 5mM 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 ℃ for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (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 enzyme inhibitory activity of sorghum was expressed as a percentage of inhibition and calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{\Delta \text{ A405(control)} - \Delta \text{ A405(extract)}}{\Delta \text{A405 (control)}} \right) \text{x } 100$$

6.3.11. Statistical analysis: Two extractions were performed for each 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. Data regarding a ll biochemical parameters of sorghum genotype were analyzed using one- way ANOVA through the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC), and the least mean square differences between genotype were compared using Tukey's test ($p<0.05$).

6.4. Results and Discussion

6.4.1. Total Soluble Phenolic Content and Phenolic Acids Characterization of Sorghum Genotypes: Millets, such as sorghum is one of the ancient crops with rich human health relevant nutritional profiles, including diverse group of phenolic phytochemicals distributed across different plant organs including grains, leaves, and stems (Dykes and Rooney 2006). In general among different grains, sorghum is extremely resilient to biotic and abiotic

stresses. Therefore, it is well accepted that sorghum has higher level of stress protective secondary metabolites such as inducible phenolic compounds when compared to other cereal grains (Chandrasekara and Shahidi 2010; Svensson et al. 2010). The same abiotic and biotic stress relevant phenolic compounds of millets have also shown nutritional relevance and cellular protective functions such as antioxidant, ant-diabetic, cardiovascular health and lipid metabolism related benefits when consumed as part of a balanced diet (Shahidi and Chandrasekara 2013). However, the phenolic content and phenolic profiles of sorghum vary widely among different genotypes and further due to differences in growing conditions (Dykes et al. 2005). In the current study, significant variation in total soluble phenolic content (TSP) was observed among fifty-nine sorghum genotypes ($p< 0.05$) (Table 6.1.). The TSP content of sorghum genotypes ranged between 1.17 (CHR FS-9) - 9.17 (Brachytic Sorghum) (mg GAE/ g DW). Previously Dykes et al. (2005) reported similar range of phenolic content (3.1-8.9 mg GAE/ g DW) in different sorghum genotypes and observed that sorghum with thick pericarp and purple and red plants had high total phenolic content when compared to thin pericarp and from tan plants. However, Brachytic sorghum which is hybrid forage sorghum with tan color plants, and M81-E which is a sweet sorghum with brown grain had significantly high TSP content when compared to other sorghum genotypes. Interestingly, sorghum genotypes CHR-SS2, Sweething, Pampa Verde Pacas, Trudan Headless, Greentreat A+, BMR 108, which are sorghum× Sudan grass hybrids had significantly higher TSP content. Therefore, these sorghum genotypes can be targeted for functional food design or to develop functional ingredients or nutraceuticals to advance biochemically relevant health benefits associated with their high phenolic content. However, not only just phenolic content, but profile of phenolic acids is also important to understand potential health benefits of these sorghum genotypes.

Table 6.1. Total soluble phenolic content (TSP) (mg GAE/g DW), total antioxidant activity (ABTS and DPPH based free radical scavenging activity-%inhibition), and protein content (%) of fifty-nine sorghum genotypes.

Genotypes	TSP	ABTS	DPPH	Protein
SPX 28313	2.10 ± 0.02hijkl	82.4 ± 0.9hijk	36.7 ± 0.8hijklm	11.9 ± 0.4vwxyz
SD 1741 BMR	1.63 ± 0.03klmno	60.4 ± 0.7opqrst	32.1 ± 0.9klmnopq	15.9 ± 0.2cdef
Special Effort	4.86 ± 0.26e	93.5 ± 0.6abcd	86.7 ± 0.6a	12.7 ± 0.1pqrstuvw
Trudan Headless	5.52 ± 0.31de	89.9 ± 4.9abcdefgh	85 ± 0.5a	13.8 ± 0.3jklmnop
SPX3952	1.43 ± 0.05mno	60.9 ± 1.8opqrst	27.6 ± 1.0mnopqrst	15.9 ± 0.001cde
SPX 3402	1.24 ± 0.03 o	61.9 ± 0.7opqrs	21.9 ± 1.2stu	14.4 ± 0.1hijklm
SS 405	1.45 ± 0.05lmno	66 ± 1.4nop	31.3 ± 0.5klmnopqr	11.4 ± 0.1stuvwxyz
Pacesetter BMR	1.18 ± 0.02o	66.7 ± 0.9mno	26.2 ± 0.8pqrstu	11.9 ± 0.0stuvwxyz
SPX 902	2.88 ± 0.22g	92.1 ± 1.2abcdef	86.9 ± 0.7a	11.4 ± 0.3stuvwxyz
CHR-FS3	1.55 ± 0.06 lmno	84.9 ± 0.5cdefghijk	35.1 ± 0.6jklmnop	15.1 ± 0.1defghi
CHR-FS9	1.31 ± 0.07mno	54.9 ± 0.9stu	26.5 ± 0.6opqrstu	15.6 ± 0.02cdefg
SPX 3903	1.92 ± 0.09hijklmn	92.5 ± 0.9abcde	56.9 ± 3.2ef	11.9 ± 0.1stuvwxyz
SPX 904	1.70 ± 0.09 klmno	79.6 ± 0.7ijkl	38.5 ± 2.0hijkl	11.5 ± 0.3stuvwxyz
SDH 2942 BMR	1.34 ± 0.09mno	60.5 ± 0.7opqrst	22.6 ± 2.1rstu	14.8 ± 0.3efghij
FS 5	1.56 ± 0.08 lmno	80.1 ± 1.0ijkl	35.5 ± 2.9jklmno	11.6 ± 0.2stuvwxyz
XAL 53	1.60 ± 0.08klmno	83.9 ± 0.9efghijk	39.5 ± 2.7hijkl	12.6 ± 0.4urstuvwx
BMR AL 31	1.66 ± 0.06klmno	83 ± 1.6fghijk	39.1 ± 2.8hijkl	13.3 ± 0.02nopqrst
CHR-SG1	1.65 ± 0.08klmno	78.3 ± 1.4ijkl	37.9 ± 2.5hijkl	11.4 ± 0.2stuvwxyz

Table 6.1. Total soluble phenolic content (TSP) (mg GAE/g DW), total antioxidant activity (ABTS and DPPH based free radical scavenging activity-%inhibition), and protein content (%) of fifty-nine sorghum genotypes (continued).

Genotypes	TSP	ABTS	DPPH	Protein
CHR-FS4	CHR-FS4	CHR-FS4	CHR-FS4	CHR-FS4
CHR-FS9	CHR-FS9	CHR-FS9	CHR-FS9	CHR-FS9
Brachytic sorghum	Brachytic sorghum	Brachytic sorghum	Brachytic sorghum	Brachytic sorghum
Pampa Mijo II	Pampa Mijo II	Pampa Mijo II	Pampa Mijo II	Pampa Mijo II
Pampa Triumfo XLT	Pampa Triumfo XLT	Pampa Triumfo XLT	Pampa Triumfo XLT	Pampa Triumfo XLT
CHR-SS2	CHR-SS2	CHR-SS2	CHR-SS2	CHR-SS2
X942	X942	X942	X942	X942
Nutri Plus	Nutri Plus	Nutri Plus	Nutri Plus	Nutri Plus
NK300	NK300	NK300	NK300	NK300
Sweet sorghum 36111	Sweet sorghum 36111	Sweet sorghum 36111	Sweet sorghum 36111	Sweet sorghum 36111
Sweething	Sweething	Sweething	Sweething	Sweething
M81-E	M81-E	M81-E	M81-E	M81-E
Honey Sweet	Honey Sweet	Honey Sweet	Honey Sweet	Honey Sweet
Sweething BMR	Sweething BMR	Sweething BMR	Sweething BMR	Sweething BMR
SPX903	1.42 ± 0.10mno	61.6 ± 3.2opqrst	27.8 ± 3.1mnopqrst	16.4 ± 0.02bc
Pampa Verde Pacas BMR 6	1.31 ± 0.07mno	48.3 ± 2.8uv	20.6 ± 2.8tu	15.5 ± 0.4cdefgh
BMR-90	1.65 ± 0.09klmno	76.1 ± 2.5klm	30.5 ± 2.9lmnopqrt	14.7 ± 0.04ghijkl

Table 6.1. Total soluble phenolic content (TSP) (mg GAE/g DW), total antioxidant activity (ABTS and DPPH based free radical scavenging activity-%inhibition), and protein content (%) of fifty-nine sorghum genotypes (continued).

Genotypes	TSP	ABTS	DPPH	Protein
Greentreat A+	4.95 ± 0.20de	97 ± 0.2a	79.7 ± 1.2ab	13.1 ± 0.2opqrstu
Enorma	2.41 ± 0.05ghij	92 ± 1.4abcdefg	68.1 ± 1.9cd	10.3 ± 0.2stuvwxyz
Pampa Centurion	1.18 ± 0.08o	52.5 ± 2.2tuv	23.7 ± 2.7qrstu	13.8 ± 0.3jklmnop
Honey Sweet BMR	1.40 ± 0.03mno	56.2 ± 0.9qrstu	24.7 ± 1.2qrstu	13.6 ± 0.4klmnopqr
SX 17	2.46 ± 0.16ghi	91.5 ± 1.8abcdefgh	84.6 ± 0.4a	13.5 ± 0.03lmnopqrs
Hay King	1.63 ± 0.10klmno	96.8 ± 0.2a	80.1 ± 0.3a	11.2 ± 0.1stuvwxyz
S9-09	1.19 ± 0.04o	54.9 ± 1.0stu	24.2 ± 1.1qrstu	11.3 ± 0.3stuvwxyz
Topper	1.27 ± 0.04no	55.8 ± 1.1qrstu	22.5 ± 1.1rstu	10.5 ± 0.1stuvwxyz
Greentreat 128	1.28 ± 0.03no	58.1 ± 1.3opqrst	24.6 ± 0.8qrstu	13.8 ± 0.1jklmnopq
Sweetie BMR	1.32 ± 0.04mno	59.2 ± 2.1opqrst	27.2± 1.2nopqrst	13.9 ± 0.03ijklmno
BMR 106	1.51 ± 0.04lmno	78.7 ± 1.4ijkl	36.2 ± 0.5ijklmn	14.3 ± 0.0hijklmn
Pampa Verde Pacas	3.87 ± 0.18f	95.4 ± 0.5ab	85.9 ± 0.4a	11.7 ± 0.0stuvwxyz
BMR 105 MS	1.52 ± 0.04lmno	85.5 ± 0.5cdefghij	45.7 ± 2.6gh	12.4 ± 0.2stuvwxyz
Greentreat Dynamo	1.56 ± 0.03lmno	82.7 ± 0.8ghijk	42.9 ± 1.1ghij	10.6 ± 0.4stuvwxyz
Piper Sudan	5.57 ± 0.25d	95 ± 0.5ab	78.5 ± 0.2ab	9.2 ± 0.03stuvwxyz
Greentreat Plus	1.81 ± 0.06ijklmno	85.4 ± 0.4cdefghijk	40.5 ± 1.8ghijk	12.5 ± 0.1rstuvwxy
Sweet sorghum 36126	1.53 ± 0.02lmno	72.9 ± 0.3lmn	30.9 ± 2.1lmnopqrs	16.0 ± 0.2cd

Table 6.1. Total soluble phenolic content (TSP) (mg GAE/g DW), total antioxidant activity (ABTS and DPPH based free radical scavenging activity-%inhibition), and protein content (%) of fifty-nine sorghum genotypes (continued).

Genotypes	TSP	ABTS	DPPH	Protein
Theis	1.70 ± 0.05klmno	91.3 ± 0.5abcdefg	49.6 ± 1.6fg	10.4 ± 0.3stuvwxyz
BMR 108	4.96 ± 0.14de	94.2 ± 0.4abc	83.7 ± 0.9a	14.5 ± 0.3ghijklm
Sordan Headless	1.68 ± 0.05klmno	84.4 ± 1.2defghijk	42.9 ± 1.7ghij	12.1 ± 0.3stuvwxyz
Dale	1.96 ± 0.07hijklm	93.4 ± 0.6abcd	59.5 ± 0.7de	12.4 ± 0.2stuvwxyz
Sweet sorghum 56111	1.28 ± 0.08mno	60.6 ± 1.5opqrst	23.3 ± 0.9qrstu	15.1 ± 0.1defgh
Sweet sorghum 54126	1.50 ± 0.11lmno	87.0 ± 0.8bcdefghi	28.7 ± 2.5mnopqrst	18.3 ± 0.03a
Pampa Karamelo	1.24 ± 0.06o	65.1 ± 1.3nopq	30.7 ± 1.3lmnopqrs	11.5 ± 0.2stuvwxyz

[a] Mean ± standard error
Different lowercase letters indicate significant differences ($p< 0.05$) between genotypes separately for individual column.

Based on the above rationale, phenolic acids of all sorghum genotypes were analyzed using high performance liquid chromatography (HPLC). The major classes of phenolic acids in millets are hydroxybenzoic and hydroxycinnamic acids and their derivatives (Dykes and Rooney 2007). In this study, major phenolic acids found in sorghum genotypes were gallic acid, catechin, protocatechuic acid, *p*-coumaric acid, benzoic acid, cinnamic acid, and ferulic acid (Table 6.2). Statistically significant differences ($p< 0.05$) in individual phenolic acid concentration were observed among fifty-nine sorghum genotypes. Among different phenolic acids, high concentration of cinnamic acid was observed in most sorghum genotypes followed by gallic acid, catechin and protocatechuic acid.

Table 6.2. Concentration of individual phenolic acid (µg/ g DW) of sorghum genotypes determined using HPLC.

Genotypes	Gallic acid	Catechin	Protocate-chuic acid	Benzoic acid	*p*-coumaric acid	Cinnamic acid	Ferulic acid
SPX 28313	1.84klmnopqr	1.49lmnopqrst	0.28nopqrstu	0.07cdefghijk	0.02ef	9.66l	0.37wxyz
SD 1741 BMR	3.37cdefg	0.92qrstuv	0.29mnopqrstu	0.06cdefghijklmnopqr	0.02f	11.77g	0.45rstuvw
Special Effort	0.69tuvwx	0.69tuv	2.58b	0.04opqrstuvw	0.02f	ND	0.43stuvw
Trudan Headless	0.85stuvwx	1.15opqrstuv	1.35d	0.05ijklmnopqrstuv	0.04ef	ND	0.51mnopqrst
SPX3952	3.53bcde	1.03pqrstuv	0.27nopqrstu	0.05efghijklmnopqrs	0.02f	9.08n	0.39uvwxyz
SPX 3402	1.96klmnopq	2.29efghijk	0.23opqrstu	0.02tuvw	0.02f	9.33m	0.14z
SS 405	3.43cdef	1.06opqrstuv	0.39lmnopq	0.05efghijklmnopqrst	ND	11.76g	ND
Paceseter BMR	0.39wx	4.15a	0.19pqrstu	0.08cde	0.84a	0.13q	ND
SPX 902	0.94rstuvwx	2.04ghijklm	0.08stu	0.02stuvw	0.31c	14.83b	ND
CHR-FS3	2.73defghijk	2.65cdefgh	0.28nopqrstu	0.09c	0.31c	16.46a	0.02z
CHR-FS9	4.79a	3.02bcde	0.54jklm	0.08cd	0.85a	13.38d	0.29z
SPX 3903	2.56fghijkl	4.07a	0.27nopqrstu	0.05defghijklmnopqrs	0.49b	7.73o	0.48opqrstu
SPX 904	2.59efghijkl	3.06bcd	0.49klmno	0.04mnopqrstuvw	0.04ef	13.28d	0.65efghijk
SDH 2942 BMR	3.41cdef	1.19opqrstuv	0.42lmnop	0.05mnopqsrtuv	0.01f	14.07c	0.53lmnopqrs

Table 6.2. Concentration of individual phenolic acid (µg/ g DW) of sorghum genotypes determined using HPLC (continued).

Genotypes	Gallic acid	Catechin	Protocate-chuic acid	Benzoic acid	*p*-coumaric acid	Cinnamic acid	Ferulic acid
FS 5	3.21cdefgh	1.82ijklmno	0.47klmnop	0.06cdefghijklmnop	0.04ef	0.49p	0.78d
XAL 53	3.81bc	2.43defghij	0.86fghi	0.07cdefghij	0.01f	0.16q	0.58hijklmno
BMR AL 31	3.85abc	3.62ab	0.75ghij	0.07cdefghijklm	0.03ef	0.15q	1.04ab
CHR-SG1	3.59bcd	1.57klmnopqr	0.40lmnopq	0.07cdefghijklmno	0.01f	11.37h	0.92c
CHR-FS4	2.92cdefghij	1.72ijklmnop	0.38lmnopq	0.05efghijklmnopqrst	0.01f	10.56j	0.57jklmnop
CHR-FS9	3.38cdefg	2.80cdefg	0.26opqrstu	0.05ijklmnopqrstuv	0.03ef	12.02f	0.36wxyz
Brachytic sorghum	ND	1.76ijklmnop	0.55jklm	0.07cdefghijklmno	0.07de	0.66p	1.09a
Pampa Mijo II	2.37hijklmn	2.67cdefgh	0.84fghi	0.03qrstuvw	0.00f	0.16q	0.27z
Pampa Triumfo XLT	4.41ab	0.89rstuv	0.28nopqrstu	0.07cdefghijklmn	0.01f	0.09q	0.37wxyz
CHR-SS2	1.51mnopqrst	1.27nopqrstu	0.38lmnopqr	0.02tuvw	0.03ef	10.98i	0.46qrstuvw
X942	ND	1.56klmnopqr	1.63c	0.07cdefghij	0.03ef	10.27k	0.94bc
Nutri Plus	3.71bc	ND	0.55jklm	0.08cdefg	0.01f	0.22q	0.73de
NK300	2.27hijklmnop	2.01hijklmn	0.54jklm	0.06defghijklmnopqrs	0.01f	8.98n	0.62efghijkl
Sweet sorghum 36111	0.64tuvwx	3.11bcd	0.41lmnopq	0.08cdefghi	0.01f	0.17q	0.62efghijkl

Table 6.2. Concentration of individual phenolic acid (µg/ g DW) of sorghum genotypes determined using HPLC (continued).

Genotypes	Gallic acid	Catechin	Protocate-chuic acid	Benzoic acid	*p*-coumaric acid	Cinnamic acid	Ferulic acid
Sweething	0.55vwx	2.23fghijkl	0.39lmnopq	0.04jklmnopqrstuv	0.03ef	0.16q	0.58ijklmno
M81-E	ND	ND	0.32mnopqrst	0.04klmnopqrstuv	0.09d	0.05q	0.45rstuvw
Honey Sweet	ND	1.16opqrstuv	1.13de	0.04mnopqrstuvw	0.01f	0.03q	0.61fghijklm
Sweething BMR	3.02cdefghi	1.53klmnopqr	0.07stu	0.05hijklmnopqrstuv	0.03ef	0.09q	0.59ghijklmn
SPX903	3.58bcd	1.78ijklmnop	0.18pqrstu	0.05ghijklmnopqrstu	0.02ef	12.28e	0.68defghi
Pampa Verde Pacas BMR 6	2.43ghijklm	0.45v	0.16qrstu	0.05hijklmnopqrstuv	0.00f	0.11q	0.37wxyz
BMR-90	1.99jklmnopq	3.14bcd	0.12rstu	0.03pqrstuvw	0.02f	9.89l	0.65efghijk
Green treat A+	1.76lmnopqrs	1.72ijklmnop	1.83c	0.01w	0.03ef	0.05q	0.66efghij
Enorma	ND	1.68jklmnopq	0.52jklmn	0.07cdefghij	0.04ef	0.05q	0.54klmnopqr
Pampa Centurion	0.76tuvwx	0.69tuv	0.07tu	0.07cdefghijklmn	0.01f	0.07q	0.39uvwxyz
Honey Sweet BMR	1.59mnopqrst	1.08opqrstuv	0.05u	0.03rstuvw	0.03ef	0.06q	0.43stuvw
SX 17	ND	2.68cdefgh	1.15de	0.07cdefghijkl	0.02ef	0.05q	0.47pqrstuvw

Table 6.2. Concentration of individual phenolic acid (µg/ g DW) of sorghum genotypes determined using HPLC (continued).

Genotypes	Gallic acid	Catechin	Protocate-chuic acid	Benzoic acid	*p*-coumaric acid	Cinnamic acid	Ferulic acid
Hay King	0.04x	0.76stuv	1.15de	0.03stuvw	0.02f	0.01q	0.41tuvwx
S9-09	2.30hijklmno	1.39mnopqrst	0.15qrstu	0.04nopqrstuvw	0.03ef	0.02q	0.39uvwxy
Topper	1.43nopqrstuv	2.85cdef	3.71a	0.32a	0.03ef	0.05q	0.14z
Greentreat 128	1.57mnopqrst	2.89bcdef	1.82c	0.06cdefghijklmnopq	0.02f	0.11q	0.48opqrstuv
Sweetie BMR	2.21ijklmnopq	2.45defghi	1.05ef	0.04mnopqrstuvw	0.02f	0.07q	0.69defg
BMR 106	1.40opqrstuv	2.43defghij	0.33mnopqrs	0.02uvw	0.01f	0.15q	0.49opqrstuv
Pampa Verde Pacas	ND	ND	1.76c	0.03stuvw	0.01f	0.05q	0.39uvwxyz
BMR 105 MS	ND	ND	0.62ijkl	0.03rstuvw	0.01f	0.13q	0.29yz
Greentreat Dynamo	1.78lmnopqrs	1.29mnopqrstu	0.96efgh	0.04nopqrstuvw	0.02f	0.08q	0.49nopqrstu
Piper Sudan	ND	ND	1.79c	0.06cdefghijklmnopq	0.01f	0.01q	0.10z
Greentreat Plus	1.75lmnopqrs	3.25bc	2.45b	0.08cdefgh	0.02ef	0.09q	0.78d
Sweet sorghum 36126	0.43wx	2.21fghijkl	0.94efgh	0.07cdefghijkl	0.003f	0.11q	0.56jklmnopq
Theis	0.57uvwx	ND	0.19pqrstu	0.06cdefghijklmnopq	0.04ef	0.09q	0.39uvwxyz
BMR 108	ND	0.55uv	1.01efg	0.02uvw	0.02ef	0.03q	0.15z

Table 6.2. Concentration of individual phenolic acid (µg/ g DW) of sorghum genotypes determined using HPLC (continued).

Genotypes	Gallic acid	Catechin	Protocate-chuic acid	Benzoic acid	*p*-coumaric acid	Cinnamic acid	Ferulic acid
Sordan Headless	1.26qrstuvw	1.40mnopqrst	0.72hijk	0.02vw	0.03ef	10.73j	0.69defgh
Dale	1.55mnopqrst	2.64cdefgh	1.33d	0.08cdef	0.03ef	0.09q	0.38vwxyz
Sweet sorghum 56111	0.44wx	1.73ijklmnop	1.32d	0.05hijklmnopqrstu	0.02f	0.21q	0.31xyz
Sweet sorghum 54126	1.32pqrstuvw	1.74ijklmnop	2.63b	0.13b	0.03ef	0.24q	0.58hijklmno
Pampa Karamelo	1.81klmnopqr	2.19fghijkl	0.47klmno	0.05fghijklmnopqrstu	0.004f	0.11q	0.72def

[a] Different lowercase letters indicate significant differences ($p < 0.05$) between genotypes separately for individual column.
ND – Not detected

Previously Hahn et al. (1983) identified gallic acid, protocatechuic acid, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, ferulic, and cinnamic acids in sorghum grain extract and reported that most of these phenolic acids were in bound form. Interestingly, genotypes with higher TSP content had high gallic acid, catechin, and protocatechuic acid concentration compared to other genotypes with high cinnamic acid concentration.Overall, results of phenolic acid profile indicated that phenolic acid composition of sorghum genotypes vary widely and probably due to different parental origin (hybrid), thickness of pericarp, and due to different pigmentation of grains. Such wide variation in phenolic composition of sorghum grains may also be associated with their functionalities such as potential antioxidant and anti-hyperglycemic properties.

6.4.2. Total Antioxidant Activity (DPPH & ABTS Free Radical Scavenging) of Sorghum Genotypes:

Sorghum grain with high phenolic content in their extracts and in derived food products also exhibited high antioxidant potential in several *in vitro* and *in vivo* studies (Awika et al. 2003; 2009; Dlamini et al. 2007; Dykes et al. 2005; Kil et al. 2009). In the current study total antioxidant activity of sorghum genotypes was determined using two different free radical scavenging assays (DPPH and ABTS based). Overall, high antioxidant activity was observed in most sorghum genotypes (Table 6.1). Furthermore, similar to TSP content and phenolic acid profile, total antioxidant activity of sorghum genotypes also varied significantly ($p< 0.05$). Total antioxidant activity of sorghum genotypes ranged from 44-97(% inhibition) in ABTS based assay, and 17-87(% inhibition) in DPPH based assay. Higher antioxidant activity of sorghum genotypes were observed with ABTS based assay when compared to the antioxidant results from DPPH based assay. Previously, Awika et al. (2009) reported that sorghum genotypes with higher tannin content had high antioxidant and anti-proliferative activities. Similarly, Kil et al (2009) also observed significant differences in antioxidant activity (DPPH based) between different sorghum genotypes. Interestingly, in the current study positive correlation was observed between TSP content and total antioxidant activity of sorghum genotypes (r= 0.60 ABTS; r= 0.76 DPPH) (Table 6.3). Therefore, the results indicated that antioxidant potential of sorghum is partly related to its phenolic content and high phenolic, high antioxidant sorghum genotypes such as Brachytic sorghum, Trudan headless, Pampa Verde Pacas, Sweething, Greentreat A+, which can now be targeted to develop functional foods to counter chronic oxidative stress commonly associated with type 2 diabetes and other NCDs. Previously, Dlamini et al. (2007) observed that removal of the pericarp and the testa during decortication process significantly reduced

antioxidant activity of both tannin and non-tannin sorghum. Therefore, selecting proper post-harvest processing method is also important to retain higher antioxidant potentials in functional foods derived from sorghum.

6.4.3. Protein Content of Sorghum Genotypes: Grain proteins are important nutritional components and have significant impact on overall food quality, especially through their interaction with other grain constituents such as starch. Therefore determining protein content and composition is important to understand overall food quality of grain, such as sorghum (Hamaker and Bugusu 2003). Previously, Lockhart and Cronje (2000) reported 6-18% protein content with 70-90% of the total protein belonging to storage protein. In the current study, protein content of sorghum genotypes varied between 9.2-18.3% with highest protein content in Sweet sorghum 54126 (Table 6.1). Furthermore, high protein content was observed in sweet sorghum genotypes when compared to other forage and sorghum hybrids. Therefore, sorghum genotypes with high protein content can also be targeted as functional food especially to support protein needs as part of balanced nutrition where global need is to address food and nutritional security challenges. Furthermore, protein from grains and other plant-based foods also have relevance in lowering the risks of type 2 diabetes and other chronic diseases (Sluijs et al. 2010). Previously, anti-diabetic functionality of millet protein fractions was observed in diabetic mice (Nishizawa et al. 2009). Therefore, sorghum genotypes with high phenolic, high antioxidant and high protein content can be rationally targeted in biochemically relevant dietary complementary strategies or to design functional foods to address both low calorie-linked malnutrition and excess calorie-linked chronic disease challenges such as type 2 diabetes.

Table 6.3. Pearson correlation (*r*) between different biochemical parameters of fifty-nine sorghum genotypes.

	ABTS	DPPH	Protein	α-Amylase	α-Glucosidase		
					Undiluted	Half diluted	One-fifth diluted
TSP	0.60	0.76	-0.25	0.73	0.84	0.86	0.87
ABTS	*	0.82	-0.35	0.48	0.61	0.57	0.87
DPPH	*	*	-0.40	0.57	0.84	0.82	0.76
Protein	*	*	*	-0.16	-0.25	-0.25	-0.19
α-amylase	*	*	*	*	0.67	0.68	0.70

6.4.4. Anti-hyperglycemic Properties (α-Amylase and α-Glucosidase Enzyme Inhibitory Activities) of Sorghum Genotypes: Anti-hyperglycemic functionality of sorghum genotypes was investigated using rapid *in vitro* α-amylase and α-glucosidase enzyme inhibition assays. Overall, low to moderate α-amylase (1.3-48.3%) and low to high (4.4-99%) α-glucosidase enzyme inhibitory activities were observed in undiluted sample of sorghum flour (Table 6.4). Similar to TSP content and antioxidant activity, α-amylase and α-glucosidase enzyme inhibitory activities vary significantly ($p< 0.05$) among sorghum genotypes investigated in this study.Significant dose response in α-glucosidase enzyme inhibitory activity was also found in most sorghum genotypes. Interestingly, high and positive correlation between TSP content and α-glucosidase enzyme inhibitory activity (r=0.84) was observed, while moderate and positive correlation was observed between TSP content and α-amylase enzyme inhibitory activity of sorghum genotypes.

Table 6.4. α-Amylase and α-glucosidase enzyme inhibitory (% inhibition) activities of fifty-nine sorghum genotypes.

Genotypes	α-Amylase	α-Glucosidase		
		Undiluted	Half diluted	One-fifth diluted
SPX 28313	11.7 ± 0.9hijklm	14.11 ± 2.5ijklm	8.5 ± 1.5lmn	4.2 ± 1.0kl
SD 1741 BMR	8.4 ± 0.8ijklm	6.6 ± 2.2lm	5.9 ± 1.7lmn	2.9 ± 1.0l
Special Effort	36.4 ± 1.2abcd	98.4 ± 0.2a	97.9 ± 0.1a	93.6 ± 0.4a
Trudan Headless	28.9 ± 0.8cdefg	99.0 ± 0.0a	97.9 ± 0.2a	91.6 ± 0.4a
SPX3952	13.8 ± 1.2fghijklm	13.3 ± 1.4ijklm	12.0 ± 2.4 klmn	7.2 ± 2.1ijkl
SPX 3402	18.5 ± 0.9eghijkl	10.3 ± 2.1jklm	9.5 ± 2.5klmn	5.9 ± 1.6ijkl
SS 405	19.9 ± 2.2defghijk	29.1 ± 4.6efghijkl	26.8 ± 7.1fghijklm	23.3 ± 7.1fghijkl
Pacesetter BMR	13.6 ± 1.4fghijklm	31.4 ± 8.5defghijk	33.2 ± 9.5fghijk	31.5 ± 9.4eghi
SPX 902	15.6 ± 2.2efghijklm	80.4 ± 2.6ab	62.0 ± 3.3cde	28.9 ± 4.9efghijk
CHR-FS3	14.9 ± 1.8efghijklm	11.9 ± 3.7jklm	8.5 ± 3.0lmn	4.6 ± 1.9kl
CHR-FS9	17.2 ± 1.3efghijklm	17.5 ± 5.6ijklm	17.3 ± 5.3ijklmn	19.5 ± 5.9fghijkl
SPX 3903	17.8 ± 1.7efghijklm	24.1 ± 7.4ghijklm	23.0 ± 7.0ghijklmn	20.5 ± 6.3fghijkl
SPX 904	14.1 ± 2.6fghijklm	12.5 ± 1.9jklm	10.5 ± 0.9klmn	9.3 ± 1.4hijkl
SDH 2942 BMR	22.3 ± 4.2cdefghijk	7.3 ± 2.1klm	5.1 ± 1.3mn	4.5 ± 1.7kl
FS 5	17.9 ± 2.9efghijklm	15.2 ± 3.7ijklm	14.3 ± 3.2jklmn	12.9 ± 3.1ghijkl
XAL 53	12.5 ± 1.4ghijklm	50.6 ± 13.3cdef	46.7 ± 13.6efg	44.2 ± 13.2def
BMR AL 31	11.4 ± 1.7hijklm	11.0 ± 4.5jklm	7.4 ± 2.8lmn	6.6 ± 2.8ijkl
CHR-SG1	13.4 ± 1.9fghijklm	21.7 ± 5.4hijklm	15.9 ± 4.6jklmn	23.2 ± 9.2fghijkl

Table 6.4. α-Amylase and α-glucosidase enzyme inhibitory (% inhibition) activities of fifty-nine sorghum genotypes (continued).

Genotypes	α-Amylase	α-Glucosidase		
		Undiluted	Half diluted	One-fifth diluted
CHR-FS4	20.6 ± 3.5efghijk	24.9 ± 4.4ghijlkm	17.1 ± 4.4ijklmn	10.9 ± 3.4ghijkl
CHR-FS9	16.3 ± 0.8efghijklm	11.1 ± 3.2jklm	6.5 ± 2.6lmn	9.2 ± 3.7hijkl
Brachytic sorghum	16.3 ± 3.9efghijklm	98.9 ± 0.1a	97.7 ± 0.2a	91.0 ± 1.3a
Pampa Mijo II	30.2 ± 3.5bcdef	21.8 ± 6.7hijklm	15.4 ± 4.5jklmn	15.1 ± 4.7ghijkl
Pampa Triumfo XLT	7.6 ± 0.8ijklm	26.2 ± 3.5fghijklm	20.5 ± 3.1hijklmn	22.5 ± 3.5fghijkl
CHR-SS2	31.2 ± 1.8bcde	98.4 ± 0.2a	96.5 ±0.5a	86.4 ± 2.0a
X942	17.6 ± 1.8efghijklm	46.4 ± 5.9cdefg	37.3 ± 6.7fghij	34.5 ± 8.5defgh
Nutri Plus	16.2 ± 1.9efghijklm	52.9 ± 3.9cde	48.6 ± 3.9def	49.5 ± 5.2cde
NK300	14.0 ± 2.9fghijklm	21.9 ± 5.7ghijklm	19.5 ± 6.3hijklmn	18.1 ± 6.8ghijkl
Sweet sorghum 36111	15.1 ± 1.3efghijklm	9.9 ± 3.5jklm	4.3 ± 1.8mn	2.4 ± 1.0l
Sweething	46.8 ± 3.6ab	98.4 ± 0.6a	95.6 ± 1.6ab	93.1 ± 3.1a
M81-E	48.3 ± 3.5a	97.1 ± 1.6a	97.4 ± 0.7a	94.2 ± 1.8a
Honey Sweet	1.9 ± 0.8m	12.9 ± 3.9ijklm	7.9 ± 2.4lmn	5.0 ± 2.8jkl
Sweething BMR	9.9 ± 0.9hijklm	19.6 ± 3.8ijklm	19.2 ± 2.8hijklmn	18.6 ± 2.9ghijkl
SPX903	9.6 ± 1.4hijklm	24.9 ± 1.6ghijklm	20.8 ± 1.7hijklmn	14.9 ± 2.6ghijkl
Pampa Verde Pacas BMR 6	10.7 ± 0.8hijklm	37.1 ± 3.2defghi	36.9 ± 4.0fghij	30.2 ± 2.7efghij
BMR-90	7.4 ± 0.9jklm	45.6 ± 2.3cdefgh	40.8 ± 2.7efghi	34.2 ± 2.5efgh

Table 6.4. α-Amylase and α-glucosidase enzyme inhibitory (% inhibition) activities of fifty-nine sorghum genotypes (continued).

Genotypes	α-Amylase	α-Glucosidase		
		Undiluted	Half diluted	One-fifth diluted
Greentreat A+	23.1 ± 7.5cdefghij	97.3 ± 0.3a	94.1 ± 1.2ab	83.3 ± 3.3ab
Enorma	2.9 ± 0.6lm	23.9 ± 6.7ghijklm	16.1 ± 4.9jklmn	13.2 ± 4.1ghijkl
Pampa Centurion	6.2 ± 0.7klm	8.8 ± 3.6jklm	11.1 ± 3.6klmn	12.9 ± 4.4ghijkl
Honey Sweet BMR	11.1 ± 0.8hijklm	7.8 ± 2.4jklm	6.3 ± 1.9lmn	3.4 ± 1.1l
SX 17	14.7 ± 2.4efghijklm	91.8 ± 1.6ab	81.1 ± 3.2abc	59.5 ± 4.8bdc
Hay King	13.5 ± 2.2fghijklm	54.2 ± 4.0cd	42.4 ± 4.1efgh	24.0 ± 4.1fghijkl
S9-09	6.6 ± 0.6jklm	19.0 ± 2.4ijklm	19.7 ± 2.6hijklmn	17.2 ± 3.6ghijkl
Topper	14.2 ± 4.4fghijklm	25.1 ± 5.8ghijklm	21.9 ± 5.8hijklmn	20.3 ± 5.3fghijkl
Greentreat 128	8.4 ± 2.7ijklm	8.1 ± 1.8jklm	11.7 ± 1.6klmn	10.8 ± 1.1ghijkl
Sweetie BMR	6.4 ± 2.0jklm	18.5 ± 1.5ijklm	23.4 ± 3.9ghijklmn	35.3 ± 7.8defg
BMR 106	9.4 ± 2.9hijklm	11.7 ± 2.2jklm	18.7 ± 1.9hijklmn	21.6 ± 1.7fghijkl
Pampa Verde Pacas	24.5 ± 6.5cdefghi	68.6 ± 9.1bc	71.8 ± 6.5bcd	69.6 ± 2.8abc
BMR 105 MS	6.2 ± 1.4klm	15.5 ± 2.0ijklm	12.3 ± 1.5klmn	8.4 ± 1.7ijkl
Greentreat Dynamo	9.1 ± 1.4hijklm	14.6 ± 1.4ijklm	11.8 ± 1.5klmn	9.9 ± 1.1hijkl
Piper Sudan	38.5 ± 4.9abc	98.8 ± 0.2a	98.0 ± 0.3a	92.4 ± 0.9a
Greentreat Plus	19.7 ± 2.5defghijkl	21.4 ± 3.8hijklm	17.3 ± 4.5ijklmn	13.3 ± 4.0ghijkl

Table 6.4. α-Amylase and α-glucosidase enzyme inhibitory (% inhibition) activities of fifty-nine sorghum genotypes (continued).

Genotypes	α-Amylase	α-Glucosidase		
		Undiluted	Half diluted	One-fifth diluted
Sweet sorghum 36126	15.8 ± 2.7efghijklm	20. ± 7.2ijklm	11.9 ± 3.2klmn	10.2 ± 2.9ghijkl
Theis	17.7 ± 2.2efghijklm	18.9 ± 2.5ijklm	12.8 ± 2.5klmn	7.6 ± 1.8ijkl
BMR 108	25.3 ± 7.2cdefgh	97.5 ± 0.8a	92.8 ± 2.2ab	85.3 ± 4.5a
Sordan Headless	17.2 ± 10.4efghijklm	31.9 ± 8.1defghi	29.4 ± 8.9fghijkl	29.0 ± 9.4efghijk
Dale	11.7 ± 1.3hijklm	7.9 ± 2.1jklm	1.8 ± 0.6n	1.1 ± 0.4l

[a] Mean ± standard error
Different lowercase letters indicate significant differences ($p< 0.05$) between genotypes separately for individual column.

Among all genotypes Sweething and M81 E had moderate α-amylase and high α-glucosidase enzyme inhibitory activities and it was statistically significant ($p< 0.05$) when compared to most sorghum genotypes.Therefore, these sorghum genotypes can be targeted for use as functional foods or to design functional ingredients to manage chronic hyperglycemia commonly associated with type 2 diabetes. Interestingly, these two sorghum genotypes also had high TSP content and antioxidant activity. Previously, Shobana et al. (2009) reported similar α-amylase and α-glucosidase enzyme inhibitory activities in seed coat phenolics of finger millet (*Eleusine coracana* L.). Similarly, high α-glucosidase and low α-amylase enzyme inhibitory activities were observed in sorghum, foxtail millet and proso millet (Kim et al. 2011). These previous studies and findings of the current study indicated that millets such as sorghum can be potentially incorporated in anti-diabetic relevant dietary support strategies to counter chronic hyperglycemia and chronic oxidative stress commonly associated with type 2 diabetes.

6.5. Conclusions

Sorghum and other millets known used as food" in food resource limited regions, especially in Asia and Africa, have diverse human health relevant nutritional qualities and associated benefits. Therefore, sorghum can be targeted in functional food design or can be incorporated in traditional cereal based foods such as flat bread, porridge, and noodles to improve the overall nutritional qualities and human health relevant functionalities to advance dietary support strategies for prevention and management of type 2 diabetes and other NCDs. However, the human health relevant bioactive profile and associated benefits such as antioxidant and anti-hyperglycemic functionalities of sorghum vary widely among several genotypes. In the current *in vitro* study, significant variation in TSP content, phenolic acid profile, antioxidant, and anti-hyperglycemic relevant α-amylase and α-glucosidase enzyme inhibitory activities were observed among fifty-nine sorghum genotypes. Therefore from this analysis, sorghum genotypes with high phenolic, high antioxidant, and high anti-hyperglycemic functionalities (moderate α-amylase and high α-glucosidase enzyme inhibitory activities) can be further targeted for clinical or animal model based *in vivo* studies to develop functional foods, ingredients or nutraceuticals to use complementarily with pharmaceutical drugs to manage type 2 diabetes and associated complications.

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