THE INFLUENCE OF THE HARVESTING MATURITY ON POLYPHENOL COMPOSITION AND ANTI-DIABETIC PROPERTIES OF HASKAP BERRIES (*Lonicera caerulea* L.)

by

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I dedicate my thesis to my ever-loving parents, my husband, and all my teachers from my childhood those who have a great contribution to steer my life into a fruitful direction

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ABSTRACT

Haskap (*Lonicera caerulea* L.) is a recently commercialized crop in Canada. Berries of four cultivars, Aurora, Rebecca, Larissa, and Evie, were harvested at five harvesting dates (H1-H5) and assessed for their polyphenol composition, antioxidant capacity, and anti-diabetic properties. The analyses revealed that the interaction effect of cultivar and harvesting date influenced the anthocyanins. Moreover, Larissa and Evie at H5 showed the significantly higher C3G content. Extracts derived from five maturity stages of four cultivars showed anti-diabetic properties including inhibition of activities of alpha-amylase, alpha-glucosidase, dipeptidyl peptidase-4, and formation of advanced glycation end-products *in vitro*. Dietary supplementation of C3G-rich haskap extract (CE) in mice fed with high-fat high-sucrose diet reduced the rate of weight gain, an improved glucose tolerance, and suppressed in gluconeogenesis regulating protein, fructose-1,6-bisphosphatase with concomitantly increased phosphorylation of 5' AMP-activated protein kinase (AMPK). In conclusion, C3G may improve the plasma glucose profile in diet-induced obesity by decreasing gluconeogenesis.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANTH	Anthocyanin
AMPK	AMP-activated protein kinase
AGE	Advanced glycation end-products
BP	Whole berry powder
CE	C3G-rich haskap extract
C3G	Cyanidin-3-*O*-glucoside
CRD	Complete randomized design
C3R	Cyanidin-3-*O*-rutinoside
C35DG	Cyanidin-3,5-diglucoside
Chlf	Chlorophyll
CVD	Cardiovascular diseases
DW	Dry weight
DM	Diabetes mellitus
DPP-4	Dipeptidyl peptidase-4
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
ESI	Electroscopy ionization
Folin-C assay	Folin-Ciocalteu assay
FBPase	Fructose-1,6-bisphosphatase
FW	Fresh weight
FRAP	Ferric reducing antioxidant power
FLAV	Flavonol
FBPase	Fructose-1,6-bisphosphatase
GLUT-4	Glucose transporter-4
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
G6Pase	Glucose-6-phosphatase
GAE	Gallic acid equivalent
HAS	Human serum albumin
HFHS	High-fat high-sucrose

ipGTT	Intraperitoneal glucose tolerance test
ipITT	Intraperitoneal insulin tolerance test
LED	Light-emitting-diode
LF	Low fat
NS	No supplement
OAA	Oxaloacetic acid
PEP	Phosphoenolpyruvate
PEP-CK	Phosphoenolpyruvate carboxykinase
Pet3G	Petunidin-3-*O*-glucoside
Peo3G	Peonidin-3-*O*-glucoside
PNP	*p*-Nitrophenol
Q3Gal	Quercetin-3-*O*-galactoside
Q3Glu	Quercetin-3-*O*-glucoside
Q3ArabinoG	Quercetin-3-*O*-arabinoside
Q3Rha	Quercetin-3-*O*-rhamnoside
Q3R	Quercetin-3-*O*-rutinoside
SV	Source of variation
TAC	Total anthocyanin content
TE	Trolox equivalent
TPC	Total phenolic content
T2D	Type 2 diabetes
UAE	Ultrasound-assisted extraction
UPLC	Ultra-pressure liquid chromatography

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CHAPTER 1: INTRODUCTION

Increasing interest in plant-based natural health products has led to numerous studies that show the therapeutic effects of fruits and vegetables in the prevention or reduction of the risk of various chronic diseases. Health benefits of berries have gained considerable attention due to the presence of polyphenols with anti-inflammatory, antioxidant, and neuroprotective properties (Paredes-Lopez et al., 2010). Haskap (*Lonicera caerulea* L.), also known as blue honeysuckles, recently commercialized in North America, has been known for its medicinal properties for centuries in Russia, China and Japan (Rupasinghe et al., 2018; Svarcova et al., 2007). The major cultivars introduced to Canada in early 2010s include 'Tundra', 'Berry Blue', 'Indigo Gem', and 'Borealis' and more recent cultivars include 'Honey bee', Aurora, 'Boreal Blizzard', 'Boreal Beauty' and 'Boreal Beast' (Khattab et al., 2016; Rupasinghe et al., 2015; Rupasinghe et al., 2018). Haskap berries have long been successfully cultivated in Russia and Japan due to many favorable characteristics of the crop including very early maturity, unique flavor and the ability to tolerate a very low temperature such as -46°C (Plekhanova et al., 1999; Svarcova et al., 2007). Polyphenols present in haskap have gained much attention due to their health-promoting properties (Rupasinghe et al., 2012; Rupasinghe et al., 2018). Physiologically-active polyphenols including flavonoids and phenolic acids in fruits contribute to the prevention of chronic diseases such as cancer, cardiovascular and neurodegenerative diseases (George et al., 2017; Rupasinghe et al., 2015; Thilakarathne & Rupasinghe, 2012; Jones et al., 2012). Among flavonoids, anthocyanins are water-soluble, ubiquitous plant secondary metabolites which are responsible for deep colors in various fruits and vegetables (Paredes-Lopez et al., 2010). Cyanidin-3-*O*-glucoside (C3G) represents about

76% of total anthocyanins present in haskap berries and possess beneficial effects in managing complications of T2D (Sasaki et al., 2007; Rupasinghe et al., 2018).

Diabetes mellitus (DM) has become one of the major chronic, non-communicable diseases in the world. Patients with DM are at high risk of developing specific pathological complications such as damage to the heart, kidneys, eyes, blood vessels, and skin (Rupasinghe et al., 2018). The prevalence of diabetes among adults is estimated at approximately 8.5% worldwide (Mahzari et al., 2018). Diabetes has mainly been categorized as Type 1 and Type 2. Type 1 diabetes is known as insulin-dependent diabetes, and it occurs due to the destruction of pancreatic β-cell islets resulting in insulin deficiency (Cnop et al., 2005). The most common, type 2 diabetes (T2D) is characterized as excessive accumulation of glucose in the circulation, as a result of poor responsiveness to insulin and subsequent development of resistance to insulin by the body (Chen et al., 2012). Preventive measures including dietary interventions and changes in lifestyle could significantly reduce the onset of diabetes. A diet containing a higher proportion of fruits and vegetables has been proposed for preventing the manifestation of T2D (Rupasinghe et al., 2017).

Glucose homeostasis is critical for healthy body functioning. In human, the blood glucose level is maintained at an optimum physiological level by the function of several hormones which show the homeostatic effect on pancreas, liver, kidney and skeletal muscles (Zhang et al., 2012; Meyer et al., 2002). The pancreas plays a pivotal role in glucose homeostasis by secreting insulin and glucagon (Zhang et al., 2012). It has been demonstrated that C3G possesses anti-diabetic activity through multiple mechanisms, such as inhibition of carbohydrate-hydrolyzing enzymes (alpha-amylase and alpha-glucosidase) and up-regulation of glucose transporter-4 (GLUT-4) (Sasaki et al., 2007). The inhibition of

carbohydrate-hydrolyzing enzymes manages T2D by controlling the rapid release of glucose into the bloodstream (McDougall et al., 2005). Incretins are intestinal hormones that potentiate insulin secretion following nutrient ingestion (Gautier et al., 2005). Incretin-based antidiabetic properties are mainly caused by two hormones, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) (Gautier et al., 2005). The AMP-activated protein kinase (AMPK) is also implicated in the regulation of blood glucose level (Hwang et al., 2009). Reduction of glucose absorption in the intestine is an effective way to prevent T2D. Polyphenols present in berries inhibit alpha-glucosidase and alpha-amylase enzymes (McDougall et al., 2005; Podsędek et al., 2014). Dipeptidyl peptidase-4 (DPP-4) is a peptidase and a pharmacological target for T2D treatment and anthocyanins present in blueberries and blackberries are potent DPP-4 inhibitors (Johnson et al., 2013). C3G significantly reduces blood glucose levels and increases cellular sensitivity to insulin (Sasaki et al., 2007). Anthocyanin as a functional food, demonstrates beneficial effects toward the prevention of obesity and diabetes. Moreover, dietary C3G extracts significantly reduced the development of obesity in high-fat diet-fed mice (Tsuda et al., 2003).

Several studies reveal that the polyphenol composition of haskap is greatly influenced by the cultivar. C3G concentration varies from 221 mg/ 100 g FW to 170 mg/ 100 g FW (82%) in cultivars of Polish-bred ‘Zielona’ and Canada-bred ‘Borealis’, respectively (Rupasinghe et al., 2015; Skupień et al., 2007). Polish cultivars, ‘Duet’ and ‘Pojack’ contain the major phenolic acid, chlorogenic acid, approximately 294 mg per 100 g of dry matter and 267 mg per 100 g of FW, respectively (Jurikova et al., 2012; Wojdyło et al., 2013). However, a comparatively lower concentration of C3G is found in cultivars grown in Canada including ‘Tundra’, ‘Berry Blue’, ‘Indigo Gem’ and ‘Borealis’ (21-44 mg of chlorogenic acid per

100 g of FW), indicating the significant influence of the cultivar and growing locality on polyphenol concentration (Khattab et al., 2016; Rupasinghe et al., 2015). However, the effect of cultivar and harvesting time on polyphenol composition and concentration as well as biological activities such as anti-diabetic properties of haskap has not yet been studied in detail. Therefore, it is important to investigate the differences among cultivars and the potential of haskap berry as a dietary source for the prevention or management of T2D.

1.1 Research hypothesis

Cultivar and harvesting date influence the polyphenol composition and biological properties of haskap berries. Haskap polyphenols, especially C3G exhibit anti-diabetic properties *in vitro* and *in vivo* through the inhibition of carbohydrate-hydrolyzing enzymes, inhibition of DPP-4 and AMPK-mediated regulation of gluconeogenesis.

1.2 Objectives

The overall goal of this research is to assess haskap berries as a potential nutraceutical source for the prevention or management of T2D. The specific objectives are:

1. To investigate the effect of cultivar and harvest date on polyphenol profiles and determination of anthocyanin content using the Multiplex fluorescence detector;
2. To investigate the anti-diabetic properties of haskap berry extracts using *in vitro* assays of carbohydrate-hydrolyzing enzymes, DPP-4 inhibition, and AGEs inhibition;
3. To study the effects of dietary supplementation of haskap on AMPK activation and plasma glucose levels using an experimental mouse model of T2D.

CHAPTER 2: LITERATURE REVIEW

2.1 Haskap (*Lonicera caerulea* L.) as a source for nutraceuticals

Haskap, also known as blue honey-suckle or honeyberry, has been a traditional medicinal plant for thousands of years, in Russia, China, and Japan, where haskap grow under natural weather conditions (Bors, 2009; Rupasinghe et al., 2018; Svarcovaa et al., 2007). Cultivated haskap plants grow 1.0 m wide, and 1.8 – 2.0 m tall and have the potential to grow as a commercial berry crop for northern latitudes since the bushes can tolerate a temperature as low as -46°C without any damage to the plant (Plekhanova et al., 1999). The flowers are light yellow-colored, tubular shaped and about 2 cm long. The fruits are edible, soft, oval-shaped and about 2 cm long, with dark blue to purple colored-tender skin which is covered by a waxy coating (Hummer, 2006). However, the shape of the haskap berries can vary within the species; cylindrical with irregular shape toward the apex and jug-shaped are the most common (Hummer, 2006). Cultivars produced during the early stages of breeding were bitter. However, breeders have begun selecting sweet types for commercial cultivation. In Nova Scotia, haskap ripens in mid to late June, a few weeks before strawberry does (Ochmian et al., 2012). The advantages of growing haskap as a commercial fruit crop are, early fruiting and ripening, being rich in bioactive flavonoids, frost tolerance, suitability for mechanized harvesting, and relative resistance to pests and diseases, such as mildew (Hummer et al., 2012). Moreover, haskap berries contain higher amount of health-promoting phytochemicals and antioxidant capacity, compared to other berries such as blueberries; thus, a potential source for manufacturing of nutraceuticals (Celli et al., 2014).

2.1.1 A brief history of haskap: origin and distribution

Haskap belongs to the *Lonicera* genus, native to Northern boreal forests, and is a widely known ornamental shrub as well as a source of folk medicine in Japan, Russia, China to treat hypertension, glaucoma and heart attack (Thompson & Barney, 2007; Rupasinghe et al., 2018). The most utilized haskap species which produce sweet berries are recognized as *Lonicera edulis* L. and *Lonicera kamtchatica* L. was originated in Russia and from Japan (Thompson & Barney, 2007). A few decades ago, haskap was initially introduced to Canada as blue honeysuckle, and it was widely used as an ornamental shrub on the Canadian prairies (Rupasinghe et al., 2012). However, in the last decade, edible, cold-hardy varieties were successfully introduced to North America and Canada (Rupasinghe et al., 2012). Initially, three major cultivars; 'Borealis', 'Indigo Gem' and 'Tundra', were introduced to Canada and most recently introduced varieties include, 'Honey Bee', 'Aurora', 'Boreal Blizzard', 'Boreal Beauty' and 'Boreal Beast' (Rupasinghe et al., 2018). Borealis has soft, large fruit, approximately 2 cm in diameter while Tundra and Indigo Gem have firmer fruit, allowing safe mechanical harvesting (Rupasinghe et al., 2012). A number of factors, including sufficient amount of sunlight, higher organic content, and soil pH 5 – 7, are required for the growth and a higher yield of haskap plants (Hummer, 2006).

2.1.2 Composition and biologically active compounds of haskap

The nutritional composition of haskap berries comprises of carbohydrates (10.19-15.59 %), proteins (4.6-8.41%), fat (2.18-4.77%) and essential minerals (Rupasinghe et al., 2012; Rupasinghe et al., 2018). A study shows that nutritional composition of three haskap cultivars grown in Canada; 'Borealis', 'Indigo Gem' and 'Tundra', were significantly different from each other. However, the values fall into the range of common berries such

as blueberries, blackberries, strawberries and raspberries (Rupasinghe et al., 2012). A similar study conducted using haskap grown in Poland demonstrates significant differences in dry matter, pectin and ash content among eight cultivars and genotypes (Wojdyło et al., 2013). Furthermore, both studies show that citric acid is the predominant organic acid in haskap berries, which represents approximately 47% of the total organic acid content. Haskap contains a higher concentration of vitamin C (186.6 mg of vitamin C per 100 g of FW), particularly in the Polish-bred haskap known as 'Pojark' (Jurikova et al., 2012). Scientific studies reveal that the nutritional composition, yield, chemical and physical characteristics depend on the genotype, plant maturity and environmental conditions (Jackson and Lombard, 1993; Prange and DeEll, 1995).

Medicinal properties of haskap are primarily related to non-nutritional chemicals including anthocyanins, flavonols, and phenolic acids (Rupasinghe et al., 2018). Haskap berries are rich in major types of phenolic compounds including anthocyanins C3G, cyanidin-3-*O*-rutinoside (C3R), petunidin-3-*O*-glucoside (Pet3G), peonidin-3-*O*-glucoside (Peo3G), cyanidin-3,5-diglucoside (C35DG)), flavonols (quercetin-3-*O*-galactoside (Q3Gal), quercetin-3-*O*-glucoside (Q3Glu), quercetin-3-*O*-arabinoglucoside (Q3ArabinoG), quercetin-3-*O*-rhamnoside (Q3Rha), quercetin-3-*O*-rutinoside (Q3R)), flavan-3-ols (catechin, epicatechin, Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG)) and phenolic acids (chlorogenic acid, ferulic acid, salicylic acid and coumaric acid) (Rupasinghe et al., 2015).

2.1.3. Anthocyanins and C3G

Anthocyanin compounds are a group of water-soluble plant pigments that are responsible for red-orange to blue-violet colors in fruits and vegetables such as blueberry, blackberry, strawberry, beet, carrot, and eggplant (Wu et al., 2006). According to the US dietary consumption, daily intake of anthocyanin in the US is approximately 180 – 225 mg per day (McGhie and Walton, 2007). Anthocyanin is found more abundantly in the skin of haskap (12.28 g/kg) compared to the flesh (4.34 g/L) (Jurikova et al., 2012). C3G contributes to the prevention of various chronic diseases, such as cancer, diabetes, and cardiovascular diseases (Olivas-Aguirre et al., 2016). Canadian-bred haskap varieties contain significantly higher C3G content (68 – 649 mg/100 g FW) compared to other common types of berries including strawberries (3.7 mg/ 10 g FW), blueberries (3.0 mg/100 g FW), cranberries (0.7 mg/ 100g FW) and chokeberries (1.7 mg/ 100g FW) (Wang et al., 2002; Zheng and Wang, 2003). The backbone of C3G consists of a benzopyran core (Benzoyl ring (A), a pyran ring (C)), a phenolic ring (B) attached to its C-2-position and a sugar moiety attached to the C-3-position (Figure 1). The C3G isolated from plants is highly unstable and extremely susceptible to degradation. Temperature, UV light, and pH show degradative effects on the stability of C3G (Sadilova et al., 2006; Jurikova et al., 2012; Pace et al., 2018). However, during storage and processing, C3G had the lowest stability compared to other types of anthocyanin present in haskap berries (Khattab et al., 2016). Therefore, lower-temperature storage and processing are recommended for retention of haskap berry anthocyanins (Khattab et al., 2016).

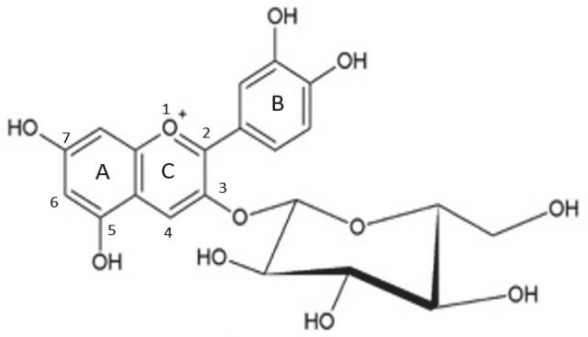

Figure 1. Chemical structure of cyanidin-3-*O*-glucoside (C3G)

In aqueous solutions, C3G undergoes a series of structural rearrangement in response to the changes in pH in four structures; the flavylium cation (pH 1-3) (red), carbinol (pH 4-5) (colorless), quinoidal base (pH 6-7) (blue) and chalcone (pH >8) (yellowish). Anthocyanins are more stable in acidic solutions (pH 1-3), where existing primarily as flavylium cations (Fang, 2014; Pace et al., 2018).

2.1.4 Changes in anthocyanin during fruit maturity

During fruit ripening, the fruits increase in size, decrease in firmness and reduce in acidity (Conde et al., 2007). The acidity of haskap berries gradually decrease during the harvesting season, mid-May to June. Several factors affect the chemical composition of berries, including maturity, genetic make-up, climatic conditions and agricultural practices (Conde et al., 2007). Adequate moisture, sunlight and soil fertility result in higher concentration of phenolics and tannins in berry fruits (Weston, 2000). Moreover, plant nutrients including, P and K are also important for the growth and productivity of haskap plants (Iheshiulo et al., 2018). Total anthocyanin content is increased with maturity of blueberries (Prior et al., 1998; Gibson et al., 2013). Ripened haskap berries are dark blue in color, which confirmed a higher content of anthocyanin compounds (Ochmian et al., 2012). The anthocyanin pigment concentration increases and undergoes a structural transformation with a change

in the acidity during fruit ripening (Cabrita et al., 2000). At the early stages of fruit development, fruit contains a higher concentration of acids. Under conditions, (pH 1-3), the anthocyanin compound transforms into the flavylium form, which gives a red color. Anthocyanin stability and color intensity then decrease at neutral pH (pH 7), and at pH 8-9, anthocyanin further turns into bluish-color with advanced ripening stage (Cabrita et al., 2000).

2.1.5 The extraction of haskap polyphenols

The extraction of bioactive substances from plant materials is the initial step in the utilization of plant polyphenols in preparing dietary supplements, food ingredients, nutraceuticals, etc. Freeze-drying is considered as the first step of extraction which retains a high content of polyphenols in plant materials than air-drying. For example, freeze-dried marionberries, strawberries and corn contained a higher content of total phenolics compared with their air-dried samples (Asami et al., 2003). Ethanol-based solvent extraction is a widely used method to produce extracts from plant materials due to the efficiency, ease of use, and acceptability. An acidified ethanol is found to be more efficient compared to methanol, in preparing anthocyanin-rich phenolic extracts from haskap berries (Myjavcová et al., 2010). The optimum extraction solvent to dried haskap sample ration of 25:1 has been reported (Celli et al., 2015). Ultrasound-assisted extraction (UAE) was used to accelerate the extraction of polyphenols in haskap berries, which requires low temperature (35 ºC) and relatively short duration (20 min). The mechanism of UAE involves the force created by bubbles formed by waves in an ultrasonic bath provide mechanical effects which resulted in the disruption of cell membranes to facilitate the release of extractable compounds (Chemat and Khan, 2011). This method increases the

contact surface area between solid and liquid phases with lower degradation of phenolic compounds (Celli et al., 2015).

2.1.6 Non-destructive method of measuring anthocyanins

The Multiplex (FORCE-A Orsay, France) portable optical sensor functions based on the screening of fruit chlorophyll fluorescence which can assess plant constituents including anthocyanin contents (Agati et al., 2013). The chlorophyll fluorescence signal is weakened by the higher anthocyanin concentration of the berry skin. The optical sensors were developed for non-destructive measurements of anthocyanin accumulation in fruit skin (Agati et al., 2013). This fluorescence detector is based on light emitting diodes (LEDs) excitation and filtered-photodiode detection which can estimate plant constituents (Cerovic et al., 2008). This instrument can be used to assess berry maturation both in the laboratory and on berry clusters in the field. The optical sensor is controlled by a computer which is based on the screening of excitation of chlorophyll fluorescence (ChlF) which uses UV light for flavonols (FLAV), or visible light for anthocyanin (ANTH) (Figure 2) (Agati et al., 2013).

Figure 2. The Multiplex fluorescence detector. A side view of the Multiplex instrument **(A),** Measurement of the haskap berry skin anthocyanin in the laboratory using the Multiplex by triggering the excitation light **(B)**

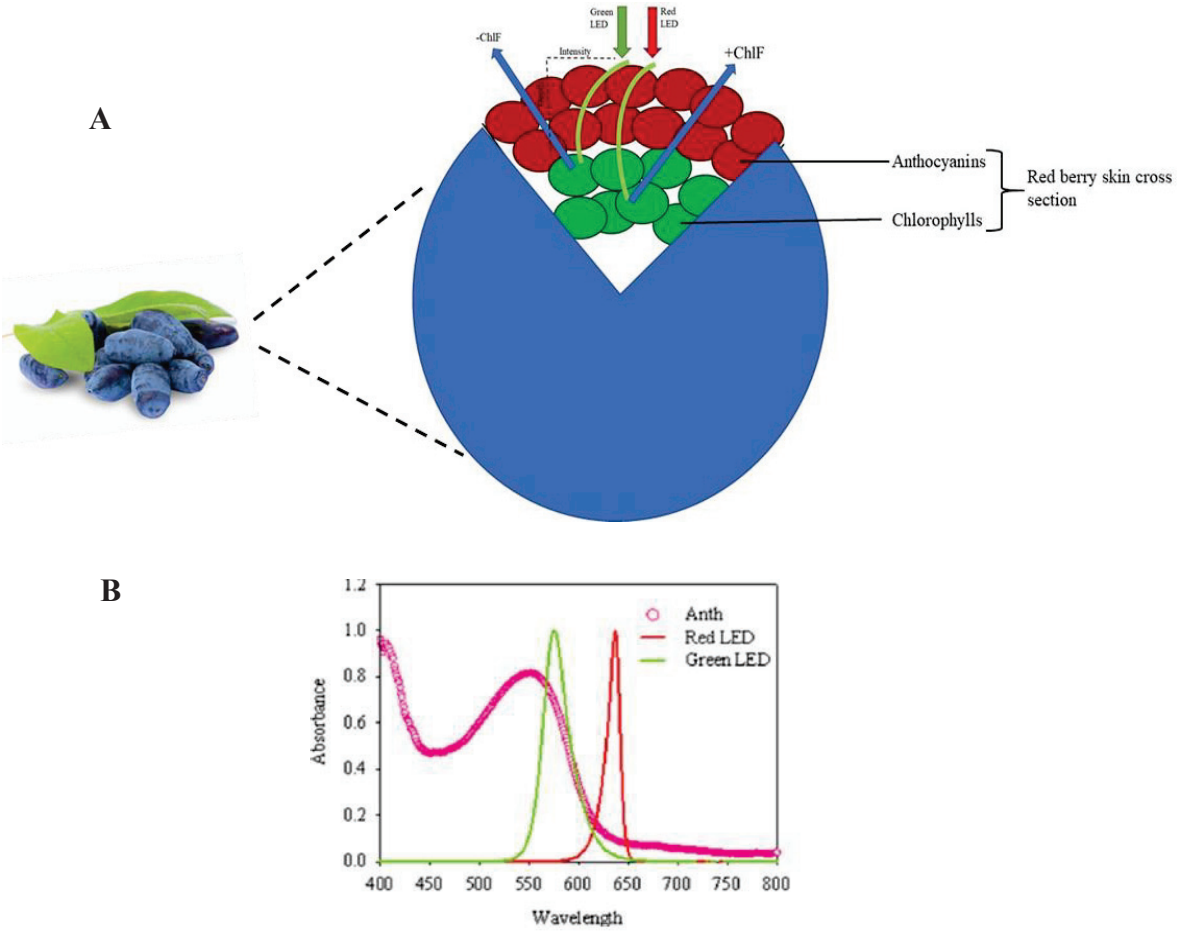

Figure 3. Diagram of chlorophyll fluorescence screening technique in Multiplex fluorescence detector. Schematic diagram of the fluorescence screening method on the effect of excitation light by compounds in haskap **(A)**, the absorption spectrum of anthocyanin and emission spectrum of green and red LEDs **(B)** (adopted from Agati et al., 2013).

The exocarp of fruit can be found on top of the chlorophyll layer of the fruit skin. Anthocyanins and chlorophylls are located in the cell vacuoles and the internal membranes of the chloroplasts respectively (Agati et al., 2013). The intensity of the excitation light is exponentially reduced as it transmits deep inside a berry. The extent of a reduction depends on the concentration of anthocyanin and the wavelength of irradiation (Figure 3). Higher chlorophyll (+ChlF) and lower chlorophyll (-ChlF) fluorescence correspond to the lesser

and greater attenuation of red and green lights, respectively (Agati et al., 2013). The higher the anthocyanin concentration, the lower excitation light transmitted to the deeper chlorophyll layers, and thus the chlorophyll signal decrease proportionally (Agati et al., 2007).

Anthocyanin mainly absorbs the green color spectrum around 520 nm (Figure 3A). The detection of chlorophyll will be significantly lower under the green excitation compared to the red-light excitation. The anthocyanin concentration present in the berry skin can be obtained by the index of comparing two fluorescence signals (Agati et al., 2013).

Anthocyanin index = $ANTH_{RG}$

$ANTH_{GR} = -ANTH_{RG} = \log (FRF_G/FRF_R)$

FRF_G and FRF_R are the far-red chlorophyll excited in the green and red respectively.

2.1.7. Concept of nutraceuticals

Several studies have investigated the therapeutic properties of various fruits and vegetables including berries as an important source of health supporting polyphenols in the human diet (Bahadoran et al., 2013; Rupasinghe et al., 2015). Certain types of food provide beneficial effects beyond the basic nutritional needs, and those are known as functional foods and nutraceuticals (Roberfroid, 2002). Nutraceuticals, in appearance similar to medicine in the form of tablets and capsules, containing the biologically active food components, which claim to have potential medical or health benefits, including the prevention of diseases (Dutta et al., 2016). There is a research interest in finding nutraceuticals derived from plant-based foods, such as various fruit crops. Due to their biological properties, polyphenols

present in berries are appropriate nutraceuticals for various chronic diseases including diabetes (Bahadoran et al., 2013; Rupasinghe et al., 2017).

2.2 Anthocyanins and health benefits

Dietary habits considered as a critical factor in determining the initiation and progression of diseases. Numerous studies have used animal models to show the health benefits of anthocyanin compounds against diabetes, cardiovascular diseases (CVD), and improvement of brain functions (Tsuda, 2012; Boeing et al., 2012; Ganesan & Xu, 2017; Rupasinghe et al., 2018; Rupasinghe and Arumuggam, 2019). Anthocyanin derived from purple corn shows a remarkable impact on pancreatic β-cells and insulin secretion (Hong et al., 2013). Intake of anthocyanin inhibits high-fat meal-induced body weight gain and body fat accumulation in mice. C57BL/6 mice fed with a C3G-rich diet demonstrates a reduction in high-fat meal-induced body fat accumulation (Tsuda et al., 2003). Body weight gain was decreased by the intake of mulberry extract in six-week-old male hamsters (Peng et al., 2011). Several studies have reported that consumption of anthocyanin inhibits the elevation of blood glucose level in mice fed with a high-fat diet (Prior et al., 2008; Grace et al., 2009). A human clinical trial reveals that the consumption of berries rich in anthocyanin significantly lowers the risk of cardiovascular diseases (CVD) related death (Rissanen et al., 2003). A human cohort study shows that the consumption of anthocyanin-rich blackcurrants, raspberries, lingonberries, and chokeberry play a role in preventing cardiovascular diseases (Erlund et al., 2008). Moreover, a study conducted by Krikorian and colleagues highlights brain-protective and memory improving activity in elderly individuals upon consumption of blueberry juice for 12 weeks (Krikorian et al., 2010).

2.2.1 Prevalence of Type 2 diabetes (T2D)

Diabetes is one of the major chronic non-communicable diseases which has become a worldwide epidemic during the last decade. World Health Organization (WHO) states that diabetes would be the 7th leading cause of death worldwide by the year 2030 (Mathers & Loncar, 2006). Currently, 424.9 million adults have diagnosed with type 1 and T2D globally, and worldwide prevalence is expected to increase to 628.6 million by 2045 (International Diabetes Federation, 2017). The most common type of diabetes is T2D, which causes a defect in insulin-mediated glucose uptake by cells (Chen et al., 2012). The prevalence of T2D among the adult population is approximately 8.5% worldwide (Mahzari et al., 2018). The current prevalence of T2D in Canada is approximately 7.6%, and it is estimated that 15,700 will die due to the disease annually (International Diabetes Federation, 2017). High-carbohydrate diets, overweight and inadequate physical activity are major non-genetic contributing factors of T2D (Chen et al., 2012). The economic burden of controlling T2D increases rapidly and the majority of the cost is allocated for the management of consequences of diabetes-related complications including retinopathy, nephropathy and cardiovascular diseases (Nathan, 1993). Early detection and preventive measures are important to reduce the risk of onset of T2D related complications, and thereby it helps to reduce the cost for health care services worldwide.

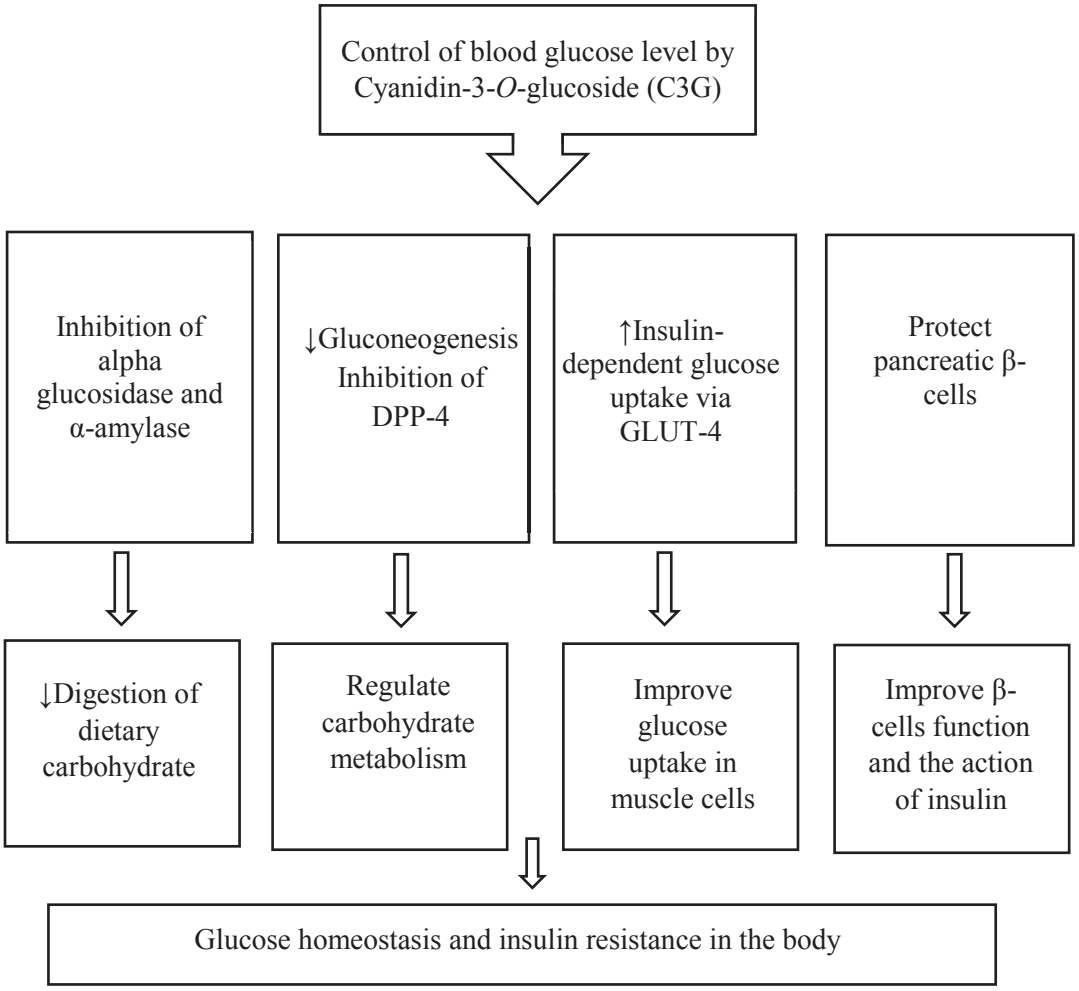

Figure 4. The effects of C3G on the management of blood glucose level under T2D conditions. GLUT-4, Glucose transporter type 4; DPP-4, Dipeptidyl peptidase-4; (↑) increase; (↓) decrease (adopted from Bahadoran et al., 2013).

2.2.2 Protective role of anthocyanin against diabetes

A diet that includes a sufficient amount of fruits and vegetables containing anthocyanins prevents the occurrence of diabetes or improve the disease condition and/or complications of individuals with diabetes (Sancho & Pastore, 2012). The consumption of anthocyanin compounds lowers diabetes complications in multiple organs and tissues including the liver, pancreas, kidney, and skeletal muscles by protecting pancreatic β-cells, improving

insulin resistance, increasing insulin secretion, improving liver function and inhibiting carbohydrate hydrolyzing enzymes (alpha-glucosidase and alpha-amylase) (Figure 5) (Sancho & Pastore, 2012). The liver plays a vital role in glucose regulation by storing glycogen and hepatic glucose production (König et al., 2012). Lack of insulin secretion by pancreatic β-cells or an inability of tissue cells to utilize the insulin leads to a failure in glucose homeostasis (Figure 5). The prevention of gluconeogenesis, glycogenolysis is the strategic targets of the prevention of diabetes (König et al., 2012). Anthocyanin extracted from cornelian cherries (*Cornus mas* L.) improves β-cell functions and decreases lipid accumulation in the liver (Jayaprakasam et al., 2006). Purple corn extracts protect β-cells, increase insulin secretion and reduce blood glucose levels (Hong et al., 2013). A study based on purple corn anthocyanin shows a protective role in diabetes-associated kidney failures (Li et al., 2012).

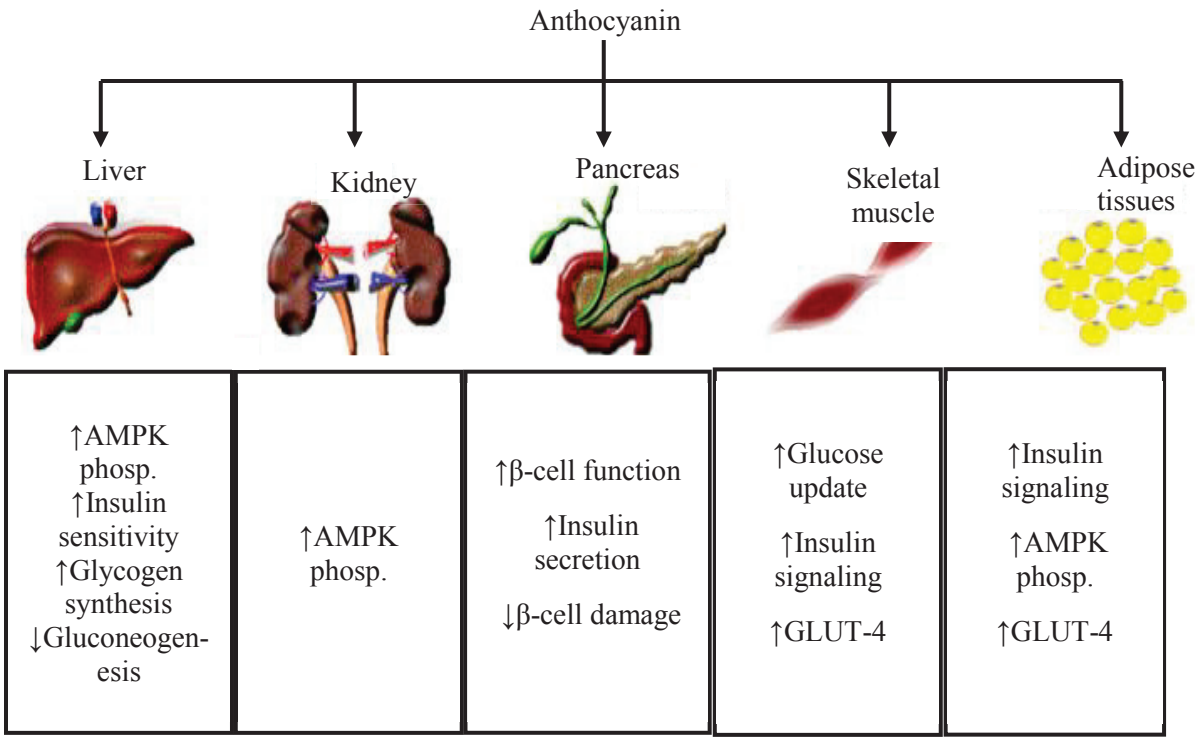

Figure 5. Protective role of anthocyanins against diabetes and associated complications in various organs including liver, kidney, pancreas, skeletal muscle, and adipose tissue (phosp., phosphorylation) (Adopted from Gowd et al., 2017).

2.2.2.1 Inhibition of carbohydrate-hydrolyzing enzymes

Human dietary carbohydrates are hydrolyzed into monosaccharides suitable for absorption by the catalytic activity of pancreatic alpha-amylase and intestinal alpha-glucosidase enzymes. The inhibition of these enzymes decreases the rapid release of glucose into the bloodstream (Podsędek et al., 2014).

The inhibitory effectiveness of anthocyanin against alpha-glucosidase activity has been reported in extracts prepared using colored fruits such as haskap, blueberry, bilberry and black currant (Podsędek et al., 2014). Among them, haskap demonstrates the strongest alpha-glucosidase inhibitory activity (IC_{50} 39.91±2.54 mg of fresh fruit/mL) (Podsędek et al., 2014). Raspberry (*Rubus idaeus* L.), strawberry (*Fragaria ananasia* L.), blackcurrant

(*Ribes nigrum* L.), and blueberry (*Vaccinium corymbosum* L.) extracts inhibit the activities of both alpha-amylase and alpha-glucosidase (McDougall et al, 2005).

2.2.2.2 Stimulation of insulin by inhibition of dipeptidyl peptidase-4 (DPP-4)

The major human incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), stimulate insulin release in a glucose-dependent manner (Gautier et al., 2005). DPP-4 is a peptidase and a novel pharmacological target for T2D treatment (Nauck et al., 2009). Incretin-based therapy has several potential sites of action for the treatment of T2D ranging from increased insulin secretion, reduced glucagon secretion, and regulation of glucose homeostasis (Gautier et al., 2005). DPP-4 cleaves both GLP-1 and GIP and transforms them into inactive metabolites which further reduce insulin secretion (Holst et al., 2009). Both hormones have short half-lives due to the rapid degradation by DPP-4 (Holst et al., 2009). The inhibition of DPP-4 results in elevated plasma insulin levels by inhibiting the degradation of active GLP-1 leads to the suppression of blood glucose elevation (Gautier et al., 2005). Therefore, the development of DPP-4 inhibitors to control blood glucose levels is a new option for the treatment of T2D (Gautier et al., 2005). A recent study shows that bilberry, also called European blueberry (*Vaccinium myrtillus*), and cranberry (*Vaccinium macrocarpon*) juices were able to inhibit DPP-4 in a dose-dependent manner (IC_{50} values; 1319 μg/ml for blueberry juice and 470 μg/mL for cranberry juice) (Cásedas et al., 2017).

2.2.2.3. Inhibition of advanced glycation end products (AGE) formation

AGEs are a diverse group of molecules generated by glycation of soluble and structural proteins, in plasma and tissues with advancing age, diabetes, and renal failure (Raj et al., 2000). AGEs considered as uremic toxins that are responsible for the normal aging process and the evolution of renal and vascular complications of diabetes (Raj et al., 2000). AGEs are produced from the oxidative reaction of free sugar molecules with amino groups in target protein (Booth et al., 1997). For several decades, plant-based medicines have been utilized to treat diabetes. The extracts of wild raspberry stem show anti-AGE activity compared to drugs that are clinically used such as aminoguanidine and metformin (Rupasinghe et al., 2015). Raspberry stems inhibit 93.5% of fluorescence-based AGE product formation (Rupasinghe et al., 2015). C3G extract prepared from skins of red grape (*Vitis vinifera* L.) also shows antiglycation properties, which further suppress the AGEs formation (Jariyapamornkoon et al., 2013). Furthermore, leaves and stems of low bush blueberries (*Vaccinium angustifolium*) demonstrate anti-glycation properties (McIntyre et al., 2009).

2.2.2.4 Inhibition of gluconeogenesis

The liver plays a vital role in the regulation of blood glucose through glycogen metabolism and gluconeogenesis (Hardie, 2008). The key enzymes responsible for the regulation of gluconeogenesis are pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEP-CK), fructose-1,6-bisphosphatase (FBPase) and glucose 6-phosphatase (G6-Pase) (Hardie, 2008; Dang et al., 2010). FBPase catalyzes a rate-limiting step in the gluconeogenesis pathway, thereby representing a potential target for controlling the excessive glucose production by the liver in patients with T2D (Dang et al., 2010). AMP-activated protein

kinase (AMPK) is a potential therapeutic target for the prevention and treatment of T2D (Hardie, 2008). Activation of AMPK by dietary polyphenols leads to a suppression of hepatic gluconeogenesis (Hwang et al., 2009). Bilberry (*Vaccinium myrtillus*) extracts rich in anthocyanin stimulate insulin sensitivity and activate the AMPK pathway which leads to a reduction of elevated blood glucose levels (Takikawa et al., 2010). Similarly, mulberry (*Morus alba* L.) fruit extract reduces hepatic glucose production, increases insulin sensitivity through AMPK phosphorylation and inhibits gluconeogenesis (Choi et al., 2016; Yan et al., 2016). Activation of AMPK in the liver by black soybean (*Glycine max* L.) seed coat extracts inhibit the gluconeogenesis by down-regulating the expression of regulatory enzymes of gluconeogenesis such as PEP-CK and G6-Pase, which leads the reduction of blood glucose concentration (Kurimoto et al., 2013).

2.2.3. Haskap and glucose homeostasis

Anti-glucosidase effects of anthocyanins have been reported for colorful fruits including blackcurrant (*Ribes nigrum* L.), strawberry (*Fragaria ananasia* L.), and raspberry (*Rubus idaeus* L.) (McDougall et al., 2005). Haskap showed strong alpha-glucosidase inhibitory activity with an IC_{50} value of 39.91 mg/mL, and order of the potency of alpha-glucosidase inhibitory activity was as follows; haskap (*Lonicera caerulea* L.) > blueberry (*Vaccinium corymbosum* L.) > bilberry (*Vaccinium myrtillus* L.) > blackcurrant (*Ribes nigrum* L.) > sweet cherry (*Prunus avium* L.) > red goose-berry (*Ribes uva-crispa* L.) (Podsędek et al., 2014). Haskap anthocyanin, mainly C3G, suppresses postprandial hyperlipidemia and hyperglycemia in rats fed a high-fat and high-sucrose diet (Takahashi et al., 2014).

Diets rich in fruits and vegetables are strongly associated with better health and reduced all-cause mortality (Nguyen et al., 2016). Studies using animal models have confirmed the

positive effect of polyphenols on metabolic health (Anhê et al., 2013). Dietary addition of anthocyanin-rich Kamchatka haskap (*Lonicera caerulea* L.) has the ability to control the disorders related to diabetes induced by a high-fructose diet in rats (Jurgoński et al., 2013). The experiment was conducted using male Wistar rats fed with high-fructose diet supplemented with Kamchatka haskap berry extract for 4 weeks. As a result, insulin resistance, impaired glucose tolerance, increased activity in alpha- and beta-glucosidase was observed (Jurgoński et al., 2013). However, there are no studies that have examined the effect of dietary interventions of C3G-rich haskap or haskap nutraceuticals on the risk factors for T2D. Therefore, a well-designed commercially grown haskap cultivar-based studies are necessary to draw a firm conclusion about the contribution of C3G-rich haskap berry for the management and prevention of T2D. Dose-response trials could be performed to ascertain the optimal intake required to reduce the risk of T2D. Further research studies are required to discover the mechanism of action of C3G as well as dietary sources of C3G.

CHAPTER 3: MATERIALS AND METHODS

3.1 Laboratory instruments

The following instruments were used for the experiments:

A class II-type A2 biological safety cabinet (LR2-452, ESCO Technologies Inc., Horsham, PA, USA), water bath (ISOTEMP 205, Fisher Scientific, Mountain View, CA, USA), centrifuge (Sorvail Legend Micro 21 R, Thermo Fisher Scientific Inc., Waltham, MA, USA), large capacity centrifuge (Damon/ IEC model CU-5000, San Diego CA, USA) microplate reader (Tecan Infinite® M200 PRO, Morriscille, NC, USA), nitrogen evaporator (N-EVAP™ 111, Organomation Associates Inc., Berlin, MA, USA), micro centrifuge (Sorvail ST 16, Thermo Fisher Scientific Inc., Waltham, MA, USA), freeze dryer (Dura-Dry™ MP FD-14-85BMP1, DJS Enterprises, Markham, ON, Canada), commercial blender (HBB909, Hamilton Beach Brands Inc., Glen Allen, VA, USA), coffee grinders (Black & Decker smart grind, Towson, Maryland, USA), rotary evaporator (Heidolph RotaChill, UVS400-115, Thermo Electron Corporation, Milford, MA, USA), ultrasonic bath of 20 kHz/ 1000 Watts (model 750D, VWR, West Chester, PA, USA), multiplex (FORCE-A, Orsay, France) hand held, multi-parametric fluorescence detector, BIO-RAD ChemiDoc MP Imaging System, digital hand-held refractometer (Digital refractometer 300016, SPER SCIENTIFIC, Scottsdale, AZ, USA), UPLC ESI MS/MS system (Model H-class system, Waters, Milford, MA, USA), Multiplex fluorescence detector (FORCE-A, Orsay, France), Bruker X-ray *in-vivo* imagining (Bruker Ltd, Milton, Ontario, Canada) and Tissue-homogenizer (Model THP115, Omni international, GA, USA).

3.2. Effect of cultivar and harvest date on the quality attributes of haskap

During the harvest season of 2017, haskap berries were (minimum of 100 g per replicate) hand harvested in triplicate from five harvesting dates (June 20 (H1), June 23 (H2), June 26 (H3), June 29 (H4), and July 02 (H5)) of four cultivars from LaHave Natural Farms, Blockhouse, NS. Four cultivars include one developed at the University of Saskatchewan (Aurora) and four imported from Poland (Rebecca (Łukaszewska), Larissa (Wojtek), and Evie (Jolanta). Berries were harvested every three days over two weeks. Each replicate represents berries harvested and pooled from a minimum of five plants. Brix levels of berries were measured in the field using a refractometer. The percentage of blue colored berries was determined by counting the number of blue colored and total number of berries in each sample.

$$Blue\ \% = \frac{Number\ of\ blue\ colored\ berries}{Total\ number\ of\ berries} \times 100\%$$

The haskap berries from four cultivars across H1-H5 were frozen (-20°C) to facilitate transport (180 km) from the farm to the Dalhousie University Agricultural Campus (Dal-AC), Truro, NS. The four cultivars have been identified as potential cultivars for expanding the commercial cultivation with mechanical harvesting in NS by the farm. The berries were stored up to 3 months at -80 ^{0}C for analyses of various fruit quality attributes. Furthermore, the effect of cultivar and the harvest time on the polyphenol composition of the haskap berries and correlations between different berry quality attributes were assessed.

3.3 Laboratory analysis

3.3.1 Preparation of haskap powder

Frozen berries were freeze-dried separately according to the harvesting dates and cultivars, 72 h in a freeze-drier (Dura-Dry™ MP FD-14-85BMP1, DJS Enterprises, Markham, ON, Canada). The freeze-dried samples were stored in a desiccator until ground. Freeze-dried samples were ground in a coffee grinder (Black & Decker smart), and the powder was used for the analysis.

3.3.2 Preparation of ethanol extract

The haskap extracts were prepared according to the method described by Celli and colleagues (Celli et al., 2015). Ground haskap berry powder (0.6 g) separately transferred to 50 mL amber glass vials according to the cultivar and harvesting date and 15 mL of extraction solution (80% ethanol, 0.5% formic acid, 19.5% distilled water) was added to each vial. The extraction procedure was conducted in an ultrasonic bath of 20 kHz/ 1000 Watts (model 750D, VWR, West Chester, PA, USA), with a temperature of 35 ºC for 20 min. After the extraction, the contents were centrifuged at 3000 rpm for 15 min (Sorvail Legend Micro 21 R, Thermo Fisher Scientific Inc., Waltham, MA, USA). The supernatant was removed and stored in 15 mL Falcon tubes. The samples were analyzed for sugar content (°Brix), total anthocyanin content, total phenolic content (TPC), the composition of polyphenols, and total antioxidant capacity.

3.3.3. Total soluble solid measurements

Approximately 10 g of frozen haskap berries from each cultivar and harvesting date, were crushed using a garlic presser and the °Brix value of the resulted juice was measured using a refractometer (Digital refractometer 300016, SPER SCIENTIFIC, Scottsdale, AZ, USA).

The °Brix values were measured in the field also using a refractometer as described in the section 3.2.

3.3.4. Determination of total anthocyanin by the pH differential method

Total anthocyanin content in the samples was determined using the pH-differential method (AOAC method 2005.02). Haskap extracts were diluted with distilled water to give absorbance values (A) between 0.0100 A and 9.000 A. Ten-fold diluted samples in both pH 1, and pH 4.5 buffer were prepared in duplicates, and the absorbance was measured at 520 nm and 700 nm using a microplate reader (Tecan Infinite® M200 PRO, Morrisville, NC, USA). The absorbance values of diluted samples were calculated using the following equation:

$$A = (\mathrm{A520} - \mathrm{A700})\text{at pH 1.0} - (\mathrm{A520} - \mathrm{A700})\text{ at pH 4.5}$$

Total anthocyanin content was calculated using the following equation:

$$\text{Total anthocyanin content} = \frac{\mathrm{A} \times \mathrm{MW} \times \mathrm{DF} \times 1000}{\mathrm{\varepsilon} \times \mathrm{L}}$$

The molar extinction coefficient (Ɛ); 26,900, molecular weight of C3G (MW); 484.83 g/mol, dilution factor (DF); path length (L) (Lee et al., 2005).

3.3.5 Determination of total phenolic content (TPC)

TPC in haskap samples was determined using the modified Folin-Ciocalteu assay in 96-well plate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada) as previously described (Singleton et al., 1999) and modified by Rupasinghe et al. (2008). Briefly, 20 µL of diluted (1/20) haskap extract was mixed with 100 µL of 0.2 N Folin-Ciocalteu reagent in the wells of the clear 96-well microplates (COSTAR 9017, Fisher Scientific, Ottawa, ON) and left to stand incubated at room temperature for 5 min in the dark. Then, 80 µL of a 7.5% sodium

carbonate solution was added and incubated for 2 h at room temperature before taking a reading at 760 nm using the microplate reader. The final reaction mixture consisted of 20 µl sample or gallic acid standard, 100 µL of Folin-Ciocalteu reagent and 80 µL of sodium carbonate solution per well. A standard curve was prepared using gallic acid (10 – 250 mg/L). The solutions were made fresh under reduced light conditions, and the reaction was carried out in dark conditions.

3.3.6 The total antioxidant capacity

The total antioxidant capacity of haskap extracts was determined by Ferric Reducing Antioxidant Power (FRAP) assay, which measures the electron donation potential of samples, as described by Benzie and Strain (1996), and modified by Rupasinghe et al. (2008). The working reagent, consisting of 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride, 1 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution (10:1:1, v/v/v), was made freshly and added (180 µL) to 20 µL of haskap extract or standard in 96-well microplate. The absorbance values were measured at 593 nm using microplate reader, after 6 min incubation at room temperature in the dark. The antioxidant capacity of extracts was calculated based on the Trolox standards (5 – 450 µM). The Trolox equivalent antioxidant capacity was expressed as µmol Trolox equivalent (TE)/ 100 g fresh weight (FW) (µmol TE/ 100 g FW).

3.3.7 UPLC-ESI-MS/MS analysis of phenolic compounds

The major individual polyphenols present in the haskap extract were identified and quantified according to the method described by Rupasinghe et al. (2008) using ultra-pressure liquid chromatography (UPLC) coupled with electrospray ionization (ESI) and mass spectrometry (MS). Briefly, extracts were dissolved (20 mg/mL) in ethanol containing

0.5% formic acid to obtain a final concentration of 20,000 mg/L. Dissolved samples were filtered through 0.22 μm nylon syringe filters and analyzed. Analyses were conducted using UPLC (Waters, Milford, MA, USA) coupled with a Micromass Quattro micro API MS/MS system and controlled with MassLynx V4.0 data analysis system (Micromass, Cary, NC, USA). An Aquity BEH C_{18} (100 mm × 2.1 mm, 1.7 μm) column (Waters, Milford, MA, USA) was used.

The separation of flavonol, flavan-3-ol and phenolic acid, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A linear gradient profile was used with the following proportions of solvent A applied at time t (min); (t, A%): (0, 94%), (2, 83.5%), (2.61, 83%), (2.17,82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), (8.75, 94%), (12, 94%). The analysis of anthocyanins was performed using the mobile phases of 5% formic acid in water (solvent A) and 5% formic acid in methanol (solvent B). The linear gradient profiles used were as follows: (t, A%): (4.76,0%), (6,70%), (.57,0%), (5.25, 0%), (12,0%), (14,48%), (17,36%), (19,10%), (22,90%). Electrospray ionization in negative ion mode (ESI-) was used for the analysis of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone. The following conditions were used: capillary voltage 3000 V, nebulizer gas (N_2) temperature 375 ºC at a flow rate of 0.35 mL/min. For the analysis of anthocyanins, electrospray ionization in positive ion mode (ESI+) was used. The settings for positive ion experiments were as follows: capillary voltage (25-50V) was optimized for each compound. Single ion-monitoring (SIM) mode was employed for quantification in comparison with standards: *m/z* 301 for quercetin (Q), *m/z* 609 for Q-3-*O*-rutinoside, *m/z* 463 for Q-3-*O*-glucoside and Q-3-*O*-galactoside, *m/z* 448 for Q-3-*O*-rhamnoside, *m/z* 593for Q-3-*O*-peltoside, *m/z* 273 for phloritin, *m/z* 435 for

phloridzin, m/z 353 for chlorogenic acid, *m/z* 179 for caffeic acid, *m/z* 193 for ferulic acid and isoferulic acid, *m/z* 449 for cyanidin-3-*O*-galactoside, *m/z* 289 for catechin, *m/z* 290 for epicatechin, and m/z 305 for epigallocatechin.

3.3.8 Non-destructive anthocyanin measurements

The haskap berry samples in each cultivar and harvesting date were measured by the Multiplex fluorescence detector. Each sample, 100 g of berries were placed on a white colored plate, and Multiplex fluorescence detector was placed on top of the berries filling the 5×10^3 mm^2 circular area of the sensor window, and three scans per replicate were measured (Figure 6). Multiplex fluorescence detector measures the anthocyanin accumulation on fruit skin based on light-emitting-diode (LED) excitation and filtered-photodiode detection. The two fluorescence signals are compared to obtain an index that is proportional to the berry skin anthocyanin and flavonol content.

$ANTH_{GR} = log\ (FRF_G / FRF_R)$

$FLAV = log\ (FRF_R / FRF_{UV})$

(Unit of the measurements – Multiplex)

FRF_G, FRF_R, and FRF_{UV} are the far-red chlorophyll excited in the green and red lights and Ultra-violet respectively.

The Multiplex fluorescence detector was calibrated by the company (FORCE-A, Orsay, France) prior to being sent to the laboratory. Non-destructive optical measurement of total anthocyanin was performed by scanning the haskap berries of four cultivars across five harvesting dates using the multiplex fluorescence detector.

Figure 6. Measurements of anthocyanin content of haskap berries using Multiplex fluorescence detector

3.4 The *in vitro* studies of investigating anti-diabetic properties

A dilution series of each cultivar across five harvesting dates (H1-H5) were used in *in vitro* assays; alpha-amylase, alpha-glucosidase, DPP-4 enzyme and advanced glycation end products (AGEs) inhibition assays. The IC_{50} value was determined using Prism8 software (Graphpad Software, Inc., San Diego, CA, USA).

3.4.1 Alpha-amylase inhibition assay

The alpha-amylase inhibition assay was performed according to the method described by Watanabe et al. (1997) using porcine pancreatic amylase (100 U, Sigma). Briefly, 20 µL of sample and 20 µL of alpha-amylase enzyme solution was dissolved in a 96-well microplate and incubated for 10 min. The substrate, *p*-nitrophenyl-α-D-maltopentoglycoside, incubated at 37 °C for 5 min. After the incubation, 20 µL of the substrate was added to the above sample-enzyme mixture and again incubated for 20 min. Finally, the trisodium phosphate solution (pH 11) was added to stop the reaction. The absorbance readings were taken at 405 nm using the microplate reader, to measure the *p*-nitrophenyl formation.

3.4.2 Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibition assay was also performed according to the method described by Watanabe et al. (1997). Briefly, alpha-glucosidase (1 U/mL) was dissolved in 0.01 M phosphate buffer (pH 6.8) containing 0.2% Bovine Serum Albumin (BSA) and used as an enzyme solution. p-4-Nitrophenyl-α-D-glucopyranoside (pNPG; 5 mM) in the same buffer (pH 6.8) was used as the substrate. The enzyme solution (20 µL) and 120 µL of sample were mixed in a 96-well microplate and incubated at 37 ℃ for 15 min. After the incubation, a substrate solution (20 µL) was added and incubated for another 15 min at 37 ºC. After 15 min, 0.2 M sodium carbonate solution was added to stop the reaction. The amount of *p*-nitrophenol released was measured using the microplate reader at 405 nm absorbance.

3.4.3 DPP-4 assay

DPP-4 enzyme activity was determined by the rate of hydrolysis of a surrogate substrate, H-Gly-Pro-AMC (AMC; 7-amino-4-methylcoumarin) (Tanaka-Amino et al., 2008). The 20 µL of DPP-4 enzyme was added to each well in a black 96-well flat-bottom plate, followed by the addition of 20 µL of assay buffer (25 mM HEPES, 140 mM NaCl, 1% RIA-grade BSA, pH 7.8) containing 80 mM $MgCl_2$, and then 20 µL of haskap extract or Sitagliptin drug. The reaction was initiated by adding 50 µL of substrate (H-Gly-Pro-AMC (AMC; 7-amino-4-methylcoumarin) solution to all the wells being used. After being incubated at the temperature of 37 ºC for 30 min, the fluorescence from liberated AMC was measured using the microplate reader (excitation 360 nm/emission 460 nm). The IC_{50} values were calculated using the PRISM8 software (Graphpad Software, Inc., San Diego, CA, USA).

3.4.4. Advanced glycation end-products (AGEs) assay

The methodology of Liu et al. (2011) was modified by using human serum albumin (HSA)-HAS solution, and glucose solution was prepared by dissolving in phosphate buffer (PBS; 50 mM, pH 7.4) to obtain the concentrations 35 mg/mL and 16.5 mM, respectively. Sodium azide (0.2 g/L) was added as an antimicrobial agent. The haskap samples of four cultivars; Aurora, Evie, Larissa, and Rebecca, across five harvesting dates (H1-H5), were dissolved in the same PBS buffer. One milliliter from each solution; HAS, glucose and respective haskap sample at a concentration range of 40 – 40,000 µg/mL, were mixed together and the mixtures were incubated at 37 ºC for a week. PBS buffer was used as the blank. HAS-glucose without any inhibitory agent was used as the control and aminoguanidine, and acarbose were used for the comparative purpose. Upon seven days of incubation completed, the fluorescence measurements were taken from each sample using an excitation of 330 nm and an emission of 410 nm, respectively, using the microplate reader. The percent inhibition of AGEs formation was calculated using the following formula:

$$AGEs\ formation\ \% = \left(1 - \frac{(fluorescence\ of\ the\ test\ group)}{(fluorescence\ of\ the\ control\ group)}\right) 100\%$$

3.5 The *in vivo* studies of investigating anti-diabetic properties

Ethical approval for animal use was obtained from the Dalhousie University Committee on Laboratory Animals (UCLA) (Protocol number: 13-077) (Appendix F). Eight-week-old C57BI/6 male mice (Jackson, Bar Harbor, ME, USA) were housed in a controlled environment (12 h day/ night cycle, lights turned off at 18:00) with food and water *ad libitum* in the animal facility of Dalhousie Medicine New Brunswick, Saint John, NB, Canada. After two weeks of acclimatization with regular chow diet, mice were randomly divided into nine groups (n=6) and fed with nine different diets for 16 weeks, comprising

of regular chow, low fat (LF), high-fat high-sucrose (HFHS) diets combination with no supplementation (NS), supplementation of C3G-rich extract (CE) or whole berry powder (BP) (Table 1).

Table 1. The types of diets used for the animal trial

Group	Type of diet	Supplement	Mouse feed	Number of mice
1	Chow	NS	Chow	6
2	Chow	CE	Chow+CE	6
3	Chow	BP	Chow+BP	6
4	LF	NS	LF	6
5	LF	CE	LF+CE	6
6	LF	BP	LF+BP	6
7	HFHS	NS	HFHS	6
8	HFHS	CE	HFHS+CE	6
9	HFHS	BP	HFHS+BP	6

NS, no supplement; LF, Low fat; HFHS, High-fat high-sucrose; CE, C3G-rich extract; BP, whole berry powder

3.5.1 The preparation of supplements containing C3G-rich extract (CE) and whole berry powder (BP) for the *in vivo* study

BP was prepared using the haskap cultivar 'Tundra' as described under the Section 3.3.1. For the preparation of CE, a crude ethanol extract was prepared using the BP prepared from 'Tundra" cultivar. Briefly, freeze-dried haskap pomace powder (250 g) was mixed with 4 L of aqueous 80% ethanol solution containing 0.5% formic acid and the extraction procedure was conducted in an ultrasonic bath of 20 kHz/ 1000 Watts (model 750D, VWR, West Chester, PA, USA), with a temperature 35 ºC for 20 min. After the extraction, the contents were centrifuged at 3000 rpm for 15 min. Next the extracts were filtered separately using Whatman No. 5 filter papers under vacuum and concentrated using a rotary evaporator (Rotavapor, R-200, Buchi, Flawil, Switzerland) at 45 ºC. To remove the sugar present in crude extract, flash chromatography was used as described by Pace et al. (2018). Briefly, a glass column (3.8 × 45 cm, Sati International Scientific Inc., Dorval, QC, Canada) was packed with 600 g adsorbent (Sorbent SP207-05 Sepabeads Resin Brominated Styrenic Adsorbent: particle size 250 μm, surface area 630 m^3/g, Sorbent Technologies, Atlanta, GA, USA) and conditioned with methanol followed by DI H_2O acidified to pH 2.5 using hydrochloric acid (HCl). The crude extract was loaded to the column and washed with approximately 1 L of acidified DI H_2O (pH 2.5) until the Brix of eluent reached 0.2%. The C3G-rich fraction was eluted using 500 mL of 100% methanol. The solvent, methanol, was removed using rotary evaporation (rotovapor, R-200, Buchi, Flawil, Switzerland) and then freeze-dried (Dura-Dry™ MP FD-14-85BMP1, DJS Enterprises, Markham, ON, Canada) to prepare dried CE.

In this study, the doses of dietary supplementation of BP and CE were determined based on the dose of total anthocyanins used in the previously reported human clinical studies (Li et al., 2015; Zhang et al., 2015). Supplementation of purified anthocyanin (320 mg total anthocyanins/day) from bilberry (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) for 24 weeks compared with placebo supplementation reduced fasting plasma glucose and LDL-cholesterol and increased HDL-cholesterol in diabetic patients (Li et al., 2015). In a similar human clinical trial, consumption of purified anthocyanin tablets (320 mg anthocyanins/day) for 12 weeks reduced fasting blood glucose and two-hour glucose level after oral glucose tolerance test of patients with non-alcoholic fatty liver disease (Zhang et al., 2015). Therefore, BP and CE supplemented diets of this study were designed to comprise of 1.69 mg total anthocyanins (C3GE) per mouse per day, which is equivalent to 320 mg total anthocyanin per day for a human. The assumptions of 70 kg body weight for the human subjects and an average weight of 30 g per mice were considered. The conversion of human dose to animal dose was based on body surface area as described by Reagan-Shaw et al., (2007). The following equation was used for the calculation.

$$\text{Human equivalent dose (HED)} \left(\frac{\text{mg}}{\text{kg}}\right) = \text{Animal dose} \left(\frac{\text{mg}}{\text{kg}}\right) \times \frac{\text{Animal } K_m \text{ factor}}{\text{Human } K_m \text{ factor}}$$

Based on the total anthocyanins present in BP (24 mg C3GE/g DW) and CE (200 mg C3GE/g DW), The percentages of BP (1.4%) and CE (0.192%) required for each diet was calculated and incorporated by the commercial diet supplier (Research Diets Inc., 20 Jules Lane, New Brunswick, NJ, USA). The online diet dose calculator of the manufacturer was used for the required calculations (https://www.researchdiets.com/opensource-diets/add-test-compounds/diet-dose-calculator). The commercial products used for the formulation of chow, LF, and HFHS diets were Purina 5001, rodent diet D12450H, and rodent diet

D12451. The total anthocyanin content of BP and CE was determined using the pH-differential method (AOAC method 2005.02) and expressed as mg C3GE/g DW. The composition of chow is provideded in appendix E.

Table 2. The detailed composition of mice diets consisting of regular, LF, and HFHS diet supplemented with C3G-rich haskap extract (CE) and whole haskap berry powder (BP).

Product	**Chow**	**LF**	**HFHS**	**Chow+BP**	**LF+BP**	**HFHS+BP**	**Chow+CE**	**LF+CE**	**HFHS+CE**
Protein (%)	29.8	19	24	N/A	19	24	N/A	19	24
Carb (%)	56.7	67	41	N/A	66	40	N/A	67	41
Fat (%)	13.4	4	24	N/A	4	24	N/A	4	24
Ingredients (g)									
Casein	N/A	200	200	N/A	200	200	N/A	200	200
L-Cystine	N/A	3	3	N/A	3	3	N/A	3	3
Corn starch	N/A	452.2	72.8	N/A	438.6	61.8	N/A	452.2	72.8
Maltodextrin 10	N/A	75	100	N/A	75	100	N/A	75	100
Sucrose	N/A	172.8	172.8	N/A	172.8	172.8	N/A	172.8	172.8
Cellulose BW 200	N/A	50	50	N/A	50	50	N/A	50	50
Soybean Oil	N/A	25	25	N/A	25	25	N/A	25	25
Lard	N/A	20	177.5	N/A	20	177.5	N/A	20	177.5

Product	Chow	LF	HFHS	Chow+BP	LF+BP	HFHS+BP	Chow+CE	LF+CE	HFHS+CE
Mineral Mix S10026	N/A	10	10	N/A	10	10	N/A	10	10
DiCalcium Phosphate	N/A	13	13	N/A	13	13	N/A	13	13
Calcium Carbonate	N/A	5.5	5.5	N/A	5.5	5.5	N/A	5.5	5.5
Potassium Citrate	N/A	16.5	16.5	N/A	16.5	16.5	N/A	16.5	16.5
Vitamin Mix	N/A	10	10	N/A	10	10	N/A	10	10
Choline Bitartrate	N/A	2	2	N/A	2	2	N/A	2	2
Purina Rodent 5001	1000	0	0	994.7	0	0	985.7	0	0

Product	Chow	LF	HFHS	Chow+BP	LF+BP	HFHS+BP	Chow+CE	LF+CE	HFHS+CE
Haskap 1 (g) (BP)	**0**	**0**	**0**	**14**	**14.7**	**12.0**	**0**	**0**	**0**
Haskap 2 (g) (CE)	**0**	**0**	**0**	**0**	**0**	**0**	**1.92**	**2.03**	**1.65**

Product	Chow	LF	HFHS	Chow+BP	LF+BP	HFHS+BP	Chow+CE	LF+CE	HFHS+CE
FD&C Red Dye #40	0	0.05	0	0	0	0.025	0.3	0.025	0
FD&C Yellow Dye #5	0	0	0.05	0	0.025	0.025	0	0	0
FD&C Blue Dye #1	0	0	0	0.3	0.025	0	0	0.025	0.05
Total	**1000**	**1055**	**858.2**	**1000**	**1056.2**	**859.26**	**1000**	**1057.1**	**859.801**
Haskap 1 (%) (BP)	**0**	**0**	**0**	**1.4**	**1.4**	**1.4**	**0**	**0**	**0**
Haskap 2 (%) (CE)	**0**	**0**	**0**	**0**	**0**	**0**	**0.192**	**0.192**	**0.192**

LF, low fat; HFHS, high-fat high sucrose; CE, C3G-rich haskap extract supplement; BP, Haskap whole berry powder; Carb, carbohydrate; N/A, not available in the data sheet which is provided by the Research Diet company.

3.5.2 Glucose homeostasis

To examine the effect of supplementations on glucose homeostasis and insulin sensitivity in ttreatments groups, mice were subjected to an intraperitoneal glucose tolerance test (ipGTT) at week 12 and intraperitoneal insulin tolerance test (ipITT) at week 14.

3.5.2.1 Intraperitoneal glucose tolerance test (ipGTT):

ipGTT was performed to determine the effect of C3G-rich diet on glucose homeostasis, particularly under obesogenic conditions. The test was performed in week 12, using all 54 mice across the nine types of different diet groups. The animals were fasted for 16 h and were weighed both pre- and post-fasting. During fasting, food was withdrawn from the animals, but free access to water was given. The amount of glucose required for each animal was measured according to the weight after fasting, and each animal was injected intraperitoneally with 2 g glucose/kg body weight. The glucose solution (20%) was prepared freshly on the day of the test and sterile filtered using a 0.2 μm polyethersulfone (PES) filter. To collect the blood, the tail vein was nicked with an 18G needle, and the tail was milked properly for a sizeable drop of blood to form. Following that, a glucose strip (Accu-Chek) was used to soak up the blood, and the glucose levels were measured using a glucometer (Accu-Chek Aviva Nano, Roche, Mannheim, Germany). The readings were recorded prior to glucose injection (0 min) and after injection at 15, 30, 60, and 120 min. The glucose homeostasis was measured in terms of total glycemia (mM).

3.5.2.2 Intraperitoneal insulin tolerance test (ipITT)

The 54 animals across the nine types of diets were subjected to ipITT at week 14, in order to analyze whether any improved glucose homeostasis was due to increased insulin sensitivity induced by the C3G-rich extract. Insulin was freshly prepared on the day of the

test and injected intraperitoneally at a dose of 1 U/kg body weight or a concentration of 0.1U/mL. Briefly, 20 μL of sterile insulin was diluted with 1.98 mL of sterile saline. The final concentration of the insulin in U/mL was obtained by diluting 2 mL of previously diluted insulin with 18 mL of sterile saline. The mice fasted for 4 h following which they were weighed, and the volume of insulin for each mice was calculated. During fasting, food was withdrawn from the animals, but free access to water was given. The tail vein was nicked to collect blood, with an 18G needle and the tail was milked properly for a sizeable drop of blood to form. Following that, a glucose strip (Accu-Chek) was used to soak up the blood, and the glucose levels were measured using a glucometer (Accu-Chek Aviva Nano). The readings were recorded prior to insulin injection (0 min) and after the insulin injection at 15, 30, 60, and 120 min. The insulin sensitivity was measured in terms of total glycemia (mM).

3.5.3 Measurements of body weight, food intake and peripheral fat mass

Body weight changes were measured every two weeks, and food intake was assessed in the 6th week. Three animals (n=3) from each type of diet were used for food intake study. The food was placed on a metal grid in each cage. Briefly, the single animal was housed per cage and initially, the weights of the animal, weights of the grids, and the weights of the mouse-cage beddings were measured on day 1 in the 6^{th} week. Food was weighed and placed on the grid. Finally, the weights of the animals, the weights of the grids and weights of the beddings were measured each day continuously in the 6^{th} week. Moreover, peripheral fat mass gain also was measured at 8^{th} and 16^{th} week using X-ray in *in-vivo* imagining (Bruker Ltd, Milton, Ontario, Canada). The mice body parameters including body weight,

food intake, peripheral fat mass, ipGTT and ipITT measurements were recorded with the help of a postdoctoral fellow at DMNB.

3.5.4 Western blot

The animals were euthanized after 16 weeks by decapitation using a guillotine (Kent Scientific Corporation), and tissues were harvested separately and immediately transferred to -80 ºC refrigerator for further use. Liver tissues were ground under liquid nitrogen, and 50 mg were homogenized using the tissue-homogenizer (Omni international, GA, USA) in lysis buffer (pH 7.4) consisting of sodium orthovanadate (Calbiochem, Cat #: 567540), phosphatase inhibitor cocktail set IV (Millipore Cat #: 524628), and protease inhibitor cocktail (Sigma, Cat #: P8340) and kept on ice for 45 min. After centrifugation at 5,000 g for 20 min, the protein content of the supernatant was quantified using a BSA protein assay kit (Thermo Scientific, Pierce Biotechnology, IL, USA). Protein lysates were boiled for five minutes in sodium dodecyl sulfate 4X (SDS; Calbiochem, Cat #: 7910-500G) with dithiothreitol (DTT; Cat #: CA97061-340). An aliquot (25 μg) of the samples were subjected to polyacrylamide gel electrophoresis (PAGE) in 4-20% gels. The gels were run at 90 volts for 30 minutes and then switched to 120 volts for 1.5 hours to separate proteins according to size. At this point, proteins were transferred from the gels to a nitrocellulose membrane (90 volts for 90 min at 4 °C with contact of transfer buffer), and membranes were subsequently blocked in 5% (w/v) milk (skimmed milk powder) in Tris-buffered saline [TBS 20×(Trisma-HCl base, sodium chloride, and distilled water, pH 8.4) and Tween-20 (Bio-Rad, Cat #: 170-6531) (TBS-T, 7.6 pH)] for 40 min. Membranes were then incubated with primary antibody overnight in 10 mL of 1% (w/v) milk in TBS-T. The primary antibodies used for Western blot analysis were pACC Ser 79, ACC, pAMPKT172,

AMPK and FBPase, which were purchased from Cell Signaling Technologies (Danvers, MA, USA). The next day, membranes were incubated for 2 h at room temperature with the corresponding secondary antibody depending on the nature of the primary antibody (anti-rabbit and anti-goat antibodies accordingly) (Santa Cruz Biotechnology, Texas, USA), and subsequently imaged using Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Cat #: NEL105001EA). Images were collected using a Bio-Rad imager and data were analyzed using Image Lab 5.0 software, and protein expression data was corrected to total Coomassie protein stain for densitometry analysis.

3.6 Statistical analysis

For field experiments and *in vitro* assays:

Repeated measures analysis was used to determine the effect of the cultivar and harvesting date on the polyphenol composition of haskap berry samples. The experimental design adopted was the completely randomized design (CRD), with three replications. Harvest time was the repeated measure time factor (5 harvests). The model has the main effects of cultivar and harvest time as well as their interaction effect. The analysis was completed using the Mixed Procedure of SAS (SAS Institute Inc. 2014), and further multiple means comparison was done for significant ($p<0.05$) effects by comparing the least squares means of the corresponding treatment combinations if the interaction effect is significant, or the levels of the factor(s) when the interaction is not significant. Letter grouping was generated using a 5% level of significance for the main effects (cultivar or harvesting date) and using a 1% level of significance for the interaction effect of cultivar and harvesting date. For each response, the validity of the model assumptions was verified by examining the residuals as described by Montgomery (2017).

For *in vivo* studies:

Statistical analysis was performed using a two-way ANOVA using PRISM8 statistical software (Graphpad Software, Inc., San Diego, CA, USA) followed by a Tukey's multiple comparison test; *p<0,05, **p<0.01, **** p<0.0001.

CHAPTER 4: RESULTS

4.1 Effect of cultivar and harvest date on polyphenol profile of haskap

The morphological color was changed from green to dark purple/blue in haskap berries harvested across five harvesting dates (H1-H5) from four cultivars including Aurora, Evie, Larissa, and Rebecca (Figure 7). All the berries at H5 were in dark purple (Figure 7). The interaction effect between cultivar and harvesting date was significant in the percentage of blue colored berry (Table 3). A pattern was observed that no significant difference was observed at the percentage of blue colored berries at H5 across all the cultivars ($p \leq 0.05$) (Table 3). The blue colored berry percentage of Evie at H1 was significantly lower compared to other cultivars (Table 3).

4.1.1. The sugar content

The sugar content (°Brix index) of haskap berries was evaluated using a hand-held digital refractometer in both the field and the laboratory. The cultivar Larissa at H5 harvesting date showed significantly highest value while the cultivar Evie at H1 showed the lowest value both in field and laboratory Brix measurements ($p \leq 0.05$). In summary, the highest field Brix: 10.75%; the lowest field Brix: 6.19%; the highest laboratory Brix: 11.3%; the lowest laboratory Brix: 4.4% (Figure 8A). Field Brix values at H2 of Aurora, H2, and H3 of Rebecca and H2, H3, and H4 of Larissa were not significantly different (Figure 8A). Overall, lower Brix measurements were observed in the cultivar Evie from H1-H5. However, a similar pattern was observed in both field and laboratory measurements of sugar content (Figure 8A). Furthermore, a positive correlation was observed between field Brix and laboratory Brix values ($r^2 = 59.5\%$) (Figure 8B).

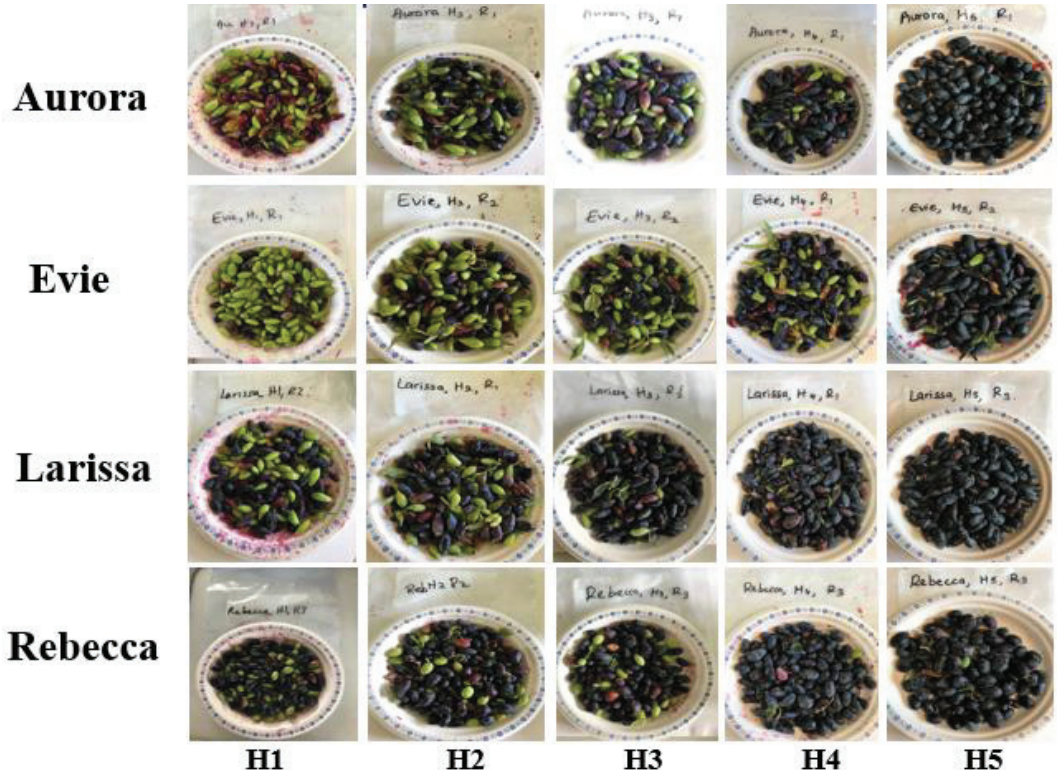

Figure 7. Photographs of the berries of five harvesting dates (H1 – H5) in four cultivars of haskap, Aurora, Evie, Larissa, and Rebecca.

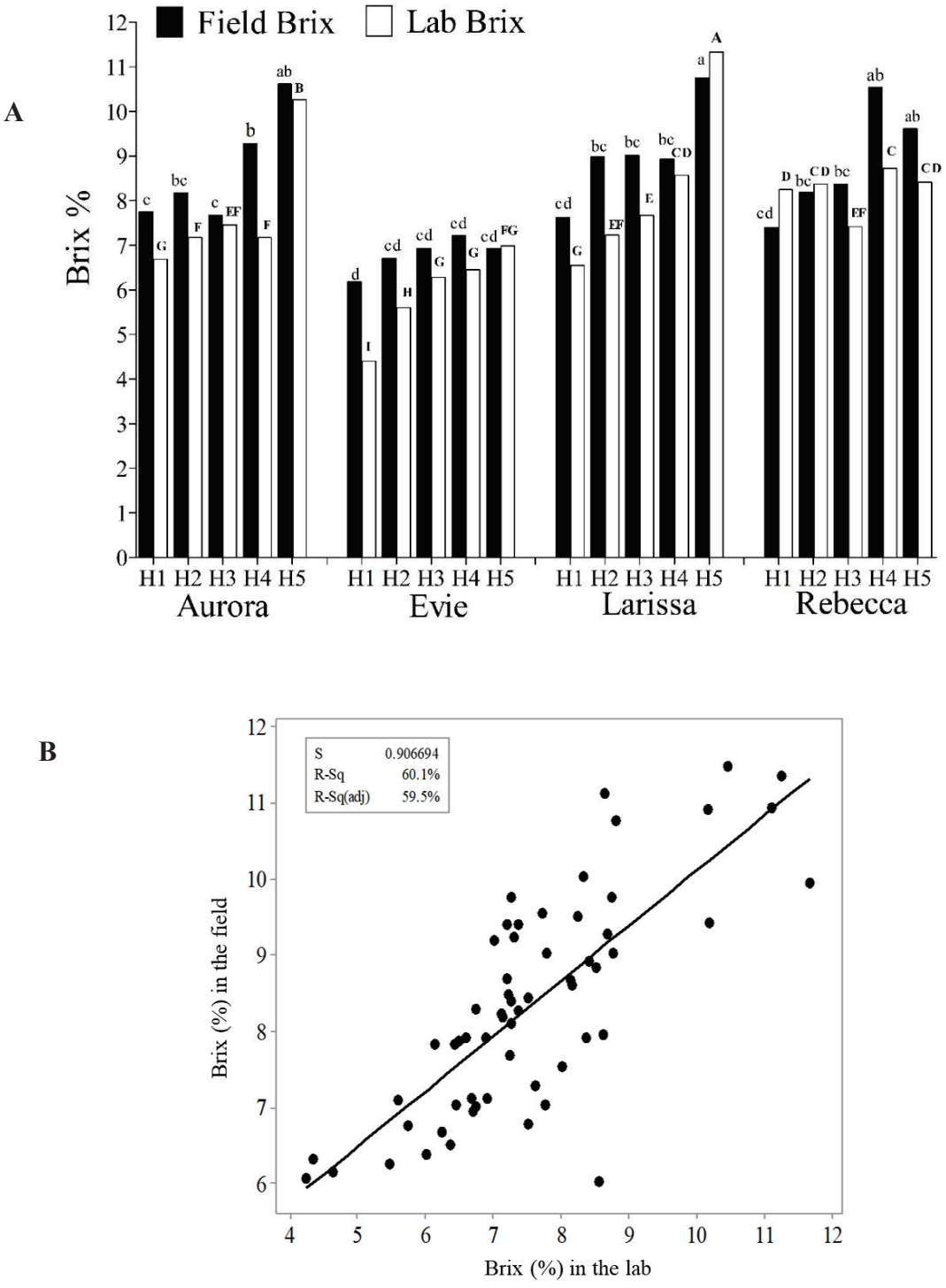

Figure 8. Brix measured in the field and laboratory for berries of H1-H5 harvesting dates of four haskap cultivars, Aurora, Evie, Larissa, and Rebecca **(A)**. Scatterplot of Brix measurements (field Vs. laboratory) using Minitab18 **(B)**. Means sharing the same letter are not significantly different. The measurements were performed in triplicate (n=3) and the mean values were calculated.

4.1.2. Distribution of anthocyanin among cultivars and different harvesting dates

4.1.2.1 Total anthocyanin content (TAC) by the pH differential method

The TAC of haskap berries across H1-H5 determined by the pH-differential method has a range between 39.2 to 294.0 mg C3GE/100g FW (Table 3). The cultivar Aurora at H5 was attributed with the highest average value of total anthocyanins (294.0 mg C3GE/100 g FW), while the cultivar Evie at H1 was found to be lower in anthocyanins (39.2 mg C3GE/100 g FW) (Table 3). However, as a pattern, TAC gradually increased from H1 to H5 of the cultivar Larissa. In contrast, no pattern was observed in TAC measured for other cultivars; Aurora, Evie, and Rebecca. Overall, H5 harvest date has the significantly higher total anthocyanin content (247.36 mg C3GE/ 100 g FW) compared with other harvest dates.

4.1.2.2 Non-destructive anthocyanin measurement

Based on the Multiplex measurements, cultivar Larissa at H5 and cultivar Rebecca at H4 demonstrated significantly higher ($p \leq 0.05$) total anthocyanin content among all the cultivars while the lowest was observed at H1 of the cultivar Evie. No statistical difference was found at H5 in both Aurora and Evie (Table 3). Total anthocyanin content at H1, H2, H3 of Aurora, H2, H3 and H4 of Evie H1, H2 of Larissa and H1 of Rebecca were not significantly different (Table 3). Multiplex fluorescence measurements of TAC were positively correlated with the TAC of the berries measured by other two methods, UPLC-ESI-MS analysis (R^2 = 68.3%) and pH differential method (R^2 = 65.9%) (Figure 9A and 9B). The strong positive correlation was represented by the pH differential method and UPLC-ESI-MS analysis of TAC of haskap berries (R^2 = 86.9%) (Figure 9C).

4.1.3 The major polyphenols measured by UPLC-ESI-MS

The polyphenols present in four commercially grown haskap cultivars, Aurora, Evie, Larissa, and Rebecca, at five harvesting dates (H1, H2, H3, H4, and H5), were extracted separately using 80% ethanol, 0.5% formic acid, 18.5% distilled water, and the concentration of major polyphenols was determined. The haskap berries contain predominantly flavonoids and phenolic acids. Anthocyanin is the major type of flavonoid. C3G is the most prominent type of anthocyanin, which accounts for 79% of total anthocyanin in all the extracts. Similar results of C3G being the most abundant anthocyanin of *L. caerulea* L. cultivars have been reported (Rupasinghe et al., 2015; Jurikova et al., 2012; Gazdik et al., 2008).

The cultivar by harvesting date interaction effect (cultivar × harvesting date) was significant ($p \leq 0.05$) for UPLC-ESI-MS measurements including five types of anthocyanins; C3G, C3R, Pet3G, Peo3G, C35DG, TAC content by the pH differential method, and TAC by Multiplex fluorescence detector (Table 3). Larissa at H5 and Evie H5 contained significantly highest C3G content compared to other harvesting dates and cultivars (Figure 10A). The average C3G content of Larissa from H1 – H5 varies from 208 – 1212 mg/100 g FW (Table 3). The TAC at H5 in both Larissa and Evie cultivars were significantly dominant compared to other cultivars (Figure 10B). The TAC and C3G concentration among the four cultivars and five harvesting dates showed similar patterns (Figure 10A and 10B). Significantly lower TAC was observed at H1, H2 of Evie and H1, H3 of Aurora. However, no significant difference was observed in TAC of H2, H4 of Aurora, H1, H2, and H5 of Rebecca, H1, H2, H3 of Larissa and H3 of Evie (Table 3). Overall, a similar pattern was observed in the measurement of TAC using all the three

methods including, UPLC-ESI-MS analysis, pH differential method, and Multiplex fluorescence measurements (Table 3). The cultivar Larissa has the overall highest mean value of anthocyanin compared to the other three cultivars.

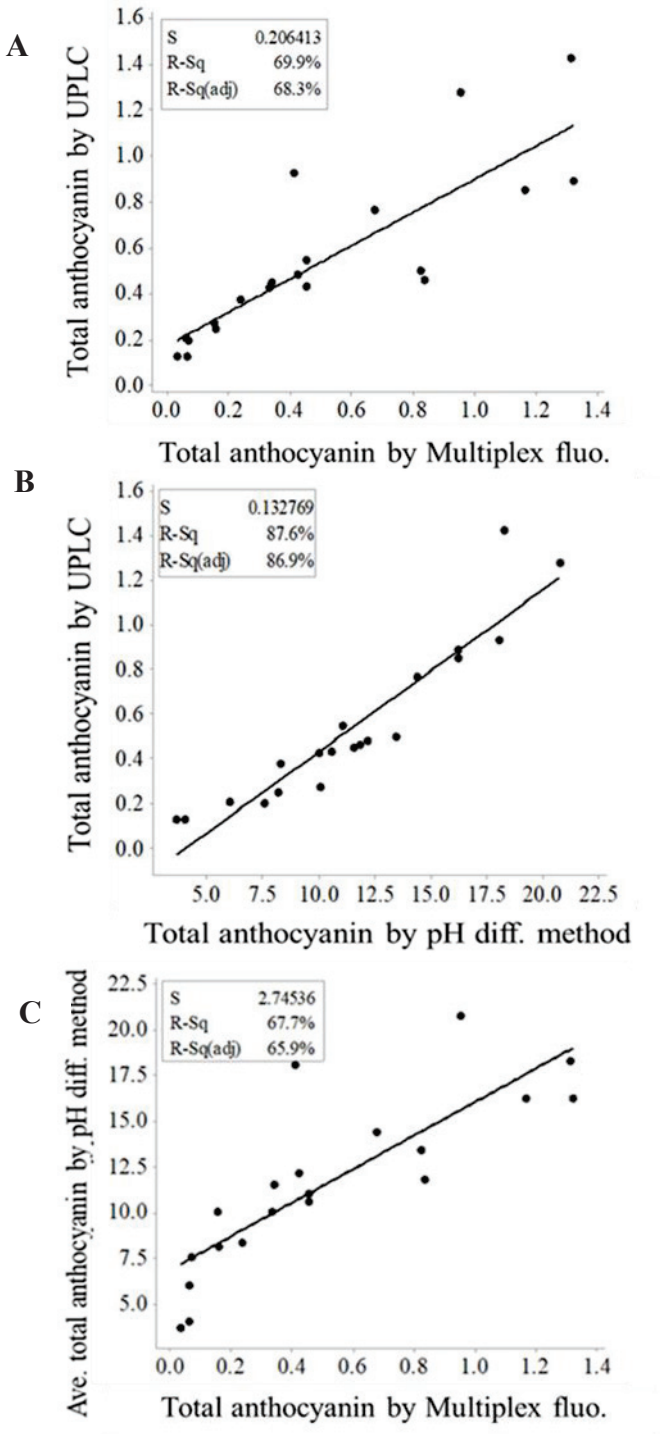

Figure 9. Correlation between average total anthocyanin measured using UPLC and Multiplex fluorescence detector (Multiplex fluo.) (R^2 = 68.3%) **(A),** pH differential method (pH diff.) and Multiplex fluorescence detector (R^2 = 65.9%) **(B),** and UPLC-ESI-MS and pH differential method (R^2 = 86.9%) **(C).**

Table 3. Indicators of anthocyanin content of berries harvested from five different harvest dates of four haskap cultivars.

Cultivar	Har. date	BB%	Anthocyanin concentration by UPLC-ESI-MS						pH diff. TAC	Multiplex TAC
			C3G	C3R	Pet3G	Peo3G	C35DG	TAC		
Aurora	H1	56.3bc	141.9ef	5.1de	16.0bcdef	6.6def	32.0cd	201.6d	85.8575	0.06cd
	H2	55.1bc	185.9ef	5.0de	12.4cdef	6.5ef	34.7cd	244.3cd	109.852	0.16cd
	H3	64.6b	91.7f	2.7e	11.1def	4.4ef	16.7d	126.3d	55.1185	0.06cd
	H4	74.1b	347.2def	9.9cde	45.3ab	16.5abcd	59.3bcd	478.4cd	163.19	0.42c
	H5	99.7a	665.1bc	21.9a	43.1abcd	20.3ab	98.7abc	849.1bc	294.005	1.16ab
Evie	H1	19.6d	96.9f	0.9e	2.5f	2.8f	18.3d	121.3d	39.1831	0.03d
	H2	40.9c	160.8ef	1.3e	4.5f	4.2f	24.7d	195.2d	93.6642	0.07cd
	H3	66.3b	301.7ef	2.7e	10.7ef	7.4cdef	49.7cd	372.0cd	88.3033	0.24cd
	H4	70.7b	760.0b	4.7e	18.5bcdef	17.2abc	125.7ab	926.3b	205.615	0.41cd
	H5	96.4a	1070.2a	6.4de	34.2abcdef	21.6a	142.0a	1274.4a	274.343	0.95ab

Cultivar	Har. date	Blue%	Anthocyanin concentration by UPLC-ESI-MS						pH diff. TAC	Multiplex TAC
			C3G	C3R	Pet3G	Peo3G	C35DG	TAC		
Larissa	H1	54.3bc	208.0ef	5.7de	12.6cdef	4.3ef	38.0cd	268.5cd	130.44	0.15cd
	H2	65.2b	306.1ef	6.8de	38.5abcde	11.7bcdef	58.0bcd	421.0cd	131.969	0.33cd
	H3	93.3ab	399.9cde	9.2cde	21.3abcdef	5.8ef	61.7bcd	497.9cd	179.735	0.82b
	H4	83.1ab	629.7bcd	14.4abcd	29.0abcdef	9.6cdef	81.7abcd	764.6bc	206.671	0.68bc
	H5	99.8a	1212.3a	17.7abc	44.0abc	16.8abc	132.3a	1422.9a	259.407	1.31a
Rebecca	H1	68.2b	322.4ef	9.6cde	26.4abcdef	14.2abcde	72.0abcd	444.8cd	165.511	0.34cd
	H2	69.3b	331.8def	10.0bcde	18.2bcdef	11.5bcdef	57.7bcd	429.5cd	149.626	0.45bc
	H3	87.5ab	402.0cde	8.8cde	27.6abcdef	16.7abc	86.3abcd	541.4c	156.359	0.45bc
	H4	95.8a	692.9bc	17.5abc	50.5a	24.0a	102.0abc	886.8b	278.306	1.32a
	H5	96.6a	341.2def	4.8de	13.2cdef	17.2abc	81.0abcd	457.3cd	161.689	0.84b
SV										
Cultivar		0.001	0.008	0.093	0.178	0.302	0.345	0.005	0.944	0.163
Date		0.001	0.001	0.001	0.003	0.001	0.001	0.001	**0.001** [b]	0.001
Cultivar*Date		**0.001**[b]	**0.001**[b]	**0.001**[b]	**0.025**[b]	**0.033**[b]	**0.037**[b]	**0.001**[b]	0.998	**0.001**[b]

SV = Source of variation. [b] Significant or marginally significant effects that need multiple means comparison are shown in bold

Mean values of blue colored berry percentage (Blue %), mean values of five types of anthocyanin (C3G, C3R, Pet3G, Peo3G, C35DG) (mg/100 g FW), total anthocyanin content by UPLC-ESI-MS analysis (mg/100 g FW), total anthocyanin content measured by pH differential method (mg C3GE /100 g FW) and total anthocyanin content measured by Multiplex fluorescence

detector (Multiplex index) obtained from four cultivars (Aurora, Evie, Larissa, and Rebecca) and five harvesting dates (H1,H2,H3,H4, and H5) in three replicates (n=3) separately and determined the mean value. ANOVA p-values show the significance of the main and interaction effects of cultivar and harvesting date. Within each column, means sharing the same letters are not significantly different. BB, Blue berry, C3G, cyanidin-3-*O*-glucoside; C3R, cyanidin-3-*O*-rutinoside; Pet3G, Petunidin-3-*O*-glucoside; Peo3G, Peonidin-3-*O*-glucoside; C35DG, Cyanidin-3,5-diglucoside; UPLC, High-performance liquid chromatography; Har. date, harvested date; TAC, Total anthocyanin content; pH diff., pH differential method of total anthocyanin content; Multiplex antho., TAC measured by Multiplex system.

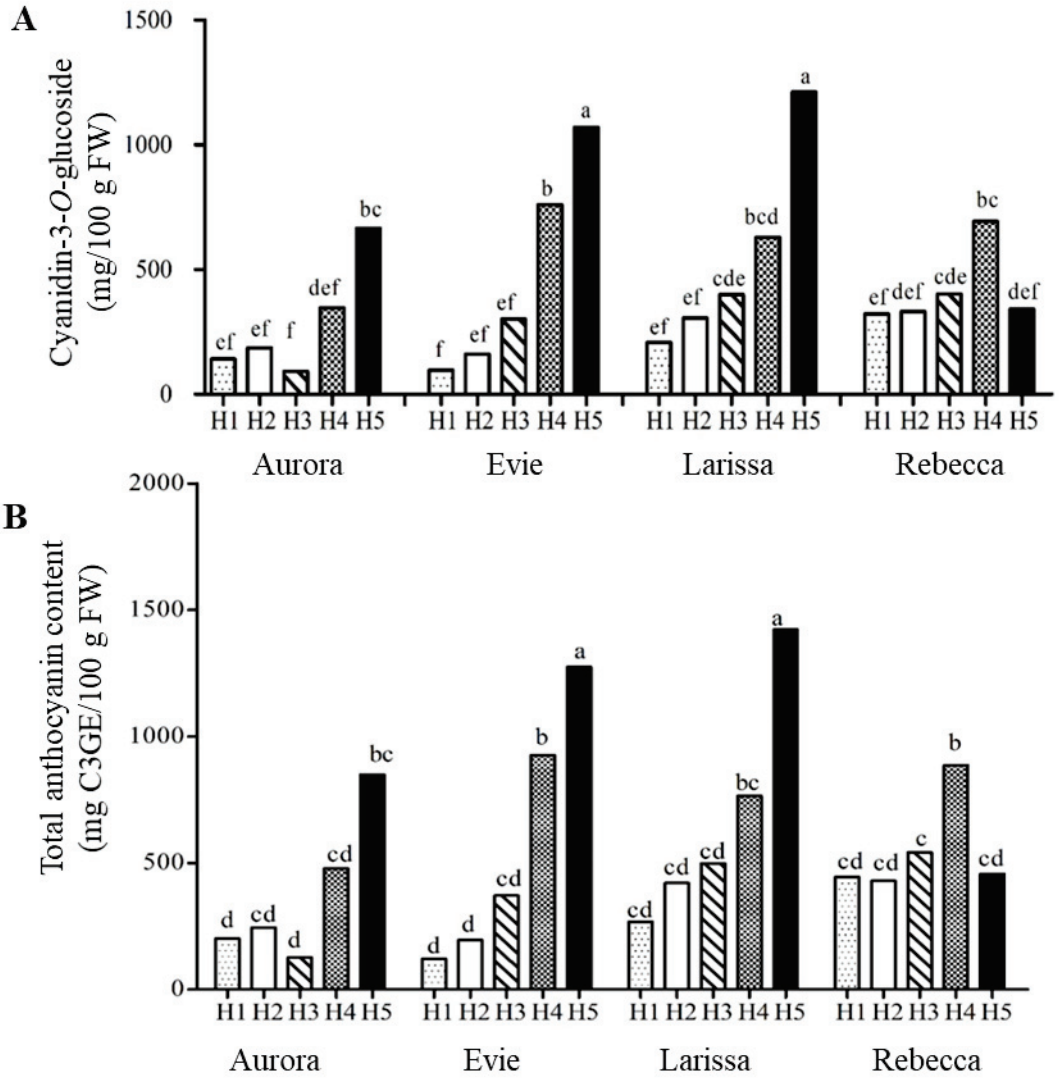

Figure 10. The variation of cyanidin-3-*O*-glucoside content (C3G) **(A)** and total anthocyanin content **(B)** among harvesting dates (H1-H5) of four haskap cultivars. Each bar with different letters (a-f) represents a significant statistical difference ($p \leq 0.05$). The measurements were done using UPLC-ESI-MS. The measurements were obtained from three replicates (n=3) separately and determined the mean value.

Q3R was the most abundant flavonol as determined by the UPLC-ESI-MS (Table 4). Harvesting dates H1, and H4 of Rebecca exhibited the highest amount of Q3R, 47.81 mg/100g FW; while cultivar Rebecca at H5 exhibited the least amount of 14.31 mg/100 g FW. These results agree with the previous studies, which reported a range of 7-17 mg/100

g FW for Q3R (Wojdyło et al., 2013). The concentration of the Q3R followed by the Q3arabinoG and Q3Glu; while Q3Gal was the least present flavonol across four cultivars (Table 4). Statistically, the interaction effect of the cultivar by harvesting date (cultivar × harvesting date) was significant on non-anthocyanin flavonoid compounds including Q3Gal, Q3Rha, Q3R, catechin and Epigallocatechin gallate (EGCG) ($p \leq 0.05$) (Table 4). The main effect, cultivar was significant on Q3arabinoG. The main effect, harvesting date was significant on chlorogenic acid and epicatechin (Table 4). Both the main effects, cultivar and harvesting date, was significant on Q3Glu and epicatechin gallate (ECG) (Table 4). The haskap berries also contained phenolic acids. Interestingly, approximately 95% of the phenolic acids determined consisted of chlorogenic acid. Moreover, H1 harvest date showed significantly higher chlorogenic acid content (70.74 mg/100g FW). Among flavan-3-ol compounds, epicatechin and catechin were observed as the most abundant in the haskap cultivars (Table 4). Larissa at H1 and Aurora at H3 exhibited the highest concentrations of catechin and epicatechin, respectively. Overall, the phenolic composition was observed to be consistent with previous reports comparing haskap to various types of berries (Rupasinghe et al., 2012; Jurikova et al., 2012).

Table 4. The concentration of non-anthocyanins from berries harvested from five different harvest dates of four haskap cultivars.

Cultivar	Har. date	Flavonols					Chlor. acid	Flavan-3-ol				Total non. antho.
		Q3Gal	Q3Glu	Q3ArG	Q3Rha	Q3R		Catechin	Epicat.	ECG	EGCG	
Aurora	H1	0.10ab	2.28	2.84	0.13d	39.91ab	67.03	3.58bc	2.57efg	1.76	0.19abc	120.4
	H2	0.10ab	2.91	3.81	0.13d	39.66ab	68.65	3.14c	4.03cd	1.39	0.2ab	124
	H3	0.10ab	2.86	4.75	0.17cd	40.13ab	65.8	1.51c	6.03a	0.99	0.19abc	122.5
	H4	0.10ab	3.54	4.13	0.27ab	38.77ab	50.09	1.66c	5.57ab	0.99	0.14bcd	105.2
	H5	0.10ab	3.45	2.19	0.20bcd	41.98ab	55.57	4.16bc	1.43h	1.4	0.15abcd	110.6
Evie	H1	0.06ab	6.64	19.71	0.17cd	38.08ab	70.09	4.79bc	4.97bc	0.91	0.10cde	145.5
	H2	0.08ab	7.05	19.44	0.19bcd	37.67ab	70.02	3.44bc	4.67bc	1.08	0.11bcde	143.8
	H3	0.09ab	7.38	18.17	0.21bcd	36.78ab	59.02	3.04c	5.03abc	0.93	0.10de	130.7
	H4	0.11ab	8.55	18.13	0.25abc	32.57b	52.37	2.54c	5.40ab	0.83	0.07de	120.8
	H5	0.11ab	9.42	16.98	0.33a	34.89ab	50.38	1.96c	5.57ab	0.83	0.10cde	120.6

Cultivar	Har. date	Flavonols					Chlor. acid	Flavan-3-ol				Total non. antho.
		Q3Gal	Q3Glu	Q3ArG	Q3Rha	Q3R		Catechin	Epicat.	ECG	EGCG	
Larissa	H1	0.13a	4.34	8.24	0.21bcd	44.22ab	74.17	7.84a	1.83gh	1.25	0.11bcde	142.3
	H2	0.11ab	4.03	5.69	0.20bcd	40.49	69.04	7.15ab	1.40h	1.32	0.11bcde	129.6
	H3	0.14a	4.18	5.91	0.21bcd	42.53ab	63.69	7.56ab	1.50h	0.95	0.11bcde	126.8
	H4	0.14a	4.78	6.58	0.19bcd	39.83ab	56.52	6.87ab	2.50efgh	0.89	0.12bcde	118.4
	H5	0.11ab	5.7	7.43	0.21bcd	35.56ab	45.94	4.99bc	2.93ef	0.88	0.05e	103.8
Rebecca	H1	0.10ab	3.66	3.73	0.17cd	47.81 a	71.67	5.41b	1.70gh	1.98	0.16abcd	135.9
	H2	0.06ab	3.4	3.94	0.17cd	40.87ab	68.34	4.27bc	1.93fgh	2.1	0.24a	125
	H3	0.04b	6.29	8.11	0.22bcd	33.52 b	59.9	3.08c	3.37de	1.87	0.13bcde	116.5
	H4	0.11ab	4.88	3.82	0.23bc	47.81 a	62.5	3.94bc	1.80gh	1.74	0.14bcd	127
	H5	0.06ab	7.07	8.17	0.23bc	14.31 b	41.35	1.13c	4.23cd	1.84	0.08 de	78.4
SV												
Cultivar		0.017	**0.001**[b]	**0.001**[b]	0.027	0.142	0.957	0.001	0.001	**0.001**[b]	0.01	0.057
Date		0.077	**0.002**[b]	0.698	0.001	0.006	**0.000**[b]	0.001	0.002	**0.001**[b]	0	**0.001**[b]
Cultivar*Date		**0.020**[b]	0.187	0.125	**0.031**[b]	**0.016**[b]	0.132	**0.003**[b]	**0.001**[b]	0.063	**0.006**[b]	0.246

SV = Source of variation. [b] Significant or marginally significant effects that need multiple means comparison are shown in bold face.

Means of non-anthocyanin polyphenols: five types of flavonol: Q3Gal (Quercetin-3-*O*-galactoside), Q3Glu (Quercetin-3-*O*-glucoside), Q3ArabinoG (Quercetin-3-*O*-arabinoglucoside), Q3Rha (Quercetin-3-*O*-rhamnoside), Q3R (Quercetin-3-*O*-rutinoside); chlorogenic acid; four types of flavan-3-ol: catechin, epicatechin, ECG (epicatechin gallate), EGCG (epigallocatechin gallate); and total non-anthocyanins (mg/ 100g FW) obtained from four cultivars (Aurora, Evie, Larissa, and Rebecca) and five harvesting dates (H1, H2, H3, H4, H5)) in three replicates (n=3) separately and determined the mean value. ANOVA p-values show the significance of the main and interaction effects of cultivar and harvesting date. Within each column, means sharing the same letter are not significantly different. Har. Date, harvested date; Chlor. acid, chlorogenic acid; Epicat., epicatechin; total non-antho; total non-anthocyanins.

4.1.4. Total phenolic content (TPC)

The TPC of aqueous ethanol extracts of the four cultivars determined by the Folin-Ciocalteu assay ranged from 425.4 to 715.7 mg GAE/100 g FW (Table 5). Previously reported values of phenolic content of haskap berries were 428.1 to 903 mg GAE/100g FW, similar to current data (Rupasinghe et al., 2012; Rop et al., 2011). However, the cultivar Larissa at H5 harvesting date showed the higher value of TPC measured by both UPLC-ESI-MS and Folin-Ciocalteu assay (Table 5). Moreover, no pattern was observed in TPC measurements by two methods, UPLC-ESI-MS and Folin-Ciocalteu assay in four cultivars across five harvesting dates (Table 5).

4.1.5. Total antioxidant capacity

The FRAP assay provides the antioxidant capacity of aqueous ethanol extracts of four cultivars. The average FRAP values varied from 773.3 to 1921.9 µmol TE/100 g FW among all the cultivars from H1- H5 harvesting dates (Table 5). However, the main effect "Day" was significant for total antioxidant capacity ($p \leq 0.05$) (Table 5).

Table 5. Total antioxidant capacity by FRAP (μmol TE/100 g FW), total phenolic content by UPLC-ESI-MS (mg/100 g FW), and total phenolic content by Folin-Ciocalteu assay (mg GAE/100 g FW) of berries harvested from five different harvest dates of four haskap cultivars.

Cultivar	Har. date	FRAP	Total phenolics by UPLC	Total phenolics by Folin-C assay
Aurora	H1	958.4	322.1 e	520.9
	H2	984.3	368.3 de	534.2
	H3	773.3	248.9 e	460.5
	H4	1239.3	583.7 de	555.1
	H5	1751.4	959.8 bd	692.7
Evie	H1	889	266.9 e	425.4
	H2	1060	339.0 e	518.9
	H3	1139.9	502.8 de	551.5
	H4	1626.3	1047.2 b	681.3
	H5	1921.9	1395.0 a	435.4
Larissa	H1	1085.6	410.9 de	573.3
	H2	1249	550.6 de	574.1
	H3	1437	624.8 de	616.8
	H4	1751.9	883.2 bd	630.9
	H5	1923.2	1526.8 a	715.7
Rebecca	H1	1505.1	581.2 de	457.5
	H2	1383.9	554.9 de	482.6
	H3	1529.8	657.9 d	512.5
	H4	1811	1013.8 b	674.4
	H5	919	535.9 de	777.1
SV				
Cultivar		0.467	0.004	0.938
Date		**0.002**[b]	0.001	**0.005**[b]
Cultivar*Date		0.2559	**0.001**[b]	0.598

SV = Source of variation. [b] Significant or marginally significant effects that need multiple means comparison are shown in bold font. Within each column, means sharing the same letters are not significantly different. Har. date, harvested date; FRAP, Ferric Reducing Antioxidant Power; Folin-C assay, Folin-Ciocalteu assay. ANOVA p-values that show the significance of the main and interaction effects of cultivar and harvesting date. The values were obtained from three replicates (n=3) separately and the mean values were calculated.

4.2 The *in vitro* studies of investigating anti-diabetic properties

4.2.1 Carbohydrate-hydrolyzing enzyme inhibitory assays

Extracts prepared from the four cultivars (Aurora, Evie, Larissa, and Rebecca) of haskap across five harvesting dates (H1-H5) inhibited alpha-amylase and alpha-glucosidase enzymes *in vitro* (Figure 11). A dose-dependent inhibitory effect of all the haskap extracts was observed in both carbohydrate-hydrolyzing enzyme inhibitory assays (Figure 11) (Appendix A and B). Clinically-used acarbose (standard drug) and epigallocatechin gallate (EGCG) were used for the comparison purpose. Acarbose and EGCG showed relatively low IC_{50} values in both enzyme inhibitory assays, indicating their high efficacy compared to haskap extracts (Figure 11). Compared to the haskap extracts, the IC_{50} value of acarbose and EGCG in alpha-amylase was 400-fold and 32-fold lower, respectively (Figure 11A). Among haskap extracts, Larissa H1-H4 showed the highest inhibition of alpha-amylase with IC_{50} values 2611, 2482, 2496, and 2376 μg/mL, respectively. These values were significantly different from the IC_{50} values obtained from other cultivars at H1-H4. The lowest inhibition of alpha-amylase enzyme was observed at H5 in Aurora, and moderate inhibition was found in Evie and Rebecca cultivars in H1-H5 harvesting dates (Figure 11A). Compared to the haskap extracts, the IC_{50} value of acarbose and EGCG in alpha-glucosidase was 10-fold and 50-fold lower, respectively (Figure 11B). The cultivar Evie from H1-H5 demonstrated the highest inhibitory activity of the alpha-glucosidase enzyme (IC_{50} values; 1131-1365 μg/mL); while, H5 in Aurora and Larissa showed the lowest inhibition (IC_{50} values; 2120 and 2066 μg/mL, respectively). Overall, among all haskap extracts, Larissa cultivar showed the lowest IC_{50} value, which indicated the highest

inhibition of alpha-amylase enzyme, whereas cultivar Evie, demonstrated the strongest inhibition against alpha-glucosidase (Figure 11A and B).

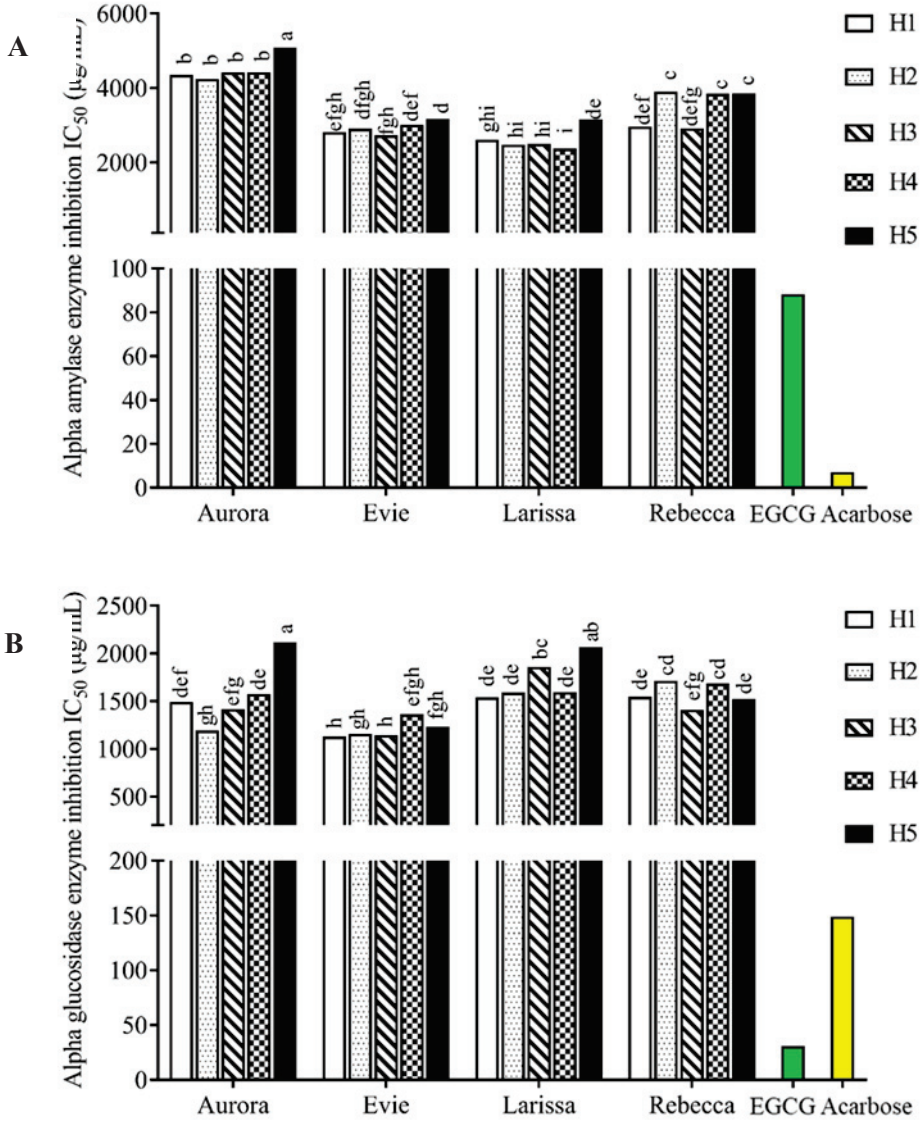

Figure 11. Carbohydrate-hydrolyzing enzymes inhibition by haskap extracts prepared from five maturity stages of four cultivars. Alpha-amylase enzyme inhibition **(A)**, alpha-glucosidase enzyme inhibition **(B)**, indicated in IC_{50} (µg/mL). The IC_{50} values were calculated using the PRISM8 software (Graphpad Software, Inc., San Diego, CA, USA). Multiple means comparison was done for significant ($p \leq 0.05$) effects by comparing the least squares means of the corresponding treatment combinations.

4.2.2 Dipeptidyl-peptidase (DPP-4) enzyme inhibition

Anthocyanin-rich polyphenols isolated from four cultivars and five harvesting dates of haskap berries were analyzed for the DPP-4 inhibitory activity. For the comparison, sitagliptin (IC_{50}, 315.7 µg/mL), a known DPP-4 inhibitor, was employed. Each haskap cultivar from five harvesting dates was tested in a concentration range within 100 to 40,000 µg/mL and exhibited IC_{50} values ranging from 2151 to 11563 µg/mL (Figure 12A). Significantly lower IC_{50} values were observed for the extracts of H5 of Larissa, H1, H2, and H4 of Evie and H1-H5 of Rebecca, which suggested a higher DPP-4 inhibition compared to other harvesting dates. The results showed that H5 of Larissa cultivar was the most effective of all cultivars and harvesting dates at reducing the activity of DPP-4 and the lowest IC_{50} value of 2151 µg/mL. Significantly high IC_{50} values were observed at H2, H4, and H5 in Aurora, and H1 in Larissa, which indicated a lower inhibition of DPP-4 enzyme (Figure 12A). The cultivar Aurora at H5 showed the highest IC_{50} value among all the other samples tested ($p \leq 0.05$). However, a certain pattern was not observed at DPP-4 enzyme inhibition among different harvesting dates of four cultivars (Figure 12A) (Appendix C). Moreover, the DPP-4 inhibitory activities are not correspondent to the TAC from four cultivars across H1-H5 harvesting dates, which was determined by UPLC-ESI-MS analysis (Table 3). For example, there is no significant difference in inhibition of DPP-4 enzyme by Evie H2 and H4, while the TAC was four times higher in H2 than that of H4 (Table 3). Additionally, a significant difference was not observed in IC_{50} values from H1-H5 in Rebecca (Figure 12A), while the TAC was comparatively higher at H4 of Rebecca (Table 3). However, the highest inhibition of DPP-4 enzyme (lowest IC_{50}) was observed at H5 of Larissa, which contained the highest TAC and C3G concentrations (Table 3).

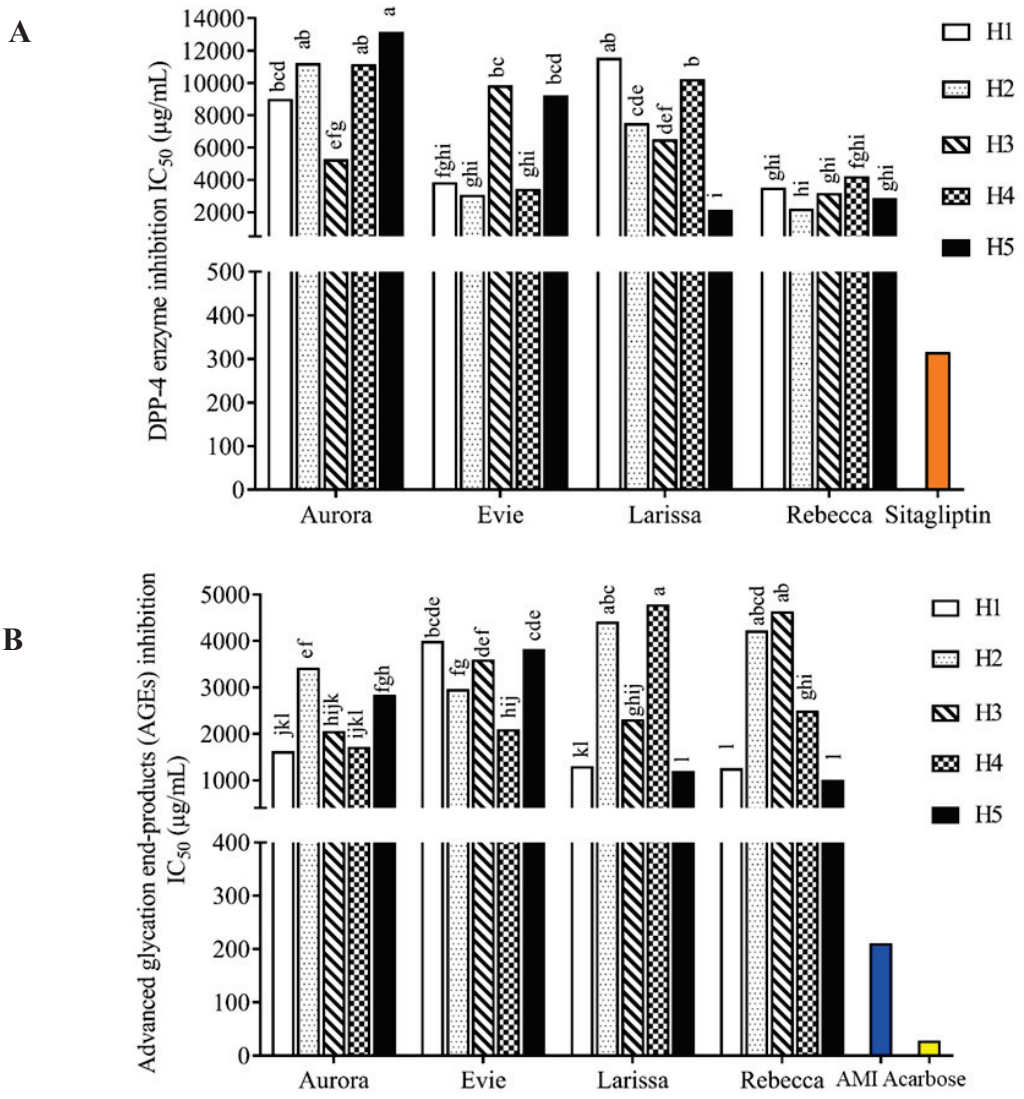

Figure 12. Inhibition of DPP-4 activity **(A),** and advanced glycation end-product formation **(B)** by haskap extracts prepared from five maturity stages of four cultivars. The clinical drug sitagliptin was employed for comparison purposes in DPP-4 assay. Aminoguanidine (AMI) and acarbose were used for the comparative purpose in AGEs inhibitory assay. The IC_{50} values were calculated using the PRISM8 software (Graphpad Software, Inc., San Diego, CA, USA). Multiple means comparison was performed for significant ($p \leq 0.05$) effects by comparing the least squares means of the corresponding treatment combinations.

4.2.3 Inhibition of advanced glycation end products (AGEs)

Fluorometric analyses of advanced glycation end-products (AGEs) inhibition by the haskap extracts exhibited the inhibitory activity with varying IC_{50} (Figure 12B). The observed IC_{50} values were ranged from 1195 – 4788 μg/mL of four cultivars across all five harvesting dates (H1-H5). Cultivars including Larissa (H2 and H4) and Rebecca (H2 and H3), showed the highest IC_{50}, hence, they were least effective compared to other cultivars and harvesting dates (Figure 12B). Clinically available anti-glycation drugs including aminoguanidine (211.3 μg/mL) and acarbose (27.6 μg/mL) showed the lowest IC_{50} values, which indicated higher inhibition of AFE formation compared to haskap extracts under the experimental conditions (Figure 12B). However, all the haskap extracts from four cultivars across H1-H5 showed AGEs inhibitory properties (Appendix D). Nevertheless, the IC_{50} values were approximately five-fold and forty-fold higher than the anti-glycation drugs, aminoguanidine, and acarbose, respectively.

4.3 The anti-diabetic properties of haskap *in vivo*

4.3.1 Effects of haskap supplementation on mice characteristics

The C57BL/6 mice (8 weeks old) were randomly assigned to nine experimental groups (n=6) and fed with different diets for 16 weeks, comprising of regular chow, low fat (LF) (10 kcal% fat; 17 kcal% sucrose), and HFHS (45 kcal% fat; 17 kcal% sucrose) diets. These three diet groups were further sub-divided based on the haskap supplementation: no haskap supplemented control group, C3G-rich extract supplementation (CE; C3G-rich extracts; 200 mg C3GE/g DW crude extract; 0.192% haskap extract), and haskap berry powder (BP; 24 mg C3GE/g DW; 1.4% haskap whole berry powder) (Table 2).

4.3.1.1 C3G-rich haskap supplement on the HFHS-fed mice protect from short term weight gain and peripheral adiposity

The mice on different diets were weighed every two weeks, to determine the effects of supplementation on body weight. The differences in body weights with C3G supplementation, particularly in the HFHS group was observed as early as six weeks (Figure 13A). Since the food intake significantly impacts weight gain, the average food intake was measured in the different groups for 7 days, at week 6. The results demonstrated a significant reduction in food intake in the CE supplemented HFHS diet fed mice (HFHS+CE) compared to the mice fed with Chow supplemented with CE (Chow+CE) ($p \leq 0.01$) (Figure 13B). Consistently, HFHS diet-fed mice with CE supplementation also showed a reduced weight gain at week 6, compared to HFHS-fed mice with no supplementation (HFHS+NS) and HFHS-fed, BP supplemented group (HFHS+BP) (Figure 13C).

A marked reduction in the peripheral fat mass was observed in the HFHS+CE group compared to HFHS+BP group and HFHS+NS group, at week 8 (Figure 14A). Further, the study indicated that the differences in body weight were also consistent with the differences in peripheral fat mass accumulation at week 8 (Figure 14A). Therefore, mice fed with HFHS+CE exhibited considerable resistance to diet-induced weight gain and fat accumulation in comparison to mice fed HFHS+BP ($p \leq 0.01$) and to a lesser extent from the HFHS diet fed mice with no supplementation, for a short period of 8 weeks ($p \leq 0.05$). The peripheral fat mass at week 16 was measured to ascertain whether the supplement-protection against body weight gain and peripheral fat accumulation was maintained for a longer period. However, CE supplementation did not confer resistance to fat mass

accumulation upon long term consumption of the HFHS diet (Figure 14B). This was also reflected in their body weight gain (Figure 14A). This observation suggested that the effect of CE supplementation is pronounced under an obesogenic milieu and it offers short term protection against HFHS diet-induced body weight gain and fat mass accretion.

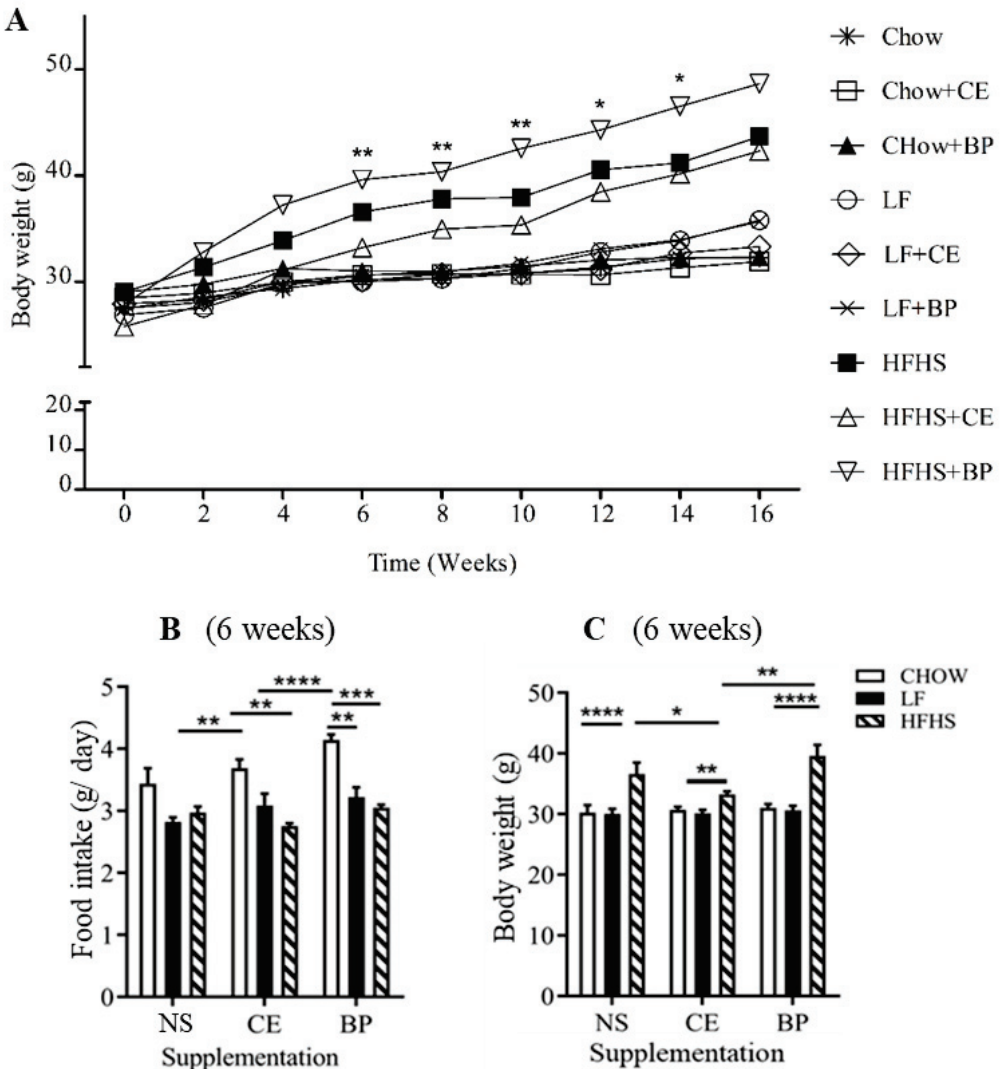

Figure 13. The impact of C3G-rich haskap extract (CE) supplementation on food intake and body weight of chow-, LF- and HFHS-fed mice. Body weight change from 0 – 16 week **(A)**, Average food intake (g/day) at week 6 **(B)**, body weight (g) at week 6 **(C)** were measured. Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple comparison test to compare the effect of HFHS diet and HFHS+supplementation; *$p \leq 0{,}05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ as indicated. NS, no

supplement; CE, C3G-rich haskap extract; BP, Haskap whole berry powder; LF, low fat; HFHS, high-fat high-sucrose.

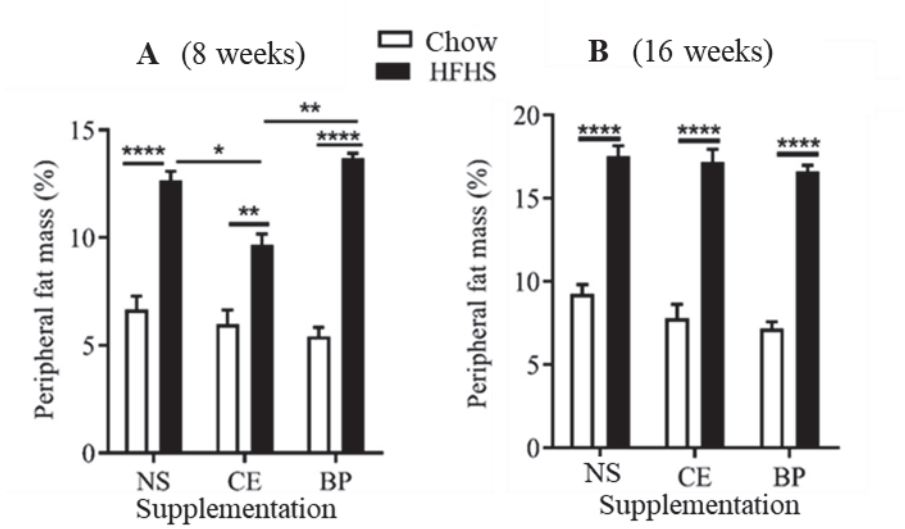

Figure 14. C3G-rich haskap extract (CE) supplemented diet reduced short-term peripheral fat mass gain in HFHS-fed mice. Peripheral fat mass gain (%) measured at 8 weeks **(A)**, and 16 weeks **(B)**. Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple comparison test; *$p \leq 0{,}05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ as indicated. NS, no supplement; CE, C3G-rich haskap extract; BP, Haskap whole berry powder; HFHS, high-fat high sucrose.

4.3.1.2. C3G-rich haskap extract supplementation improves glucose tolerance and insulin-stimulated glucose disposal in obese mice

In order to determine the effects of CE and BP on glucose homeostasis and insulin sensitivity, the animals were subjected to an intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT) at week 12 and week 14, respectively. Mice fed with HFHS diet presented with considerable hyperglycemia than the chow-fed mice, at 60 and 120 min following glucose administration (Figure 15A). Prominently, this difference in blood glucose levels was not observed between the chow and HFHS groups supplemented with CE at 60 min and was only moderately significant at 120 min (Figure 15B). However, the mice fed with HFHS supplemented with BP exhibited hyperglycemia

at earlier time points of 30, 60, and 120 min, compared to the other diet groups on CE supplementation (Figure 15C). Significantly higher blood glucose level was observed in Chow+BP supplemented group in 15 min after the glucose injection (Figure 15D). Moreover, no significant differences were observed in the LF diet fed group (Figure 15E). However, significantly higher blood glucose level was determined in HFHS+BP group in 30 min after the glucose injection (Figure 15F).

The effect of CE and BP supplementation on insulin sensitivity was also examined in the study. Mice in the chow diet group supplemented with BP exhibited a lower level of glycemia at 15 - 120 min, followed by an insulin injection at 0 min (Figure 16A). Moreover, the baseline blood glucose level was found to be higher in the LF+CE group compared to LF+BP group (Figure 16B). However, there were no pronounced differences in baseline blood glucose levels between HFHS+CE and HFHS+BP groups (Figure 16C). Moreover, the blood glucose level was significantly higher at 60 and 90 min after the insulin injection in HFHS+BP group (Figure 16C). Therefore, HFHS+CE group demonstrated lower blood glucose level compared to HFHS+BP group throughout 0-120 min period (Figure 16C).

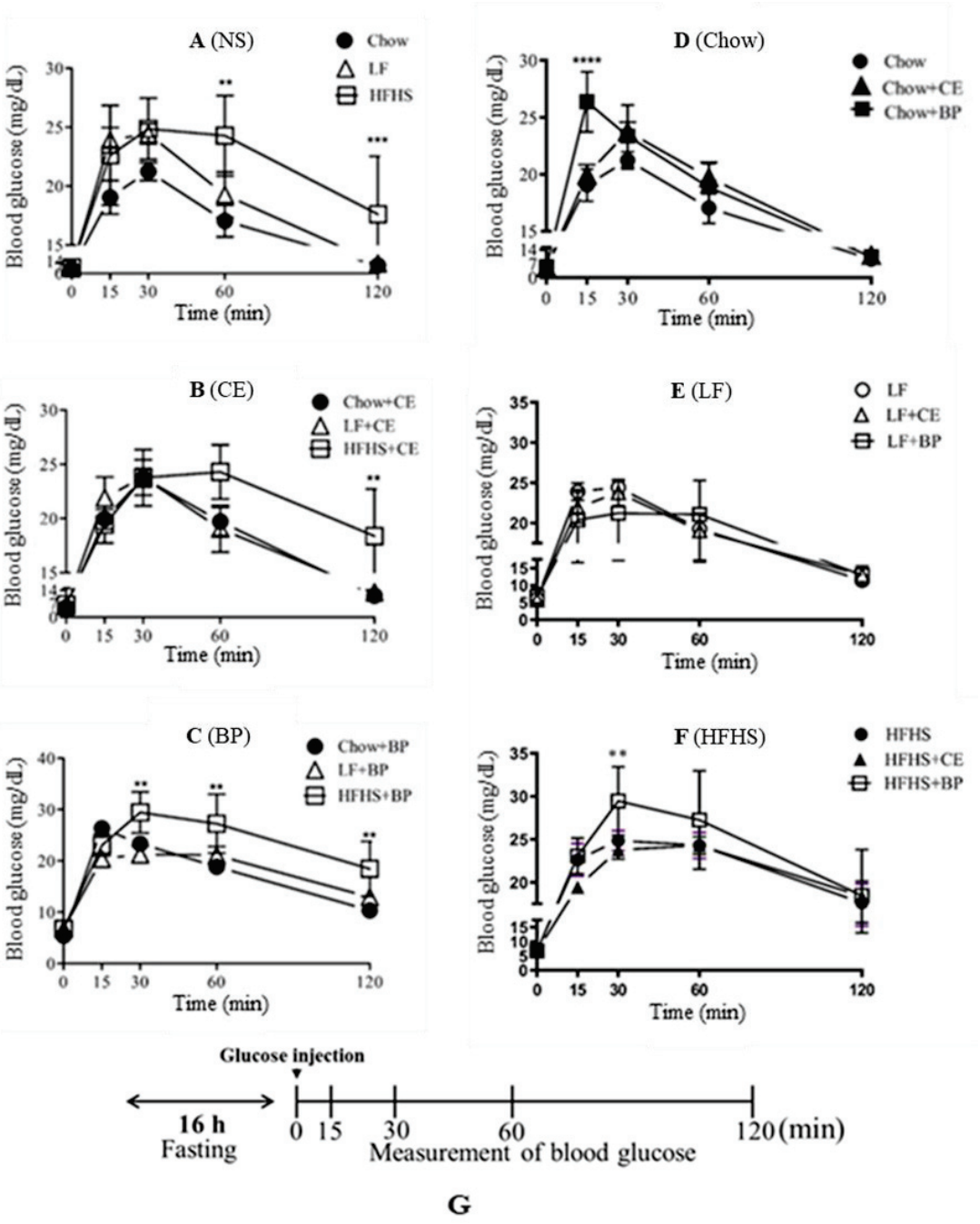

Figure 15. C3G-rich haskap extract (CE) supplementation improves glucose homeostasis in diet-induced obese mice. At week 12, mice were fasted for 16 h and subjected to intraperitoneal glucose tolerance tests (ipGTT, 2 g/kg glucose). Blood glucose levels were determined in the mice fed with no dietary supplements, supplemented with CE or BP (**A** to **F**). A schematic diagram of the timeline of glucose injection and blood collection **(G)**. Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple comparison test; *$p \leq 0{,}05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ as indicated. NS, no supplement; CE, C3G-rich extract; BP, Haskap berry powder; LF, low fat; HFHS, high-fat high sucrose.

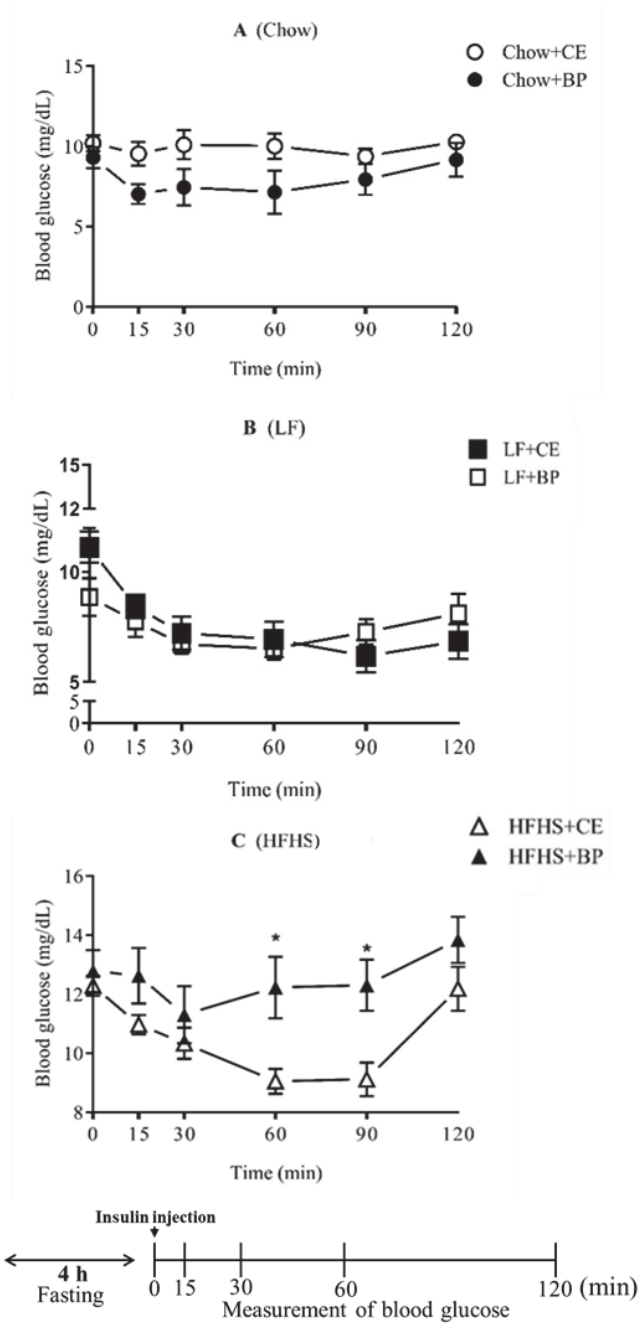

Figure 16. C3G-rich extract (CE) supplementation improves insulin sensitivity in diet-induced obese mice. At week 14 mice were fasted for 4 h and insulin tolerance tests (ipITT) were carried out after intraperitoneal insulin injection (1IU/kg). The graphs are chow diet group **(A)**, LF diet group **(B)** and HFHS diet group **(C).** A schematic diagram of the timeline of insulin injection and blood collection **(D).** Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple mean comparison test; *$p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ as indicated. CE, C3G-rich haskap extract; BP, Haskap whole berry powder; LF, low fat; HFHS, high-fat high sucrose.

4.3.2 Effect of haskap on hepatic gluconeogenesis and fat metabolism

The study investigated the *in vivo* effects of dietary supplementation of CE and BP on fatty acid synthesis-related protein acetyl CoA carboxylase (ACC) abundance. The immunoblot analysis revealed suppression of ACC phosphorylation in the HFHS diet-fed mice group (Figure 17B). CE-supplementation increased pACC expression in the HFHS-fed animals, while BP significantly suppressed the expression (Figure 17B). Moreover, no response was observed in the LF fed mice group (Figure 17B).

AMP-activated kinase (AMPK) is the major regulator of ACC phosphorylation. The phosphorylation of the ACC1 Ser 79 residue inactivates ACC. Immunoblot analysis revealed that a significant reduction in pAMPK T172 level in HFHS+NS compared to Chow+NS. Moreover, a significant increase in pAMPK T172 levels in the HFHS+CE group compared to HFHS diet-fed no supplemented group (HFHS+NS) (Figure 17A). Furthermore, LF+BP group showed significantly higher pAMPK level compared to that of HFHS+BP group (Figure 17A).

The fructose-1,6-bisphosphatase (FBPase) is an important enzyme in the hepatic-gluconeogenesis pathway. In order to ascertain whether CE supplementation affects the protein expression of FBPase, liver tissues from one hour fasted mice (sacrificed at 16 weeks post feeding), were harvested and subjected to Western blot analysis. The current analysis showed a significant increase in FBPase protein levels in the HFHS-fed mice (HFHS+NS) compared to the Chow-fed mice (Chow+NS) (Figure 17C). The HFHS+CE group lowered the FBPase expression compared to HFHS+NS group (Figure 17C). Mice fed HFHS diet supplemented with BP (HFHS+BP) showed a further reduction in FBase expression, although mice had higher baseline blood glucose levels, reduced insulin

tolerance and poor glucose clearance (Figure 17C and 16C). However, no response was observed in the LF diet group (Figure 17C). The study also revealed that the AMPK activation is also been associated with decreased hepatic glucose production with a reduced FBPase expression in the HFHS+CE group (Figure 17A and 17C). However, no difference in either ACC or AMPK phosphorylation was observed upon CE supplementation in the Chow-fed group (Figure 17A and B).

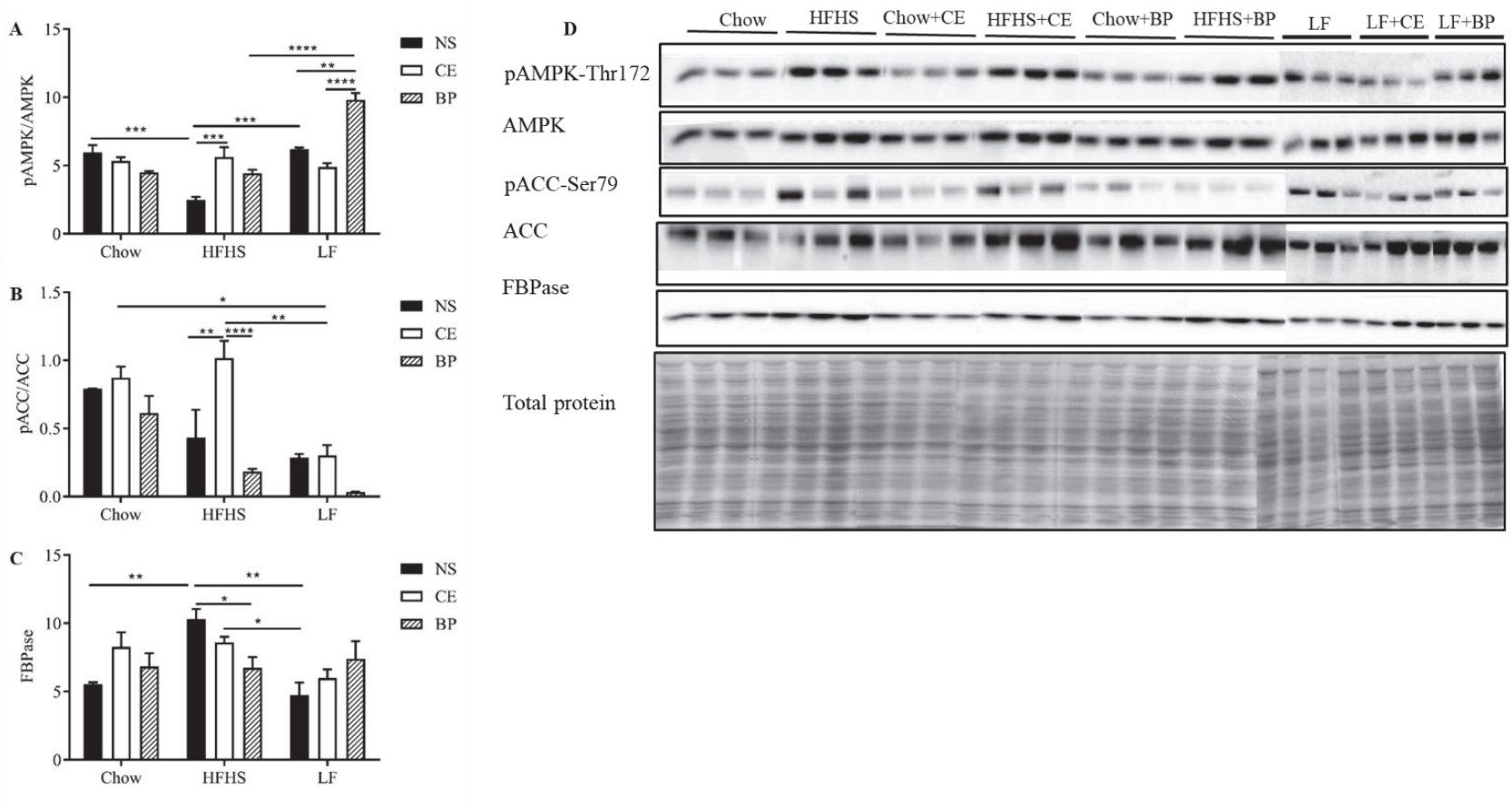

Figure 17. The effect of BP and CE supplementation on AMPK activation, ACC inactivation and FBPase protein in liver tissue of Chow, LF, and HFHS-fed mice. Immunoblot and densitometric analysis of phosphorylated AMPKThr172/AMPK ratio **(A)**, phosphorylated ACC-Ser79/ACC ratio **(B)**, and FBPase protein **(C)** in the liver tissues of mice. Representative immunoblots for phosphorylated AMPK, total AMPK, phosphorylated ACC, total ACC, total proteins of FBPase and the immunoblot of total proteins of the gel **(D)**. Each protein was normalized to total protein levels. Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple comparison test; *$p \leq 0{,}05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ as indicated (Prism8 version). ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; FBPase, fructose-1,6-bisphosphatase; NS, no supplement; CE, C3G-rich haskap extract; BP, Haskap whole berry powder; LF, low-fat; HFHS, high-fat high-sucrose.

CHAPTER 5: DISCUSSION

5.1 Effect of cultivar and harvest date on polyphenol profile

Berries of *Lonicera caerulea* L., are known for health-promoting effects due to the higher concentration of polyphenols. The haskap industry needs to answer the question when is the best time for mechanical harvesting of haskap berries for nutraceutical manufacturing. This study attempted to generate some basic understanding to address the above research question. In the current study, the haskap from Aurora, Evie and Larissa cultivars at fifth harvest date (H5) showed a higher concentration of anthocyanins. In line with previous studies, late-harvested Polish haskap berries are significantly bigger in size, softer, with a higher concentration of polyphenols and lower acidity (Skupień et al., 2007; Ochmian et al., 2013). The same trend was observed in mid-late and late ripened cultivars of apricot (*Prunus armeniaca* L.) and black currants (*Ribes nigrum* L.) (Mratinić et al., 2012; Krüger et al., 2012). Late harvested haskap berries are sweet but lower firmness affects fruit handling and quality for the commercial fresh fruit market. Haskap berries grown in North-Western Poland, show very early ripening under their climatic conditions, and the berries harvest in mid-May (Ochmian et al., 2013). In the process of ripening, the berries switch from a status of hard and acidic to a status where they get softer in texture with strong flavor and color. Increased duration of gaining sunlight and moderate fertility are important factors for the development of fruits which leads to the formation of plant secondary metabolites including polyphenols, which are also important for fruit color, taste, and odor (Conde et al., 2007). Therefore, the determination of harvesting date is an important factor to optimize level of polyphenols when aiming for nutraceutical manufacturing.

Polyphenol composition of haskap is greatly affected by the cultivar and growing locality (Rupasinghe et al., 2018). C3G, the most prominent anthocyanin in haskap represents 79% to 92% of total anthocyanin (Rupasinghe et al., 2018). The C3G concentrations of Polish-bred, 'Zielona' and Canada-bred 'Borealis' are 221 mg/ 100 g FW and 170 mg/ 100 g FW, respectively (Rupasinghe et al., 2015; Skupień et al., 2007). Another study indicated that Canada-bred haskap varieties contained C3G with the range of 68 – 649 mg/100 g fresh weight (FW) (Khattab et al., 2016; Rupasinghe et al., 2015). Moreover, Polish cultivars 'Duet' and 'Pojack' contained higher content of chlorogenic acid (approximately 294 mg/100 g of dry matter and 267 mg/100 g of FW respectively) compared to Canadian cultivars such as 'Tundra', 'Berry Blue' (chlorogenic acid 21-44 mg/100 g of FW) indicating the significant influence of growing location and cultivar on the chemical composition of haskap (Jurikova et al., 2012; Wojdyło et al., 2013; Khattab et al., 2016; Rupasinghe et al., 2015). Previous reports also showed that the Q3R represented the highest concentration of flavonol, which accounts for 27% - 88% of the total flavonols determined by UPLC (Rupasinghe et al., 2015). This followed by the presence of Q3ArabinoG and Q3Glu. The least present flavonol was Q3Gal which also showed the evidence in a previous report (Rupasinghe et al., 2015). Among the flavan-3-ols, catechin and epicatechin showed the highest concentration, and it also was demonstrated in previous studies which reported around 25% to 77% and 15% to 70% of the total flavan-3-ols, respectively (Rupasinghe et al., 2015). The concentrations of polyphenols were found to be consistent with previous reports (Rupasinghe et al., 2012; Rupasinghe et al., 2015; Rupasinghe et al., 2018). Overall, the study agreed to the hypothesis of the influence of the cultivar and harvesting date on polyphenol composition of haskap berries.

The Folin-Ciocalteu assay is a reproducible and simple technique that has been widely used for quantification of phenolic compounds in plant extracts. Previous studies showed that the total phenolic content of haskap berries measured by Folin-Ciocalteu assay ranged from 140.5 to 1142 mg GAE/100 g of FW (Skupień et al., 2007, Fan et al., 2011, Rop et al., 2011; Rupasinghe et al., 2012). These values are higher than the other fruits including wild red raspberry, blackberry, and blueberry (Bakowska-Barczak et al., 2007; You et al., 2011). Previously reported data of the phenolic content of *L. caerulea* L. have been 428.1 to 622.5 mg GAE 100/g FW (Rupasinghe et al., 2012) and 575 to 903 mg GAE 100/g FW (Rop et al., 2011) support the current data. Total anthocyanin content also evaluated by the pH differential method. In line with previous studies, haskap berries showed the average of 1300 mg C3G equivalents per 100 g FW (Bakowska-Barczak et al., 2007; Fan et al., 2011; Lefèvre et al., 2011). These values are higher than the other fruit sources including raspberry (Chen et al., 2013), and blueberry (Skrede et al., 2000). Antioxidant capacity measured by FRAP assay range between 7.57 to 113 µmol of TE/g of FW (Rop et al., 2011; Sánchez-Moreno, 2002). Some haskap cultivars contain higher antioxidant capacity compared to other fruits such as blueberry (*V. angustifolium* L.) 16.24 µmol TE/ g), blackberry (*R. fruticosus* L.) 15.03 µmol TE/g and raspberry (*R. idaeus* L.) 7.57 µmol TE/g) (Rupasinghe et al., 2012). Moreover, sugar content in haskap was quantified using the refractometer. Brix index was evaluated in the range of 8 to 12% in ripened berries both in the field and laboratory measurements, and it agrees with the previous finding ranged from 10 to 17% (Thompson, 2006). A study showed that the monosaccharides including, glucose and fructose, predominated the haskap berries and together accounted for more than 95% of the total sugars quantified (Rupasinghe et al., 2015). Moreover, a positive correlation was observed between Multiplex fluorescence detector and the other methods of measuring

total anthocyanin content using wet chemistry related methods (pH differential method/UPLC-ESI-MS analysis). Multiplex fluorescence detector is an environmentally friendly, time efficient technique, which is an alternative to the pH differential method and UPLC analysis of total anthocyanin measurements. The non-destructive technique of evaluating anthocyanins present in fruit skin is an innovative method to understand the seasonal evolution of phenolic compounds.

5.2 The *in vitro* studies of investigating anti-diabetic properties

The inhibition of carbohydrate-hydrolyzing enzymes involved in controlling starch breakdown and intestinal glucose absorption may contribute to the management of postprandial hyperglycemia (Rupasinghe et al. 2017; Podsędek et al., 2014). The results suggest that there is a relationship between anthocyanin, phenolic acid concentrations and enzyme inhibitory activities. The tested extracts revealed that Larissa and Evie contained higher total anthocyanin and phenolic acid (chlorogenic acid) which corresponded to the highest inhibition of carbohydrate hydrolyzing enzymes (Table 3 and 4). Overall, constituents of haskap extracts could be considered as natural alpha-amylase and alpha-glucosidase inhibitors, which further leads to reduce blood glucose level. However, in alpha amylase and alpha glucosidase assays, compared to tested haskap extracts, the clinical drug acarbose showed 400-fold and 10-fold higher inhibition respectively. Several studies demonstrate alpha-glucosidase and alpha-amylase enzyme inhibitory activity of plant polyphenols. Anthocyanin fractions from black carrots (*Daucus carota* L.) show the highest inhibitory activity of alpha-glucosidase and alpha-amylase enzymes in a dose-dependent manner (Esatbeyoglu et al., 2016). Black rice (*Oryza sativa* L.) contains a high anthocyanin content (3.83 mg anthocyanin/ g of extract) and shows a high alpha-glucosidase enzyme

inhibitory (IC_{50} = 13.56 µg/mL) as well suggesting a link between anthocyanin content and antidiabetic effects of plant polyphenols (Yao et al., 2009). A study revealed that highbush blueberry (*Vaccinium corombosum*) showed alpha amlylase and alpha glucosidase inhibitory capacity relative to acarbose, range from 91.8 to 103.3% for alpha amylase and from 103.2% to 190.8% for alpha-glucosidase (Johnson et al., 2011). Fruits including, chokeberry (*Aronia melanocarpa* L), red currant (*Ribes nigrum* L.), red and green gooseberries (*Ribes uva-crispa* L.) show alpha-amylase inhibitory activities (Podsędek et al., 2014). C3G demonstrates 1.8 times higher alpha-glucosidase inhibitory activity compared to C3Gal, indicating that the type of sugar moiety attached to the anthocyanidin plays a major role in inhibitory activities (Bräunlich, et al., 2013). Haskap berries also contain a higher amount of C3G; therefore, it suggests that the higher C3G content in haskap berry might have played a major role in haskap-induced enzyme inhibitory activities. The chlorogenic acid also shows strong alpha-glucosidase inhibitory properties (Xu et al., 2015). Moreover, flavan-3-ol compounds including catechin and epicatechin contained in almond seed skin exhibit strong alpha-amylase inhibitory properties (Tsujita et al., 2013). Overall, polyphenols show the ability of controlling postprandial glucose level associated with T2D (Esatbeyoglu et al., 2016; Rupasinghe et al. 2017).

The enteroendocrine cells secrete glucagon-like peptide 1 (GLP-1), which plays a major role in managing glucose homeostasis by inducing glucose-dependent stimulation of insulin secretion, and subsequent reduction of blood glucose level (Drucker, 2013). GLP-1 is degraded by DPP-4, thus, inhibition of DPP-4 leads to controlling elevated blood glucose (Drucker, 2013). The findings of the current study indicates that polyphenols extracted from haskap berries of four cultivars harvested at five harvesting dates exhibited DPP-4

inhibitory effect *in vitro*. The cultivar Larissa at H5 showed the highest anthocyanin concentration, which also suggest that the biological activity could be dependent on the anthocyanin content of haskap berries. These results also indicated that C3G, the most abundant type of anthocyanin, could involve in the DPP-4 inhibition. However, other types of anthocyanin compounds also could contribute to inhibitory activities. Further studies need to be performed in order to understand the mechanism of inhibition of DPP-4 enzyme. Overall, the results obtained from this study indicated the efficacy of haskap polyphenols as a natural DPP-4 inhibitor. Several studies show the DPP-4 inhibitory effect of polyphenols. Anthocyanin present in blueberry (*Vaccinium angustifolium* L.) and blackberry (*Rubus lanciniatus* L.) have been shown to reduce DPP-4 activity *in vitro* (Fan et al., 2013). Flavonoid-rich extract of *Pilea microphylla* L. inhibits DPP-4 enzyme with IC_{50} 520.4±15.4 µg/mL by dose-dependent manner (Bansal et al., 2012).

Results indicate that the haskap extracts of four cultivars (Aurora, Evie, Larissa, and Rebecca) demonstrated AGEs inhibitory properties. However, the anti-glycation properties of haskap samples were very low compared to commonly available drugs including acarbose and aminoguanidine (Figure 12B). Several previous experiments suggest the anti-AGE activity of plant polyphenols (Wu and Yen, 2005; McIntyre et al., 2009). Flavonoids including catechin, epicatechin, ECG, EGCG, quercetin, show anti-AGE activity (Wu and Yen, 2005). Leaves and stems of lowbush blueberries (*Vaccinium angustifolium*) collected in different seasons demonstrate anti-glycation effects (McIntyre et al., 2009). Fruits and seed extracts of *Byrsonima crassifolia* L. exhibited significant inhibitory properties against AGEs formation with IC_{50} values ranging from 94.3 – 138.7 µg/mL (Perez-Gutierrez et al., 2010). Canadian berries including, black bearberry (*Arctous alpine* L.), crowberry

(*Empetrum nigrum* L.), lingonberry (*Vaccinium vitis-idaea* L.) and bog cranberry (*Vaccinium oxycoccus* L.) show AGEs inhibitory activities and it strongly correlates with radical scavenging activity, total phenolic content and weakly correlated with total anthocyanin content (Harris et al., 2014). Anthocyanin compounds are effective AGEs inhibitors, but total phenolic content appears more relevant than total monomeric anthocyanin compounds to antiglycation activities (Harris et al., 2014). However, the results of the current study did not show any correspondence between anti-glycation properties and total phenolic content as well as antioxidant properties of haskap extracts. Further studies need to be performed in order to understand the mechanism of anti-glycation properties of haskap polyphenols. Overall, haskap extracts could be considered as natural inhibitors of carbohydrate-hydrolyzing enzymes, DPP-4 enzyme and AGE formation. Therefore, haskap berries can be used for manufacturing nutraceuticals and food ingredients targeted for managing T2D.

5.3 The effect of dietary supplementation of haskap on metabolic indicators in mice, AMPK activation and plasma glucose level using a mice model of T2D.

The study demonstrated that the administration of C3G ameliorates HFHS-induced complications in diabetic mice. The effect of dietary CE supplement was associated with the reduction of food intake, body weight gain, and peripheral fat mass accumulation which further improves glucose homeostasis and insulin sensitivity. In line with previous studies, anthocyanin compounds used as a dietary supplement ameliorates metabolic disorders including obesity, weight gain, insulin resistance, and fasting hyperlipidemia (Tsuda et al., 2003, Tsuda et al., 2006, Mazza, 2005). Anthocyanins, particularly C3G, delphinidin-3-*O*-glucoside, and pelargonidin-3-*O*-galactoside, enhance the secretion of insulin from islets

of Langerhans β-cells (Jayaprakasam et al., 2005). Furthermore, dietary anthocyanin-rich extracts reverse high fructose diet-induced hyperglycemia by improving insulin sensitivity in rats (Sprague-Dawley) and experimental diabetic Male KK-A^y mice (Guo et al., 2007, Takikawa et al., 2010). Plant extracts show antidiabetic properties through the modulation of insulin sensitivity and glucose homeostasis, and the activities are frequently ascribed to anthocyanidins and anthocyanins present in plant materials (Domitrovic, 2011). Dietary C3G significantly reduces the serum glucose concentration and enhances insulin sensitivity in diabetic mice as a result of antioxidant properties (Sasaki et al., 2007). The current field study also showed a higher antioxidant capacity of haskap berry polyphenols which may have a positive impact on haskap berry polyphenols-induced anti-diabetic properties. Overall, HFHS diet-induced obese mice supplemented with C3G-rich haskap extract demonstrated short term protection from body weight gain, improved glucose tolerance, and enhanced insulin sensitivity. The study showed C3G, a natural polyphenol, may work as an alternative intervention in reducing higher blood glucose levels.

AMPK is a major metabolic-sensing protein which is essential for energy metabolism in cells, particularly glucose and lipid metabolism (Hardie 2006). AMPK also serves as a potential target for treating T2D (Hardie, 2008) as the anti-diabetic drugs, including metformin and thiazolidinediones, significantly activate AMPK (Zang et al., 2004). The current study showed the decreased phosphorylation of AMPK in the HFHS diet-fed mice compared to the chow controls. This agrees with a previous study where male C57BL/6 mice fed with an HFHS diet decreased AMPK phosphorylation in the liver (Yang et al., 2012). Several studies have also demonstrated that plant polyphenols activate AMPK signaling pathway which leads to the reduction of hyperglycemia (Hwang et al., 2009;

Kurimoto et al., 2013). For example, anthocyanin-rich cranberry (*Vaccinium* sp.) extract reduces hyperglycemia and insulin sensitivity through activating AMPK in high-fat-diet-fed mice (Shabrova et al., 2011). Polyphenols including quercetin present in several fruits and vegetables, epigallocatechin gallate from green tea, theaflavin from black tea demonstrate the ability to activate AMPK (Hwang et al., 2009). Black soybean seed coat extract rich in C3G ameliorates insulin sensitivity by activation of AMPK in liver tissues of diabetic mice (Kurimoto et al., 2013). Our study demonstrates that CE supplementation activates hepatic AMPK protein levels in obese mice. Moreover, the activation of AMPK regulates several processes including fatty acid synthesis and gluconeogenesis (Figure 18) (Musi et al., 2002; Gruzman et al., 2009).

Furthermore, impairment in the ability to oxidize dietary fat has also been associated with insulin resistance and metabolic imbalance (Riccardi et al., 2004). Therefore, it is important to determine whether CE supplement affects fat metabolism in the HFHS diet fed animals. ACC is a multi-subunit protein, residing in two isomeric forms ACC1 and ACC2, and fatty acid oxidation promoted by ACC inhibition (Figure 18) (Luo et al., 2012). Phosphorylation of ACC1 on its Ser 79 subunit renders its inactive form, and it can no longer suppress fat oxidation. The current study indicated that the increased insulin sensitivity observed in the HFHS fed mice supplemented with CE, could partly be due to its effect on ACC inhibition. AMPK inhibits fat accumulation by downregulating signaling components including ACC, which is highly expressed in lipogenic tissues as well as in liver (Saha and Ruderman, 2003; Gruzman et al., 2009). A study showed that bilberry extracts fed KK-A^y mice also showed inactivation of ACC via Ser 79 phosphorylation through AMPK activation (Takikawa et al., 2010). The current study demonstrated that the ACC level was lower at HFHS fed mice compared to a chow-fed diet without any supplementation. This also revealed in other

studies which determined a lower level of hepatic ACC levels in rodents fed with high-fat diet (Li et al., 2011; Friedman et al., 2012). Consistently, our study showed a probable AMPK-mediated inhibition of hepatic ACC levels in the CE supplemented HFHS diet fed group, suggesting a potential regulation of hepatic fat metabolism by the haskap polyphenols (Figure 17B).

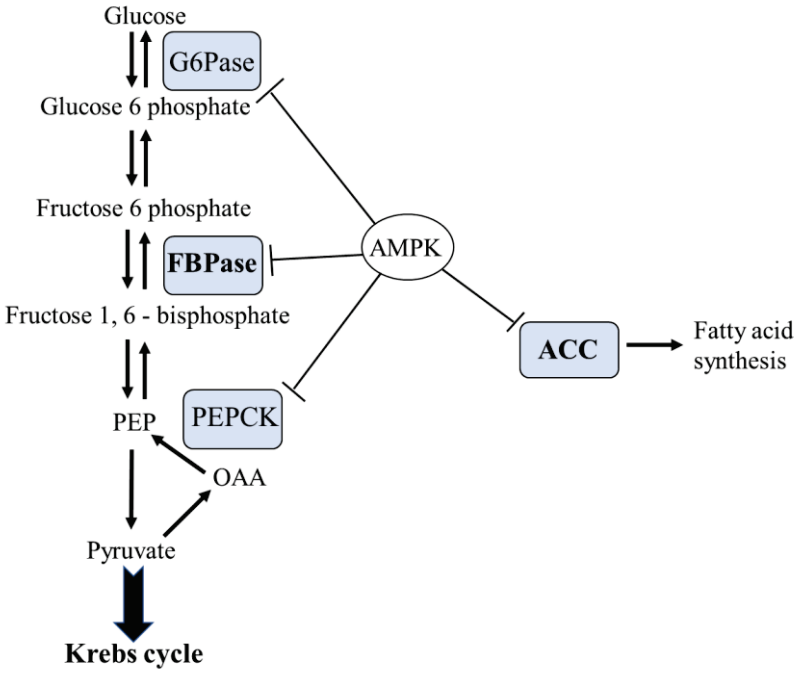

Figure 18. AMPK regulates fatty acid synthesis and gluconeogenesis. AMPK inhibits fatty acid synthesis by downregulating ACC which is responsible for fatty acid synthesis. AMPK suppresses the activities of enzymes involved in gluconeogenesis including FBPase, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEP-CK). PEP, phosphoenolpyruvate; OAA, oxaloacetic acid; ACC, Acetyl CoA carboxylase.

Hepatic gluconeogenesis is a metabolic process of producing glucose from non-carbohydrate sources, such as fat, and prior studies have shown that diets enriched in fat results in increased gluconeogenesis (Commerford et al., 2002, Song et al., 2001). Excessive endogenous glucose production is a major contributor to fasting hyperglycemia in diabetes and obesity (Radziuk and Pye, 2004). Therefore, it is important to determine

whether the improvement in glucose and insulin tolerance in our model was contributed by the reduced hepatic gluconeogenic flux in the CE supplemented, HFHS diet-fed mice. A crucial regulatory step in the hepatic gluconeogenesis is the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, which is catalyzed by FBPase (Erion et al., 2005).

AMPK activation in the liver also controls hyperglycemia in diabetic mice by inhibiting hepatic gluconeogenesis (Foretz et al., 2005) through modulation of the key regulatory enzymes, PEP-CK, and glucose-6-phosphatase (G6Pase) (McGee et al., 2008). Several studies in the past, have highlighted the effect of dietary C3G from different plant sources to modulate hepatic gluconeogenesis. For instance, purple corn (*Zea mays* L.) extracts rich in C3G, enhanced AMPK phosphorylation and reduced the expression of PEP-CK and, G6Pase in liver (Huang et al., 2015). Dietary C3G significantly reduced the gene expression level of G6Pase compared to the control group without any supplementation in KK-A^y diabetic mice (Sasaki et al., 2007). The current study revealed that AMPK activation caused by CE supplementation, down-regulate the expression of FBPase, and result in a reduction of endogenous glucose production, in agreement with a previous study (Huang et al., 2015; McGee et al., 2008). However, it is currently still unclear the complete mechanism how polyphenols are involved in reducing gluconeogenesis. Furthermore, future investigation on *in vivo* role of haskap polyphenols is needed in order to understand the mechanism of anti-diabetic properties including the activation of AMPK, suppression of hepatic lipogenesis and gluconeogenesis. These findings may provide a new therapeutic approach for the management of T2D. Overall, the Western blot data suggest that CE supplementation under an existing obesogenic millieu activates AMPK with concomitant

inhibition of ACC thus modulating fat metabolism and subsequent reduction in endogenous glucose production by inhibiting hepatic gluconeogenesis.

5.4 The Summary

Haskap (*Lonicera caerulea* L.), is a recently commercialized berry crop in Canada. The cultivars and harvesting time can influence the polyphenol composition thus the biological properties of haskap berries. The polyphenols present in berries of four commercially grown haskap cultivars, Aurora, Evie, Larissa, and Rebecca harvested at five different dates (H1-H5), were extracted separately in acidified 80% ethanol and analyzed to characterize their polyphenol profiles. The haskap berries contain various types of polyphenols including flavonoids and phenolic acids. Anthocyanin is the major type of flavonoid, which are known for potential health-promoting effects. C3G is the most prominent anthocyanin of haskap berries, which contribute to about 79% of total anthocyanin in all extracts. The cultivar Larissa at H5 possessed the highest C3G content (1212.3±63.9 mg/100 g FW). The Q3R is the most predominant flavonol of haskap berries, and the highest was observed in Rebecca at H4 (47.81 mg/100 g FW). The haskap berries also contained phenolic acids, among them approximately 95% was chlorogenic acid. The highest, total phenolic content is observed in Evie at H5 (38.9 mg/100 g FW) while the lowest in Rebecca at H1 (21.3 mg/100 g FW). Overall, among the selected haskap cultivars in the current study, Larissa at H5 was found to be rich in C3G and total anthocyanin contents, total sugar content, and total polyphenols content.

The study was also extended to determine the potential anti-diabetic properties of haskap polyphenols *in vitro*. The results indicated that haskap extracts exhibit a dose-dependent inhibition of alpha-amylase, alpha-glucosidase, DPP-4, and AGE formation. Extracts of Larissa and Evie demonstrated the highest inhibition of activities of alpha-amylase and alpha-glucosidase enzymes, respectively at the tested concentrations. Moreover, Larissa at

H5 exhibited the highest DPP-4 inhibitory activity. The results indicate that haskap polyphenols are natural glucosidase inhibitors which influence the digestion of dietary carbohydrates. Furthermore, haskap berry extract is also a natural inhibitor of DPP-4 and AGEs formation which is associated with T2D.

Increasing energy expenditure and regulation of glucose and lipid metabolism are important for the management of T2D. Our study demonstrated that supplementation with a C3G-rich haskap extract in an obesogenic milieu improves glucose homeostasis by ameliorating glucose tolerance and insulin sensitivity. This was supported by a reduction in AMPK driven expression of FBPase, a key regulatory enzyme of gluconeogenesis. Furthermore, CE supplementation also conferred protection against peripheral fat mass accumulation and weight gain in obese mice. Although this effect was not seen in the long term feeding of HFHS diet, a significant reduction in the active form of hepatic ACC was observed which was also driven by AMPK. Further studies are required to confirm the observations on the physiological impact of C3G supplementation in ameliorating metabolic imbalance in obesity and insulin resistance. Overall, haskap berry polyphenols are a locally available source of food bioactive that could help manage T2D and related complications. Moreover, haskap polyphenols could be used as a value-added food ingredient to produce functional foods and nutraceuticals.

5.5 Future recommendations

The current study was performed only for a single harvesting season (2017), and it is recommended to perform the study for more than one harvesting season in order to compare the polyphenol composition variation between different harvesting seasons. The study conducted using haskap berries harvested from very young plants of about two years old. However, in future studies, it is suggested to collect berries from fully established (three to four years) plants since the age of the plant may have an impact on the polyphenol composition.

This study is the first preliminary step for the use of non-destructive field measurement of anthocyanins (Multiplex) as a harvesting indicator of haskap berry for nutraceutical manufacturing. In the future, it is recommended to expand this research by applying fluorescence related technology to monitor the anthocyanin concentration of haskap berries for deciding the harvest date.

The results of the current study unveil the potential to utilize haskap polyphenols in managing T2D. Dose-response trials could be performed to ascertain the optimal intake required to reduce the risk of T2D. However, more scientific evidence and human clinical trials are needed in order to make the recommendations and to understand the mechanism of specific polyphenol compounds at the metabolic regulations associated with the pathogenesis of T2D. The future studies should employ properly designed placebo-controlled double-blind cross-over human studies permitting oral administration of haskap and isolated polyphenols, which will provide more insight on the potency of haskap berry polyphenols in managing metabolic disorders. Moreover, long-term human intervention studies, particularly with diabetic patients, are necessary to draw a firm conclusion about

the contribution of C3G for the management of T2D. Based on the new knowledge, haskap berries could be used in the development of nutraceuticals and functional foods for reducing the risk of T2D.

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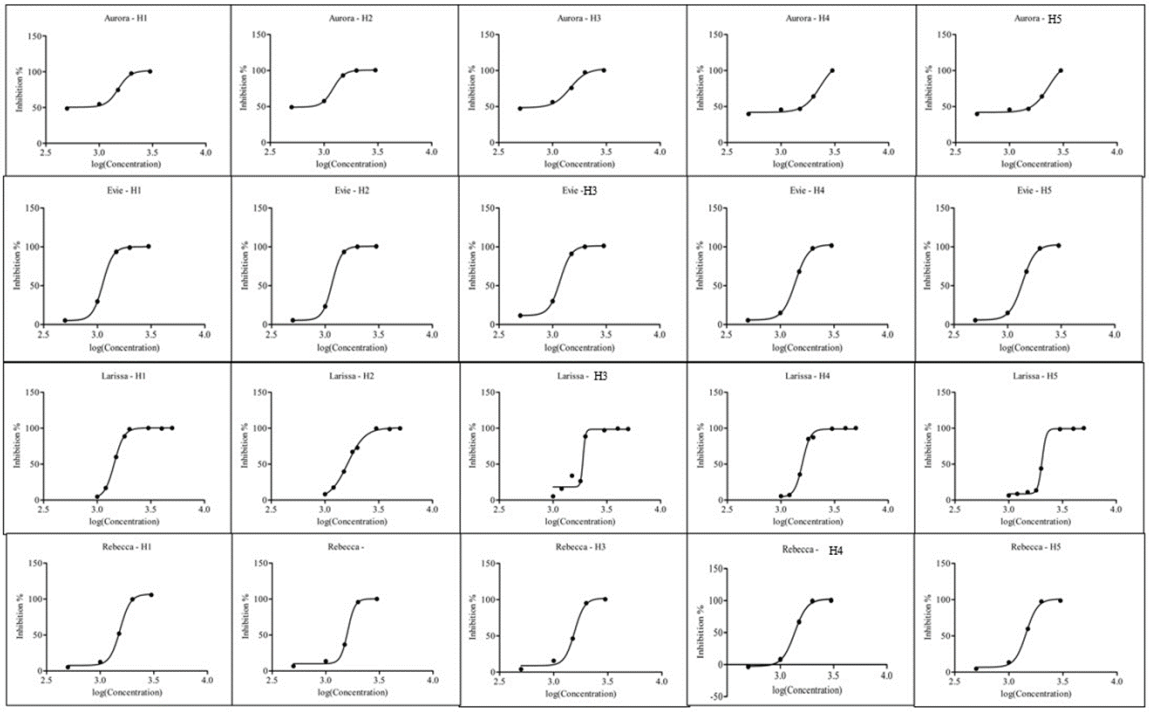
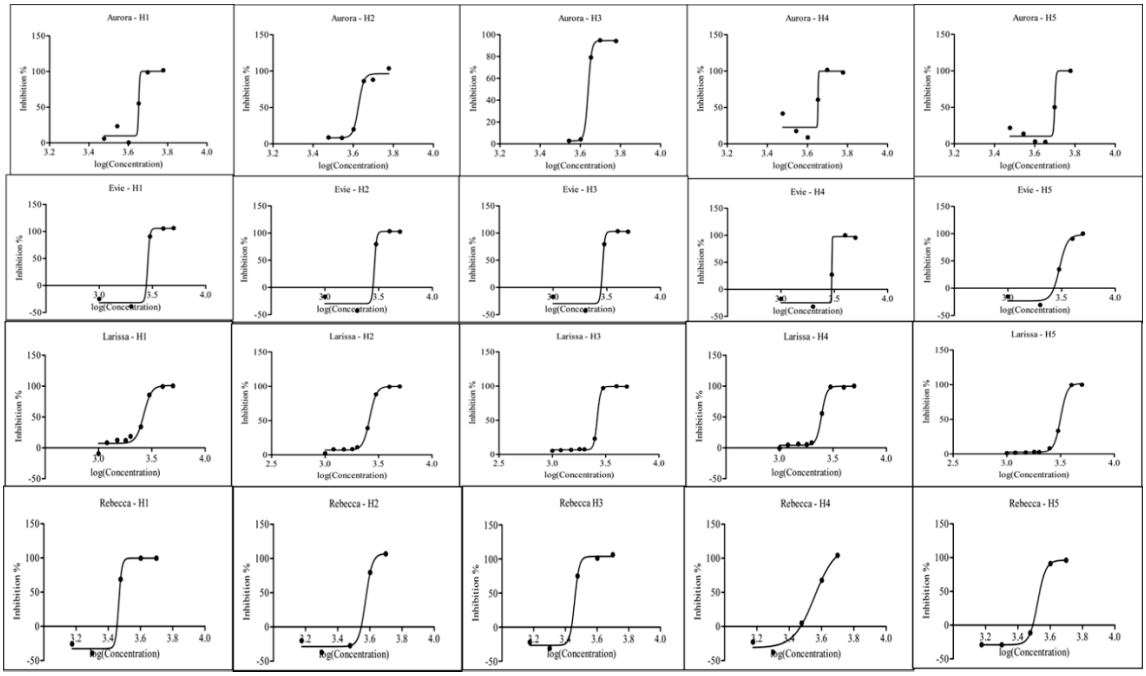
Appendix A

The IC_{50} values of alpha glucosidase assay for four cultivars across five harvest dates



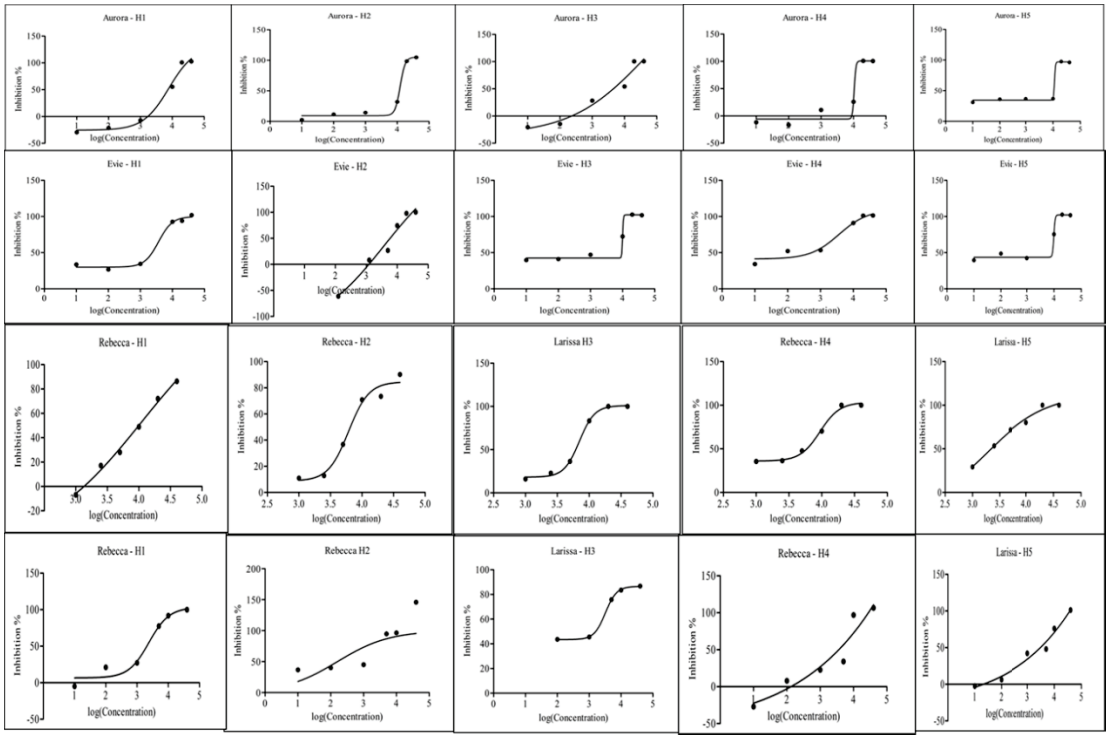
Appendix B

The IC_{50} values of alpha amylase assay for four cultivars across five harvest dates



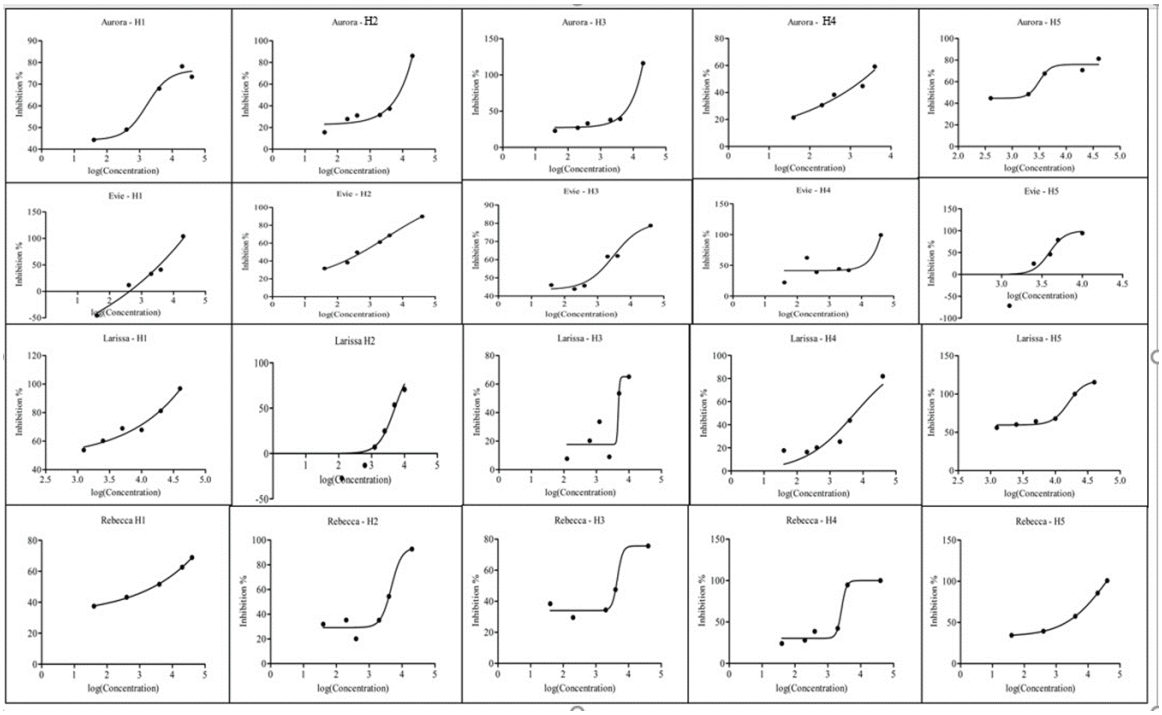
Appendix C

The IC_{50} values of DPP-4 assay for four cultivars across five harvest dates



Appendix D

The IC_{50} values of AGEs inhibtion assay for four cultivars across five harvest dates



Appendix E

The Chow diet composition (The scanned detailed sheet from the LabDiet.com)

Laboratory Rodent Diet 5001*

DESCRIPTION

Laboratory Rodent Diet is recommended for rats, mice, hamsters and gerbils. This diet is a complete life cycle diet formulated using managed formulation, delivering Constant Nutrition®. This is paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. It is formulated for life-cycle nutrition; however, it is not designed for maximizing production in mouse breeding colonies. This product has been the standard of biomedical research for over 70 years.

Features and Benefits

- Managed Formulation delivers Constant Nutrition®
- High quality animal protein added to create a superior balance of amino acids for optimum performance
- Formulated for multiple species for single product inventory
- The rodent diet standard for biomedical research

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets)

Other Versions Available

- 5L0D PicoLab® Laboratory Rodent Diet (Minimum order required)

GUARANTEED ANALYSIS

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	8.0%
Moisture not more than	12.0%

INGREDIENTS

Dehulled soybean meal, ground corn, dried beet pulp, fish meal, ground oats, dehydrated alfalfa meal, cane molasses, brewers dried yeast, wheat germ, whey, porcine animal fat preserved with BHA and citric acid, wheat middlings, porcine meat and bone meal, salt, calcium carbonate, DL-methionine, choline chloride, cholecalciferol, folic acid, vitamin A acetate, menadione dimethylpyrimidinol bisulfite (source of vitamin K), pyridoxine hydrochloride, thiamine mononitrate, biotin, nicotinic acid, calcium pantothenate, dl-alpha tocopheryl acetate (form of vitamin E), vitamin B_{12} supplement, riboflavin supplement, ferrous sulfate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times.

Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters-Adults will eat up to 14 grams per day.

For information regarding shelf life please visit www.labdiet.com.

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**25.0**
Arginine, %	1.57
Cystine, %	0.39
Glycine, %	1.28
Histidine, %	0.62
Isoleucine, %	1.06
Leucine, %	1.89
Lysine, %	1.48
Methionine, %	0.59
Phenylalanine, %	1.11
Tyrosine, %	0.77
Threonine, %	0.97
Tryptophan, %	0.28
Valine, %	1.16
Serine, %	1.18
Aspartic Acid, %	2.81
Glutamic Acid, %	4.74
Alanine, %	1.44
Proline, %	1.47
Taurine, %	0.03
Fat (ether extract), %	**5.0**
Fat (acid hydrolysis), %	**6.4**
Cholesterol, ppm	209
Linoleic Acid, %	1.05
Linolenic Acid, %	0.09
Arachidonic Acid, %	0.02
Omega-3 Fatty Acids, %	0.30
Total Saturated Fatty Acids, %	1.48
Total Monounsaturated Fatty Acids, %	1.62
Fiber (Crude), %	**5.3**
Neutral Detergent Fiber[3], %	16.7
Acid Detergent Fiber[4], %	6.9
Nitrogen-Free Extract (by difference), %	**47.5**
Starch, %	21.0
Glucose, %	0.19
Fructose, %	0.27
Sucrose, %	3.83
Lactose, %	2.01
Total Digestible Nutrients, %	**73.8**
Gross Energy, kcal/gm	**4.09**
Physiological Fuel Value[5], kcal/gm	**3.35**
Metabolizable Energy, kcal/gm	**2.91**
Minerals	
Ash, %	**7.0**
Calcium, %	0.95
Phosphorus, %	0.70
Phosphorus (non-phytate), %	0.42
Potassium, %	1.28
Magnesium, %	0.23
Sulfur, %	0.36
Sodium, %	0.39
Chloride, %	0.64
Fluorine, ppm	15
Iron, ppm	240
Zinc, ppm	85
Manganese, ppm	75
Copper, ppm	15
Cobalt, ppm	0.91
Iodine, ppm	0.99
Chromium (added), ppm	0.01
Selenium, ppm	0.41
Vitamins	
Carotene, ppm	2.3
Vitamin K,ppm	1.3
Thiamin Hydrochloride, ppm	16
Riboflavin, ppm	4.7
Niacin, ppm	120
Pantothenic Acid, ppm	24
Choline Chloride, ppm	2250
Folic Acid, ppm	7.1
Pyridoxine, ppm	6.0
Biotin, ppm	0.30
B_{12}, mcg/kg	51
Vitamin A, IU/gm	15
Vitamin D_3 (added), IU/gm	4.6
Vitamin E, IU/kg	42
Ascorbic Acid, mg/gm	—
Calories provided by:	
Protein, %	29.829
Fat (ether extract), %	13.427
Carbohydrates, %	56.744

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

Appendix F

The scanned copy of the approval for the animal study

DALHOUSIE UNIVERSITY
Inspiring Minds

NOTICE OF PROTOCOL APPROVAL
UNIVERSITY COMMITTEE ON LABORATORY ANIMALS

Protocol Number:	**16-099**
Previous Protocol Number:	**New**
Principal Investigator:	**Dr. Thomas Pulinilkunnil**
Start Date/Expiry Date:	**August 1, 2016/August 1, 2017**
Title of Study:	**Cardioprotective properties of haskap polyphenols using experimental models of diet induced obesity**
Species:	**Mice**

Jennifer Wipp
UCLA@dal.ca
University Committee on Laboratory Animals
902-494-1270
WEBSITE: http://www.dal.ca/dept/animal-ethics.html

In compliance with granting agency and Dalhousie University policy, Dalhousie Research Services is not permitted to release funding instalments into research accounts until documentation of all necessary approvals are submitted (i.e. Human ethics, animal ethics, biohazard and radiation permits).

IMPORTANT FUNDING INFORMATION:

To ensure the research funds related to this protocol are released, fill out the information below and

Scan and send to caroline.sequeira@dal.ca or fax the entire page to Dal Research Services 494-1595

For IWK funded projects fax to IWK Research Office - FAX 470-6767

***** If this protocol covers more than one research project, include information on all related grants.**

Principal Investigator: Dr. Vasantha Rupasinghe

Grant Project title(s): Optimization of haskap juice manufacturing Process

Granting Agency(ies): Mitacs / Haskapa™

Granting Agency / Award #: FR11266

Dalhousie Account # (if known) to which this protocol applies: 34158

Signature of Grant Holder: