A comparative study of the antioxidant potential and metabolic profiling of lingonberry *(Vaccinium vitis idaea)* from Northern Manitoba and Newfoundland

By

Aakangchhya Bhusal

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ABSTRACT

Lingonberries *(Vaccinium vitis-idaea)* are edible fruit that belong to the Ericaceae plant family. A growing body of evidence suggests that consumption of berries provide considerable health benefits due to their high polyphenols, antioxidants, vitamins and mineral content. Therefore, it is beneficial to evaluate the antioxidant capacity of berries in order to select lines with higher antioxidant capacity. The aim of this study was to determine the antioxidant capacity, total phenolics, total anthocyanins, anti-apoptotic effect and the metabolomic profile of lingonberries from Northern Manitoba (wild) and Newfoundland (greenhouse-grown). A total of 159 lingonberry samples were collected from Northern Manitoba (Lynn Lake and Flin Flon) and Newfoundland. Samples were freeze dried, lyophilized, ground into powder and extracted with solvent methanol. Total anthocyanins were determined by the pH differential method. Total phenolics were evaluated by the Folin-Ciocalteau's assay, the anti-apoptotic effect was measured fluorescently and the oxygen radical absorbance capacity (ORAC) assay was utilized to evaluate the antioxidant capacity. Our study showed that Northern Manitoba-grown lingonberries contain a higher level of anthocyanins and phenolics compared to Newfoundland greenhouse-grown lingonberries. Results show that Northern Manitoba-grown lingonberries have higher antioxidant capacity compared to ones grown in Newfoundland. Findings from the study suggest that berries grown in green house conditions and extreme climatic conditions have different antioxidant capacity, anti-apoptotic effects, total phenolics and total anthocyanins; that is, more extreme climates may result in berries with higher antioxidant capacity, total phenolics and anthocyanins.

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DEDICATION

I would like to dedicate this thesis to my family.

To my mom and dad, sisters and my husband

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ABBREVIATIONS

AAPH	2,2'-azobis-2-methyl-propanimidamide,dihydrochloride
ACCCRC	Atlantic Cool Climate Crop Research Centre
AP	Activator protein
ATCC	American Type Culture Collection
AUC	Area under curve
CCARM	Canadian Centre for Agri-Food Research in Health and Medicine
CE	Capillary electrophoresis
DNA	Deoxyribonucleic acid
DW	Dry weight
ET	Electron transfer
FDC	Food development centre
FFLB	Flin Flon lingonberry
GAE	Gallic acid equivalent
GC	Gas chromatography
HAT	Hydrogen atom transfer
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
IR	Ischemia reperfusion
LC	Liquid chromatography
LDL	Low density lipoprotein
LLLB	Lynn Lake lingonberry
MS	Mass spectrometry

NF-κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PAI	Plasminogen activator inhibitor
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acids
qTOF	Quadruple time of flight
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAC	Total anthocyanins content
TE	Trolox equivalent
TPC	Total polyphenols content
TROLOX	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

CHAPTER I: LITERATURE REVIEW

1.1. Lingonberry

Berries are a good source of many bioactive compounds such as anthocyanins, phenolics, flavonoids and vitamin C (Wang & Lin, 2000; Kahkonen, Hopia, & Heinonen, 2001). They also contain a number of essential nutrients including folic acid, potassium, fiber and antioxidants (Torronen, Kolehmainen, Sarkkinen, Mykkanen, & Niskanen, 2012). Lingonberry *(Vaccinium vitis idaea)* belongs to the family Ericaceae (Kylli et al., 2011) and belongs to the same family as cranberry and blueberry. It is an evergreen shrub that bears red fruit in summer or fall. The fruit is a little smaller, less tart and juicier than the cranberry. The flowers of lingonberries are like that of blueberry which is either white or pink. It is believed to be native to arctic and subarctic regions and it can be found in different parts of world including North America and Northern Europe. The natural habitat of lingonberry comprises densely wooded areas, mountain peaks and rocky exposed cliffs (Isaak, Petkau, O, Debnath, & Siow, 2015). This particular fruit is known by different other names such as partridgeberry, fox berry, cowberry, wolfberry and northern mountain cranberry. One of the peculiar features of this plant is to withstand harsh winter conditions, extreme heat and windy exposures (Kylli et al., 2011). The attributed beneficial effect of consuming different berries like blueberry, strawberry, cranberry, blackberry, raspberry and their hybrids are diverse and ranges from protection against inflammation to enhanced cognitive functions (Bakowska-Barczak, Marianchuk, & Kolodziejczyk, 2007).

Lingonberry has been incorporated into human diet since long time. It is utilized in making different items like jams, sauce, juice and yoghurt. Indigenous people of Canada have been using this berry to treat urinary tract infection and other disorders like diabetes (Harbilas et al., 2009). The plant is also used as an ornamental groundcover. Lingonberry is good source of natural antioxidants and has higher antioxidant capacity than other berries like blueberry, cranberry, strawberry, raspberry and bilberry (Bakowska-Barczak et al., 2007; Zheng & Wang, 2003).

Lingonberries propagate by means of seed or underground rhizomes. Different commercial methods like tissue culture and stem cuttings can also be used in the propagation of lingonberry plant. The tissue culture technique is a modern micropropagation method that results in the rapid clonal production of numerous plants. In addition, production of lingonberries at the commercial level is well-established in countries such as Finland, Germany, Switzerland, Austria and Sweden. In Canada, most lingonberries are harvested from the wild. Newfoundland and Labrador produces lingonberries in the largest quantities in Canada (Bhullar & Rupasinghe, 2015).

1.2. Nutritional Composition of Lingonberry

Lingonberry is utilized in different forms in diets. It is well appreciated by the consumers for its tart taste. Studies have reported that lingonberry contains 0.7 grams of protein per 100 grams of berries, 0.5 grams of fat per 100 grams of berries and 10.9 grams of carbohydrates per 100 grams of the berries (Bere, 2007). Similarly, 0.32 grams of polyunsaturated fatty acids per 100 grams of berries and 0.06 grams of monounsaturated fatty acids per 100 grams berries (Bere, 2007). Likewise, the study also included the composition of individual polyunsaturated fatty acids (PUFA) as 0.14 grams

of n-6 fatty acid (linoleic acid) and 0.18 grams of n-3 fatty acid (linolenic acid) per 100 grams berries (Bere, 2007). Other studies reported that cyanidin-3-glucoside, cyanidin-3-galactoside and cyanidin-3-arabinoside were the key anthocyanins in the lingonberry (Ek, Kartimo, Mattila, & Tolonen, 2006). It has been reported that lingonberries contain plant sterols; 160 mg per 100 grams dry weight (Szakiel, Paczkowski, Koivuniemi, & Huttunen, 2012). Several studies have reported that lingonberries exhibit higher antioxidant capacity in comparison to other berries like raspberries, cranberries, strawberries and blueberries (Bakowska-Barczak, Marianchuk, & Kolodziejczyk, 2007; Zheng & Wang, 2003).

1.3. Potential Health Benefits of Lingonberry

Lingonberry is a well-accepted fruit in Nordic countries and is utilized in the diet in different forms such as jellies, jams, juices and baking (Lehtonen et al., 2013). Several studies have shown that lingonberry contains a high polyphenol content exhibiting high antioxidant and antimicrobial activity (Lehtonen et al., 2013). These berries are rich in a number of polyphenols such as *p*-coumaric acid and quercetin (Lehtonen et al., 2013). Cranberry and lingonberry both belonging to the *Vaccinium* genus, are believed to be useful in treating urinary tract infection (Ek et al., 2006).

In another study by Kivimaki, Ehlers, Turpeinen, Vapaatalo, & Korpela, (2011), hypertensive rats were supplemented with lingonberry, cranberry and blackcurrant juice for a period of 8 weeks. Findings from the study concluded that lingonberry juice but not cranberry or blackcurrant juice helped to reduce blood pressure and improved vascular function in spontaneously hypertensive rats. The study also reported that, lingonberry, cranberry and blackcurrant differed in their phenolic content. Lingonberries had higher

content of flavonols, flavan-3-ols and procyanidins. The results suggest that the potential health benefits may be due to the prevalence of phenolic compounds in the lingonberry. However, the question of whether the anti-hypertensive property of the lingonberry is the result of one of the phenolic compounds or combined effect of the detected compounds or the effect of other undetected compounds is yet unknown (Kivimaki et al., 2011; Kivimaki, Siltari, Ehlers, Korpela, & Vapaatalo, 2013; Kivimaki et al., 2013).

Studies have been done on different berries including blackberry, raspberry, lingonberry, blackcurrant, bilberry, crowberry, acaiberry and prune to evaluate whether these berries provide any protective effects against diet-induced obesity. Mice were fed with three different forms of a diet; namely, a low-fat diet, a high-fat diet and a high-fat diet supplemented with berries. Results obtained from the study showed that the high-fat diet supplemented with lingonberry, bilberry and blackcurrant; were able to decrease the fat content, markers of inflammation like plasminogen activator inhibitor (PAI-1) and build-up of hepatic lipids (Heyman et al., 2014). The diet supplemented with lingonberries and blackcurrants contained the polyphenols quercetin-3-O-galactoside and quercetin-3-O-glucoside. The study suggests that a diet supplemented with berries like lingonberry and blackcurrant help mitigate the harmful effects caused by diet-induced obesity and type 2 diabetes (Heyman et al., 2014).

Along the same line, *in vitro* and *in vivo* studies on a diet-induced obesity mice model (Harbilas et al., 2009; Eid et al., 2014) have shown the anti-diabetic property of lingonberry. Results from those studies suggest that the lingonberry supplementation was able to bring back the blood glucose level to the normal in the high-fat diet fed mice. The proposed mechanism behind this effect is the enhanced expression of GLUT 4 in the

skeletal muscle by the involvement of both insulin-dependent and insulin-independent pathways (Eid et al., 2014). Antioxidant potential of polyphenols comes as a result of an aromatic structure and attachment of different functional and reactive groups. A polyphenol has an aromatic structure to which different functional groups are attached which results in its antioxidant activity (Eid et al., 2014). Berries are very rich source of antioxidants which are largely phenolics. The phenolics present in the berries provide various health benefits by supressing the expression of nuclear factor-kappa B (NF-kB) and activator protein, AP-1 (Yang & Kortesniemi, 2015). Consumption of berries also helps reduce the production of pro-inflammatory cytokines which in turn provide protection against inflammation (Joseph, Edirisinghe, & Burton-Freeman, 2014).

Simeonov et al. (2002) has shown that intake of chokeberry juice reduced the glucose level in both type 1 and type 2 diabetic patients. In separate studies, Chamber and Camire (2003) have shown no effect on glucose level when adults with diabetes were fed cranberry juice. These data suggests different juices have different activities and factors such as gender or age of the patients may affect the outcome. In a randomized clinical trials, (Erlund et al., 2008) showed that intake of different type of berries improved the cardiovascular function in subjects with cardiovascular risk. Similarly, another study showed that intake of anthocyanin capsules for 90days elevated high density lipoprotein (HDL) level and reduced low density lipoprotein (LDL) cholesterol level in dyslipidemia subjects (Qin et al., 2009). Another study showed the reduction of neoplastic lesions in oral cancer upon administration of black raspberry gel in patients (Shumway et al., 2008). Taken together, these studies suggested the beneficial effects of berries in human health.

Future studies are needed to understand the underlying mechanism and larger patient samples are needed to validate these findings.

1.4. Berry Phenolics

Growth and development of plants is determined by a number of factors. Secondary metabolites in plants are considered not essential for growth and development but play a vital role in providing protection against plant pathogens (Winkel-Shirley, 2001), helps in seed dispersion, provide protection against UV damage and provide defense against microbial agents (Winkel-Shirley, 2001). Phenolic compounds are a type of secondary metabolite that bears an aromatic ring with one or more hydroxyl groups. These compounds are synthesized from phenylalanine and the shikimate pathway (Paredes-Lopez, Cervantes-Ceja, Vigna-Perez, & Hernandez-Perez, 2010).

The general public has turned a lot of attention to berries as a natural source of antioxidants. Health benefits of berries can be attributed to their phenolics which may contain flavonoids, stilbenes, or tannins (Seeram, 2008). It is believed that if the antioxidant defense mechanism of the body is weakened then there is a greater risk of cardiovascular diseases, inflammation and diabetes (Jaakola & Hohtola, 2010). A lot of phenolic compounds have shown a range of bioactivity like anti-inflammatory properties, anti-cancer properties and have shown to provide protection against cardiovascular disease (Seeram, 2008). There are different types of polyphenols present in the berries as listed in Fig. 1.

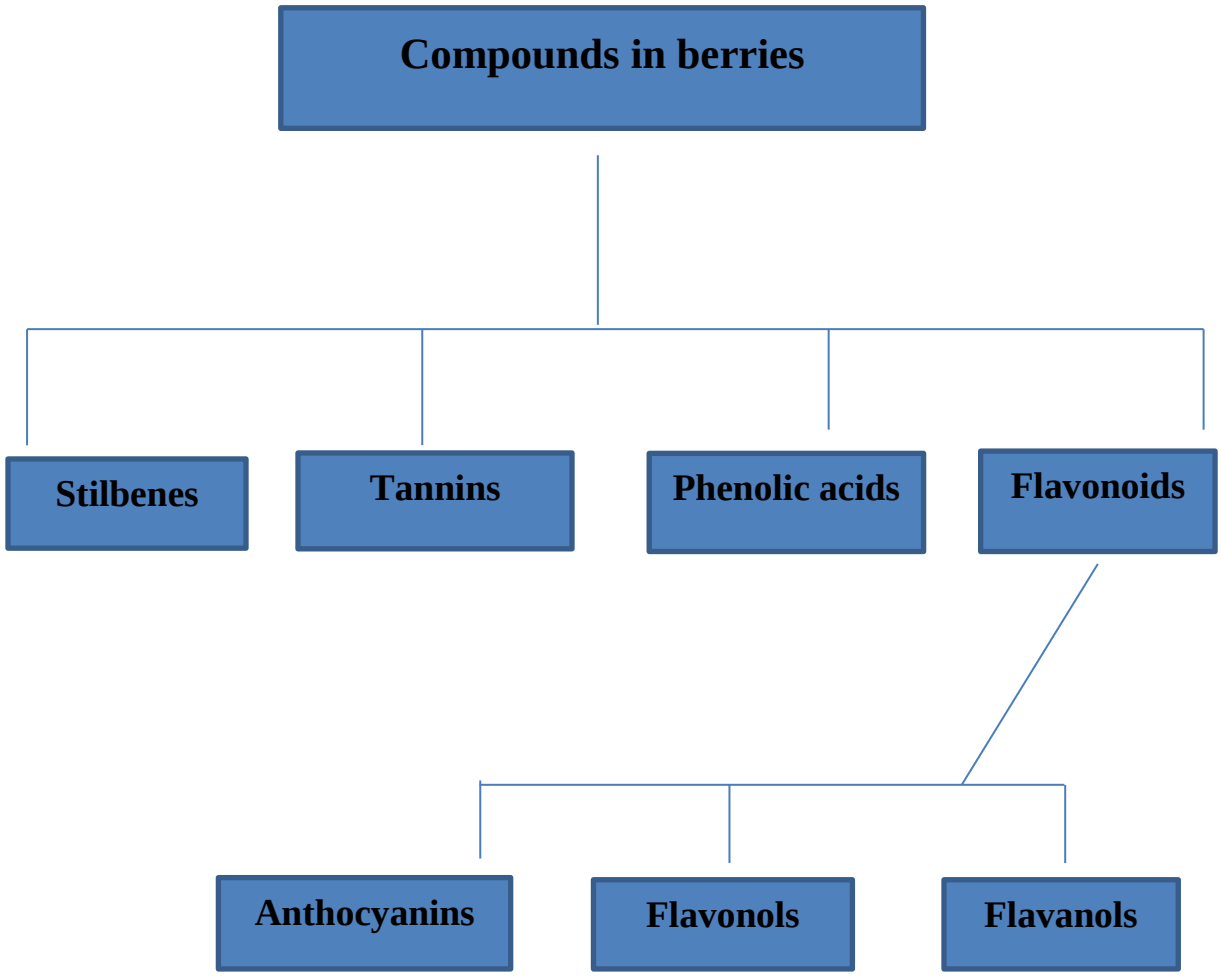


Fig. 1: List of phenolic compounds in berries -Adapted from (Paredes-Lopez et al., 2010)

Table 1 shows the different types of polyphenolic compounds with the sources of food in which they are found, basic skeleton of the compounds and the total number of carbon atoms these compounds have.

Table 1: List of polyphenolic compounds with food sources, Basic skeleton and Number of carbon atoms Adapted from (de Mello Andrade & Fasolo, 2014)

Type	Sources	Number of carbon atoms	Basic skeleton
Stilbenes	Grape skin Peanuts Blackberries	14	C6-C2-C6
Phenolic acids	Coffee Red wine	7	C6-C1
Anthocyanins	Blackberry Blueberry Strawberry	15	C6-C3-C6
Flavanols	Green tea Chocolate Cherry	15	C6-C3-C6
Flavonols	Apple Kale Leek	15	C6-C3-C6

1.4.1. Stilbenes

Stilbenes are naturally occurring polyphenols in plants. They are found in variety of fruits like cowberry, lingonberry and blueberry. Resveratrol, pterostilbene and piceatannol are the three different known types of stilbenes (Lyons et al., 2003) and the structures of these compounds are shown in Fig. 2. Resveratrol is the most common type of stilbene. Numerous studies have shown the biological properties of stilbene (Ali, Maltese, Choi, & Verpoorte, 2010). Studies have reported the diverse biological properties of resveratrol as anti-inflammatory, anti-cancer and anti-aging in animal models and humans (Lyons et al., 2003; Rimando, Kalt, Magee, Dewey, & Ballington, 2004; Wang, Catana, Yang, Roderick,

& van Breemen, 2002; Gambini et al., 2015). It has been shown to have antifungal activities against different types of pathogens including *Cladosporium cuccumerinum, Pyricularia oryzae, Plasmopara viticola*. The presence of stilbene in grapevine was shown to contribute to the resistance of fungal diseases (Husken et al., 2005).

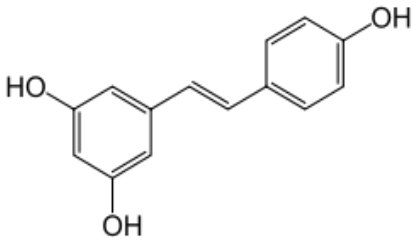
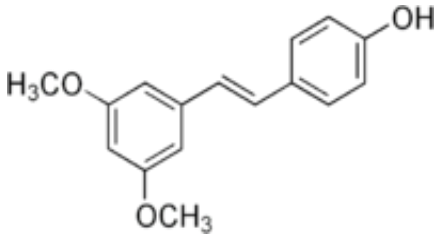
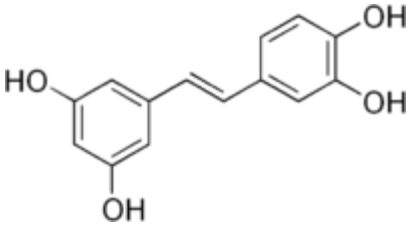
Type	Chemical Structure
Resveratrol	
Pterostilbene	
Piceatannol	

Fig. 2: Structure of examples of stilbenes: resveratrol, pterostilbene and piceatannol

1.4.2. Tannins

Tannins are tart in taste and are crucial for evaluation of the sensory property of fruit and juices. They usually occur in two forms: as esters of gallic acid and ellagic acid as non-hydrolysable tannins in condensed form (Chung, Wong, Wei, Huang, & Lin, 1998). The condensed form of tannins is named as proanthocyanidins; whereas, the ones that occur in ester form are named as hydrolysable tannins (Kolniak-Ostek, Kucharska, Sokol-Letowska, & Fecka, 2015). Chokeberry is reported to contain high levels of condensed tannins. In another study (Da Silva, Lajolo, & Genovese, 2007) it was reported that there is a lower quantity of tannins present in honeyberry and blackberry. Berries like strawberry, raspberry and blackberry contain hydrolysable tannins which appear less frequently in plants (Da Silva et al., 2007; Gulcin et al., 2011).

Plants contain measurable amount of tannins. There are contradictory reports on the uses of tannins and tannin associated products. Some experimental animal models have suggested that tannins are carcinogenic in nature; whereas, others showed anti-carcinogenic properties. The anti-carcinogenic property found in tannins was correlated to their anti-oxidative property (Kono, Ikeda, Tokudome, & Kuratsune, 1988; Yamane et al., 1991). In addition to anti-carcinogenic properties, antimicrobial properties of tannins were also reported. However, there is limited literature to support the direct impact of tannins on human health (Chung et al., 1998).

1.4.3. Phenolic acids

Phenolic acids are secondary metabolites found in plants and fungi. Phenolic acids are aromatic acids that contain a phenolic ring and an organic carboxylic acid; that is, a C6-C1+ skeleton. There are mainly two different types of phenolic acids in berries; namely,

benzoic acid and cinnamic acid derivatives (Zadernowski, Naczk, & Nesterowicz, 2005). In berries, benzoic acid derivatives like gallic acid, ellagic acid, salicyclic acid and *p*-hydroxybenzoic acids are present (Taruscio, Barney, & Exon, 2004). Likewise, cinnamic acid derivatives such as caffeic acid, ferulic acid and *p*-coumaric acid are also present. Cinnamic acid derivatives occur in esterified form and hydroxybenzoic acid derivatives occur in glycosylated form (Zadernowski et al., 2005; Taruscio et al., 2004). Some of the examples of phenolic acids along with their chemical structures are shown in Fig. 3. Phenolic acids were shown to have antimicrobial and anti-insecticide properties (Alves et al., 2013). Phenolic acids obtained from mushrooms were shown to have antitumor and antioxidant properties from *in vitro* studies (Heleno et al., 2013).

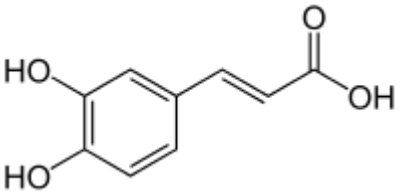
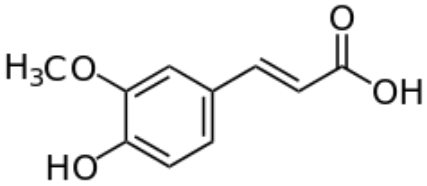
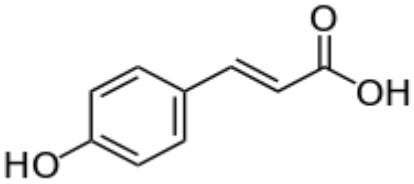
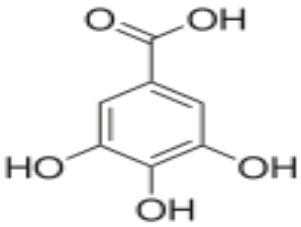
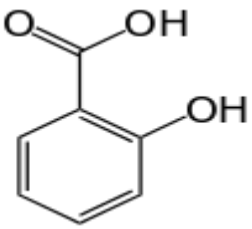
Type	Sub-type	Chemical Structure
Cinnamic acid derivatives	Caffeic acid	
	Ferulic acid	
	p-coumaric acid	
Benzoic acid derivatives	Gallic acid	
	Salicylic acid	

Fig. 3: Structure of phenolic acids (caffeic acid, ferulic acid, *p*-coumaric, gallic acid and salicylic acid)

1.4.4. Flavonoids

Flavonoids are obtained from natural sources such as vegetables, fruits, tea and wine (Middleton E Jr, 1998). A positive correlation between flavonoid intake through red wine and lower cardiovascular mortality were shown in Mediterranean populations (Formica & Regelson, 1995). Apart from the cardio-protective property, flavonoids were also shown to have antioxidant (Pietta, 2000), anti-tumour (Loft & Poulsen, 1996), and anti-inflammatory properties (Ferrandiz & Alcaraz, 1991). Among different types of berry phenolics, flavonoids are the largest group which is further divided into three different sub-groups: anthocyanins, flavonols and flavanols.

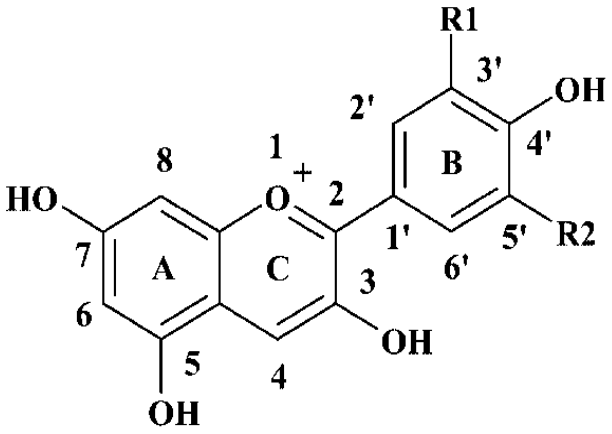
1.4.4.1. Anthocyanins

Among the 3 different types of flavonoids in berries, anthocyanin is a subgroup. It is the water soluble compound that gives blue, purple and red colour to the fruits and vegetables (Li, Wang, Luo, Zhao, & Chen, 2015). These pigments are prevalent in different parts of the plants and; mostly found, in fruits, flowers, roots, leaves and the epidermal skin. The major pathway involved in the synthesis of anthocyanins is the phenylpropanoid pathway; a pathway which is involved in synthesis of different secondary metabolites such as flavonoids and phenolic acids (Li, Wang, Luo, Zhao, & Chen, 2015). Anthocyanins are generally attached to a different sugar moiety like glucose, galactose, fructose, arabinose, rhamnose and xylose (Prior & Wu, 2006).

There are six major types of anthocyanins: cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin (Fig. 4) (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002). The most common anthocyanin is the cyanidin followed by delphinidin, peonidin, pelargonidin, petunidin and malvidin (Moyer, Hummer, Finn, Frei, &

Wrolstad, 2002). Cyanidin is responsible for the red color. Likewise, delphinidin gives a bluish color and pelargonidin gives a characteristic purple color to the plants (Stintzing et al., 2002; Moyer et al., 2002; Pappas & Schaich, 2009).

Cyanidin-3-glucoside is the key anthocyanin found in most fruits (Pappas & Schaich, 2009). For example, it is present in blackberry, chokeberry, elderberry, cranberry, cabbage, spinach and red radishes. Glycosidic derivatives of malvidin are mostly found in red grapes. Anthocyanins have gained a huge attention owing to their antioxidant potential. Evidence suggests that anthocyanins play a crucial role in diminishing the occurrence of different cancers (Nile & Park, 2014) in epidemiological, and both *in vitro* and *in vivo* animal studies (Carlton et al., 2001). Anthocyanins act as antioxidants and provide several health benefits (Howell, 2007). They help to mitigate the harmful effects caused by free radicals during the oxidative stress and they have anti-inflammatory properties as well (Hosseinian & Beta, 2007). In addition to this, anthocyanins have received tremendous attention from food scientists as an alternative to the synthetic colorants. They have potential to be utilized as natural colorants in the food industry (Hosseinian & Beta, 2007; Nabuurs, McCallum, Brown, & Kirby, 2015). Three major anthocyanins in lingonberry as reported in the literature are cyanidin-3-glucoside, cyanidin-3-galactoside and cyanidin-3-arabinoside. Fig. 4 shows different types of anthocyanins with the associated chemical structure.



Aglycone	R1	R2
Cyanidin (Cy)	OH	H
Peonidin (Pn)	OCH3	H
Pelargonidin (Pg)	H	H
Malvidin (Mv)	OCH3	OCH3
Dephinidin (Dp)	OH	OH
Petunidin (Pt)	OCH3	OH

Fig. 4: List of different anthocyanins -Adapted from (Hosseinian & Beta, 2007)

1.4.4.2. Flavonols

Flavonols are the other common secondary plant metabolite within the flavonoids group and are found in different fruits and vegetables. Major sources of flavonols are apple skins, berries and broccoli. Quercetin is the widely described flavonol (Nijveldt et al., 2001). Some of the common examples of flavonols are quercetin, myricetin and kaempferol (Paredes-Lopez et al., 2010). One flavonol is different from the other based on the number and the position of hydroxyl groups on the B ring (Fig. 4). They normally occur in the form of glycosides in food. Glycosides and, in particular sugars, are mainly attached at the 3rd carbon position (Rodriguez-Mateos, Heiss, Borges, & Crozier, 2014).

Light plays a crucial role in the formation of the flavonol glycosides. Plant parts like leaves and other outer parts have a higher content of flavonols (Rodriguez-Mateos et al., 2014). Epidemiological and *in vitro* cellular studies have shown the beneficial effects of flavonols on cardiovascular health (Buijsse, Feskens, Kok, & Kromhout, 2006; Schewe et al., 2001). In addition, anti-inflammatory and antioxidant effects of flavonols have been shown in both *in vivo* animal and *in vitro* cellular studies (Rimbach, Melchin, Moehring, & Wagner, 2009; Baba et al., 2000). Example of different types of flavonols with their structures is as shown in Fig. 5.

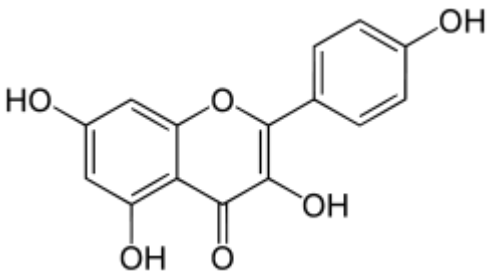
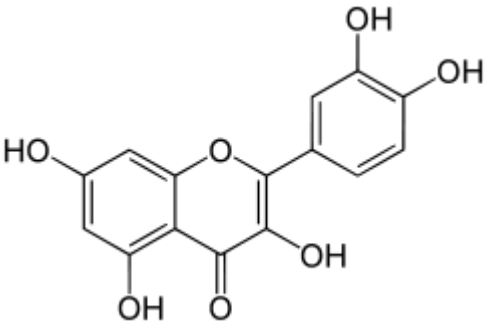
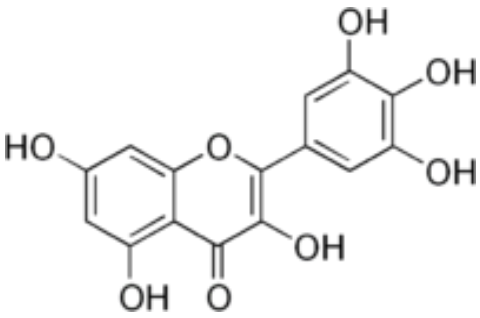
Type	Chemical Structure
Kaempferol	
Quercetin	
Myricetin	

Fig. 5: Structure of flavonols (Kaempferol, quercetin and myricetin)

1.4.4.3. Flavanols

Among different types of flavonoids, flavanols are another of the subclasses. Catechin, epicatechin, gallocatechin and epigallocatechin are the some examples of flavanols (Paredes-Lopez et al., 2010). Flavanols are one of the sub-classes of flavonoids with a complex structure. Major sources of flavanols are citrus fruits and major compounds are: Narigin and Taxifolin (Nijveldt et al., 2001). Animal models have shown the cardio-protective property of flavanols; however, there are limited clinical data available to show their beneficial effects (Mulvihill et al., 2010) . It is not fully understood as how the flavanols exhibit the protective effect in cardiovascular diseases (Chanet, Milenkovic, Manach, Mazur, & Morand, 2012). Some of the examples of flavanols with the chemical structure are shown in Fig. 6.

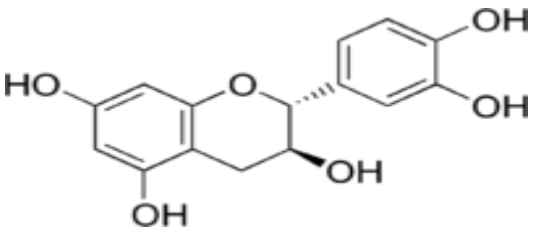
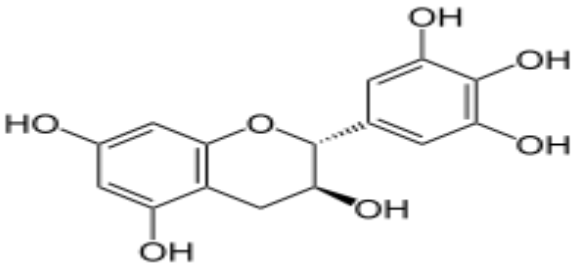
Type	Chemical Structure
Catechin	
Gallocatechin	

Fig. 6: Structure of flavanol

1.5. Effects of cool climate on production of secondary metabolites

Plant secondary metabolites are compounds that are considered non-essential in the growth and development of the plant. However, in response to pathogens, insects and cold stress, plants respond by activating defense mechanisms which includes the synthesis of secondary metabolites (Ramakrishna & Ravishankar, 2011; Kasote, Katyare, Hegde, & Bae, 2015). There are various factors that influence the synthesis of secondary metabolites in plants. They can be broadly classified into two groups: biotic and abiotic factors (Manganaris, Goulas, Vicente, & Terry, 2014). Both biotic and abiotic factors play a crucial role in determining the profile and the content of secondary metabolites.

A temperate zone woody plant like lingonberry is able to withstand the harsh climatic conditions. The plant modifies its physiological and metabolic pathways by synthesizing secondary metabolites some of which are believed to have antioxidant properties (Zhu, Dong, & Zhu, 2007; Chinnusamy, Zhu, & Zhu, 2007). Modification of metabolic pathways by synthesizing different secondary metabolites leads the plants to acclimatize to the harsh cold conditions and is called cold acclimation (Chinnusamy et al., 2007). Studies on rice revealed that those plants under stress, in particular, cold stress, bear an ability to adapt to the cold. During stress condition, plants increase the antioxidant defense mechanism by which it provides protection to the plant against free radicals (Fowler & Thomashow, 2002).

Plants respond to environmental stress by producing antioxidants as a defense mechanism. It has been suggested that extreme cold climatic conditions turns on the expression of numerous genes that significantly affects phytochemical content (Guy,

Kaplan, Kopka, Selbig, & Hincha, 2008). It has been reported that cold stress is associated with the modification of various metabolic pathways. For instance, apple trees were found to up-regulate the synthesis of chlorogenic acid in response to cold stress (Ramakrishna & Ravishankar, 2011). In addition to this, anthocyanin accumulation was observed to be higher in maize seedlings exposed to cold temperature treatments (Christie, Alfenito, & Walbot, 1994). This study suggested that there was up-regulation of the phenylpropanoid and anthocyanin pathway gene expression and an increase in anthocyanin accumulation.

Other studies suggest that grapes and apples had a higher content of anthocyanins under cold stress and nutrient-deprived conditions (limiting phosphate); however, the mechanisms behind the effect is yet to be elucidated (Christie et al., 1994). Different factors like temperature, soil conditions, rainfall, moisture, harvest time, post-harvest conditions have an influence on the contents of secondary metabolites (Lehtonen et al., 2013). Local geo-climatic conditions directly determine the contents of secondary metabolites both qualitatively and quantitatively. In addition, factors like light, geographical location and ripeness affect the berry phenolic content and quality (Szakiel et al., 2012). This study will provide insight into the anthocyanin content, phenolic content and antioxidant capacity of lingonberries grown in two different conditions: wild and controlled greenhouse conditions.

1.6. Free radicals

Any atom or molecule which poses an unpaired electron in its valence shell is known as a free radical. It can be produced from endogenous sources as well as exogenous sources (Phaniendra, Jestadi, & Periyasamy, 2015). Free radicals can also

be produced by various enzymatic sources. Table 2 summarizes a few sources of free radicals (Phaniendra et al., 2015).

Table 2: Various sources of free radicals

External sources of free radicals	Cellular sources of free radicals	Enzymatic sources of free radicals
Smoking	Mitochondria	Xanthine oxidase
Alcohol	Phagocytic cells	Cytochrome oxidase
UV radiation	Endoplasmic reticulum	Membrane NADPH oxidase
Pollution		

Adapted from (Phaniendra et al., 2015).

As free radicals lack an electron at their valence shell, they are unstable, highly reactive and are able to cause damage to various biomolecules by taking away electrons from the donor molecules. Important classes of biomolecules that are directly affected by the action of the free radicals are deoxyribonucleic acid (DNA), proteins and lipids (Droge, 2002). Free radicals attack these biomolecules by stealing the electrons and these donor molecules, in turn, end up becoming free radicals. As a result of which these molecules are unable to function properly and the result is in cell damage and cell death (Lobo, Patil, Phatak, & Chandra, 2010).

Free radicals are not always deleterious and undesired as they play a crucial role in normal cellular metabolism in processes like gene expression, cellular growth and can provide protection against infections (Droge, 2002). Different immune cells like

macrophages and neutrophils produce reactive oxygen species (ROS) which kill bacteria and provide protection to the body (Droge, 2002). A balance needs to be maintained between the production of free radicals and antioxidants (Lobo, Patil, Phatak, & Chandra, 2010). The condition where production of free radical overwhelms the body's antioxidant system is known as oxidative stress (Lobo et al., 2010). Oxidative stress is thought to be responsible for inducing a number of chronic diseases like diabetes mellitus, hypertension, multiple sclerosis, Parkinson's disease and arthritis (Phaniendra et al., 2015).

Free radicals can either give or gain an electron to become stable. However, this process leads to the formation of other free radicals leading to a chain reaction. Both enzymatic and non-enzymatic reactions are responsible for the generation of free radicals (Reiter et al., 2003). Free radicals can be broadly classified into the two categories of reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Reiter et al., 2003). Some of the molecules derived from oxygen are O-2(superoxide), $HO^{\cdot}$ (hydroxyl), HO_2 (hydroperoxyl), $ROO^{\cdot}$ (peroxyl), Similarly, $NO^{\cdot}$ (nitric oxide), $ONOO^{\cdot}$ (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide) are some examples of nitrogen-derived free radicals (Reiter et al., 2003). There are two different groups into which reactive nitrogen species (RNS) and reactive oxygen species (ROS) are placed; namely, radicals and non-radicals (Weidinger & Kozlov, 2015).

1.7. Antioxidants

A molecule that is able to prevent oxidation of other molecules is called an antioxidant (Bazinet & Doyen, 2015). Antioxidants are able to scavenge free radicals. An antioxidant donates an electron to the free radical and stabilizes the free radicals. There

are two different types of antioxidants: enzymatic and non-enzymatic antioxidants (Ashraf et al., 2015). These molecules help to neutralize free radicals in the body. Free radicals are generated in the body as a result of normal cellular metabolism. However, there needs to be a balance between the bodies' antioxidant system and the production of free radicals. In a diseased condition or as we grow older, production of free radicals is much higher and body is unable to maintain a balance. In such case, dietary antioxidants can play a significant role to restore the balance (Shahidi, 2000). Some examples of enzymatic antioxidants are superoxide dismutase (SOD), catalase, coenzyme Q10 and glutathione; whereas, some of non-enzymatic antioxidants are tocopherols, vitamin C, vitamin E, polyphenol (Bazinet & Doyen, 2015). Antioxidants can be obtained either from food itself or in the form of supplements which helps to mitigate the effects of free radicals. Dietary antioxidants are abundantly present in fruits and vegetables (Valko et al., 2007).

Some of the examples of antioxidants are flavonoids which act by chelating redox-active metals and by scavenging free radicals (Ginter, Simko, & Panakova, 2014). It is supposed that antioxidants work by two different mechanisms. One of the mechanisms includes an antioxidant donating an electron to the free radical to break the chain reaction while other includes removal of reactive oxygen (ROS)/reactive nitrogen species (RNS). Antioxidants in diets are able to neutralize the adverse effects caused by free radicals (Ginter et al., 2014).

1.8. Oxidative stress and diseases

When the body's capacity to balance reactive oxygen species (ROS) is hindered the condition is termed as oxidative stress (Hermes-Lima et al., 2015). Reactive oxygen

species are beneficial when they are produced during the normal metabolic process but are deleterious when produced at high levels during infections or other environmental stress (Hermes-Lima et al., 2015). Under normal conditions, reactive oxygen species play a fundamental role in immunomodulation, apoptosis, cell signalling and hormonal regulation (Reshi, Su, & Hong, 2014). During oxidative stress there is an imbalance in the body's ability to provide antioxidant defense mechanism and the production of the free radicals. A decrease in the level of antioxidants is associated with dysfunctions of the immune system. Researchers have also shown the decrease in antioxidants levels of vitamin C, glutathione, flavonoids, catalases, SOD during the disease condition (Eisele, Markart, & Schulz, 2015; Caruana & Vassallo, 2015; Singh & Pai, 2015).

1.9. Ischemia-reperfusion

Ischemia is the condition in which oxygen and nutrient supply to an organ or tissue is obstructed (Hausenloy & Yellon, 2013). A number of biochemical and metabolic changes occur. As a consequence of deprived oxygen conditions, anaerobic cellular metabolism takes place. Production of lactate and lowering of intracellular pH is the outcome of ischemia. The electron transport chain is disturbed; in particular, the Na-K-ATPase pump (Hausenloy & Yellon, 2013). If these conditions prevail and are not treated then it can result in an organ failure. During reperfusion, the electron transport chain goes back to normal resulting in generation of reactive oxygen species (ROS*)* (Hausenloy & Yellon, 2013). An outburst of reactive oxygen species (ROS) after the restoration of blood flow can then result in apoptosis (Kumar & Jugdutt, 2003).

Caspases belongs to cysteine proteases protein family and have known to play central role in programmed cell death known as apoptosis (Czerski & Nunez, 2004;

Degterev & Yuan, 2008). One of the characteristics features of apoptosis is the activation of caspases (Czerski & Nunez, 2004). In all healthy cells, caspase exists in an inactive form called caspase zymogens. Upon being triggered by an activating signal, the inactive form of caspase (pro-caspase) gets converted into the activate state (caspase) (Fuchs & Steller, 2011). Morphologically, apoptosis is characterized by DNA fragmentation, membrane blebbing (a bulge or protrusion of the plasma membrane of a cell), nuclear condensation and cell shrinkage (Czerski & Nunez, 2004). Caspases are activated by various pathways which include: the Extrinsic pathway (Death receptor pathway), Intrinsic pathway (Mitochondrial pathway) and endoplasmic reticulum signalling pathway (Czerski & Nunez, 2004).There are two types of caspase based on their nature of activation and function. Initiation caspases (caspase 2, 8, 9, 10, 11 and 12) receive the pro-apoptotic signals (Riedl & Shi, 2004). The pro-apoptotic signal is generated from number of activities in cells. The induction of death ligands such as FASL (extrinsic apoptosis pathway), DNA damage and cytochrome c released from mitochondria (intrinsic pathway) all contribute to generate pro-apoptotic signal that in turn activate initiation caspase (Fuchs & Steller, 2011). Upon activation of initiation caspase, the signal is relayed to downstream caspase known as effector caspase (Caspase 3 and caspase 7) for execution of apoptosis by targeting cellular proteins (Riedl & Shi, 2004).

1.10. Methods to determine antioxidant capacity

There are different methods to evaluate the antioxidant capacity of plants *in vitro*. Two of the commonly utilized methods to evaluate antioxidant capacity *in vitro* are: oxygen radical absorbance capacity (ORAC) (Isaak, Petkau, O, Debnath, & Siow, 2015; Prior et al., 2003) and the Folin-Ciocalteau's method (Prior, Wu, & Schaich, 2005).

There is no single method that is able to effectively and completely evaluate the antioxidant capacity of the sample (Prior et al., 2005). A specific method utilized to determine the antioxidant capacity simply represents the certain specific conditions under which a specific assay is used; therefore, it will be improper to draw a definitive conclusion on total antioxidant activity based on results of single method employed (Prior et al., 2005) and caution is in order to compare values from different studies. It is therefore recommended to use more than one method to evaluate the antioxidant capacity (Huang, Ou, & Prior, 2005). Different chemical methods to determine the antioxidant capacity involves two mechanisms: hydrogen atom transfer (HAT) which involves competition between the substrate and antioxidant for the free radicals (Huang et al., 2005), and the other mechanism is known as the electron transfer (ET) where the oxidant is reduced and is indicated by a change in the color (Huang et al., 2005).

1.10.1. Oxygen radical absorbance capacity (ORAC)

ORAC is one of the widely used methods to determine the ability of antioxidants to scavenge free radicals. This method is based on a hydrogen atom transfer (HAT) mechanism (Niki, 2010). Fluorescein is used as the probe and 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) is used as the peroxyl radical generator (Niki, 2010). By measuring the net area under the fluorescence decay curve generated during assay the potential of an antioxidant to scavenge free radicals is determined (Niki, 2010). The reaction condition is close to biological system which includes the temperature of 37^0C and pH 7; therefore, this method is considered as one of the standard methods to determine the antioxidant capacity (Lopez-Alarcon & Denicola, 2013). Trolox is used as the standard to determine the antioxidant capacity. Results are expressed in terms of trolox

equivalents. One of the major limitations of ORAC assay is that the ORAC assay is temperature sensitive so a slight fluctuation in the temperature may affect the overall reproducibility of the assay. Further, as the fluorescein is sensitive to light, if it exposed to light, it might degrade and affect the result outcome (Amorati & Valgimigli, 2015).

1.10.2. Total Phenolic Content

Total phenolic content of fruits and vegetables is determined by a method called the Folin-Ciocalteau's method. It is a widely utilized method in which Folin-Ciocalteau's reagent; a yellow colored acidic solution containing phosphotungstic and phosphomolybdic acids, in presence of the phenolic compounds undergoes a complex redox reaction (Gulcin, 2012; Singleton & Rossi, 1965). When there is the reaction between the acidic complex and the phenolic compounds, phenolic compounds transfer electrons to the acid complexes resulting in a blue complex which is quantified by measuring absorbance with the help of spectrophotometer (Gulcin, 2012; Singleton & Rossi, 1965). The intensity of light absorption is directly proportional to the concentration of the phenolics (Singleton & Rossi, 1965). The Folin-Ciocalteau's method is considered to be a simple and inexpensive method to determine the total phenolic content. The limitation of the method is that it does not measure the specific phenolic content as the method accounts for total phenolic content only (Amorati & Valgimigli, 2015).

1.11. Metabolomics

Metabolomics is the systematic documentation and study of the complete chemical constituents of a sample at one point in time, and a powerful tool with the diverse applications. It has been widely utilized in the various fields like discovery of drugs,

animal and plant physiology, nutrition and diseases (Wishart, 2008; Yuliana, Khatib, Choi, & Verpoorte, 2011; Fillet & Frederich, 2015; Gika, Theodoridis, Plumb, & Wilson, 2014). Application of a metabolomics approach provides a comprehensive analysis of all the metabolites present in the sample at one time point (Gika et al., 2014). A typical metabolomics methodology follows four steps. The first step is the preparation of the samples followed by a separation step which can be done by different methodologies, data reduction by employing statistics, and finally the identification of individual metabolites (Xu, Zou, Liu, Zhang, & Ong, 2011; Alonso, Marsal, & Julia, 2015).

Sample preparation in metabolomics is a very crucial step as it can impact the outcome. In general, the sample preparation step is based on the desired metabolite of interest as per the design of the experiment. A typical sample preparation approach starts with the harvest of plant material, drying of the sample and the extraction. Drying of the sample is very important as the non-optimal water content in the sample could possibly lead to decomposition of metabolites which eventually affects the overall result. Drying of the sample helps to inhibit the enzyme activity and further degradation by microbial growth. Regardless of the compound of interest and the analytical method, the process of harvesting a plant material and drying process remains the same. The choice of solvent for the extraction process depends on the metabolite of interest. There are few parameters that need to be taken into consideration during the extraction process such as the temperature at which extraction is to be carried out, duration of the extraction and the ratio of the sample and the solvent (Huie, 2002). To date, there is no single solvent available that can dissolve all of the compounds in a sample and this is one limitation of a metabolomics study.

Some other challenges and limitations of metabolomics study are listed below.

(i) Diversity: It is difficult to detect the diverse nature of metabolites, their isomers; slight modification in the chemical structure of compound using similar extraction and fractionation process may affect result outcome. One protocol may not be suitable for detecting these minor changes and may require multiple other extraction process which may be tedious and time consuming (Hill, Czauderna, Klapperstuck, Roessner, & Schreiber, 2015).

(ii) Detection sensitivity: In a given pool of samples, some metabolites may be found in low level compared to other metabolites. Detecting these low abundance metabolites is difficult as high abundant metabolites overshadow these compounds. Also there is a chance that these minor metabolites are lost during the extraction process (Roessner & Pettolino, 2007).

(iii) Quantification of metabolites: To accurately quantify the metabolites present in sample pools, an internal standard curve is required. Due to the enormous chemical diversity present in samples it is not feasible to generate the internal calibration curve for all compounds. Therefore, absolute quantification of metabolites by current MS based technology remains issue therefore metabolomics experts are using relative change in abundance as semi-quantification approach (Hegeman, 2010).

For the separation process, different methods like gas chromatography (GC), high performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) and capillary electrophoresis (CE) can be utilized (Alonso et al., 2015). Each method is able to detect specific types of compounds. NMR and MS are widely used

analytical tools in metabolomics. However, each of these tools has its own limitations. For example, NMR can provide the structure and structural diversity of range of metabolites but is not as sensitive as MS. GC-MS is sensitive instrument but is limited in detecting volatile compounds (Bottcher, Roepenack-Lahaye, Willscher, Scheel, & Clemens, 2007; Eisenreich & Bacher, 2007). Polar compounds are better separated by high performance liquid chromatography (HPLC). Capillary electrophoresis is better suited for the separation of charged analytes (De Vos et al., 2007).

One of the advantages of using liquid chromatography coupled to mass spectrometry (LC-MS) is that this method is suitable for a low to high molecular weight compounds and compounds can be of hydrophilic to hydrophobic nature. In addition to this, choice of column and mobile phase can be done based upon the need (Putri, Yamamoto, Tsugawa, & Fukusaki, 2013). There is no ideal method to be utilized for the separation step. Depending upon the objective of the study the selection of a method needs to be carried out; each instrument comes with certain strength and weakness. Combination of two or more systems provides a more comprehensive result (Putri et al., 2013).

There are basically two different approaches to metabolomics; a targeted approach and a non-targeted approach. A targeted metabolomics approach is based upon the identification of a specific group of compounds under predefined conditions (Vinayavekhin & Saghatelian, 2010). This approach requires identification and quantification of the metabolites. Whereas, a non-targeted metabolomics approach provide comprehensive and unbiased information about the metabolites present in the sample (Vinayavekhin & Saghatelian, 2010). This approach helps to detect as many

compounds as possible without the need of identification and quantification of the metabolites.

CHAPTER II: RESEARCH PLAN

2.1. Knowledge gap

There is wealth of information on berry anthocyanins, phenolics and antioxidant activity. However, there is very little information linking chemical properties and bioactivity to a metabolomic profile of lingonberries. In addition to this, there has been no comparison of lingonberries grown under different climatic condition especially those from Northern Manitoba and other Canadian provinces. Also, there is no knowledge regarding the antioxidant potential and chemical composition of lingonberry grown under wild and controlled greenhouse conditions. There is a belief that plants grown under extreme (stressful) climatic conditions have some compositional advantage; however, there is very little scientific evidence to support the belief. Therefore, a study of this kind will provide insight into the berries grown under the two different climatic conditions, with one being extreme and stressful and the other controlled greenhouse conditions.

2.2. Aim of the study

The study will provide information whether berries grown in different locations have differences in phenolics, anthocyanins and antioxidant capacity. In addition to this, the study will provide information on the metabolic profiles of different lines and cultivars of berries grown in two different locations. Previously unexplored Northern Manitoba wild grown lingonberry will be studied and a wealth of information will be obtained.

2.3. Hypotheses

1. Northern Manitoba lingonberries will have a higher content of total anthocyanins, phenolics and a higher antioxidant capacity.
2. Northern Manitoba berries will have higher levels of metabolites associated with antioxidant activity.
3. Northern Manitoba berries will have unique compounds which may not be present in greenhouse grown Newfoundland samples.

2.4. Overall Objective

To compare lingonberries from Northern Manitoba and greenhouse grown from Newfoundland in terms of phenolics, anthocyanins content, antioxidant potential and metabolomics profile.

2.5. Specific Objectives

1. To compare total anthocyanin content, total phenolic content, antioxidant capacity and anti-apoptotic effect of wild lingonberries from Northern Manitoba and greenhouse grown lingonberries from Newfoundland.
2. To determine the metabolomics profile of lingonberries from Northern Manitoba and compare it with greenhouse grown lingonberries from Newfoundland.

CHAPTER III: METHODOLOGY

3.1. Plant Material

Lingonberrries *(Vaccinium vitis ideaea)* used in the study were obtained from Northern Manitoba, Newfoundland and Labrador, Canada. Berries were collected from the wild by harvesters in Lynn Lake, Flin Flon and Food Development Centre (FDC) for Manitoba samples. Newfoundland samples were obtained from Dr. Samir Debnath at the Atlantic Cool Climate Crop Research Centre (ACCCRC) which were grown and maintained in the ACCCRC green house. The green house conditions are outlined in Table 3 as described by (Isaak et al., 2015) and the annual precipitation, altitude and GPS locations of Lynn Lake, Flin Flon and Newfoundland is listed in Table 4. Only fully ripened berries were harvested and dispatched to Winnipeg. Immediately upon the receipt, berries were frozen and stored at -80^{0}C.

Table 3: Greenhouse growing conditions for Newfoundland lingonberry

Light	Natural light conditions with maximum photosynthetic photon flux of 90 µmol^{-2}s^1
Temperature	20 ± 2^{0}C
Humidity	85 % relative humidity
Plastic pots	Diameter of 25 cm 18 cm deep equivalent to 6 liters

Table 4: Average annual precipitation, altitude with GPS location sites for the harvested sites of wild Manitoba (Lynn Lake and Flin Flon) lingonberry and cultivated lingonberry from Newfoundland

Location	Altitude (m)	Precipitation (mm)	Latitude (N)	Longitude (W)
Lynn Lake	357	492.4	56°51'	101°4'
Flin Flon	304	484.1	54°46'	101°51'
Newfoundland	6	679	56°32'	61°40'

3.2. Sample preparation

Frozen berries were dried by lyophilisation, cut into pieces and ground with the help of a mortar and pestle. Then, the samples were extracted by following the dried product extraction protocol established at the Canadian Centre for Agri-Food Research in Health and Medicine (CCARM) as described by (Isaak et al., 2015). 0.5 grams of sample were weighed out into 30 mL centrifuge tube then 6 mL of 80% methanol and 2% formic acid (v/v) were added. The mixture was left at room temperature for 15 minutes. The mixture was vortexed gently every 5 minutes and sonicated for 15 minutes at 37^0C. The mixture was then centrifuged for 15 minutes at 3000 X g and the supernatant was transferred to 50 mL Falcon tubes. The extraction procedure was repeated and the supernatants from both extractions were combined. Samples were stored at -20^0C overnight and filtered with a 0.2 µm syringe filter into a new 15 mL Falcon tube. Samples were then stored at -20^0C until further use. The background information on

samples with the genotype, propagation type, province and growing condition is shown in Table 5.

Table 5: Background information on lingonberry samples with genotype, propagation type, growing condition and province

Group	Sample	Genotype	Propagation Type	Province	Growing Condition
	FFLB	Unknown	Background Information unknown	Manitoba	Wild
	FDC	Unknown	Background Information unknown	Manitoba	Wild
	LLLB1	Unknown	Background Information unknown	Manitoba	Wild
	LLLB2	Unknown	Background Information unknown	Manitoba	Wild
	Dave Buck	Unknown	Background Information unknown	Manitoba	Wild
1	17	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
1	18	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	6	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	7	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	9	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
1	10	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	12	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	13	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	14	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	15	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	16	E1-1 (clone from Estonia)	Micro-propagated	Newfoundland	Greenhouse grown
1	19	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	20	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	21	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	22	H1 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
1	23	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	24	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	25	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	26	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	27	Regal	Micro-propagated	Newfoundland	Greenhouse grown

1	28	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	29	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	30	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	31	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	32	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	33	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	34	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	35	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	36	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	37	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	38	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
1	39	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	40	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	41	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	42	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	43	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
1	44	Regal	Micro-propagated	Newfoundland	Greenhouse grown
1	46	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
1	47	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
1	48	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
1	49	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	7	H2 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	12	H3 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	13	H4 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	19	H5 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	34	H6 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	41	E1-2 (a clone from Estonian)	Micro-propagated	Newfoundland	Greenhouse grown
2	43	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	45	H7 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown

2	11	H8 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	17	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	20	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	25	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	33	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	40	H9 (hybrid Splendor x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	46	H10 (hybrid Splendor x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	9	Ida	Stem Cutting	Newfoundland	Greenhouse grown
2	10	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
2	14	H11 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	15	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	16	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	18	H12 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	21	H13 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	22	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	23	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	24	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	26	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	27	H14 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	29	H15 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	30	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	31	H16 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	32	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	35	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	36	Erntedank	Micro-propagated	Newfoundland	Greenhouse grown
2	37	Ida	Stem Cutting	Newfoundland	Greenhouse grown
2	38	Entrekrone	Stem Cutting	Newfoundland	Greenhouse grown
2	39	ES1 (an European selection)	Stem Cutting	Newfoundland	Greenhouse grown

2	42	H17 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	44	H18 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	47	H19 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	48	Erntredank	Micro-propagated	Newfoundland	Greenhouse grown
2	49	H20 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	50	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	60	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	52	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	56	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	61	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	54	Ida	Stem Cutting	Newfoundland	Greenhouse grown
2	76	Splendor	Stem Cutting	Newfoundland	Greenhouse grown
2	85	H21 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	88	H22 (hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	51	H23 (hybrid Splendor x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	53	Sussi	Stem Cutting	Newfoundland	Greenhouse grown
2	55	H24 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	57	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	58	H25 (hybrid Splendor x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	59	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	62	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	63	Regal	Stem Cutting	Newfoundland	Greenhouse grown
2	64	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	65	H26 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	66	Entredank	Stem Cutting	Newfoundland	Greenhouse grown

2	67	Splendor	Stem Cutting	Newfoundland	Greenhouse grown
2	68	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	69	H27 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	70	Splendor	Unknown	Newfoundland	Greenhouse grown
2	71	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	72	H28 (hybrid Splendor x Erntekrone	Micro-propagated	Newfoundland	Greenhouse grown
2	73	H29 (hybrid DA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	74	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	75	Splendor	Stem Cutting	Newfoundland	Greenhouse grown
2	77	Regal	Stem Cutting	Newfoundland	Greenhouse grown
2	78	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	79	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	80	H30 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	81	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	82	Sussi	Stem Cutting	Newfoundland	Greenhouse grown
2	83	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	84	H31 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	86	H32 (hybrid Splendor x Erntekrone	Micro-propagated	Newfoundland	Greenhouse grown
2	87	H33 (hybrid Splendor x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	89	H34 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	90	Koralle	Stem Cutting	Newfoundland	Greenhouse grown
2	91	H35 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	92	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	93	H36 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	94	H37 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	95	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	96	Erntekrone	Stem Cutting	Newfoundland	Greenhouse grown

2	97	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	98	H38 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	99	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	100	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	101	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	102	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	103	H39 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	104	Ida	Stem Cutting	Newfoundland	Greenhouse grown
2	105	H40 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	106	H41 (Hybrid Splendor X Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	107	E1-3 (a clone from Estonia)	Micro-propagated	Newfoundland	Greenhouse grown
2	108	H42 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	109	Regal	Stem Cutting	Newfoundland	Greenhouse grown
2	110	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
2	111	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
2	112	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
2	113	Splendor	Micro-propagated	Newfoundland	Greenhouse grown
2	114	Regal	Micro-propagated	Newfoundland	Greenhouse grown
2	115	Regal	Stem Cutting	Newfoundland	Greenhouse grown
2	116	H43 (hybrid IDA x Erntekrone)	Micro-propagated	Newfoundland	Greenhouse grown
2	117	Entredank	Micro-propagated	Newfoundland	Greenhouse grown
2	118	Koralle	Stem Cutting	Newfoundland	Greenhouse grown
2	119	Sanna	Stem Cutting	Newfoundland	Greenhouse grown
2	120	Koralle	Stem Cutting	Newfoundland	Greenhouse grown
2	121	Koralle	Stem Cutting	Newfoundland	Greenhouse grown

3.3. Determination of Total anthocyanins

Total anthocyanin content of the lingonberry extracts were determined using the pH differential method using a UV-spectrophotometer for use in a microplate, based on methods by (Isaak et al., 2015; Lee, Durst, & Wrolstad, 2005; Isaak et al., 2015). First samples were diluted in each of potassium chloride (pH 1.0) and sodium acetate (pH 4.5) at pre-determined suitable dilution. Then, the samples were added to a 96 well plate. The absorbance was measured at 520 nm and 700 nm using a SpectraMax M5 microplate reader. Analysis of the data was done by the use of Softmax Pro software (version 6.2). Total anthocyanin content was calculated using a formula.

$$\text{mg cyanidin} - 3 - \text{glucoside equivalents/L} = \left[\frac{\text{A} * \text{MW} * \text{DF} * 1000}{\text{molar extinction constant} * 0.6}\right]$$

Where, A= [(A520−A700) $pH_{1.0}$− (A520−A700) $pH_{4.5}$],

0.6= path length in cm,

MW= 449.2 g/mol for cyanidin-3-glucoside

Molar extinction constant = 26900

DF= Dilution factor

1000=conversion from g to mg

All the samples were run in triplicate and results were expressed as mg cyanidin-3-glucoside equivalents per 100 grams dry weight.

3.4. Determination of antioxidant capacity

Antioxidant capacity of berries was determined by using two different *in vitro* methods; namely, a total phenolics assay and an oxygen radical absorbance capacity (ORAC) assay.

3.4.1. Total phenolic assay

In order to determine total phenolics, Folin-Ciocalteau's method as described by (Ainsworth & Gillespie, 2007; Isaak et al., 2015) was used with slight modifications. 40 µL of 25% Folin-Ciocalteau's solution was added to a 96 well plate followed by 20 µL standard (10-400 mg/L Gallic acid) and sample or blank to the respective wells. The plates were shaken at 500 rpm for 30 seconds followed by 5 minutes without shaking at 37^0C. Then, 140µL of 700 mM sodium carbonate was added to the plate and again the plate was shaken at 500 rpm for 30 seconds at 37^0C. Finally, after addition of the reagent, the plate was incubated at room temperature for 2 hours. Absorbance was measured at 765 nm using SpectraMax M5 microplate reader. Analysis of the data was done by the use of Softmax Pro software (version 6.2). Gallic acid was used as the standard. All samples and standards were run in triplicate and the results were expressed as milli-grams gallic acid equivalent (GAE) per 100 grams dry weight.

3.4.2. ORAC assay

Oxygen radical absorbance capacity (ORAC) was evaluated using Trolox as a standard. It is a water-soluble analogue of vitamin E. The ORAC assay was carried out using the method as described by (Gillespie, Chae, & Ainsworth, 2007; Isaak et al., 2015) with minor modifications. For the ORAC assay, all the reagents were prepared in 75 mM

phosphate buffer (pH 7.4). As fluorescein is sensitive to light, reactions were protected from light. 75 µL of fluorescein was added to the 96 well plate, 25 µL of Trolox standards (5-50 µm) followed by samples or blank to the corresponding wells. In a pre-warmed 37^0C micro-plate shaker, the plate was shaken for 3 minutes at 500 rpm. The plate was allowed to sit on a micro-plate shaker without shaking for 7 minutes followed by addition of 100 µL of AAPH prepared in pre-warmed 37^0C buffer. For the generation of peroxyl radical, AAPH was utilized as the source. Then, fluorescence was recorded for 1 hour every 1.5 minutes using SpectraMax M5 microplate reader. Net area under curve for the sample and standard was determined by subtracting the area under curve (AUC) of the blank. The blank lacks the presence of antioxidants compounds. Final results were attained by comparing net AUC of the standard and the sample. Data analysis was carried out using Softmax Pro software (version 6.2). All determinations were carried out in quadruplicate and results were expressed as micromole of Trolox equivalent (TE) per 100 grams dry weight.

3.5. Cell culture

A H9c2 rat cardiomyoblast cell line was used and obtained from ATCC (Manassas, VA, USA). Cells were cultured in 100 mm plates at 5% humidified CO_2 maintained at 37^0C. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% Antibiotic- Antimycotic. Cells were grown until they reach 70-80% confluency. Cells were pre-incubated with the lingonberry extract for 24 hours at desired concentration before starting ischemia- reperfusion (IR).

3.6. Ischemia reperfusion model *in vitro*

The ischemia reperfusion model used in the experiment is based on (Mockridge, Marber, & Heads, 2000; Isaak et al., 2015) with slight modifications. Ischemia condition was mimicked by placing the cells in ischemia buffer maintained at pH 6.3. Composition of the ischemic buffer is shown in the Table 6. During ischemia, cells were incubated in a Modular Incubator Chamber (Billups-Rothenberg-Delmar CA) maintained at 5% CO_2, 95% nitrogen and 37^0C for 1 hour. Then, for the reperfusion, cells were incubated in DMEM maintained at 5 % CO_2 for 30 minutes. Finally, for the control groups, cells were incubated in DMEM at atmosphere of 5 % CO_2 for a total of 1 hour 30 minutes.

Table 6: Composition of Ischemic Medium

	Concentration
NaCl	137 mM
KCl	15.8 mM
MgCl2: 6H2O	0.49 mM
CaCl2: 2H2O	0.9 mM
HEPES	4 mM
Deoxyglucose	10 mM
Sodium Lactate	20 mM
Sodium-Dithionite	1 mM

3.7. Determination of caspase-3-activity

In order to measure the caspase-3 activity, a Caspase-3-assay Kit (AnaSpec SensoLyte™, Fremont, CA) was utilized. First, H9c2 cardiomyoblast cells were cultured to a density of 4 X 10^5 cells then pre-treated with the lingonberry extracts 24 hours prior to

the assay. Cells were treated with lingonberry extracts (High dose = 1:500 dilution in media, Low dose = 1:1000 in media) for 24 hours prior to ischemia-reperfusion (IR). Cells were washed twice with warm PBS. Media for the control plates were changed with DMEM clear media; whereas, in order to mimic ischemia-reperfusion (IR), cells were incubated in ischemic medium for 1 hour for ischemia. It was then followed by placing cells in clear DMEM/normal conditions to mimic reperfusion for 30 minutes. Then, to all cell culture plates, 1 mL cold PBS was added. The plates were then placed on ice and cells were scraped from the plates. Cells were transferred to microfuge tubes and spun down at 800g for 5 minutes at 4^0C. Supernatant was aspirated and 600 µL lysis buffer added to each microfuge tube. The mixture was then incubated on ice for 30 minutes with gentle agitation. The process was followed by spinning the mixture at 3000 g for 3 minutes. Resulting pellet was discarded and only the supernatant was kept. Each well was plated with the supernatant to which the caspase-3 substrate solution was added. The plate was then incubated at 37^0C for 2 hours. It was then followed by reading fluorescence at Ex/Em =380/500 with a Spectra Max M5 microplate reader. Analysis of data was performed using Softmax Pro software (version 6.2).

3.8. Metabolomics - Untargeted Approach

3.8.1. Selection of samples

Frozen berries were lyophilized, cut into pieces and ground with mortar and pestle as described in the previous section. Due to the limited availability of the samples and the feasibility of the study, the extraction procedure was carried out for the pooled samples only. Five different samples with the most abundance were selected for Northern Manitoba

samples (Sample ID: Dave Buck, FDC, FFLB, LLLB1 and LLLB2). Similarly, samples with higher abundance were selected for Newfoundland samples (Sample ID: 2-37, 2-49, 2-85, 2-91 and 2-93). The pooled mixture comprised 50 mg of the sample from five different samples each for both Northern Manitoba and Newfoundland samples. The pooled samples were then mixed thoroughly by constant vortex and shaking. A total of 50 mg was then taken from the well-mixed pooled sample for extraction for each Northern Manitoba and Newfoundland sample.

3.8.2. Sample preparation- Lingonberry water soluble metabolite extraction:

Extraction of the sample for metabolite profiling was based on the method by (Chang et al., 2012) with the slight modifications. 50 mg of powdered lingonberry from the pooled samples (for both Manitoba and Newfoundland) were placed into 8 mL tubes. To each tube, 2 mL methanol and water combination was added (3:2, volume/volume). Then, 10 µL of internal standard Norvaline was added to the tubes. The mixture was then vortexed for 1 minute. After that, the mixture was sonicated for 1 hour in a Branson 1200 sonicator bath containing deionized water. The water in the sonicator was changed every 15 minutes. The mixture was then vortexed for 10 seconds followed by centrifugation at 14000 rotation per minute (RPM) for 10 minutes. Supernatant obtained after centrifugation was then transferred into 2 mL Eppendorf tubes. The extraction was repeated three times to get the clear supernatant. The pooled supernatant was dried under a gentle flow of nitrogen gas until the extract was visibly dried. Dried extract was then reconstituted in 1 mL water to acetonitrile solution (4:1, volume/volume). It was then vortexed followed by centrifugation for 10 minutes at 14000 X g. The clear supernatant was then transferred into

a clean liquid chromatography (LC) vial. The samples were then analyzed with liquid chromatography-quadrople-time of flight-mass spectrometry (LC-Q-TOF-MS) as described below. Samples were run in both the positive and negative mode.

3.8.3. LC-QTOF-MS Conditions and Analysis:

The non-targeted metabolomics was done after (Mayengbam, House, & Aliani, 2015). Briefly, samples were analyzed using Agilent 6538 UHD quadruple time of flight (q- TOF) liquid chromatography mass spectrometry (LC-MS) system consisting of 1290 infinity Agilent high performance liquid chromatography (HPLC). Metabolites were separated using a 3 X 50 mm, 2.7-Micron Agilent Poroshell column maintained at 60^0C. (Phase A) comprised of 0.1 % formic acid and water and 0.1 % formic acid and acetonitrile (phase B). The injection volume of the sample was 2 µL at a flow rate of 0.7 mL/ min. The LC- qTOF was equipped with an electrospray source and run both positive ESI (+) and negative ESI (-) ionization mode. Maintenance of auto-sampler was carried out at 50^0C. The following settings were applied during MS analysis: Nebulizer pressure 50 psig; drying nitrogen flow rate at 11 L/min; gas temperature maintained at 300^0C; fragmentor voltage of 175V; followed by shimmer voltage of 50V and OCTRF Vpp voltage of 750 V.

CHAPTER IV: RESULTS

4.1. Basis for selection of samples

A total of 159 lingonberry samples were collected from Newfoundland and Northern Manitoba. Samples obtained were not uniform in quantity. Since, all of the assays needed to be carried out in triplicates, not all samples were large enough for every assay to be performed. First, 144 of the samples were subjected to anthocyanins assay and based on the results obtained and availability of the sample to carry out further assays in triplicate, selection of samples were done. As indicated in the flowchart shown in Fig.7, the phenolics assay was carried out after the anthocyanin assay. A total of 40 lingonberry samples were selected from the Newfoundland group and 5 lingonberry samples from the Northern Manitoba group for phenolics and ORAC assay. Then, again based on the results from phenolics and ORAC assay, only 5 lingonberry samples were selected based on the highest availability of the samples to carry out other assays in triplicates. Finally, for the untargeted metabolomics study, 5 lingonberry samples from Northern Manitoba were pooled. Similarly, 5 randomly selected samples from Newfoundland based on the availability were pooled. The basis for the selection of samples for different assays is as shown in Fig. 7.

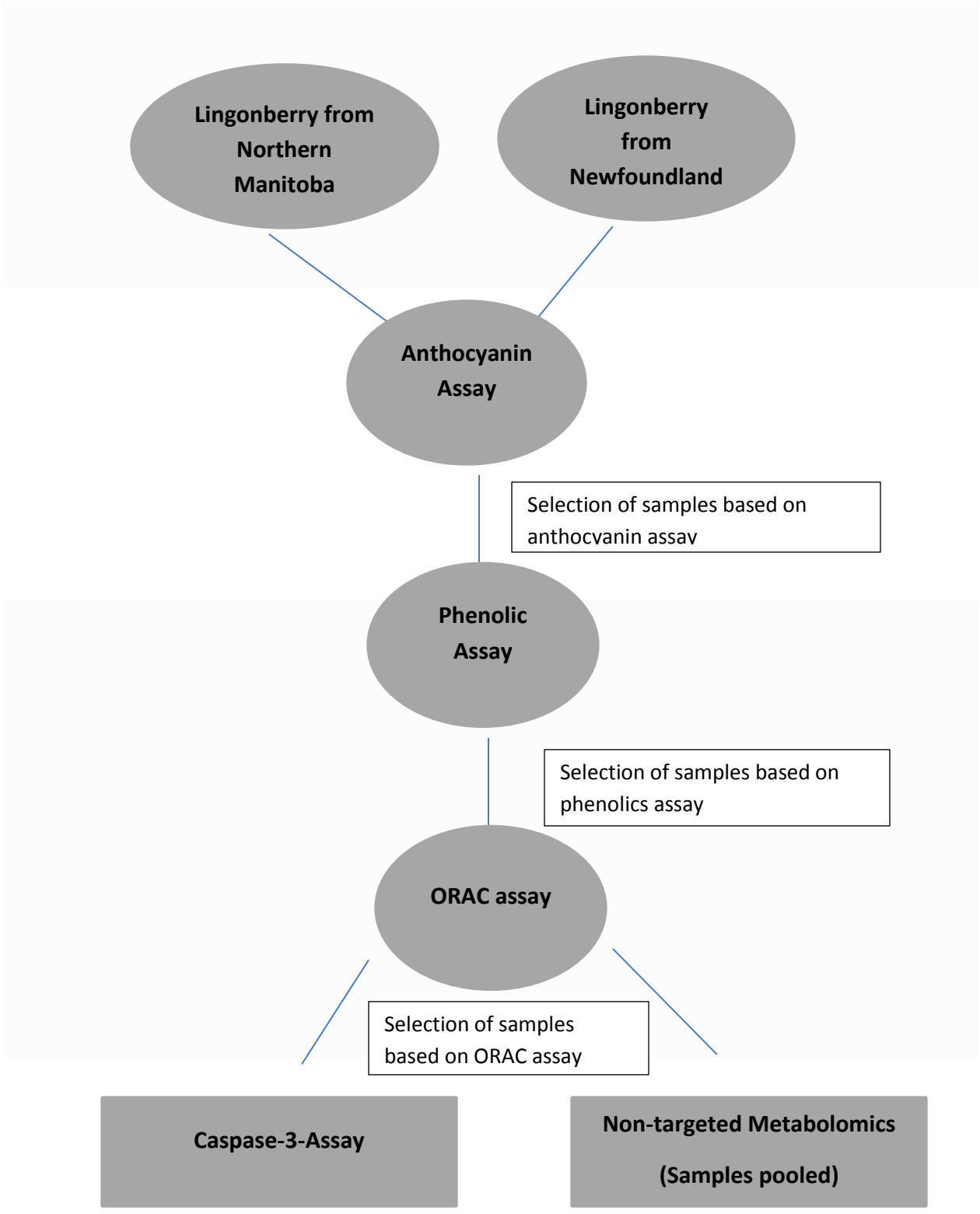
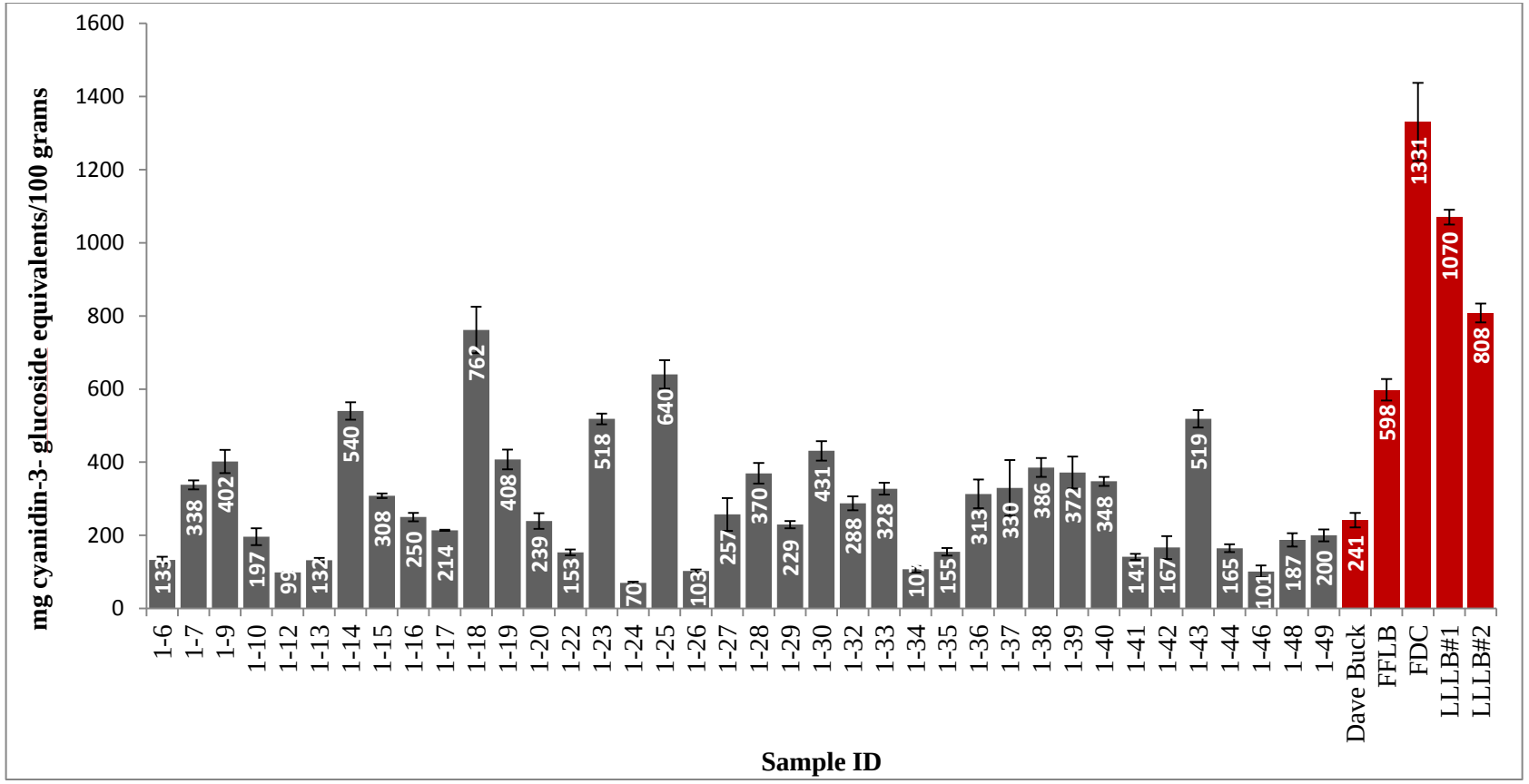


Fig. 7: Flowchart showing the basis of selection of sample

4.2. Total anthocyanins content

Total anthocyanins in lingonberry extracts were evaluated by the use of the pH differential method. A wide range of variation was observed within the groups and between the berries from two different locations. The average total anthocyanins value for Manitoba berries was 810 ± 40 mg cyanidin-3-glucoside equivalents per 100 grams. There was a huge variation within the group where values ranged from 241-1331 mg/100g (Fig. 8). Likewise, there was a huge variation within the Newfoundland berries as well. The average total anthocyanins value for Newfoundland berries were 376 ±18 mg cyanidin-3-glucoside equivalents per 100 grams. Values ranged from as high as 855 mg per 100 g and as low as 70 mg per 100g (Fig. 8). When comparing the means of total anthocyanins between the groups, Manitoba berries were found to have significantly higher content of anthocyanins in comparisons to Newfoundland berries (Fig. 9). There was > 1 -fold higher total anthocyanin content of Manitoba berries in comparison to ones from Newfoundland.


mg cyanidin-3- glucoside equivalents/100 grams
1600
1400
1200
1000
800
600
400
200
0
133
338
402
197
99
132
540
308
250
214
762
408
239
153
518
70
640
103
257
370
229
431
288
328
107
155
313
330
386
372
348
141
167
519
165
101
187
200
241
598
1331
1070
808
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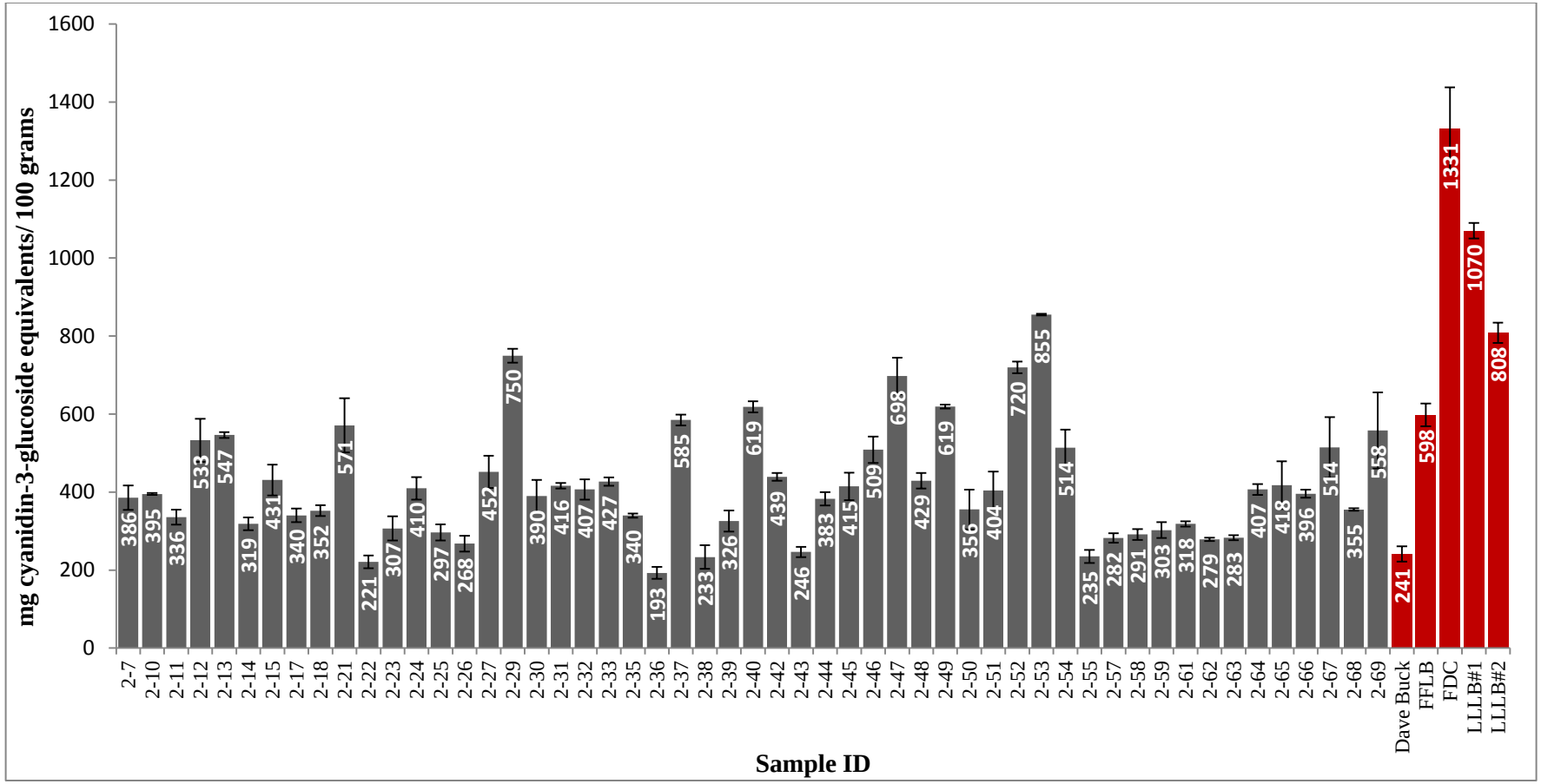
1-36
1-37
1-38
1-39
1-40
1-41
1-42
1-43
1-44
1-46
1-48
1-49
Dave Buck
FFLB
FDC
LLLB#1
LLLB#2
Sample ID


mg cyanidin-3-glucoside equivalents/ 100 grams
1600
1400
1200
1000
800
600
400
200
0
386
395
336
533
547
319
431
340
352
571
221
307
410
297
268
452
750
390
416
407
427
340
193
585
233
326
619
439
246
383
415
509
698
429
619
356
404
720
855
514
235
282
291
303
318
279
283
407
418
396
514
355
558
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1070
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Dave Buck
FFLB
FDC
LLLB#1
LLLB#2
Sample ID

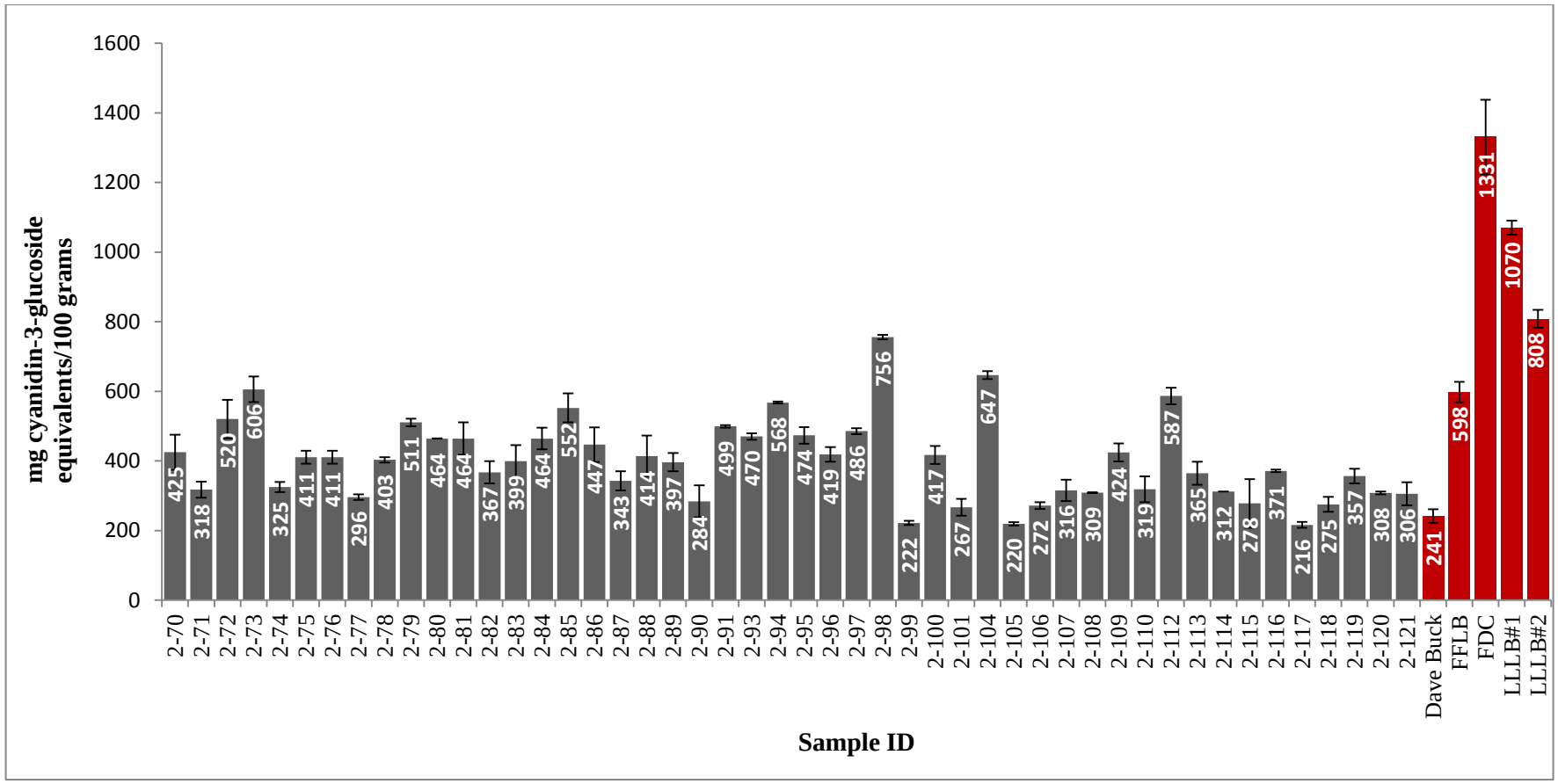

Fig. 8: Total anthocyanin content in Northern Manitoba and Newfoundland berries. Data are expressed as mean ± SD from triplicate assays.

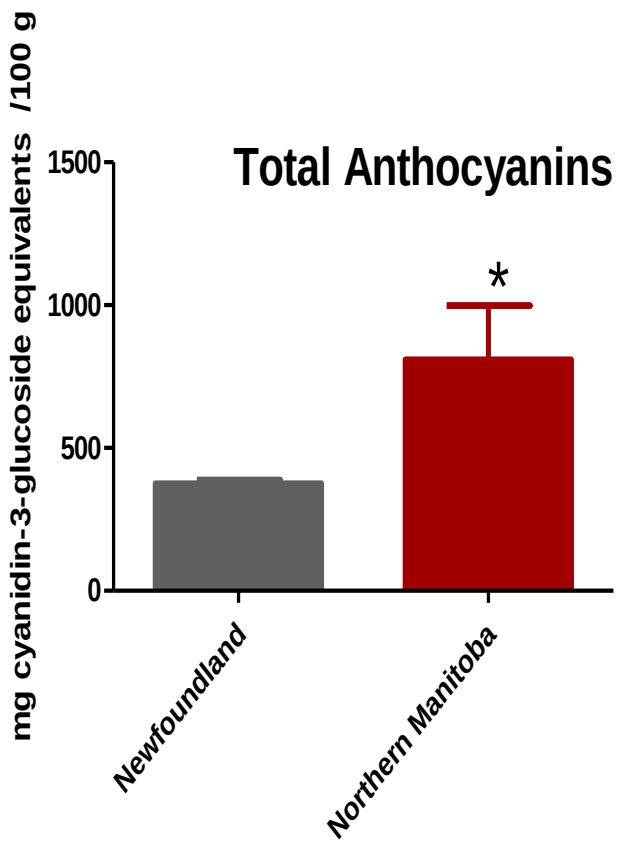

Fig. 9: Northern Manitoba berries have significantly higher anthocyanins content as compared to Newfoundland berries. Statistical analysis was performed using unpaired *t* test N=139 (Newfoundland), N= 5 (Northern Manitoba); **P*<0.0001. Data are expresses as mean± SD.

The parent genotype and hybrid genotype of greenhouse grown lingonberry from Newfoundland were compared by determining total anthocyanin content. Results from the study shows that the anthocyanin content for the hybrids falls between the ranges of values from the parent genotypes (Fig.10). For instance, genotype IDA had total anthocyanin content of 582±24 mg cyanidin-3-glucoside equivalents/100 grams dry weight. Entrekrone genotype had total anthocyanin content of 326 ± 26 mg cyanidin-3-glucoside equivalents/100 grams dry weight. Hybrid of the two genotypes IDA and Entrekrone had total anthocyanin content of 474±28 mg mg cyanidin-3-glucoside equivalents/100 grams dry weight which was between the ranges of parent genotypes.

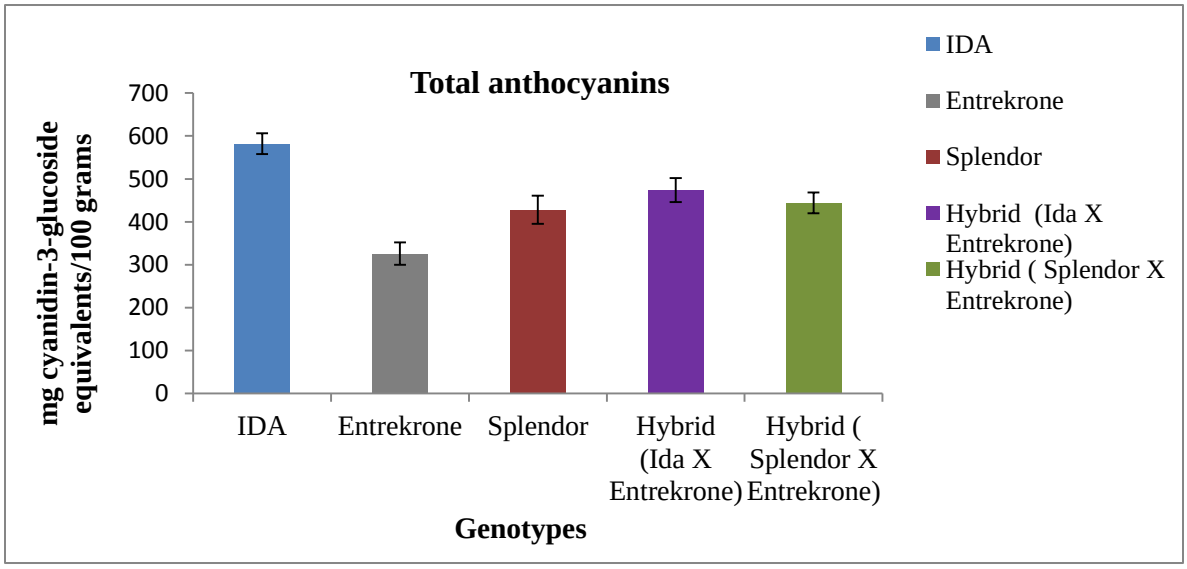

Fig. 10: Total anthocyanin content of different genotypes and its hybrid. Anthocyanin content is expresses as mg cyanidin-3-equivalents per 100 grams dry weight. Data are expressed as mean ± SD.

Results from our study shows that micro-propagation and stem cutting had no effect on the total anthocyanin values as measured by the pH differential method. There was no statistical significance in total anthocyanins content between the lingonberry fruit extract obtained by two different propagation methods; stem cutting and micro-propagation (Fig. 11).

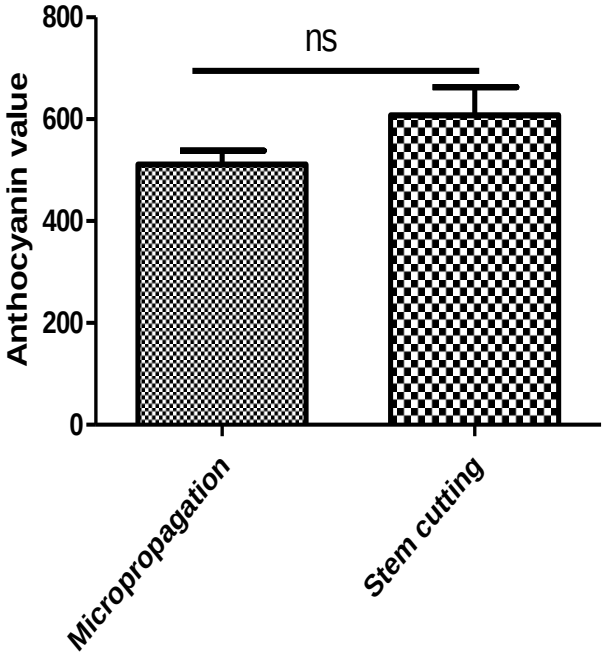

Fig. 11: Effect of propagation type (micro-propagation and stem cutting) on total anthocyanin values as measured by pH differential method. Statistical analysis was done using unpaired t-test. ns = not significant. Data are expressed as mean ± SD.

4.3. Total phenolic content

A simple and widely used method, Folin-Ciocalteau's method was utilized to evaluate the total phenolic content in the berries. As shown in Table 6, total phenolic contents ranged from 1567 - 4794 mg GAE/100g for Newfoundland berries. Total phenolic content ranged from 4067- 5189 mg GAE/100 g for Manitoba berries. The average total phenolic content for Manitoba berries was 4526 ± 80 mg GAE per 100 grams dry weight. In contrast, average total phenolic contents for Newfoundland berries were 2811 ± 51 mg GAE per 100 grams dry weight. Fig. 12 shows the wide range of phenolic content of the Newfoundland berries in contrast to that of the Manitoba berries. While comparing the means of total phenolics content from Manitoba with that of the Newfoundland ones, Manitoba berries were found to have significantly higher content (close to one fold increase) of total phenolics (Fig. 13).

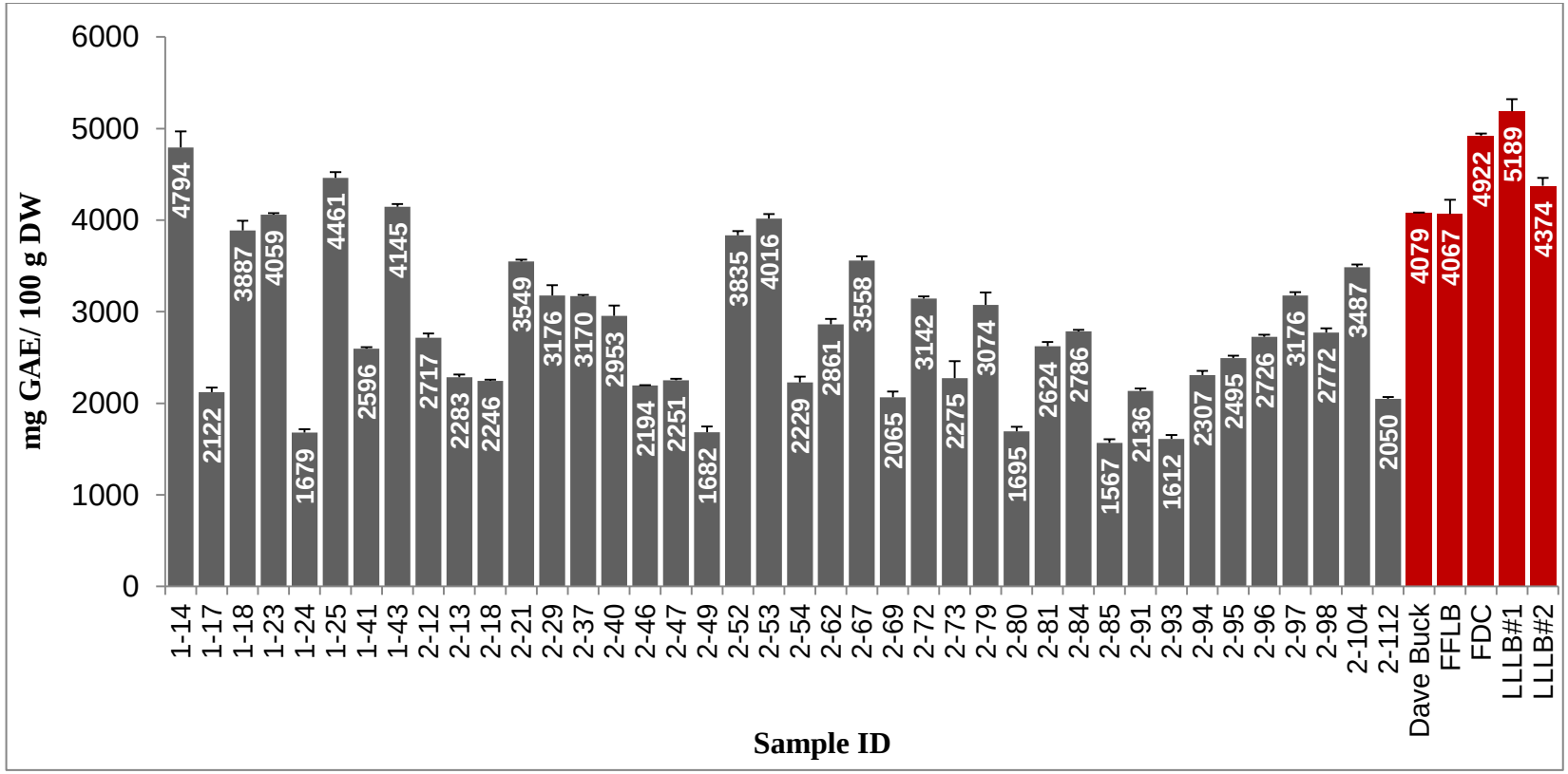

Fig. 12: Total Phenolic content. Results expressed in terms of GAE= Gallic Acid Equivalents. Data are expressed as mean ± SD from triplicate assays.

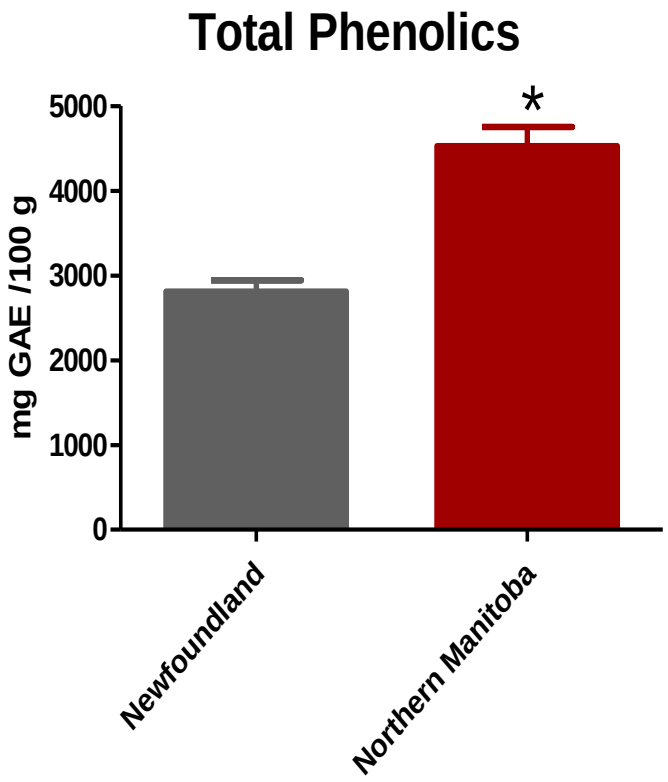

Fig. 13: Northern Manitoba berries have significantly higher total phenolic content as compared to Newfoundland berries. Statistical analysis was done using unpaired t test N=40 (Newfoundland), N= 5 (Northern Manitoba); *P<0.0001. Data are expressed as mean ± SD.

In our study, data shows that micro-propagation and stem cutting had no effect on the total phenolic content as measured by Folin-Ciocalteau's method. There was no statistical significance in total phenolic values between the lingonberry fruit extract as obtained by two different propagation methods; stem cutting and micro-propagation (Fig. 14).

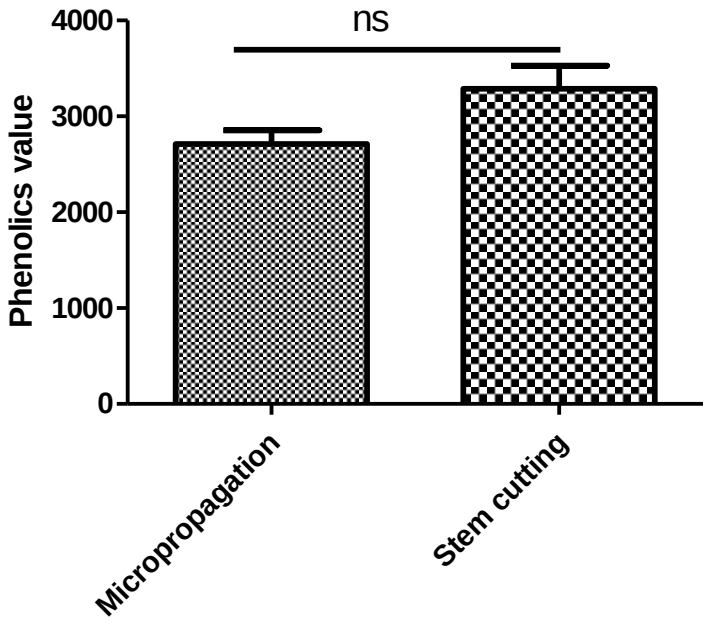

Fig. 14: Effect of propagation type (micro-propagation and stem cutting) on total phenolic content as measured by Folin-Ciocalteau's method. Statistical analysis was done using unpaired t-test ns = not significant. Data are expressed as mean ± SD.

4.4. Antioxidant activity

The antioxidant capacity as measured by ORAC differed between the Manitoba and Newfoundland berries. As listed in Table 8, ORAC values ranged from as low as 106,482 µmol TE/100 g and as high as 127,108 µmol TE/100g in Manitoba berries. Similarly, variability in ORAC values was observed in berries from Newfoundland as well. The lowest ORAC values for the berries from Newfoundland was 27,524 µmol TE/100g and the highest ORAC value was 134,460 µmol TE/100g. The average of ORAC values for Manitoba berries were 119,666 ± 3,428 µmol TE/ 100g dry weight; whereas, the average ORAC values for Newfoundland berries were 61,267 ± 1,680 µmol TE/100g dry weight. While comparing the average of ORAC values between the two groups, Manitoba berries had significantly higher ORAC values (> 1.5 fold increase) as compared to those of Newfoundland berries. ORAC values differed markedly between the groups (Fig. 15 and Fig. 16).

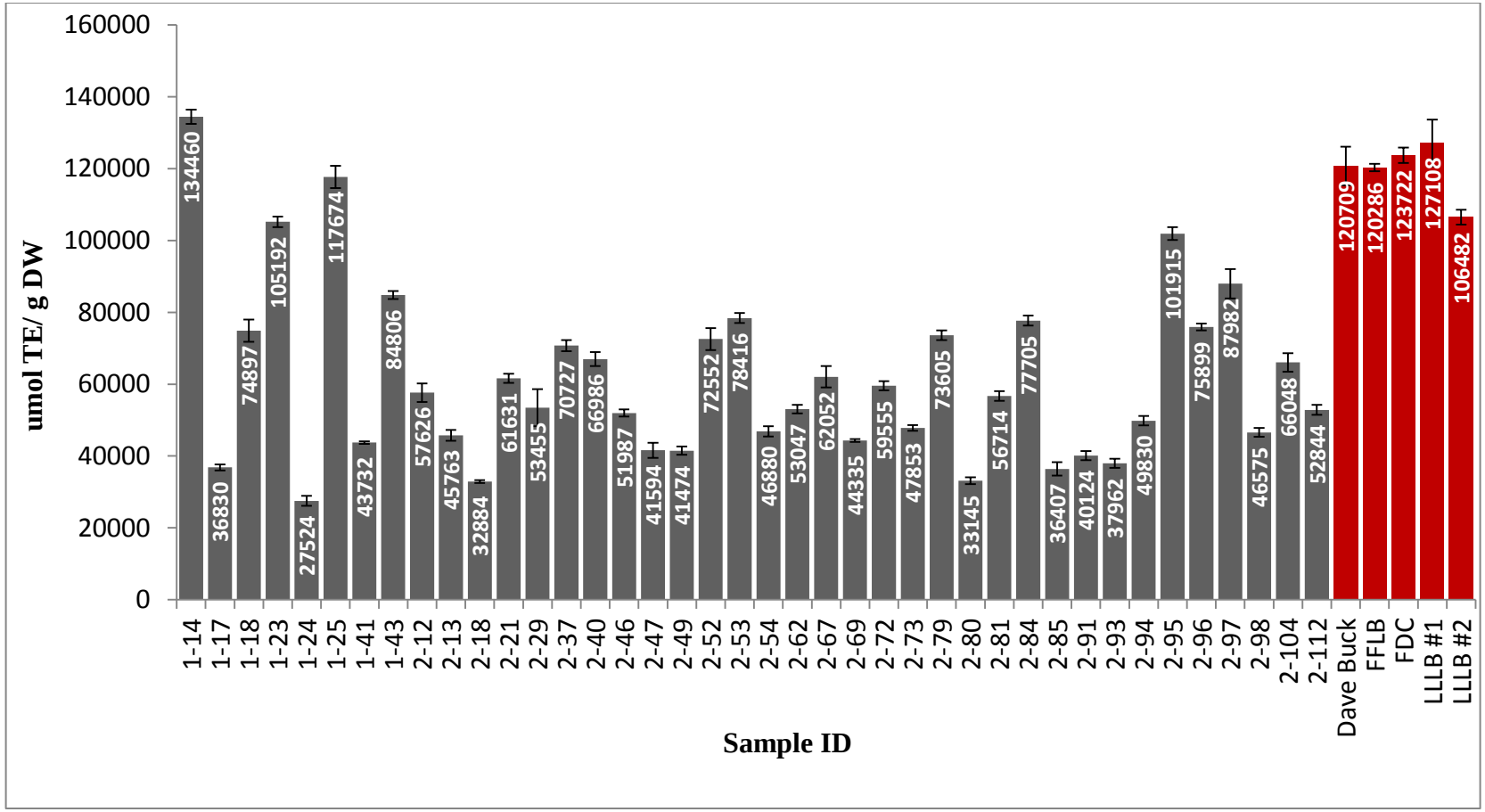

Fig. 15: ORAC values for Manitoba and Newfoundland berries and are expressed in TE= Trolox Equivalents. Data are expressed as mean ± SD from quadruplicate assays

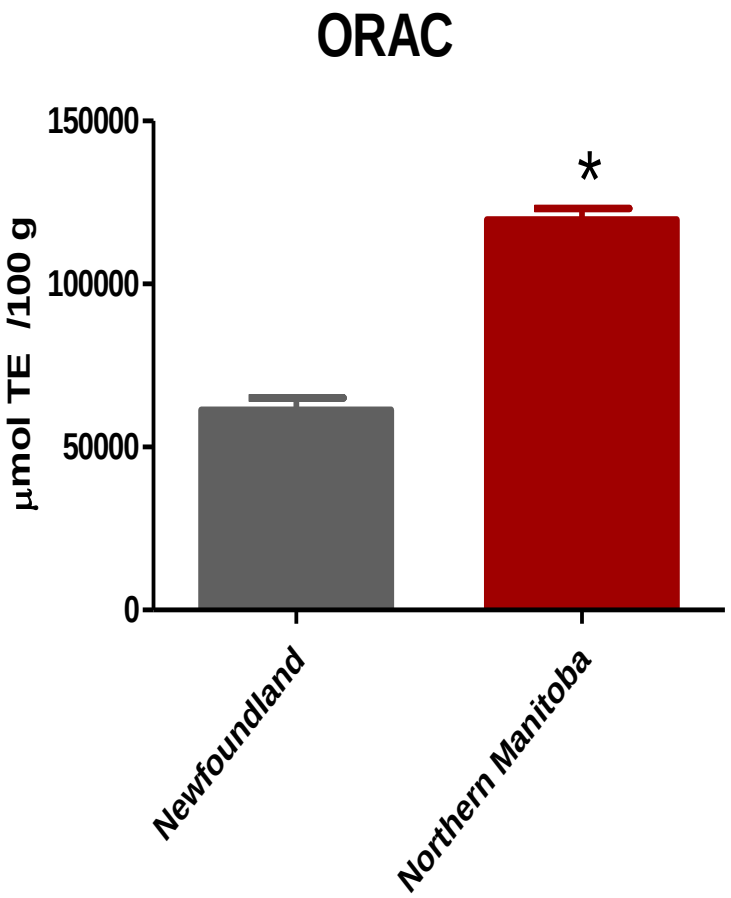

Fig. 16: Northern Manitoba berries have significantly higher ORAC value as compared to Newfoundland berries. Statistical analysis was done using unpaired t test N=40 (Newfoundland), N= 5 (Northern Manitoba); *P<0.0001. Data are expressed as mean ± SD.

Results from our study shows that micro-propagation and stem cutting had no effect on the antioxidant capacity as measured by ORAC. There was no statistical significance in ORAC values between the lingonberry fruit extract as obtained by two different propagation methods; stem cutting and micro-propagation (Fig. 17).

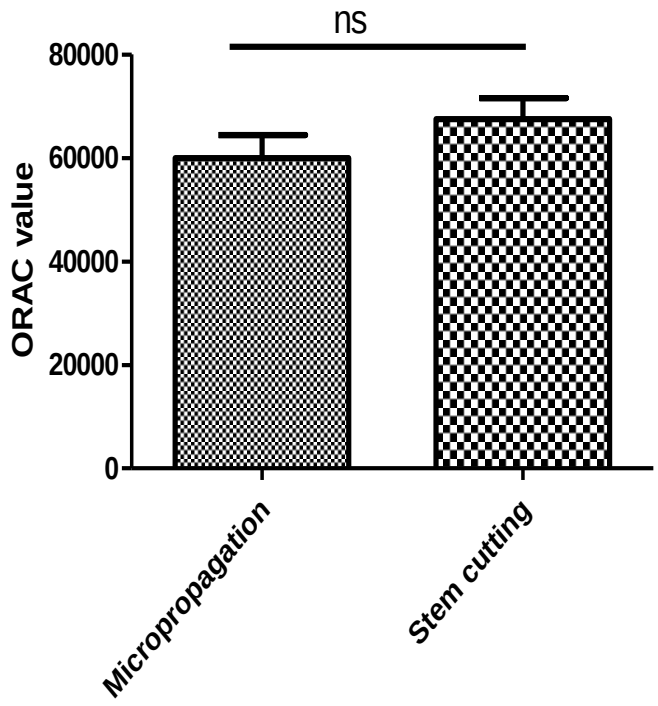

Fig. 17: Effect of propagation type (micro-propagation and stem cutting) on antioxidant capacity as measured by ORAC. Statistical analysis was done using unpaired t-test. ns = not significant Data are expressed as mean ± SD.

.

Table 7: Total Anthocyanin Content (ACY), Total phenolics (TP), Antioxidant Capacity (ORAC) and Total Anthocyanins/ Phenolics in lingonberry from Manitoba and Newfoundland

Sample ID	ACY (mg/100g)	TP (mg/100g)	ORAC (µmolTE/100g)	ACY/TP
1-14	540 ± 24	4794 ± 17	134460 ± 2001	0.11
1-17	214 ± 20	2122 ± 50	36830 ± 8150	0.10
1-18	762 ± 64	3887 ± 10	74897 ± 3063	0.19
1-23	518 ± 15	4059 ± 19	105192 ± 1459	0.12
1-24	70 ± 30	1679 ± 36	27524 ± 1364	0.04
1-25	640 ± 39	4461 ± 64	117674 ±3092	0.14
1-41	141 ± 80	2596 ± 15	43732 ± 3260	0.05
1-43	519 ± 24	4145 ± 32	84806 ± 1124	0.12
2-12	533 ± 55	2717 ± 45	57626 ± 2607	0.19
2-13	547 ± 70	2283 ± 32	45763 ± 1478	0.23
2-18	352 ± 14	2246 ± 12	32884 ± 4070	0.15
2-21	571 ± 70	3549 ± 19	61631 ± 1292	0.16
2-29	750 ± 18	3176 ± 11	53455 ± 5187	0.23
2-37	585 ± 14	3170 ± 12	70727 ± 1556	0.18
2-40	619 ± 14	2953 ± 11	66986 ± 1933	0.20
2-46	509 ± 34	2194 ± 50	51987 ± 9890	0.23
2-47	698 ± 47	2251 ± 16	41594 ± 2120	0.30
2-49	619 ± 50	1682 ± 64	41474 ± 1151	0.36
2-52	720 ± 15	3835 ± 45	72552 ± 3030	0.18
2-53	855 ± 20	4016 ± 50	78416 ± 1364	0.21
2-54	514 ± 47	2229 ± 63	46880 ± 1418	0.23
2-62	279 ± 40	2861 ± 62	53047 ± 1180	0.09
2-67	514 ± 78	3558 ± 48	62052 ± 2997	0.14
2-69	558 ± 98	2065 ± 63	44335 ± 3660	0.29
2-72	520 ± 56	3142 ± 24	59555 ± 1255	0.16
2-73	606 ± 37	2275 ± 18	47853 ± 7970	0.26
2-79	511 ± 11	3074 ± 13	73605 ± 1333	0.16
2-80	464 ± 0	1695 ± 48	33145 ± 9270	0.27
2-81	464 ± 47	2624 ± 46	56714 ± 1349	0.17
2-84	464 ± 31	2786 ± 15	77705 ± 1397	0.16
2-85	552 ± 42	1567 ± 42	36407 ± 1881	0.35
2-91	499 ± 30	2136 ± 25	40124 ± 1262	0.23
2-93	470 ± 90	1612 ± 43	37962 ± 1268	0.29
2-94	568 ± 30	2307 ± 46	49830 ± 1327	0.24
2-95	474 ± 24	2495 ± 26	101915 ± 1812	0.18
2-96	419 ± 21	2726 ± 22	75899 ± 9570	0.15
2-97	486 ± 90	3176 ± 38	87982 ± 4095	0.15
2-98	756 ± 70	2772 ± 47	46575 ± 1254	0.27
2-104	647 ± 12	3487 ± 29	66048 ± 2572	0.18
2-112	587 ± 24	2050 ± 20	52844 ± 1399	0.28
Dave Buck	241 ± 20	4079 ± 40	120709 ± 5354	0.05
FFLB	598 ± 29	4067 ± 15	120286 ± 1035	0.14
FDC	1331 ± 10	4922 ± 23	123722 ± 2170	0.27
LLLB 1	1070 ± 20	5189 ± 13	127108 ± 6518	0.20
LLLB 2	808 ± 26	4374 ± 86	106482 ± 2063	0.18

Values expressed as Mean± SD where n= 3 for total anthocyanins, n = 3 for total phenolics and n = 4 for ORAC assay

Table 7 above lists the values for total anthocyanins, phenolics, ORAC values and the ratio of anthocyanins to the phenolics. Even though the values for anthocyanins, phenolic content and ORAC values were significantly higher for Northern Manitoba lingonberry in comparisons to the ones in the Newfoundland, the ratio of anthocyanins to phenolics is comparable between the groups.

Table 8: Total anthocyanins (ACY), Total phenolics (TPH) and Antioxidant capacity (ORAC) for pooled samples

	ACY (mg/100 g)	**TPH (mg/100 g)**	**ORAC (µmol TE/100 g)**
Northern Manitoba	585 ± 12	3695 ± 18	69874 ± 2311
Newfoundland	450 ± 17	1985 ±78	59555 ± 1255

Values expressed as Mean± SD where n= 3 for total anthocyanins, n=3 for total phenolics and n=4 for ORAC assay

Table 8 shows the values for total anthocyanin content, total phenolic content and the ORAC values for the pooled samples. As mentioned in the earlier section 3.8.1, 5 lingonberry samples from Newfoundland and 5 lingonberry form Northern Manitoba were pooled. The pooled sample is the same samples that were subjected to the

metabolomics study. While comparing the anthocyanin content of pooled samples from Northern Manitoba to Newfoundland, the Manitoba sample had one-fold greater values to that of Newfoundland. Similarly, phenolic content for Northern Manitoba samples were also higher (> 1.5 fold) than Newfoundland. Likewise, ORAC values for Northern Manitoba lingonberries were higher (> 1 fold) than Newfoundland for the pooled samples as well.

It has been reported in the literature (Wu, Gu, Prior, & McKay, 2004) that there exists a positive correlation between the total phenolics and antioxidant capacity. Our study also shows a good correlation between phenolics and an antioxidant capacity (Fig. 18) and supports earlier observations.

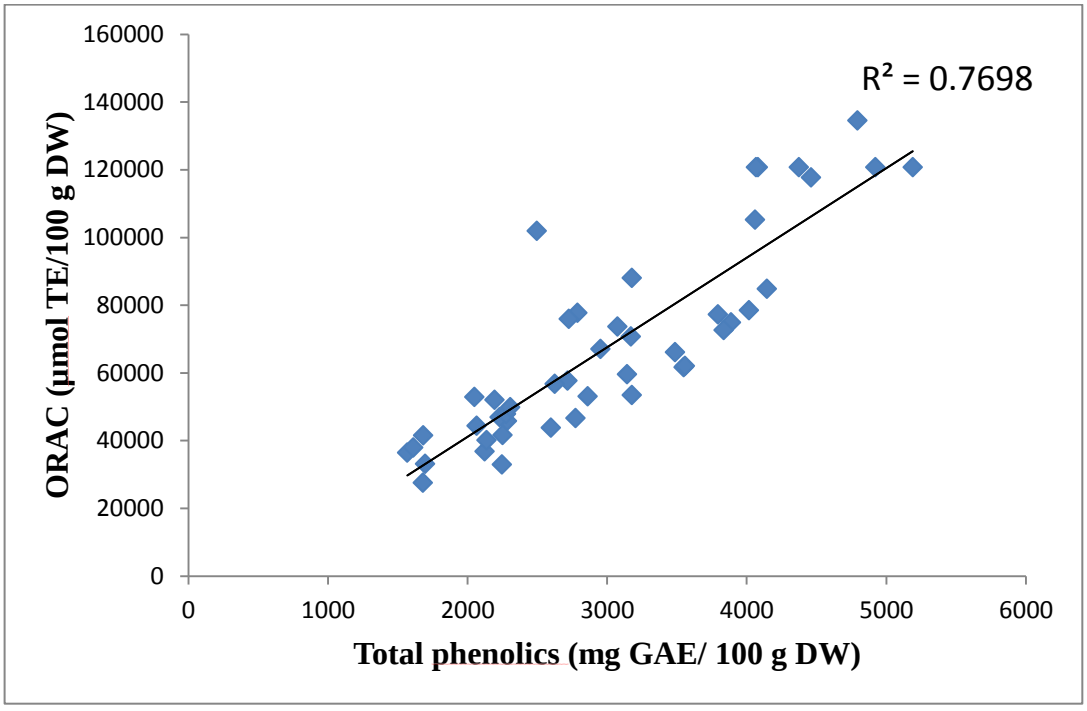

Fig. 18: Correlation plot between total phenolics and antioxidant capacity as measured by ORAC

4.5. Measurement of caspase -3- activity

One of the characteristics features of apoptosis is caspase-3-activation. Caspase-3-activation was measured with and without lingonberry extracts treatment in our *in vitro* system as described in method section (3.7). Caspase-3-activity was found to be remarkably increased in the IR groups which were not treated with lingonberry extract. Lingonberry extract was administered in two different dilutions; that is, at 1:500 and 1:1000 dilutions. At a dilution of 1:1000, most of the lingonberry extract from both Manitoba and Newfoundland were able to inhibit caspase-3-activation and seem to provide a protective effect against IR in rat cardiomyocyte (H9c2). Samples like FFLB, 2-37, 2-93 and FDC were able to significantly decrease the levels of caspase-3-activity (Fig. 19). Sample LLLB 2 had a similar effect to that of the IR with no inhibition of caspase-3-activity.

Similarly, when IR cells were treated with lingonberry at 1: 500 dilutions, samples such as 2-37, 2-49, 2-85, 2-91, 2-93 (Newfoundland), D Buck and FFLB (Northern Manitoba) showed a significant decrease in the level of caspase-3- activity (Fig. 20). Sample 2-37 was statistically significant among the different samples. This result indicates a protective effect of lingonberry against IR in cardiomyocytes.

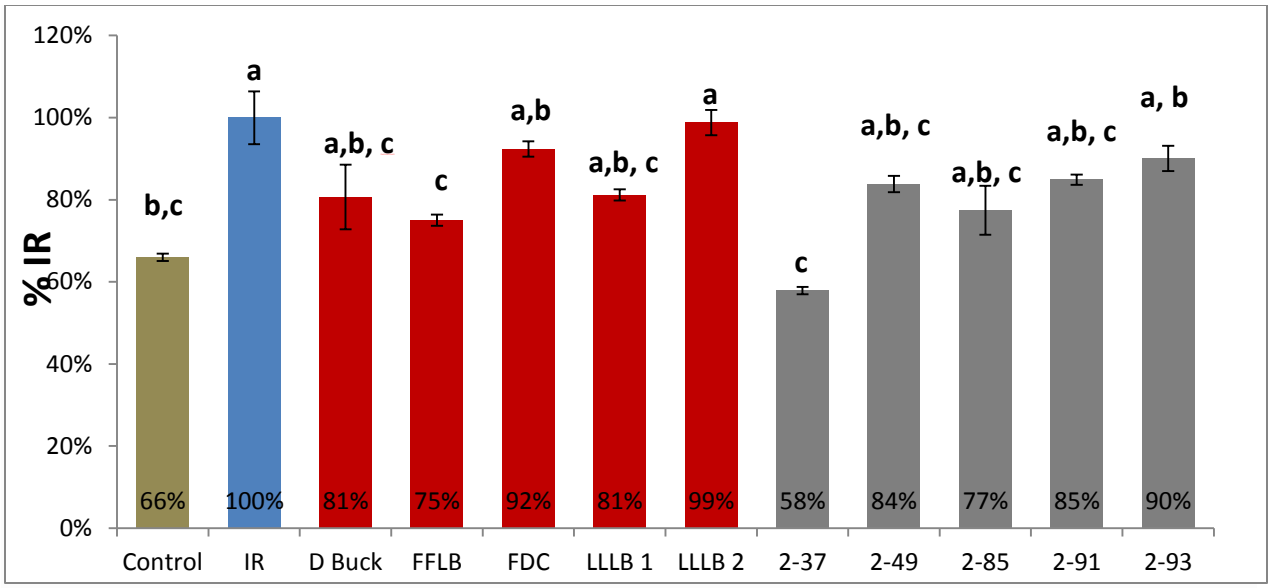

Fig. 19: Effects of lingonberry extract on ischemia/reperfusion-induced caspase-3 activation at low dose (1:1000 dilutions in cell culture media). Groups specified by same letters are not significantly different at $P < 0.05$. Enzyme activity was measured by fluorescence assay. Results are expressed as the mean ± SEM (n=3). Statistical analysis was done using one way - ANOVA.

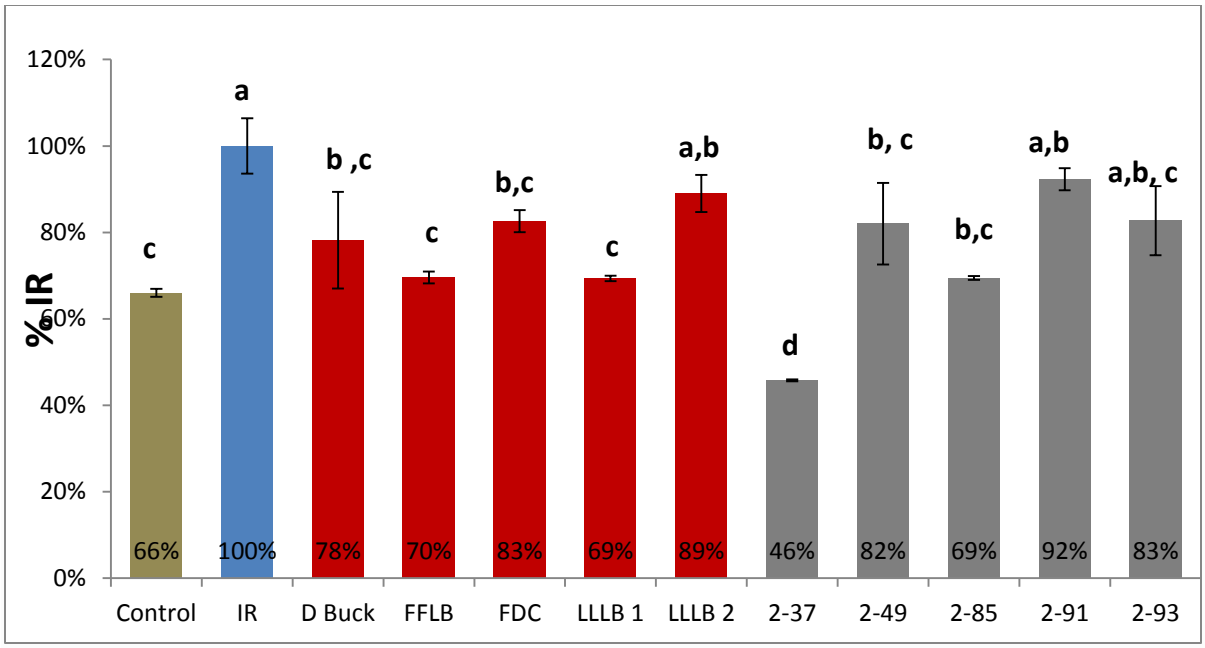

Fig. 20: Effects of lingonberry extract on ischemia/reperfusion-induced caspase-3 activation at medium dose (1:500 dilutions in cell culture media). Groups specified by same letters are not significantly different at $P < 0.05$. Enzyme activity was measured by fluorescence assay. Results are expressed as the mean ± SEM (n=3). Statistical analysis was done using one way - ANOVA.

4.6. Caspase-3-activity for the pooled samples

Caspase-3- activity as measured by fluorescence assay for Northern Manitoba and Newfoundland lingonberry extract were pooled. The caspase activity obtained for 5 Manitoba samples were pooled and individual caspase activity measured for 5 Newfoundland lingonberry extracts on simulated ischemia reperfusion on a H9c2 cell line were pooled. Then, pooled results were compared by t-test. There was no statistical significance between the Northern Manitoba and Newfoundland in terms of caspase activity measured at two dilutions (1:1000 and 1:500) (Fig. 21).

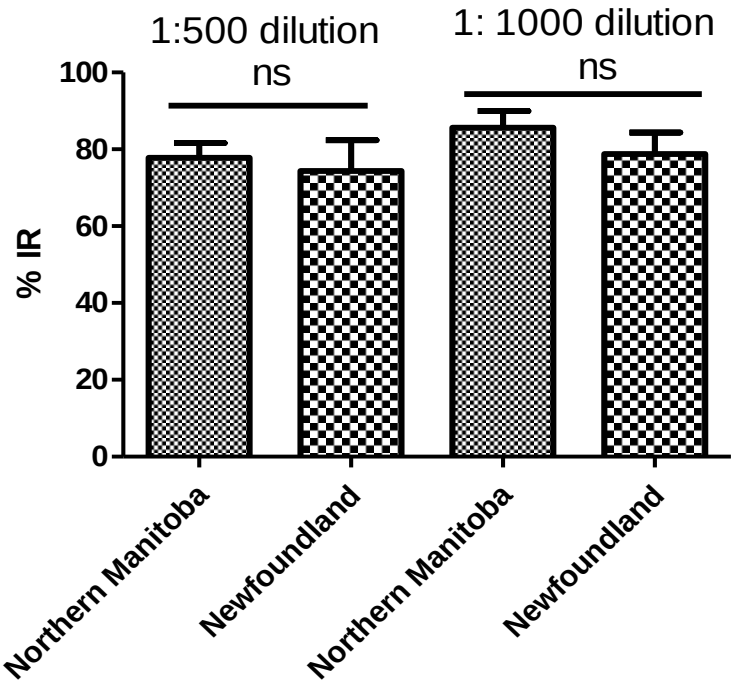

Fig. 21: Effect of lingonberry extract on ischemia/reperfusion-induced caspase-3 activation at 1:500 and 1:1000 dilutions. Statistical analysis was done using t-test. ns = not significant Data are expressed as mean ± SD.

4.7. Metabolomics Results

Lingonberry samples from Northern Manitoba and Newfoundland were run in liquid chromatography quadruple time of flight mass spectrometry (LC-QTOF-MS). A total of 4400 entities were detected (Fig. 23). All the samples were run in triplicate. Samples were separated based on retention time and molecular mass. There was a statistical significance in the number of metabolites present in the berry samples from Manitoba and Newfoundland (Table 9). Between the lingonberries from Manitoba and Newfoundland, a total of 84 compounds in positive mode and a total of 62 compounds in negative mode were significantly different between the groups. When the compounds with the highest abundance from both Manitoba and Newfoundland were searched in the literature; these compounds were shown to have demonstrated bioactivities such as anti-oxidant, anti-inflammatory, anti-apoptotic and anti-viral properties. Some of the examples of the compounds present in lingonberries with higher abundance with reported bioactivities are: zingerone, cinnamtannin $B_{1,}$ theaflavin, chlorogenic acid and epigallocatechin gallate (Table 10 and 11).

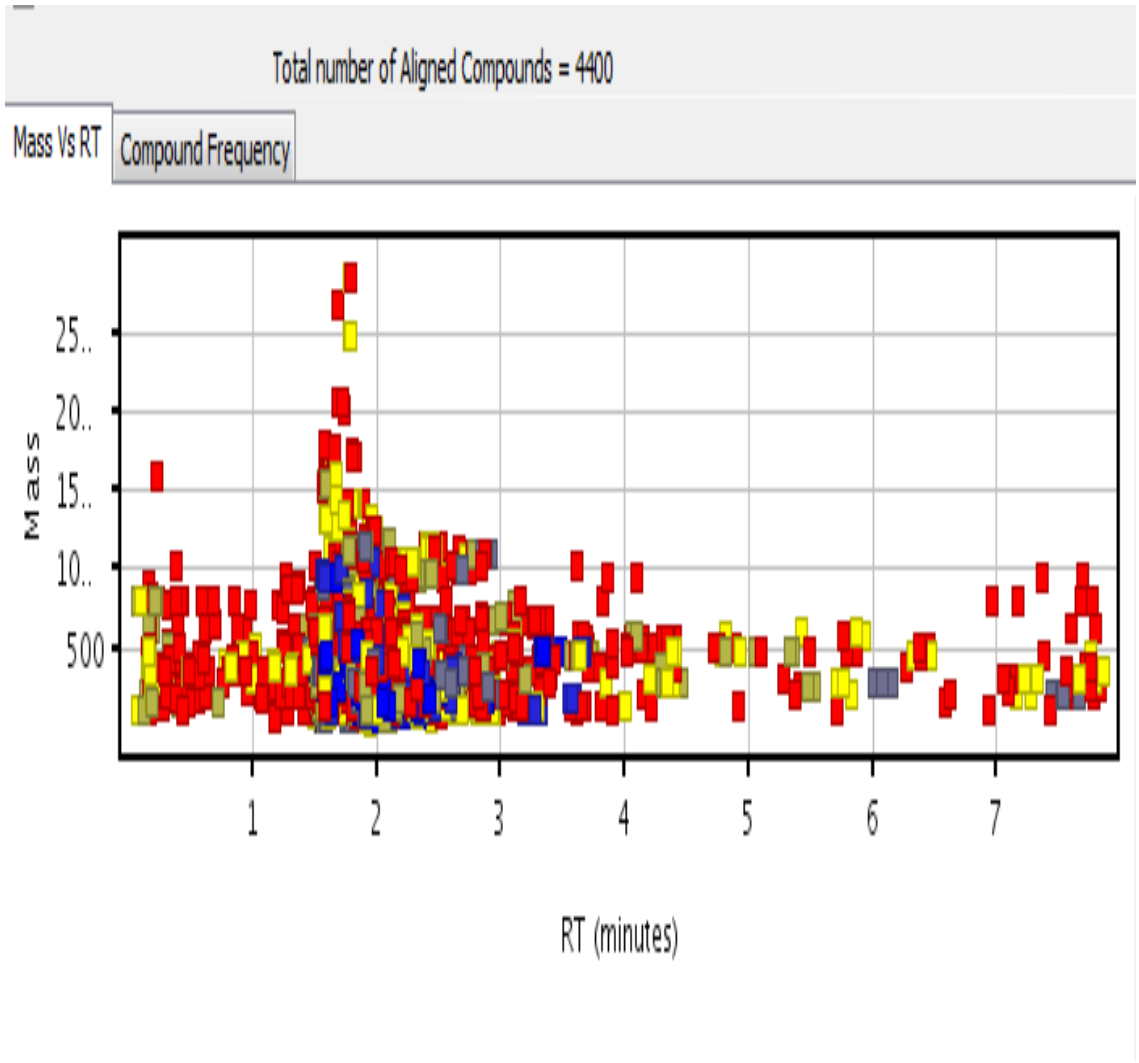

Fig. 22: A total of 4400 compounds were detected when samples were run for LC-qTOF-MS.

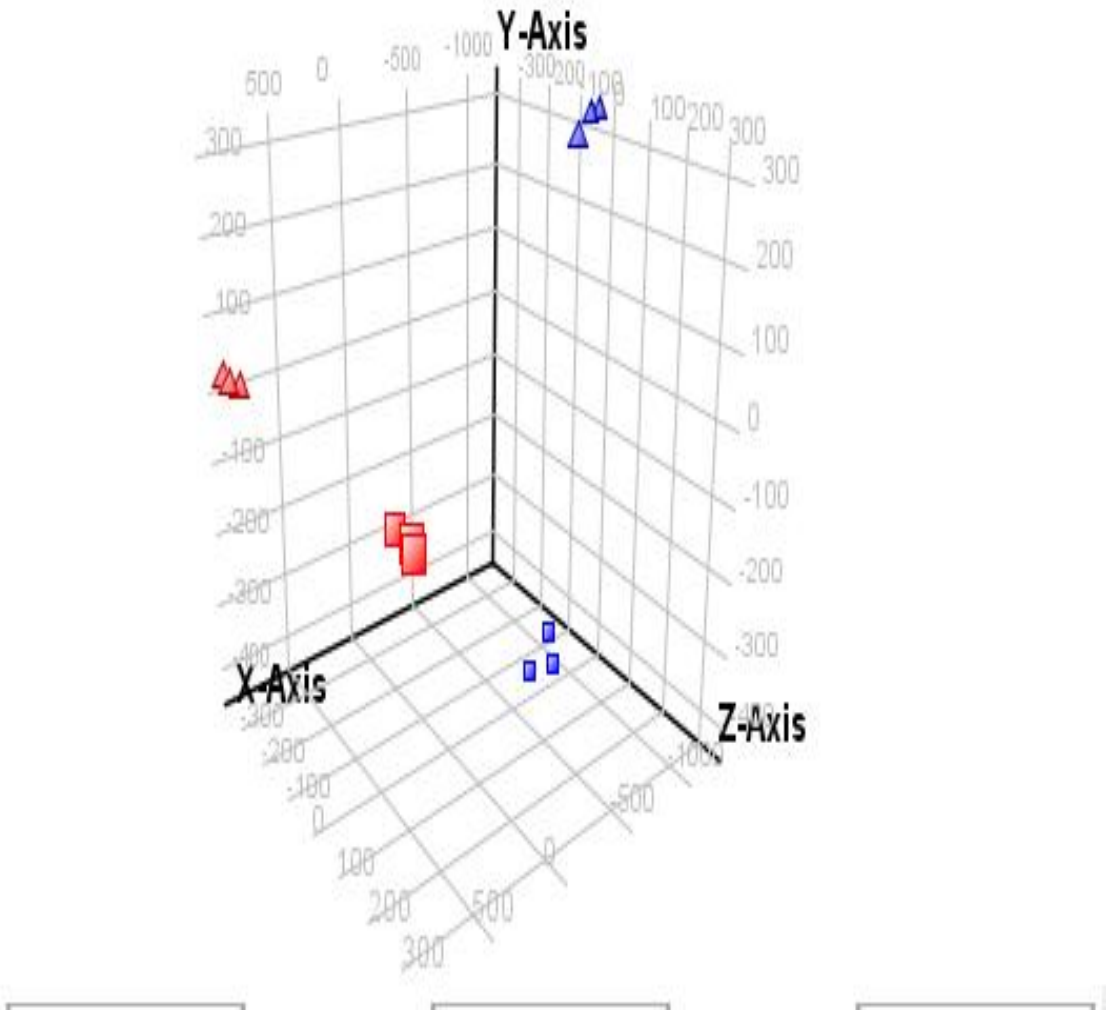

Fig. 23: Principle Component Analysis (PCA) plot obtained from LC-qTOF-MS using both positive and negative electrospray ionization. Berry samples separated into different groups based on regions. Blue and red color represents samples from two different regions. Triangle and square represents samples run in positive and negative mode. Each sample was run in triplicate.

Table 9: A total of 4400 entities were detected in both the samples. In positive mode, 84 entities were statistically significant between the samples from Manitoba and Newfoundland. In negative mode, 62 entities were statistically significant between the groups.

	Positive mode	**Negative mode**
Total entities detected	4400	
Total number of compounds unique to Northern Manitoba	28	35
Total number of compounds unique to Newfoundland	29	22
Total number of compounds common to both regions	27	5
Total	84	62

Table 10: Bioactivity of some tentatively identified compounds unique to Northern Manitoba lingonberry

Tentative compounds	Abundance in terms of Log2 values	Property	References
Cinnamtannin B1	17.86	Anti-oxidant, anti-apoptotic	(Lopez, Jardin, Salido, & Rosado, 2008; Bouaziz et al., 2007)
Quercetin 4'-glucuronide	17.73	Vasodilator, inhibit LDL oxidation	(Janisch, Williamson, Needs, & Plumb, 2004; Perez et al., 2014).
Zingerone	15.23	Anti-oxidant, anti-apoptotic, chemo-preventative effect	(Rao & Rao, 2010; Vinothkumar, Vinothkumar, Sudha, & Nalini, 2014; Rajan, Narayanan, Rabindran, Jayasree, & Manish Kumar, 2013)
Ebselen	14.29	Anti-inflammatory, anti-oxidant, protection against oxidative damage	(Azad & Tomar, 2014; Park et al., 2014; Mukherjee et al., 2014)
Chlorogenic acid	14.2	Anti-oxidant, anti-carcinogenic	(dos Santos, Almeida, Lopes, & de Souza, 2006)

Table 11: Bioactivity of some tentatively identified compounds unique to Newfoundland lingonberry

Tentative compounds	Abundance in terms of Log2 values	Property	References
Epigallocatechin Gallate	17.04	Anti-oxidant	(Fernando & Soysa, 2015)
Bergenin	16.44	Anti-inflammatory role, anti-oxidative property	(Gao et al., 2015; Yun, Lee, Yun, & Oh, 2015)
Theaflavin	14.97	Anti-oxidant, anti-fungal, anti-inflammatory	(Leung et al., 2001; Fatima, Kesharwani, Misra, & Rizvi, 2013)
Tremulacin	14.69	Anti-inflammatory	(Cheng et al., 1994)
Chamazulene	14.34	Anti-inflammatory, anti-oxidative effects	(Safayhi, Sabieraj, Sailer, & Ammon, 1994; Capuzzo, Occhipinti, & Maffei, 2014)

CHAPTER V: DISCUSSION

DISCUSSION

This is the first study where the wild grown Manitoba lingonberries were compared to the greenhouse grown lines from Newfoundland in terms of their antioxidant property and metabolic profile. The findings from the study showed that lingonberry from Manitoba had higher anthocyanins content, phenolics and *in vitro* antioxidant capacity when compared to that green-house grown lingonberry from Newfoundland. Lingonberry extracts from both Manitoba and Newfoundland demonstrated comparable anti-apoptotic effects suggesting similar inhibition of caspase-3 activation on a simulated ischemia-reperfusion (IR) model when tested on a H9c2 rat cardiac myoblast cell line. In addition to this, results from the untargeted metabolomics approach suggest that the berries grown in two different conditions; i.e, wild and greenhouse grown are different in terms of metabolites.

Results from our study suggest that there is a trend where total anthocyanin content, phenolics and ORAC values were higher in lingonberries obtained by stem cutting compared to micro-propagation (Fig. 11, Fig. 14 and Fig. 17); however, the data were not statistically significant. Thus, our study suggests that the propagation type used does not affect the anthocyanins, phenolics and antioxidant capacity. In contrast, a study by (Goyali, Igamberdiev, & Debnath, 2013) reported that in lowbush blueberry, antioxidant capacity, phenolics and morphology were affected by the method of the propagation type used. Tissue culture plants had higher number of stems and branches per plant compared to stem cutting. In addition, when leaves of blueberry plants were compared in regards to propagation type, stem cutting blueberry plant resulted in higher levels of total phenolic content, total flavonoid content and antioxidant activity compared to ones obtained by

tissue culture. The result is speculated as a result of different culture conditions used for the propagation and growth of the plants. The reported observation in the study is not widespread and was not observed in our study. In case of lingonberry, findings from our study suggest that propagation type does not affect the antioxidant capacity, total phenolic content and total anthocyanin content. Some of the factors that might have caused discrepancy in the results from the previous study by Goyali et al.(2013) and our study could be as a result of different plant material (blueberry vs. lingonberry) and growing conditions of the plant.

While comparing the total anthocyanin content of different berries like Saskatoon berry, raspberry, wild blueberry, strawberry and sea buckthorn with that of the lingonberry, Manitoba-grown lingonberry had the highest total anthocyanins content. A study by (Hosseinian & Beta, 2007) reported that Manitoba-grown Saskatoon berry and wild blueberry had total anthocyanin content of 562.4 mg per 100 grams and 558.3 mg per 100 grams dry weight, respectively. In this study, individual anthocyanin content was quantified by running the samples in high performance liquid chromatography (HPLC) along with the anthocyanin standards. The total anthocyanin content reported in the study was the sum of all individual anthocyanin present. Our study showed that the average total anthocyanin content for the Manitoba-grown lingonberry is 810 mg per 100 grams dry weight. However, in our study we determined the total anthocyanin content by the use of the pH differential method. Therefore, use of two different methods to determine anthocyanin content may have contributed towards the 30% difference in the results of the two studies. Since recovery was not compared, therefore it is not known as which of the methods are most accurate. Future studies aimed to identify the individual

anthocyanin present in lingonberry need to be done. Whether the higher total anthocyanin content of lingonberry grown in Northern Manitoba is the result of higher presence one individual anthocyanin or a combination of different individual anthocyanin is unknown. It is also possible that the variation in the results could be because of the genetic factors, temperature, light, type of the soil, harvest time or post-harvest storage as well as analysis methodology (Naczk & Shahidi, 2004). There is a report in the literature about the variability in the values from batch to batch and yearly variation which also might contribute to the variation in the phenolic composition and the antioxidant activity observed (Naczk & Shahidi, 2004; Lee, Durst, & Wrolstad, 2005). Results from our study show that there is variability in the total anthocyanin content between the different genotypes (Fig. 10). When the parent genotypes IDA and Entrekrone; were compared to their hybrid, total anthocyanin content for the hybrid falls between the ranges of the parents (Fig. 10); suggesting that there is not a hybrid vigour effect with respect to anthocyanin content.

Earlier studies reported that, phenolic compounds present in the berries significantly contribute to their antioxidant capacity. An antioxidant property is dependent on the structure of the phenolic compounds. Phenolic compounds are characterized by the presence of one or more aromatic rings with the presence of hydroxyl groups (Bors & Michel, 2002). The presence of hydroxyl groups help to neutralize free radicals (Rice-Evans, Miller, & Paganga, 1996; Bors & Michel, 2002). The choice of assay used for the preliminary selection of cultivar needs to be simple, inexpensive and easy to perform. If the assays are to be performed for the preliminary selection of the cultivars with the higher antioxidant capacity then the methods to be

utilized should be able to be performed with the limited availability of the sample. The choice of acidified methanol as solvent for the extraction in our study is based on the fact that methanol helps to extract poly-phenolic compounds from the dried lingonberry (Isaak et al., 2015; Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009). In addition to this, methanol is suitable solvent for other methods like high performance liquid chromatography (HPLC), liquid chromatography- mass spectrometry (LC-MS) (Isaak et al., 2015; Dudonne et al., 2009).

Several factors such as environmental conditions, level of maturation at the time of harvest, pre and post-harvest storage and processing methodology greatly influence the levels of antioxidants in berries (Manganaris, Goulas, Vicente, & Terry, 2014). Previously, it was reported that temperature, soil condition and light to have an effect on total antioxidant composition in plants (Naczk & Shahidi, 2004). In our study, lingonberries were collected from two different locations. Lingonberry from Newfoundland was grown in greenhouse whereas lingonberry from Northern Manitoba was harvested from the wild. Therefore, of the different factors we speculate that temperature and soil conditions may have influenced the level of antioxidant in our results. In addition, results from our study suggest that there is variability in the total anthocyanin content among lines grown under the controlled greenhouse conditions.

In our study, significant differences were observed in total phenolics content, total anthocyanins content and antioxidant capacity of the berries from different locations. A wide variation in terms of anthocyanin content, phenolic content and antioxidant capacity was noted between the cultivars of lingonberries grown in two different conditions; wild and greenhouse-grown. When compared between the average values for the

anthocyanins, phenolics and antioxidant capacity, Manitoba berries had higher values. A study by (Uleberg et al., 2012) has reported that berries grown at the Northern latitude with cold temperatures and at higher latitude have higher content of phenolic compounds and anthocyanins. In addition, earlier study by (Graglia et al., 2001) reported the difference in flavonoids levels from different sites even though plant leaves were taken from the similar latitude. The authors concluded that even in similar latitude, other local regional factors such as local environmental conditions might have contributed to the observed difference in flavonoids level. Both of our samples were taken from the similar latitude but the altitude was different (Table 4). Previously, several studies have demonstrated that compounds such as quercetin and kaempferol were present at markedly higher levels in *S. hexandrum* (Chinese medicinal plant) grown at higher altitude and the altitude difference between different locations was around 400-500 meters (Fuglevand, Jackson, & Jenkins, 1996; Wilson, Thompson, Huner, & Greenberg, 2001; Liu, Liu, Yin, & Zhao, 2015). These findings showed that not only latitude but also altitude affects the secondary metabolite production in plants. Therefore, we speculate that higher anthocyanin level observed in Manitoba samples could have resulted from the difference in altitude (300 meters) beside other environmental factors (i.e., temperature, soil, moisture conditions).

Results from our study shows that there is a good correlation between the total phenolics and ORAC which is in agreement with the reported literature (Wu et al., 2004). This correlation between total phenolics and ORAC suggests that antioxidant activity of the berries is a result of presence of compounds in the phenolics fraction. It is however unknown whether the antioxidant capacity is due to specific individual phenolic or the

results of many combined phenolic compounds as a whole. Future studies are required for the validation and selection of productive lines and cultivars with higher antioxidant capacity.

Apoptosis is defined as programmed cell death. One of the characteristic features of apoptosis is the activation of caspases (Czerski & Nunez, 2004). In the present study, lingonberry extract demonstrated an anti-apoptotic effect by inhibiting the activation of caspase-3. This shows that lingonberry is a potential candidate that provides a cardio-protective effect in ischemia/reperfusion injury. Several lines of evidence suggested that polyphenols have crucial effect on limiting the incidence of coronary heart disease (Renaud & de, 1992; Nardini, Natella, & Scaccini, 2007)**.** Oxidation of low density lipoprotein (LDL) is regarded as the crucial mechanism in the cardiovascular disease such as atherosclerosis (Aviram et al., 2000). It was shown that polyphenols inhibit the LDL (low-density lipoprotein) oxidation which, in turn, prevents the development of atherosclerosis (disease where arteries are blocked due to plaque formation). In addition, polyphenols were also shown to inhibit expression of metalloproteinase 1 and prevention of endothelial dysfunction, thus providing protection from cardiac failure (Garcia-Lafuente, Guillamon, Villares, Rostagno, & Martinez, 2009). Earlier it was reported that Colombian blueberry with higher levels of anthocyanin and total phenolic compounds attributed to the cardio-protective effect in rat in ischemia reperfusion injury (Lopera et al., 2013). In this study, berries were provided to the animals in the form of juice so it is unknown as the effect is due to one individual compound present or the combination of different compounds present in the berry. Therefore, it is not clear whether these berries contain some unique compounds or the same compounds similar to other berries

belonging to *Vaccinium* species. Taken together, these data suggested the beneficial effects of phenolics, anthocyanin and antioxidants to a cardio-protective effect. Future studies are essential to provide further mechanistic insights as how the protection is provided.

Results from the metabolomics study as mentioned earlier showed that two different pooled samples were separated clearly as shown in the Fig. 24. The clustering of triangles and squares together suggests that samples run in triplicate were consistent with the method of extraction and the instrumentation setup. This provides evidence to support that there was no procedural error which is a crucial factor for the overall outcome of the study. Error in sample preparation and the instrumentation might result in false or misleading results. In this study, results from the untargeted metabolomics approach utilizing liquid chromatography mass spectrometry (LC-MS) have shown that lingonberries that grow in the wild and in green house conditions differ in the metabolite compositions.

Earlier it was reported that low temperature induced the anthocyanin pathway whereas high temperature was shown to switch off this pathway via the MYB transcription factors (Lin-Wang et al., 2011). The other study showed that anthocyanin content was lower in Texas-grown potatoes compared to potatoes grown in Alaska (Payyavula, Navarre, Kuhl, Pantoja, & Pillai, 2012). Further, they correlated the lower expression of key flavonoids gene expression such as chalcone synthase (CHS), dihydroflavonol 4- reductase (DFR) and UDP glucose flavonol glucosyl transferase (UFGT) with lower anthocyanin content in Texas-grown potatoes. Surprisingly, they observed a higher amount of lutein in Texas potatoes which was correlated with higher

expression of LCY-e transcript (Payyavula et al., 2012). For the expression of LCY-e and LCY-b, a substrate called lycopene is essential which helps in activating the branch of β-carotene metabolic pathway. Previously, it was also reported that reduced production of lutein when LCY-e was silenced (Diretto et al., 2006). Here they reported the higher production of lutein which was correlated with higher expression of LCY-e that suggested the metabolic pathway was channelled towards lutein production via LCY-e-LUT1 pathway. Authors also reported a significant production of anthocyanin in red and purple-flesh potatoes grown in Alaska compared to white-flesh potatoes (Payyavula et al., 2012). Therefore, this indicates that understanding of different metabolic pathways activated, involvement of different pre-cursor molecules and genes during secondary metabolites production in plants is essential. Growth of the plants can be manipulated to customise the desired phytonutrient production by varying the environmental and genetic factors. Even though these kinds of studies were focussed on comparing environmental effects on the metabolic pathway, there is no evidence on which metabolic pathways gets activated and which pathways may differ in the berries grown in green house and in wild conditions. In our study, we observed, tentative compounds such as cinnamtannin B1, zingerone, ebselen and chlorogenic acid were unique to Manitoba lingonberry; whereas, other tentative compounds detected such as epigallocatechin gallate, bergenin, tremulacin, theaflavin and chamazulene were unique to Newfoundland lingonberry. It is unknown as which metabolic pathways get activated during the synthesis of these compounds. It is an open area of future research to compare the biosynthesis pathway of the berries grown in green house conditions and in the wild. Future studies are warranted

to address this area of interest, characterize and quantify the bioactive compounds present in the lingonberry.

CHAPTER VI: FUTURE DIRECTIONS AND LIMITATIONS

Future Directions:

Results from this study support the view that berries are promising sources of natural antioxidants. The antioxidant properties and phenolic content differed significantly between the berry samples from Manitoba and Newfoundland and between wild collected (Manitoba) and green-house grown (Newfoundland). Future investigations on interplay of genetics and environment on berry phenolic need to be carried out. Determination of anthocyanins and phenolic profile of lingonberry may help explain the reason behind higher antioxidant capacity of Northern Manitoba berries harvested in wild. Further studies are needed to characterize the bioactive compounds and biological activities of the lingonberries for the better appreciation of the health benefits. Further conformational studies are essential for tentatively detected compounds which can be done by nuclear magnetic resonance (NMR). Findings from our studies warrant further investigations into selecting lines to obtain a line or cultivar that produces berries with higher antioxidant property which in turn may translate to greater health benefits.

Limitations:

The major limitation of the study is the sample size and availability. There was some lingonberry samples with just one or two berry which if included in the study would not be representative sample. Therefore some lingonberry samples from Newfoundland were excluded from the study. Not all of the samples were available in equal amount which limits the inclusion of samples for various assays. Since the lingonberry collected from Newfoundland was grown in green house conditions, there was limited availability of the samples. Therefore, there was discrepancy in the number of samples to be compared between the two groups (Manitoba vs Newfoundland).

Depending on what assays are to be performed, method of extraction, samples size should be enough to carry out the triplicate or quadruplicate assays. For instance, in this study 0.5 grams of powdered lingonberry was extracted with 6 ml of solvent which was enough to carry out total anthocyanins assay in triplicates. In addition, lack of readily available commercial standards for anthocyanins and phenolic limit the study in elucidating the profiles for anthocyanins and phenolic compounds. If the standards are available, they can be used to profile an individual anthocyanins and phenolic. However, if standards are not available then nuclear magnetic resonance (NMR) can be used for the elucidation and confirmation of the detected compounds. Furthermore, lack of well-established mass spectral libraries for food database is another limiting factor of metabolomics part of this study.

Reference List

Ainsworth, E. A. & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nat. Protoc., 2,* 875-877.

Ali, K., Maltese, F., Choi, Y. H., & Verpoorte, R. (2010). Metabolic constituents of grapevine and grape-derived products. *Phytochem.Rev., 9,* 357-378.

Alonso, A., Marsal, S., & Julia, A. (2015). Analytical methods in untargeted metabolomics: state of the art in 2015. *Front Bioeng.Biotechnol., 3,* 23.

Alves, M. J., Ferreira, I. C., Froufe, H. J., Abreu, R. M., Martins, A., & Pintado, M. (2013). Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *J.Appl.Microbiol., 115,* 346-357.

Amorati, R. & Valgimigli, L. (2015). Advantages and limitations of common testing methods for antioxidants. *Free Radic.Res., 49,* 633-649.

Ashraf, U., Kanu, A. S., Mo, Z., Hussain, S., Anjum, S. A., Khan, I. et al. (2015). Lead toxicity in rice: effects, mechanisms, and mitigation strategies-a mini review. *Environ.Sci.Pollut.Res.Int.*.

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R. et al. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to

LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am.J.Clin.Nutr., 71,* 1062-1076.

Baba, S., Osakabe, N., Natsume, M., Yasuda, A., Takizawa, T., Nakamura, T. et al. (2000). Cocoa powder enhances the level of antioxidative activity in rat plasma. *Br.J.Nutr., 84,* 673-680.

Bakowska-Barczak, A. M., Marianchuk, M., & Kolodziejczyk, P. (2007). Survey of bioactive components in Western Canadian berries. *Can.J.Physiol Pharmacol., 85,* 1139-1152.

Bazinet, L. & Doyen, A. (2015). Antioxidants, Mechanisms, and Recovery by Membrane Processes. *Crit Rev.Food Sci.Nutr.,* 0.

Bere, E. (2007). Wild berries: a good source of omega-3. *Eur.J.Clin.Nutr., 61,* 431-433.

Bhullar, K. S. & Rupasinghe, H. P. (2015). Antioxidant and cytoprotective properties of partridgeberry polyphenols. *Food Chem., 168,* 595-605.

Bors, W. & Michel, C. (2002). Chemistry of the antioxidant effect of polyphenols. *Ann.N.Y.Acad.Sci., 957,* 57-69.

Bottcher, C., Roepenack-Lahaye, E. V., Willscher, E., Scheel, D., & Clemens, S. (2007). Evaluation of matrix effects in metabolite profiling based on capillary liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry. *Anal.Chem., 79,* 1507-1513.

Buijsse, B., Feskens, E. J., Kok, F. J., & Kromhout, D. (2006). Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study. *Arch.Intern.Med., 166,* 411-417.

Carlton, P. S., Kresty, L. A., Siglin, J. C., Morse, M. A., Lu, J., Morgan, C. et al. (2001). Inhibition of N-nitrosomethylbenzylamine-induced tumorigenesis in the rat esophagus by dietary freeze-dried strawberries. *Carcinogenesis, 22,* 441-446.

Caruana, M. & Vassallo, N. (2015). Tea Polyphenols in Parkinson's Disease. *Adv.Exp.Med.Biol., 863,* 117-137.

Chambers, B. K. & Camire, M. E. (2003). Can cranberry supplementation benefit adults with type 2 diabetes? *Diabetes Care, 26,* 2695-2696.

Chanet, A., Milenkovic, D., Manach, C., Mazur, A., & Morand, C. (2012). Citrus flavanones: what is their role in cardiovascular protection? *J.Agric.Food Chem., 60,* 8809-8822.

Chang, Y., Zhao, C., Zhu, Z., Wu, Z., Zhou, J., Zhao, Y. et al. (2012). Metabolic profiling based on LC/MS to evaluate unintended effects of transgenic rice with cry1Ac and sck genes. *Plant Mol.Biol., 78,* 477-487.

Chinnusamy, V., Zhu, J., & Zhu, J. K. (2007). Cold stress regulation of gene expression in plants. *Trends Plant Sci., 12,* 444-451.

Christie, P., Alfenito, M., & Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta, 194,* 541-549.

Chung, K. T., Wong, T. Y., Wei, C. I., Huang, Y. W., & Lin, Y. (1998). Tannins and human health: a review. *Crit Rev.Food Sci.Nutr., 38,* 421-464.

Czerski, L. & Nunez, G. (2004). Apoptosome formation and caspase activation: is it different in the heart? *J.Mol.Cell Cardiol., 37,* 643-652.

Da Silva, P. M., Lajolo, F. M., & Genovese, M. I. (2007). Bioactive compounds and antioxidant capacity of strawberry jams. *Plant Foods Hum.Nutr., 62,* 127-131.

de Mello Andrade, J. M. & Fasolo, D. (2014). Chapter 20 - Polyphenol Antioxidants from Natural Sources and Contribution to Health Promotion. In R.R.Watson, V. R. Preedy, & S. Zibadi (Eds.), *Polyphenols in Human Health and Disease* (pp. 253-265). San Diego: Academic Press.

De Vos, R. C., Moco, S., Lommen, A., Keurentjes, J. J., Bino, R. J., & Hall, R. D. (2007). Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat.Protoc., 2,* 778-791.

Degterev, A. & Yuan, J. (2008). Expansion and evolution of cell death programmes. *Nat.Rev.Mol.Cell Biol., 9,* 378-390.

Diretto, G., Tavazza, R., Welsch, R., Pizzichini, D., Mourgues, F., Papacchioli, V. et al. (2006). Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. *BMC.Plant Biol., 6,* 13.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev., 82,* 47-95.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Merillon, J. M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial

interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J.Agric.Food Chem., 57,* 1768-1774.

Eid, H. M., Ouchfoun, M., Brault, A., Vallerand, D., Musallam, L., Arnason, J. T. et al. (2014). Lingonberry (Vaccinium vitis-idaea L.) Exhibits Antidiabetic Activities in a Mouse Model of Diet-Induced Obesity. *Evid.Based.Complement Alternat.Med., 2014,* 645812.

Eisele, H. J., Markart, P., & Schulz, R. (2015). Obstructive Sleep Apnea, Oxidative Stress, and Cardiovascular Disease: Evidence from Human Studies. *Oxid.Med.Cell Longev., 2015,* 608438.

Eisenreich, W. & Bacher, A. (2007). Advances of high-resolution NMR techniques in the structural and metabolic analysis of plant biochemistry. *Phytochemistry, 68,* 2799-2815.

Ek, S., Kartimo, H., Mattila, S., & Tolonen, A. (2006). Characterization of phenolic compounds from lingonberry (Vaccinium vitis-idaea). *J.Agric.Food Chem., 54,* 9834-9842.

Erlund, I., Koli, R., Alfthan, G., Marniemi, J., Puukka, P., Mustonen, P. et al. (2008). Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *Am.J.Clin.Nutr., 87,* 323-331.

Ferrandiz, M. L. & Alcaraz, M. J. (1991). Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions, 32,* 283-288.

Fillet, M. & Frederich, M. (2015). The emergence of metabolomics as a key discipline in the drug discovery process. *Drug Discov.Today Technol., 13,* 19-24.

Formica, J. V. & Regelson, W. (1995). Review of the biology of Quercetin and related bioflavonoids. *Food Chem.Toxicol., 33,* 1061-1080.

Fowler, S. & Thomashow, M. F. (2002). Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell, 14,* 1675-1690.

Fuchs, Y. & Steller, H. (2011). Programmed cell death in animal development and disease. *Cell, 147,* 742-758.

Fuglevand, G., Jackson, J. A., & Jenkins, G. I. (1996). UV-B, UV-A, and blue light signal transduction pathways interact synergistically to regulate chalcone synthase gene expression in Arabidopsis. *Plant Cell, 8,* 2347-2357.

Gambini, J., Ingles, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L. et al. (2015). Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans. *Oxid.Med.Cell Longev., 2015,* 837042.

Garcia-Lafuente, A., Guillamon, E., Villares, A., Rostagno, M. A., & Martinez, J. A. (2009). Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm.Res., 58,* 537-552.

Gika, H. G., Theodoridis, G. A., Plumb, R. S., & Wilson, I. D. (2014). Current practice of liquid chromatography-mass spectrometry in metabolomics and metabonomics. *J.Pharm.Biomed.Anal., 87,* 12-25.

Gillespie, K. M., Chae, J. M., & Ainsworth, E. A. (2007). Rapid measurement of total antioxidant capacity in plants. *Nat.Protoc., 2,* 867-870.

Ginter, E., Simko, V., & Panakova, V. (2014). Antioxidants in health and disease. *Bratisl.Lek.Listy, 115,* 603-606.

Goyali, J. C., Igamberdiev, A. U., & Debnath, S. C. (2013). Morphology, phenolic content and antioxidant capacity of lowbush blueberry (Vaccinium angustifolium Ait.) plants as affected by in vitro and ex vitro propagation methods. *Canadian Journal of Plant Science, 93,* 1001-1008.

Graglia, E., Julkunen-Tiitto, R., Shaver, G. R., Schmidt, I. K., Jonasson, S., & Michelsen, A. (2001). Environmental control and intersite variations of phenolics in Betula nana in tundra ecosystems. *New Phytologist, 151,* 227-236.

Gulcin, I. (2012). Antioxidant activity of food constituents: an overview. *Arch.Toxicol., 86,* 345-391.

Gulcin, I., Topal, F., Cakmakci, R., Bilsel, M., Goren, A. C., & Erdogan, U. (2011). Pomological features, nutritional quality, polyphenol content analysis, and antioxidant properties of domesticated and 3 wild ecotype forms of raspberries (Rubus idaeus L.). *J.Food Sci., 76,* C585-C593.

Guy, C., Kaplan, F., Kopka, J., Selbig, J., & Hincha, D. K. (2008). Metabolomics of temperature stress. *Physiologia Plantarum, 132,* 220-235.

Harbilas, D., Martineau, L. C., Harris, C. S., Adeyiwola-Spoor, D. C., Saleem, A., Lambert, J. et al. (2009). Evaluation of the antidiabetic potential of selected medicinal plant extracts from the Canadian boreal forest used to treat symptoms of diabetes: part II. *Can.J.Physiol Pharmacol., 87,* 479-492.

Hausenloy, D. J. & Yellon, D. M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J.Clin.Invest, 123,* 92-100.

Hegeman, A. D. (2010). Plant metabolomics--meeting the analytical challenges of comprehensive metabolite analysis. *Brief.Funct.Genomics, 9,* 139-148.

Heleno, S. A., Stojkovi-ç, D., Barros, L., Glamo-ìlija, J., Sokovi-ç, M., Martins, A. et al. (2013). A comparative study of chemical composition, antioxidant and antimicrobial properties of Morchella esculenta (L.) Pers. from Portugal and Serbia. *Food Research International, 51,* 236-243.

Hermes-Lima, M., Moreira, D. C., Rivera-Ingraham, G., Giraud-Billoud, M., Genaro-Mattos, T. C., & Campos, E. G. (2015). Preparation for oxidative stress under hypoxia and metabolic depression: Revisiting the proposal two decades later. *Free Radic.Biol.Med.*.

Heyman, L., Axling, U., Blanco, N., Sterner, O., Holm, C., & Berger, K. (2014). Evaluation of Beneficial Metabolic Effects of Berries in High-Fat Fed C57BL/6J Mice. *J.Nutr.Metab, 2014,* 403041.

Hill, C. B., Czauderna, T., Klapperstuck, M., Roessner, U., & Schreiber, F. (2015). Metabolomics, Standards, and Metabolic Modeling for Synthetic Biology in Plants. *Front Bioeng.Biotechnol., 3,* 167.

Hosseinian, F. S. & Beta, T. (2007). Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba berries. *J.Agric.Food Chem., 55,* 10832-10838.

Howell, A. B. (2007). Bioactive compounds in cranberries and their role in prevention of urinary tract infections. *Mol.Nutr.Food Res., 51,* 732-737.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *J.Agric.Food Chem., 53,* 1841-1856.

Huie, C. W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Anal.Bioanal.Chem., 373,* 23-30.

Husken, A., Baumert, A., Milkowski, C., Becker, H. C., Strack, D., & Mollers, C. (2005). Resveratrol glucoside (Piceid) synthesis in seeds of transgenic oilseed rape (Brassica napus L.). *Theor.Appl.Genet., 111,* 1553-1562.

Isaak, C. K., Petkau, J. C., O K, Debnath, S. C., & Siow, Y. L. (2015). Manitoba Lingonberry (Vaccinium vitis-idaea) Bioactivities in Ischemia-Reperfusion Injury. *J.Agric.Food Chem., 63,* 5660-5669.

Jaakola, L. & Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in plants. *Plant Cell Environ., 33,* 1239-1247.

Joseph, S. V., Edirisinghe, I., & Burton-Freeman, B. M. (2014). Berries: anti-inflammatory effects in humans. *J.Agric.Food Chem., 62,* 3886-3903.

Kahkonen, M. P., Hopia, A. I., & Heinonen, M. (2001). Berry phenolics and their antioxidant activity. *J.Agric.Food Chem., 49,* 4076-4082.

Kasote, D. M., Katyare, S. S., Hegde, M. V., & Bae, H. (2015). Significance of antioxidant potential of plants and its relevance to therapeutic applications. *Int.J.Biol.Sci., 11,* 982-991.

Kivimaki, A. S., Ehlers, P. I., Turpeinen, A. M., Vapaatalo, H., & Korpela, R. (2011). Lingonberry juice improves endothelium-dependent vasodilatation of mesenteric arteries in spontaneously hypertensive rats in a long-term intervention. *Journal of Functional Foods, 3,* 267-274.

Kivimaki, A. S., Siltari, A., Ehlers, P. I., Korpela, R., & Vapaatalo, H. (2013). Lingonberry juice lowers blood pressure of spontaneously hypertensive rats (SHR). *Journal of Functional Foods, 5,* 1432-1440.

Kolniak-Ostek, J., Kucharska, A. Z., Sokol-Letowska, A., & Fecka, I. (2015). Characterization of phenolic compounds of thorny and thornless blackberries. *J.Agric.Food Chem., 63,* 3012-3021.

Kono, S., Ikeda, M., Tokudome, S., & Kuratsune, M. (1988). A case-control study of gastric cancer and diet in northern Kyushu, Japan. *Jpn.J.Cancer Res., 79,* 1067-1074.

Kumar, D. & Jugdutt, B. I. (2003). Apoptosis and oxidants in the heart. *J.Lab Clin.Med., 142,* 288-297.

Kylli, P., Nohynek, L., Puupponen-Pimia, R., Westerlund-Wikstrom, B., Leppanen, T., Welling, J. et al. (2011). Lingonberry (Vaccinium vitis-idaea) and European cranberry (Vaccinium microcarpon) proanthocyanidins: isolation, identification, and bioactivities. *J.Agric.Food Chem., 59,* 3373-3384.

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *J.AOAC Int., 88,* 1269-1278.

Lehtonen, H. M., Lindstedt, A., Jarvinen, R., Sinkkonen, J., Graca, G., Viitanen, M. et al. (2013). 1H NMR-based metabolic fingerprinting of urine metabolites after consumption of lingonberries (Vaccinium vitis-idaea) with a high-fat meal. *Food Chem., 138,* 982-990.

Li, D., Wang, P., Luo, Y., Zhao, M., & Chen, F. (2015). Health Benefits of Anthocyanins and Molecular Mechanisms: Update from Recent Decade. *Crit Rev.Food Sci.Nutr.,* 0.

Lin-Wang, K., Micheletti, D., Palmer, J., Volz, R., Lozano, L., Espley, R. et al. (2011). High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. *Plant Cell Environ., 34,* 1176-1190.

Liu, W., Liu, J., Yin, D., & Zhao, X. (2015). Influence of ecological factors on the production of active substances in the anti-cancer plant Sinopodophyllum hexandrum (Royle) T.S. Ying. *PLoS One, 10,* e0122981.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn.Rev., 4,* 118-126.

Loft, S. & Poulsen, H. E. (1996). Cancer risk and oxidative DNA damage in man. *J.Mol.Med.(Berl), 74,* 297-312.

Lopera, Y. E., Fantinelli, J., Gonzalez Arbelaez, L. F., Rojano, B., Rios, J. L., Schinella, G. et al. (2013). Antioxidant Activity and Cardioprotective Effect of a Nonalcoholic Extract of

Vaccinium meridionale Swartz during Ischemia-Reperfusion in Rats. *Evid.Based.Complement Alternat.Med., 2013,* 516727.

Lopez-Alarcon, C. & Denicola, A. (2013). Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. *Anal.Chim.Acta, 763,* 1-10.

Lyons, M. M., Yu, C., Toma, R. B., Cho, S. Y., Reiboldt, W., Lee, J. et al. (2003). Resveratrol in raw and baked blueberries and bilberries. *J.Agric.Food Chem., 51,* 5867-5870.

Manganaris, G. A., Goulas, V., Vicente, A. R., & Terry, L. A. (2014). Berry antioxidants: small fruits providing large benefits. *J.Sci.Food Agric., 94,* 825-833.

Mayengbam, S., House, J. D., & Aliani, M. (2015). Investigation of vitamin B inadequacy, induced by exposure to the anti-B factor 1-amino D-proline, on plasma lipophilic metabolites of rats: a metabolomics approach. *Eur.J.Nutr.*.

Middleton E Jr (1998). Effect of plant flavonoids on immune and inflammatory cell function. *Adv.Exp.Med.Biol., 439,* 175-182.

Mockridge, J. W., Marber, M. S., & Heads, R. J. (2000). Activation of Akt during simulated ischemia/reperfusion in cardiac myocytes. *Biochem.Biophys.Res.Commun., 270,* 947-952.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: vaccinium, rubus, and ribes. *J.Agric.Food Chem., 50,* 519-525.

Mulvihill, E. E., Assini, J. M., Sutherland, B. G., DiMattia, A. S., Khami, M., Koppes, J. B. et al. (2010). Naringenin decreases progression of atherosclerosis by improving dyslipidemia

in high-fat-fed low-density lipoprotein receptor-null mice. *Arterioscler.Thromb.Vasc.Biol., 30,* 742-748.

Nabuurs, M. H., McCallum, J. L., Brown, D. C., & Kirby, C. W. (2015). NMR characterization of novel pyranoanthocyanins derived from the pulp of Panax quinquefolius L. (North American ginseng). *Magn Reson.Chem.*.

Naczk, M. & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *J.Chromatogr.A, 1054,* 95-111.

Nardini, M., Natella, F., & Scaccini, C. (2007). Role of dietary polyphenols in platelet aggregation. A review of the supplementation studies. *Platelets., 18,* 224-243.

Nijveldt, R. J., van, N. E., van Hoorn, D. E., Boelens, P. G., van, N. K., & van Leeuwen, P. A. (2001). Flavonoids: a review of probable mechanisms of action and potential applications. *Am.J.Clin.Nutr., 74,* 418-425.

Niki, E. (2010). Assessment of antioxidant capacity in vitro and in vivo. *Free Radic.Biol.Med., 49,* 503-515.

Nile, S. H. & Park, S. W. (2014). Edible berries: bioactive components and their effect on human health. *Nutrition, 30,* 134-144.

Pappas, E. & Schaich, K. M. (2009). Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability. *Crit Rev.Food Sci.Nutr., 49,* 741-781.

Paredes-Lopez, O., Cervantes-Ceja, M. L., Vigna-Perez, M., & Hernandez-Perez, T. (2010). Berries: improving human health and healthy aging, and promoting quality life--a review. *Plant Foods Hum.Nutr., 65,* 299-308.

Payyavula, R. S., Navarre, D. A., Kuhl, J. C., Pantoja, A., & Pillai, S. S. (2012). Differential effects of environment on potato phenylpropanoid and carotenoid expression. *BMC.Plant Biol., 12,* 39.

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J.Clin.Biochem., 30,* 11-26.

Pietta, P. G. (2000). Flavonoids as antioxidants. *J.Nat.Prod., 63,* 1035-1042.

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L. et al. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. *J.Agric.Food Chem., 51,* 3273-3279.

Prior, R. L. & Wu, X. (2006). Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic.Res., 40,* 1014-1028.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J.Agric.Food Chem., 53,* 4290-4302.

Putri, S. P., Yamamoto, S., Tsugawa, H., & Fukusaki, E. (2013). Current metabolomics: technological advances. *J.Biosci.Bioeng., 116,* 9-16.

Qin, Y., Xia, M., Ma, J., Hao, Y., Liu, J., Mou, H. et al. (2009). Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. *Am.J.Clin.Nutr., 90,* 485-492.

Ramakrishna, A. & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal.Behav., 6,* 1720-1731.

Reiter, R. J., Tan, D. X., Mayo, J. C., Sainz, R. M., Leon, J., & Czarnocki, Z. (2003). Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim.Pol., 50,* 1129-1146.

Renaud, S. & de, L. M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet, 339,* 1523-1526.

Reshi, M. L., Su, Y. C., & Hong, J. R. (2014). RNA Viruses: ROS-Mediated Cell Death. *Int.J.Cell Biol., 2014,* 467452.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic.Biol.Med., 20,* 933-956.

Riedl, S. J. & Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nat.Rev.Mol.Cell Biol., 5,* 897-907.

Rimando, A. M., Kalt, W., Magee, J. B., Dewey, J., & Ballington, J. R. (2004). Resveratrol, pterostilbene, and piceatannol in vaccinium berries. *J.Agric.Food Chem., 52,* 4713-4719.

Rimbach, G., Melchin, M., Moehring, J., & Wagner, A. E. (2009). Polyphenols from cocoa and vascular health-a critical review. *Int.J.Mol.Sci., 10,* 4290-4309.

Rodriguez-Mateos, A., Heiss, C., Borges, G., & Crozier, A. (2014). Berry (poly)phenols and cardiovascular health. *J.Agric.Food Chem., 62,* 3842-3851.

Roessner, U. & Pettolino, F. (2007). The importance of anatomy and physiology in plant metabolomics. In J.Nielsen & M. Jewett (Eds.), *Metabolomics* (18 ed., pp. 253-278). Springer Berlin Heidelberg.

Schewe, T., Sadik, C., Klotz, L. O., Yoshimoto, T., Kuhn, H., & Sies, H. (2001). Polyphenols of cocoa: inhibition of mammalian 15-lipoxygenase. *Biol.Chem., 382,* 1687-1696.

Seeram, N. P. (2008). Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *J.Agric.Food Chem., 56,* 627-629.

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Nahrung, 44,* 158-163.

Shumway, B. S., Kresty, L. A., Larsen, P. E., Zwick, J. C., Lu, B., Fields, H. W. et al. (2008). Effects of a topically applied bioadhesive berry gel on loss of heterozygosity indices in premalignant oral lesions. *Clin.Cancer Res., 14,* 2421-2430.

Simeonov, S. B., Botushanov, N. P., Karahanian, E. B., Pavlova, M. B., Husianitis, H. K., & Troev, D. M. (2002). Effects of Aronia melanocarpa juice as part of the dietary regimen in patients with diabetes mellitus. *Folia Med.(Plovdiv.), 44,* 20-23.

Singh, G. & Pai, R. S. (2015). Dawn of antioxidants and immune modulators to stop HIV-progression and boost the immune system in HIV/AIDS patients: An updated comprehensive and critical review. *Pharmacol.Rep., 67,* 600-605.

Singleton, V. L. & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture, 16,* 144-158.

Stintzing, F. C., Stintzing, A. S., Carle, R., Frei, B., & Wrolstad, R. E. (2002). Color and antioxidant properties of cyanidin-based anthocyanin pigments. *J.Agric.Food Chem., 50,* 6172-6181.

Szakiel, A., Paczkowski, C., Koivuniemi, H., & Huttunen, S. (2012). Comparison of the triterpenoid content of berries and leaves of lingonberry Vaccinium vitis-idaea from Finland and Poland. *J.Agric.Food Chem., 60,* 4994-5002.

Taruscio, T. G., Barney, D. L., & Exon, J. (2004). Content and profile of flavanoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest Vaccinium berries. *J.Agric.Food Chem., 52,* 3169-3176.

Torronen, R., Kolehmainen, M., Sarkkinen, E., Mykkanen, H., & Niskanen, L. (2012). Postprandial glucose, insulin, and free fatty acid responses to sucrose consumed with blackcurrants and lingonberries in healthy women. *Am.J.Clin.Nutr., 96,* 527-533.

Uleberg, E., Rohloff, J., Jaakola, L., Trost, K., Junttila, O., Haggman, H. et al. (2012). Effects of temperature and photoperiod on yield and chemical composition of northern and southern clones of bilberry (Vaccinium myrtillus L.). *J.Agric.Food Chem., 60,* 10406-10414.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int.J.Biochem.Cell Biol., 39,* 44-84.

Vinayavekhin, N. & Saghatelian, A. (2010). Untargeted metabolomics. *Curr.Protoc.Mol.Biol., Chapter 30,* Unit-24.

Wang, S. Y. & Lin, H. S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J.Agric.Food Chem., 48,* 140-146.

Wang, Y., Catana, F., Yang, Y., Roderick, R., & van Breemen, R. B. (2002). An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. *J.Agric.Food Chem., 50,* 431-435.

Weidinger, A. & Kozlov, A. V. (2015). Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. *Biomolecules., 5,* 472-484.

Wilson, K. E., Thompson, J. E., Huner, N. P., & Greenberg, B. M. (2001). Effects of ultraviolet-A exposure on ultraviolet-B-induced accumulation of specific flavonoids in Brassica napus. *Photochem.Photobiol., 73,* 678-684.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol, 126,* 485-493.

Wishart, D. S. (2008). Applications of metabolomics in drug discovery and development. *Drugs R.D., 9,* 307-322.

Wu, X., Gu, L., Prior, R. L., & McKay, S. (2004). Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity. *J.Agric.Food Chem., 52,* 7846-7856.

Xu, F., Zou, L., Liu, Y., Zhang, Z., & Ong, C. N. (2011). Enhancement of the capabilities of liquid chromatography-mass spectrometry with derivatization: general principles and applications. *Mass Spectrom.Rev., 30,* 1143-1172.

Yamane, T., Hagiwara, N., Tateishi, M., Akachi, S., Kim, M., Okuzumi, J. et al. (1991). Inhibition of azoxymethane-induced colon carcinogenesis in rat by green tea polyphenol fraction. *Jpn.J.Cancer Res., 82,* 1336-1339.

Yang, B. & Kortesniemi, M. (2015). Clinical evidence on potential health benefits of berries. *Current Opinion in Food Science, 2,* 36-42.

Yuliana, N. D., Khatib, A., Choi, Y. H., & Verpoorte, R. (2011). Metabolomics for bioactivity assessment of natural products. *Phytother.Res., 25,* 157-169.

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *J.Agric.Food Chem., 53,* 2118-2124.

Zheng, W. & Wang, S. Y. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *J.Agric.Food Chem., 51,* 502-509.

Zhu, J., Dong, C. H., & Zhu, J. K. (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr.Opin.Plant Biol., 10,* 290-295.