Manitoba lingonberry (*Vaccinium vitis-idaea*) protects against ischemia-reperfusion injury

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Abstract

Lingonberry (*Vaccinium vitis*-idaea) harvested in Manitoba, Canada contains very high levels of anthocyanins and other polyphenols. These compounds are bioavailable in the kidney and, since other antioxidant compounds have been shown to protect against ischemia-reperfusion (IR) injury in various models, we hypothesized that the anthocyanins and polyphenols in Manitoba lingonberry would be bioactive against IR injury. Due to the significance of cardiomyocyte IR injury in cardiovascular disease and the prevalence of renal IR injury following major surgeries and kidney transplants, we selected three IR models to test for protective effects of Manitoba lingonberry against these types of injuries: simulated IR in H9c2 cardiomyoblasts, acute kidney injury (AKI) in Sprague-Dawley rats, and simulated IR in HK-2 kidney proximal tubule epithelial cells.

First, the study showed the strong antioxidant capacity of Manitoba lingonberry and determined the major anthocyanins present. Second, the results showed that Manitoba lingonberry prevented apoptosis in H9c2 cells caused by IR injury or hydrogen peroxide-induced oxidative stress by inhibiting chromatin condensation, caspase activation, and MAPK signaling. Third, the study showed that rats who consumed lingonberry juice daily for three weeks had improved kidney function, reduced renal MAPK signaling, and an attenuated inflammatory response following AKI. Fourth, the link between renal MAPK signaling and inflammatory response was confirmed in HK-2 cells and pure lingonberry anthocyanins were shown to be effective at modulating this response.

These results provide the first characterization of Manitoba lingonberry and highlight the importance of dietary berry compounds for cardiovascular and renal health. This study is the first to demonstrate the use of Manitoba lingonberry juice as a functional food and the first to demonstrate that physiological nanomolar doses of anthocyanins can protect heart and kidney cells from apoptosis.

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Dedication

This thesis is dedicated to the rats who gave their lives for this research.

I would like to thank my supervisor, Dr. Siow, for giving me the privilege of working in his laboratory for the past several years. I have learned from Dr. Siow that research must first be fair and ethical for all involved, that understanding what went wrong in an experiment can be just as important as understanding what went right, and that we must have a passion for sharing the things we create in the lab if we wish to have an impact. I am very grateful for his generosity with his time and resources, his informative and entertaining conversation, his patience when experiments did not go right, and his willingness to demonstrate and teach in the lab.

I would also like to thank my committee members, Janice Dodd, Grant Pierce, and Michel Aliani, for their time and effort attending committee meetings and carefully reviewing my research. Because of each of them, I can now defend my ideas, think creatively to produce the next hypothesis, and most importantly recognize that which I do not know. Their research accomplishments and varied expertise have been very inspiring.

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List of Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
AP-1	Activator protein
AKI	Acute kidney injury
ATP	Adenosine triphosphate
ARF	Anthocyanin-rich fraction
ASC	Apoptosis-associated speck-like protein
JNK	c-Jun N-terminal kinase
CE	Complete extract
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
DMAC	Dimethylaminocinnamaldehyde
DAD	Diode array detector
DW	Dry weight
DMEM	Dulbecco's modified Eagle's medium
ESI-MS	Electrospray ionization mass spectrometer
FADD	Fas-associated protein with death domain
FRAP	Ferric reducing antioxidant power
FID	Flame ionization detector
FLR	Fluorescent detector

FC	Folin–Ciocalteu
FW	Fresh weight
GAE	Gallic acid equivalents
GC	Gas chromatography
GLC	Gas-liquid chromatography
GRF	Glomerular filtration rate
GSK	Glycogen synthase kinase
H&E	Hematoxylin and eosin
HPLC	High performance liquid
IRS	Insulin receptor substrate
IFN	Interferon
IL	Interleukin
IR	Ischemia-reperfusion
KC	Keratinocyte chemoattractant
MS	Mass spectrometer
MMP	Matrix metalloproteinase
MCLP	Methylcellulose precipitation
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MCP	Monocyte chemoattractant protein
NGAL	Neutrophil gelatinase-associated lipocalin
NLRP	NLR family pyrin domain containing

Nrf	Nuclear factor erythroid 2–related factor
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
ORAC	Oxygen radical absorbance capacity
PRR	Pattern recognition receptor
PRF	Phenolic-rich fraction
PBS	Phosphate-buffered saline
PDA	Photodiode array detector
PARP	Poly-ADP ribose polymerase
PCR	Polymerase chain reaction
PK	Protein kinase
QTOF	Quadrupole time of flight
RIP	Receptor-interacting serine/threonine-protein kinase
TSP	Thrombospondin
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TE	Trolox equivalents
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPLC	Ultra-performance liquid chromatography

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Lingonberry anthocyanins protect cardiac cells from oxidative stress-induced apoptosis
Isaak CK, Petkau JC, Blewett HJ, O K, Siow YL
Canadian Journal of Physiology and Pharmacology 2017, 95: 904-910

Supplementing diet with Manitoba lingonberry juice reduces kidney ischemia-reperfusion injury
Isaak CK, Wang P, Prashar S, O K, Brown DC, Debnath SC, Siow YL
Journal of the Science of Food and Agriculture 2017, 97: 3065-3076

Manitoba lingonberry (*Vaccinium vitis-idaea*) bioactivities in ischemia-reperfusion injury
Isaak CK, Petkau JC, O K, Debnath SC, Siow YL
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Figure 2.2. Mesovia lingonberry grown in a greenhouse (A) and in the wild (B).
Figure 2.2 A is used with permission from Dr. Samir Debnath. The plant is grown and maintained in the greenhouse of St. John's Research and Development Centre, Agriculture and Agri-Food Canada, St. John's, Newfoundland and Labrador, Canada.

Chapter 1

Statement of Intent

1.1 Hypotheses and intent

Berries are globally available, nutritious foods that provide a wealth of benefits to the consumer. They come in an abundance of flavours to appeal to all appetites, can be frozen for transport and storage without a loss in potency or taste, and can be used for cooking and baking or consumed raw. Epidemiological and intervention studies have examined the health benefits of consuming berries and found that people who consume them regularly have lower risk of cardiovascular and other chronic diseases. Lingonberry is especially remarkable because it contains more anthocyanins and antioxidants than other commonly consumed berries. These types of compounds protect against chronic conditions like cardiovascular disease, type 2 diabetes, and Alzheimer's. However, the effects on acute injuries have not been studied and most research has focused on the European subspecies of lingonberry rather than the lesser-known North American variety. Consuming functional foods like lingonberry would be an inexpensive and simple way to improve the health of Canadians while also promoting a local Northern fruit crop.

This research was guided by the general hypothesis that Northern Manitoba lingonberry possesses unique health benefits due to its exceptionally high levels of bioactive compounds. The following thesis will examine these health benefits and test for protective effects against ischemia-reperfusion (IR) injury, a type of acute injury that can occur in the heart and kidney due to a range of morbidities. The author performed experiments for, wrote, and revised each of the manuscripts contained in the following chapters.

The next chapter will provide a review of the literature concerning the compounds found in lingonberry, the link between berry compounds and human health, and the contexts

and mechanisms of IR injury in the heart and kidney. This chapter contains Manuscript I, a review article which explores c-Jun N-terminal kinase (JNK)-mediated inflammation and examines the evidence for moderating inflammation with polyphenol-rich berries.

Chapter 3 contains Manuscript II published in the Journal of Agriculture and Food Chemistry (Isaak *et al*. 2015). This study tested the first specific hypothesis that Manitoba lingonberry would protect heart cells from ischemia reperfusion-induced apoptotic cell death due to exceptionally high levels of polyphenolic compounds, including anthocyanins, which are known to have cardioprotective effects. The specific objectives of the study were 1) to characterize the antioxidant capacity and anthocyanin profile of Manitoba lingonberry and compare the Manitoba variety to Eastern Canada and European cultivars and 2) to test a Manitoba lingonberry extract as well as pure anthocyanins for effects on apoptotic cell death and apoptotic signaling in cardiomyoblasts after simulated IR injury.

Chapter 4 contains Manuscript III published in the Canadian Journal of Physiology and Pharmacology (Isaak *et al*. 2017a). This study tested the second specific hypothesis that Manitoba lingonberry anthocyanins would protect heart cells from oxidative stress-induced apoptotic cell death even at physiological concentrations. The objective of the study was to test lingonberry anthocyanins at bioavailable concentrations for effects on necrosis and apoptosis in cardiomyoblasts after exposure to oxidative stress.

Chapter 5 contains Manuscript IV published in the Journal of the Science of Food and Agriculture (Isaak *et al*. 2017b). This study tested the third and fourth specific hypotheses: that Manitoba lingonberry juice consumption would be protective against ischemic AKI because lingonberry compounds are bioavailable and metabolized in the kidney and have been shown

to have protective effects in IR injury; and that anthocyanins would be responsible for the bioactive effects of lingonberry in kidney cells because they inhibit stress-activated signaling and the subsequent inflammatory response. The specific aims of the study were 1) to develop and characterize a commercially-relevant juice product from Manitoba lingonberry for use in an animal feeding study, 2) to compare post-AKI kidney function in rats fed lingonberry juice with rats fed a sucrose-matched control beverage, 3) to test the effects of lingonberry juice consumption on renal JNK signaling pathways and systemic and renal inflammation caused by ischemic AKI, 4) to test the effects of lingonberry anthocyanins on JNK signaling and inflammation induced by simulated IR in cultured human proximal tubule cells, and 5) to test the link between JNK signaling and the inflammatory response in kidney cells.

Chapter 6 presents the author's conclusions regarding the included studies and discusses potential future directions for Manitoba lingonberry research.

1.2 Ethical considerations

This study utilized both wild plants and laboratory animals and ethical consultation was sought for each of these methodologies. For wild lingonberry collection, authorization was not required as there is no prohibition against picking or harvesting wild berries including lingonberries, as confirmed by the Department of Conservation and Water Stewardship of the Province of Manitoba. To the best of our knowledge, there was no involvement of endangered or protected species. Care was taken to not disturb any plants or animals and only the berries needed for research were taken.

For animal research, the protocol (#13-019) was approved by the University of Manitoba Animal Care Committee, in agreement with the Canadian Council on Animal Care guidelines. Specific ethical considerations for the animal research included oral syringe feeding as a more humane alternative to gavage, surgical procedures performed under isoflurane gas anesthesia and only after the rats reached stage 3 anesthesia, monitoring of body temperature, heart rate, and oxygen saturation during surgery, use of incubators and provision of food and water after surgery, careful monitoring for signs of distress during recovery after surgery, and euthanasia via sodium pentobarbital overdose according to the approved protocol.

1.3 Other guiding principles

While this study did not involve human research participants in any way, the author of this thesis drew inspiration from Dr. Hermann Michell's essay using berry-picking as a metaphor for community-based research (Michell. 2009). The following excerpts provided the author with valuable insight into the research process and the importance of community involvement at all stages of research, from hypothesis to knowledge dissemination.

"Like research, discovering the right time to gather berries usually begins by consulting and visiting with knowledgeable people in the community. Once a site is selected, researchers need to prepare to go through the boreal forest, up and down the hills, valleys, and rocky terrain with their berry pails."

"It is not uncommon to come across black bears in berry picking sites. Observation, keeping an open mind, and being alert are three important aspects of survival in the north. Bears are considered great teachers and healers. They teach us to confront danger head on or we can simply walk around it to avoid problems and conflict."

"Berries are selected based on ripeness, while others are left to go back to the earth in a continuous cycle of renewal. We study the data that is discarded and we must remember that even these seemingly senseless pieces of information also tell a story of something."

"In the research dissemination phase, the berries are shared, exchanged, and consumed in whatever final form they become. The berries will nourish just as much as they will cause many different reactions. The black wolf spirit will quietly dance around and sniff before taking a bite. Scholars, researchers, and readers will quietly trek back and forth across worldviews like the great barren ground caribou taking what they need and leaving the rest for further thought and decay. White weasels, black minks, red squirrels, and yellow-bellied birds will come in and out of their disciplinary sanctuaries and nests taking tiny bits back to their peers. Many will interpret, critique, and aggressively oppose the results based on their fields of expertise. Still others will take action, initiate dialogue and talk, build on the research, reconstruct the knowledge base leading to further research, in a never-ending cycle of truth, refinement, and change."

1.4 Knowledge dissemination

In addition to publication of Manuscript II, the results of that study were also shared via poster presentations at the Life Science Alley Conference in Minneapolis, Minnesota, USA in 2012 and the Experimental Biology Conference in San Diego, California, USA in 2014, and shared on the "Health Effects of Manitoba Fruit" episode of CTV Modern Medicine, Winnipeg, Manitoba Canada, in November 2013.

The results of Manuscript III were also shared via poster presentation at the Experimental Biology Conference in Chicago, Illinois, USA in 2017.

In addition to publication of Manuscript IV, the results of that study were also shared as an oral presentation at the Therapeutic Applications of Functional Foods and Bioactives Conference, Winnipeg, Manitoba, Canada, and via poster presentations at the Functional Foods and Natural Health Products Graduate Student Symposium in Winnipeg, Manitoba, Canada and the European Nutrition Conference in Berlin, Germany in 2016. Highlights of the study were also communicated via interview on Global TV Morning News, Winnipeg, Manitoba Canada in March 2016.

Manitoba lingonberry horticulture techniques and potential health benefits were published in the 2016 Prairie Garden published by the Prairie Garden Committee as a book chapter entitled "Lingonberry: a super-food for Canada's climate" (Isaak. 2016).

Chapter 2

Literature Review

2.1 Lingonberry botany, horticulture, and applications

2.1.1 Botany and horticulture

Lingonberry (*Vaccinium vitis-idaea*) is a cold-climate shrub that grows in arctic, sub-arctic, boreal, and even temperate regions around the world. Lingonberry belongs to the *Vaccinium* genus, along with cranberry and blueberry. While generally referred to by the species name, V. vitis-idaea, there are actually 2 sub-species of lingonberry: the larger lowland race, V. vitis-idaea ssp. vitis-idaea (L.) Britton, which grows in Europe, and the dwarf arctic-montane race, V. vitis-idaea ssp. minus (Lodd) Hulten, that spreads over Iceland, Greenland, North America, northern Asia and parts of Scandinavia (Hulten. 1949). This low-growing, woody shrub can be found in densely wooded areas, sandy bogs, rocky mountain cliffs, and mossy grasslands (Hulten. 1949; Penhallegon. 2006). Its many names reflect the extensive distribution of this hardy plant (Table 2.1).

Table 2.1 Lingonberry names around the world

Language(s)	Name	Country or Region
English	Lingonberry	Worldwide
	Partridgeberry	Newfoundland & Labrador
	Lowbush cranberry	Manitoba
	Foxberry	Quebec, Nova Scotia
	Mountain cranberry	British Colombia
	Rock cranberry	Alaska, Washington
	Cowberry	United Kingdom
French	Airelle rouge	France, Quebec
German	Preiselbeere	Germany
Swedish	Lingon	Sweden
Norwegian	Tyttebær	Norway
Finnish	Puolukka	Finland
Cree	Wisakimin, ᐐᐧᐦᑭᒼ	Alberta, Saskatchewan, Manitoba
	Wiishichimanaanh	Eeyou Istchee, Northern Quebec
Anishinaabemowin	Mashkiigimin, Muhkooseemeenuhn	Manitoba, Ontario
Inupiat	Keepmingyuk	Alaska
Qawiaraq	Keepmik	Alaska
Inuktitut	Kimminnaq, Kallak, ᑲᓕᓗᒃ	Nunavut, Nunavik, Northwest Territories

Lingonberries are generally reported to be best suited to Canada's Plant Hardiness Zones 1-6, which would mean they should grow in all parts of Canada except Southwestern British Colombia. However, in Manitoba, lingonberry is rarely seen south of Zone 3a, around Lake Winnipeg. Lingonberry is incredibly hardy and can survive temperatures below -40°C, thick

snow-cover, and strong winds, so long as temperatures do not drop below freezing during blooming or fruiting (Penhallegon. 2006). Fruiting plants have even been observed in Churchill Manitoba, which is in Hardiness Zone 0. Wild lingonberry growing near Flin Flon, Manitoba is shown in Figure 2.1. Many soil types support lingonberry growth; however well-drained, acidic, sandy, loam is preferred. The shrubs are generally low-growing (under a foot tall) with dark green, oval leaves. They spread by rhizomes, meaning that their below-ground stems grow outwards under the soil and then sprout new shrubs (Penhallegon. 2006). The dwarf subspecies in Canada generally has one bloom and generates fruit once per year, usually in September or October. The European subspecies can produce two crops, in late summer and in late October or November (Heidenreich. 2010).

The bell-shaped flowers are white or light pink and the berries are dark red and about one centimeter in diameter. Good-sized berries will only grow on shrubs that have been pollinated. Wind does not seem to help pollinate lingonberry, so flying insects like bees and butterflies are very important (Penhallegon. 2006). In Europe, where lingonberry is cultivated, high-yield cultivars are usually grown near pollinizer cultivars for the best fruit yield. Established European cultivars include Red Pearl and Sussi, which are very popular because they provide high fruit yields, Erntesegen, which has a milder flavour, and Koralle, which has a sharper flavour (Penhallegon. 2006). Lingonberry, like other cultivated fruit plants, will produce better fruit when at least two varieties are planted together (Heidenreich. 2010; Penhallegon. 2006). Figure 2.2 shows green-house grown and wild Mesovia lingonberries.

Figure 2.1 Wild Manitoba lingonberry near Flin Flon

Figure 2.2 Mesovia lingonberry grown in a greenhouse (A) and in the wild (B).

A

B

2.1.2 Traditional and commercial uses for lingonberry

Lingonberry has been an important part of many cultures for hundreds of years. In Scandinavia, lingonberries are one of the most commonly consumed fruits and, while still picked from the wild, many varieties have been cultivated for higher fruit yield, pollinizer capabilities, and appearance. In fact, lingonberry was recommended in 1651 as edging in the royal gardens of Sweden's Queen Christina by the royal gardener André Mollet, author of *Le Jardin de Plaisir* (Hjalmarsson and Ortiz. 2002). Currently, lingonberry products are very common in Scandinavia and the berries are used to make jams, juices, sauces, ice creams, baked goods, wines, and even cosmetics.

In North America, lingonberry has not been commercialized as extensively as in Europe, but the berry has still been utilized for a very long time. Cree, Déné, and Inuit peoples eat wild lingonberry and use the berries and leaves medicinally to treat a variety of ailments. The berries may be consumed to relieve fever and upset stomach, or used in a hot pack to treat swelling and headache (Kuhnlein and Turner. 1991). Cree Elders from the Eeyou Istchee region of Quebec recommended lingonberry as a remedy for symptoms of diabetes when interviewed by researchers from the University of Montreal (Leduc *et al*. 2006). Using this ethnomedicinal knowledge, the researchers then tested the antidiabetic potential of the remedies suggested by the Elders and found that lingonberry stimulated glucose uptake in muscle cells and adipocytes and enhanced adipogenesis, providing evidence for the anti-diabetic properties predicted by the Elders (Harbilas *et al*. 2009). The research group also interviewed Inuit elders from the Nunavik and Nunatsiavut territories of Canada, who recommended lingonberry for the

treatment of thrush, cough, and sore throat (Clark. 2012). Yup'ik Elders from Central Alaska reported using lingonberry to treat cold, fever, flu, and breathing problems (Active *et al*. 2014).

It is currently difficult to find wild lingonberry in Canada outside of communities engaged in berry-picking; however, some Manitoba berry-pickers are now advertising their products online and shipping frozen berries to urban centers. The exception would be Newfoundland and Labrador, where a cottage industry has emerged and "partridgeberry" candies and jams can be bought in many stores.

2.2 Health benefits of lingonberry

2.2.1 Nutrition and bioactive components

Lingonberry is considered a healthy food because it is high in fibre, low in sugar and fat, and contains important vitamins and minerals. A 100 g serving of lingonberry, which is about a handful, contains only 50 calories and 1 g of fat, while supplying 4 g of dietary fibre (Technical University of Denmark. 2016). Beyond their nutritional value, lingonberry contains a variety of bioactive phytochemicals that may provide additional specific health benefits. The most studied bioactive compounds in lingonberry are the polyphenols.

Polyphenols are compounds that contain phenol units, or aromatic rings containing phenolic hydroxyl groups, that allow them to act as antioxidants. This means they can accept an electron from or donate a hydrogen atom to a reactive species while remaining as a stable entity. Antioxidant activity will be discussed further in Section 2.2.2. There are several classes of polyphenols including phenolic acids, stilbenes, lignans, and flavonoids (Figure 2.4).

Polyphenols are generally water-soluble and are often conjugated to sugars or organic acids. They are secondary metabolites formed in plants to provide pigmentation to berries and flowers, to deter herbivores through sensory properties, to combat microbial infections, to act as signaling molecules, and to protect against UV damage. In lingonberry, the five major genes controlling polyphenol biosynthesis are highly expressed during fruit colour development as the berry matures (Jaakola et al. 2002). The metabolic pathway starts with phenylalanine being converted to naringenin and then dihydrokaempferol. From these compounds, specific enzymes can catalyze conversion to quercetin and myricitin, and then cyanidin and delphinidin. Cyanidin and delphinidin are anthocyanidins that can by glycosylated and methylated to produce all five major anthocyanins, which are the glycosylated forms of anthocyanidins that give berries their colour (Jaakola *et al*. 2002). Different berries will produce different combinations and concentrations of polyphenols, as reviewed by Del Rio et al. (2010). Blueberry contains dozens of different anthocyanins each present in low to moderate concentrations while raspberry contains larger amounts of only a few anthocyanins. Cranberry contains mainly red-coloured cyanidin glycosides while strawberries contain mostly orangey-pink-coloured pelargonidin glycosides (Del Rio *et al*. 2010).

Metabolomic analyses have revealed that lingonberry contains thousands of unique compounds including amino acids, carbohydrates, organic acids, fatty acids, plant hormones, and of course, polyphenols. A list of phenolic compounds reported in lingonberry can be found in Table 2.2 and a list of non-phenolic compounds reported in lingonberry, including lipids, hormones, and amino acids, can be found in Table 2.3.

Table 2.2 Lingonberry phenolic compounds

Compound	Amount per 100 g	Origin	Detection method	Reference
Anthocyanins				
Cyanidin-3-arabinoside	6.3 mg	Cultivated, Newfoundland, Canada	HPLC-DAD	(Zheng and Wang. 2003)
	4 mg	Cultivated, Oregon, USA		(Lee and Finn. 2012)
	21 mg	Wild, British Colombia, Canada		(Brown *et al*. 2012)
	82 mg	Wild, Alaska, USA		(Grace *et al*. 2014)
	95 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Cyanidin-3-galactoside	49 mg	Cultivated, Newfoundland, Canada	HPLC-DAD	(Zheng and Wang. 2003)
	30 mg	Cultivated, Oregon, USA		(Lee and Finn. 2012)
	22.5 mg	Wild, British Colombia, Canada		(Brown *et al*. 2012)
	107 mg	Wild, Alaska, USA		(Grace *et al*. 2014)
	122 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Cyanidin-3-glucoside	1.4 mg	Cultivated, Newfoundland, Canada	HPLC-DAD	(Zheng and Wang. 2003)
	4 mg	Oregon, USA, cultivated		(Lee and Finn. 2012)
	3 mg	Wild Haida Gwaii BC		(Brown *et al*. 2012)
	5.9 mg	Wild, Alaska, USA		(Grace *et al*. 2014)
	6.6 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Peonidin-3-glucoside	4.1 mg	Cultivated, Newfoundland, Canada	HPLC-DAD	(Zheng and Wang. 2003)
Total anthocyanins	45 mg	Cultivated, Newfoundland, Canada	pH differential method	(Zheng and Wang. 2003)
	60 mg	Cultivated, Oregon, USA		(Wang *et al*. 2005)

	94 mg	Wild, Alberta, Canada		(Bakowska-Barczak *et al*. 2007)
	24 mg	Cultivated, Oregon, USA		(Lee and Finn. 2012)
	195 mg	Wild, Alaska, USA		(Grace *et al*. 2014)
	575 mg	Wild Manitoba, Canada		(Isaak *et al*. 2015)
	35-690 mg	Cultivated, Newfoundland, Canada		(Isaak *et al*. 2015)
	810 mg	Commercial, Finland	HPLC-DAD	(Viljanen *et al*. 2004)
	223 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
	257 mg	Wild, Finland	UPLC-PDA-FLR	(Kylli *et al*. 2011)
Flavanols				
Catechin	13 mg	Wild, Europe	UPLC-MS/MS	(Jungfer *et al*. 2012)
	7 mg	Wild, China		
	0.34 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Epicatechin	2.5 mg	Wild, Europe	UPLC-MS/MS	(Jungfer *et al*. 2012)
	10.7 mg	Wild, China		
	0.39 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Kaempferol	0.5 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
Kaempferol-3-glucoside	0.8 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	0.04 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	0.01 mg	Wild, Finland		
Quercetin	14.6 mg	Wild, Finland	HPLC	(Häkkinen *et al*. 1999)
	0.2-1.5 mg	Wild, Alaska, USA		(Leiner *et al*. 2006)
	0.23 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Quercetin-3-arabinoside	3 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	1.4 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	4.1 mg	Wild, Slovenia	HPLC-DAD-ESI-MS	(Mikulic-Petkovsek *et al*.

				2012b)
Quercetin-3-diglucoside	0.03 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Quercetin-3-galactoside	8.6 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	1.7 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	1.6 mg	Wild, Slovenia	HPLC-DAD-ESI-MS	(Mikulic-Petkovsek *et al*. 2012b)
	0.25 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Quercetin-3-glucoside	0.23 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	0.4 mg	Wild, Slovenia	HPLC-DAD-ESI-MS	(Mikulic-Petkovsek *et al*. 2012b)
Quercetin-3-rhamnoside	8.2 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	2 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	0.06 mg	Wild Nunavik	UPLC-MS/MS	(Dudonné *et al*. 2015)
Quercetin-3-rutinoside	0.05 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Quercetin-3-xyloside	0.34 mg	Wild, Finland	UPLC-MS/MS	(Lehtonen *et al*. 2010)
	0.6 mg	Wild, Slovenia	HPLC-DAD-ESI-MS	(Mikulic-Petkovsek *et al*. 2012b)
Quercetin-rhamnoside	2.5 mg	Wild, Slovenia	HPLC-DAD-ESI-MS	(Mikulic-Petkovsek *et al*. 2012b)
Total flavonols	76 mg	Commercial, Finland	HPLC	(Viljanen *et al*. 2004)
	163 mg	Wild, Finland	UPLC-PDA-FLR	(Kylli *et al*. 2011)
Total flavanols and proanthocyanidins	1230 mg	Wild, Finland	UPLC-PDA-FLR	(Kylli *et al*. 2011)
Total flavonols and flavanols	1.35 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Phenolic acids				
Caffeic acid	6.3 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	4 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Caffeic acid glucoside	0.07 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)

Chlorogenic acid	18.3 mg	Wild, Alaska, USA	HPLC	(Grace *et al*. 2014)
Ellagic acid	1.36 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Ferulic acid	0.9 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Gallic acid	2.85 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Hydroxybenzoic acids	15 mg	Commercial, Finland	HPLC	(Viljanen *et al*. 2004)
	9 mg	Wild, Finland	UPLC-PDA-FLR	(Kylli *et al*. 2011)
	1.43 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Hydroxycinnamic acids	32 mg	Commercial, Finland	HPLC	(Viljanen *et al*. 2004)
	78 mg	Wild, Finland	UPLC-PDA-FLR	(Kylli *et al*. 2011)
m-coumaric acid	6.7 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
p-coumaric acid	6.2 mg	Cultivated, Newfoundland, Canada	HPLC	(Zheng and Wang. 2003)
	8.3 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Protocatechuic acid	0.24 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Shikimic acid	0.08 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Total phenolics acids	26 mg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
Proanthocyanidins				
Monomers	89 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
	41 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
A-type dimers	8 mg	Wild, Europe	UPLC-MS/MS	(Jungfer *et al*. 2012)
	9.8 mg	Wild, China		
	22 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
B-type dimers	27.5 mg	Wild, Europe	UPLC-MS/MS	(Jungfer *et al*. 2012)
	21 mg	Wild, China		
	46 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
Total dimers	89 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
A-type trimers	10 mg	Wild, Europe	UPLC-MS/MS	(Jungfer *et al*. 2012)
	7.7 mg	Wild, China		
	7.3 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
B-type trimers	2.3 mg	Wild, Europe	UPLC-MS/MS	(Zheng and Wang. 2003)

	3 mg	Wild, China		
	36 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
Total trimers	43 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Tetramers	20 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
	29 mg	Wild, Nunavik, Canada		(Dudonné *et al*. 2015)
Pentamers	19 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Hexamers	14 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Heptamers	5.8 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Pentamers-heptamers	30 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
Octamers	3.7 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Nonamers	2.7 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Decamers	0.6 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Polymers	27 mg	Wild, Alaska, USA	HPLC-DAD	(Grace *et al*. 2014)
	5.5 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Total proanthocyanidins	151 mg	Commercial, Finland	HPLC-DAD	(Viljanen *et al*. 2004)
	279 mg	Wild, Alaska, USA	DMAC method	(Grace *et al*. 2014)
	253 mg	Wild, Nunavik, Canada	HPLC-DAD	(Dudonné *et al*. 2015)
Stilbenes				
Resveratrol	0.09 mg 0.01 mg	Cultivated, Newfoundland, Canada Wild, Nova Scotia, Canada	GC-MS	(Rimando *et al*. 2004)
Tannins				
Total tannins	517 mg	Cultivated, Oregon, USA	MCLP method	(Lee and Finn. 2012)
Antioxidants				
Antioxidant activity	3810 µmol TE	Cultivated, Newfoundland, Canada	ORAC method	(Zheng and Wang. 2003)
	10,600 µmol TE	Cultivated, Oregon, USA		(Wang *et al*. 2005)
	17,100 µmol TE	Wild, Alaska, USA		(Leiner *et al*. 2006)

	32,000 µmol TE	Wild, Alaska, USA		
	< 25,000 µmol TE	Cultivated, Newfoundland, Canada		(Debnath and Sion. 2009)
	15060 µmol TE	Wild, Manitoba, Canada		(Isaak *et al*. 2015)
All polyphenols				
Total phenolic compounds	1080 mg	Commercial, Finland	HPLC	(Viljanen *et al*. 2004)
	652 mg GAE	Cultivated, Newfoundland, Canada	FC reagent method	(Zheng and Wang. 2003)
	663 mg GAE	Cultivated, Oregon, USA		(Wang *et al*. 2005)
	387 mg GAE	Wild, Slovenia		(Mikulic-Petkovsek *et al*. 2012b)
	566 mg GAE	Cultivated, Oregon, USA		(Lee and Finn. 2012)
	200-800 mg GAE	Cultivated, Newfoundland, Canada		(Debnath and Sion. 2009)
	624 mg GAE	Wild, Alaska, USA		(Grace *et al*. 2014)
	577 mg GAE	Wild, Manitoba, Canada		(Isaak *et al*. 2015)

All amounts are shown per 100 g fresh weight. When amounts were reported per dry weight, a moisture content of 85% was used for conversions. Methods include high performance liquid (HPLC) coupled to diode array detector (DAD) and/or electrospray ionization mass spectrometer (ESI-MS), ultrahigh performance liquid chromatography (UPLC) coupled to photodiode array detector (PDA) or fluorescent detector (FLR), UPLC coupled to mass spectrometer (MS) or quadrupole time of flight (QTOF) MS, gas chromatography (GC) coupled to MS or flame ionization detector (FID), gas-liquid chromatography (GLC), dimethylaminocinnamaldehyde (DMAC) colourimetric detection, methylcellulose precipitation (MCLP) method, oxygen radical absorbance capacity (ORAC) assay, Folin–Ciocalteu reagent (FC) colourimetric detection, and pH differential method. Antioxidant activities were reported as trolox equivalents (TE) and phenolic contents were reported as gallic acid equivalents (GAE).

Table 2.3 Lingonberry non-phenolic compounds, including lipids, carbohydrates, and amino acids

Compound	Amount per 100 g	Origin	Detection method	Reference
Amino acids				
Alanine	1 mg	Cultivated, Oregon, USA	HPLC-DAD	(Lee and Finn. 2012)
Arginine	6.3 mg			
Asparagine	12.5 mg			
Aspartic acid	0.9 mg			
Citrulline	0.3 mg			
GABA	4.7 mg			
Glutamic acid	1 mg			
Glutamine	3.7 mg			
Glycine	0.2 mg			
Histidine	0.7 mg			
Hydroxyproline	3.1 mg			
Isoleucine	0.2 mg			
Leucine	0.3 mg			
Lysine	0.3 mg			
Methionine	0.1 mg			
Phenylalanine	0.4 mg			
Proline	2 mg			
Serine	1.8 mg			
Threonine	0.8 mg			
Tryptophan	1.4 mg			
Tyrosine	1.3 mg			
Valine	1 mg			
Total essential amino acids	3.7 mg			
Total free amino acids	44 mg			

Carbohydrates				
Fructose	2.9 g	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Glucose	3.8 g	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Sucrose	0.4 g	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Total sugar	38 mmol	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Organic acids				
Citric acid	2.2 g	Cultivated, Oregon, USA	HPLC-DAD	(Lee and Finn. 2012)
	2 g	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Fumaric acid	3.6 g	Wild, Slovenia	HPLC	(Mikulic-Petkovsek *et al*. 2012a)
Malic acid	0.2 g			
Shikimic acid	4.1 g			
Tartaric acid	0.3 g			
Total organic acids	14 mmol			
Triterpenoids				
α-amyrin	8.6 mg	Wild, Finland	GC-MS/FID	(Szakiel *et al*. 2012)
	39 mg	Wild, Poland		
α-amyrin ester	0.9 mg	Wild, Finland		
	3 mg	Wild, Poland		
β-amyrin	7 mg	Wild, Finland		
	9.6 mg	Wild, Poland		
β-amyrin ester	0.6 mg	Wild, Poland		
Betulin	1.3 mg	Wild, Poland		
Campesterol	10 mg	Wild, Finland		
	15 mg	Wild, Poland		
Campesterol ester	1.7 mg	Wild, Poland		
Cycloartanol	6 mg	Wild, Finland		
	10.3 mg	Wild, Poland		
Cycloartanol ester	7.8 mg	Wild, Finland		
	2.6 mg	Wild, Poland		
Erythrodiol	0.3 mg	Wild, Finland		

	0.1 mg	Wild, Poland		
Fernenol	30 mg	Wild, Finland		
Fernenol	24 mg	Wild, Poland	GC-MS/FID	(Szakiel *et al*. 2012)
Fernenol ester	2.4 mg	Wild, Finland		
	3.8 mg	Wild, Poland		
Friedelin	8.5 mg	Wild, Finland		
	7.4 mg	Wild, Poland		
Lupeol	9.1 mg	Wild, Finland		
	26 mg	Wild, Poland		
Oleanolic acid	215 mg	Wild, Finland		
	209 mg	Wild, Poland		
Oleanolic acid ester	0.1 mg	Wild, Finland		
	0.5 mg	Wild, Poland		
Sitosterol	133 mg	Wild, Finland		
	136 mg	Wild, Poland		
Sitosterol ester	58 mg	Wild, Finland		
	28 mg	Wild, Poland		
Stigmastadienone	9.7 mg	Wild, Finland		
	24.5 mg	Wild, Poland		
Stigmasterol ester	21 mg	Wild, Finland		
	43 mg	Wild, Poland		
Swertenol	2 mg	Wild, Finland		
	2 mg	Wild, Poland		
Taraxasterol	1.9 mg	Wild, Poland		
Ursenal	8.8 mg	Wild, Finland		
	5.6 mg	Wild, Poland		
Ursolic acid	676 mg	Wild, Finland		
	604 mg	Wild, Poland		
Ursolic acid ester	4.8 mg	Wild, Finland		
	0.9 mg	Wild, Poland		

Uvaol	0.4 mg	Wild, Finland		
	1 mg	Wild, Poland		
Sterols				
Esterified sterols	3.6 mg	Wild, Finland	GC-MS	(Yang *et al*. 2003)
Free sterols	3.6 mg			
Plant hormones				
Abscisic acid ester	17 µg	Wild, Nunavik, Canada	UPLC-MS/MS	(Dudonné *et al*. 2015)
cis-abscisic acid	4 µg			
Dihydrophaseic abscisic acid	15 µg			
Hydroxyabscisic acid	7 µg			
Neophasic abscisic acid	0.1 µg			
Phaseic abscisic acid	7 µg			
trans-abscisic acid	4.4 µg			
Neurotransmitters				
Melatonin	0.4 mg	Wild, British Colombia, Canada	LC-MS-QTOF	(Brown *et al*. 2012)
Serotonin	1.8 mg			
Fatty acids				
C12 lauric acid	0.4 mg	Wild, Alberta, Canada	GLC	(Bakowska-Barczak *et al*. 2007)
C13 myristic acid	0.6 mg			
C16 palmitic acid	3.4 mg			
C16:1 palmitoleic acid	0.1 mg			
C18 stearic acid	0.9 mg			
C18:1 oleic acid	48 mg			
C18:2 linoleic acid	79 mg			
C18:3n3 linolenic acid	96 mg			
C20 eicosanoic acid	0.7 mg			
C20:1 eicosenoic acid	0.5 mg			
C20:2 eicosadienoic acid	0.2 mg			
C22 behenic acid	1.1 mg			

C22:1 erucic acid	8 mg			
C24 lignoceric acid	1.2 mg			
Total monounsaturated fatty acids	60 mg	Commercial, Finland	Not stated	(Bere. 2007)
Total n3 polyunsaturated fatty acids	180 mg			
Total n6 polyunsaturated fatty acids	140 mg			
Total polyunsaturated fatty acids	320 mg			
Vitamins				
Ascorbic acid	7.5 mg	Wild, Finland	HPLC-DAD	(Mikulic-Petkovsek *et al*. 2012a)
	0.8 mg	Wild, British Colombia, Canada		(Brown *et al*. 2012)
Macronutrients				
Total carbohydrates	11 g	Commercial, Finland	Not stated	(Bere. 2007)
Total fat	0.5 g			
Total protein	0.7 g			
Total saturated fat	20 mg			

All amounts are shown per 100 g fresh weight. When amounts were reported per dry weight, a moisture content of 85% was used for conversions. Methods include high performance liquid (HPLC) coupled to diode array detector (DAD) and/or electrospray ionization mass spectrometer (ESI-MS), ultrahigh performance liquid chromatography (UPLC) coupled to photodiode array detector (PDA) or fluorescent detector (FLR), UPLC coupled to mass spectrometer (MS) or quadrupole time of flight (QTOF) MS, gas chromatography (GC) coupled to MS or flame ionization detector (FID), and gas-liquid chromatography (GLC).

2.2.2 Antioxidant compounds and oxidative stress

The bioactive compounds found in berries were first thought to provide health benefits due to their strong antioxidant capacity. If these compounds can act as antioxidants in the body, they may be able to reduce oxidative stress, which underlies many pathological conditions (Valko et al. 2007). Oxidative stress is defined as the imbalance between the damaging effects of reactive oxygen species and reactive nitrogen species and the body's ability to counteract these effects through detoxification or repair (Valko et al. 2007). Reactive oxygen species are highly reactive chemical compounds that contain oxygen. Oxygen is reactive because its electron configuration consists of two unpaired electrons in different orbitals. The molecule is therefore a diradical with high electron affinity (Valko et al. 2007). Some reactive oxygen species are formed naturally through metabolism of oxygen and others are produced to act as cell signaling molecules. Hydrogen peroxide for example is produced as a cell signaling molecule and through the detoxification of superoxide anions. Low levels of reactive oxygen species are needed for homeostasis, however, when reactive oxygen species overwhelm the cell's ability to dispose of them, they can damage proteins, lipids, and DNA, leading to cell dysfunction. The most common reactive oxygen species are hydrogen peroxide, superoxide, hydroxyl radical, peroxyl radical, and singlet oxygen (Valko *et al*. 2007). Cells have many ways of keeping oxidative stress in check, including antioxidant and detoxification enzymes and small antioxidant peptides. For example, catalase can break the superoxide anion down into water and the less-reactive hydrogen peroxide (Valko *et al*. 2007). Pathophysiological oxidative stress will be described further in Section 2.4.2.

The phenolic hydroxyl groups on polyphenols are excellent hydrogen donors and can react with reactive oxygen species to break the cycle of radical generation. The phenolic ring structure allows the compound to donate a hydrogen atom or accept an electron while remaining stable by sharing the charge throughout the phenolic ring structure, thus breaking the chain of free radical reactions before cell structures are damaged (Prior. 2015). Since many polyphenols can directly scavenge reactive oxygen species, consuming berries that are rich in polyphenols may provide antioxidant effects *in vivo* and protect against the damaging effects of oxidative stress (Prior. 2015; Valko *et al*. 2007). The direct antioxidant capacity of berry compounds can be measured using *in vitro* chemical assays like the Oxygen Radical Absorbance Capacity (ORAC) assay, the Ferric Reducing Antioxidant Capacity (FRAP) assay, and the Total Phenolics assay. These assays are designed so that the rate of free radical scavenging can be colourimetrically or fluorescently determined and compared to an antioxidant standard (Huang *et al*. 2005). Whole berry extracts or individual compounds can be tested for antioxidant activity and comparisons can be made between samples (Balogh *et al*. 2010). Generally, berry extracts have high direct antioxidant activity compared to other fruits and vegetables because they contain more polyphenols.

However, just because a compound acts as an antioxidant in vitro does not mean that the compound will directly influence oxidative stress in vivo. The compound may not even reach tissues or organs in its intact form due to post-absorption metabolism (Croft. 2016). However, it is possible that direct reactive oxygen species scavenging by either intact compounds or phenolic metabolites may contribute to the protective effects of polyphenols, since *in vitro* studies show that intracellular oxidative stress is inhibited after treatment with

polyphenols (Mane *et al*. 2011) and dietary polyphenol intervention studies in humans have demonstrated positive effects on oxidative stress biomarkers, including urinary by-products of lipid oxidation (Annuzzi *et al*. 2014; Prior. 2015). Other mechanisms of the protective effects of polyphenols will be discussed in Section 2.2.4.

2.2.3 Evidence for the health benefits of berry compounds and lingonberries

Epidemiological studies have demonstrated an inverse association between higher flavonoid and anthocyanin intakes and risk of chronic diseases including type 2 diabetes, cardiovascular disease, Parkinson's disease, and cancer. The Health Professionals Follow-Up Study of 43,880 healthy men found that higher intakes of flavonoids from fruit were associated with a lower risk of myocardial infarction and ischemic stroke (Cassidy *et al*. 2016). The Nurses' Health Study of 82,643 healthy women found that higher intakes of flavones and proanthocyanidins were associated with lower risk of depression (Chang *et al*. 2016). A substudy of the TwinsUK Registry of 1,997 women found that higher intakes of anthocyanins and flavones were associated with lower biomarkers for insulin resistance and inflammation (Jennings *et al*. 2014). A second substudy of the TwinsUK registry of 2,734 women found that higher anthocyanin intakes were associated with lower limb-to-trunk fat mass ratios (Jennings *et al*. 2017). A cross-sectional analysis of 2,375 participants in the Framingham Heart Study Offspring Cohort found that higher anthocyanin intakes were associated with lower levels of twelve inflammatory biomarkers (Cassidy *et al*. 2015). The Veterans Affairs Normative Aging Study of 839 elderly men found that higher anthocyanin intakes were associated with lower risk of age-related lung function decline (Mehta *et al*. 2016).

A meta-analysis of prospective cohort studies, including three that reported anthocyanin intake for a total of 200,894 participants and five that reported berry intake for a total of 194,019 participants, found that higher intakes of anthocyanins and berries were associated with lower risk of type 2 diabetes (Guo *et al*. 2016). A meta-analysis of fourteen prospective cohort studies found that higher intakes of flavonoids including anthocyanins were associated with lower risk of cardiovascular disease events (Wang *et al*. 2014b). A meta-analysis of seven prospective cohort or case-control studies found that higher intakes of anthocyanins were associated with lower risk of esophageal cancer (Cui *et al*. 2016). A meta-analysis of the Health Professionals Follow-Up Study, the Nurses' Health Study, and the Nurses' Health Study II including a total of 124,086 participants found that increased consumption of anthocyanins was associated with better weight management (Mehta *et al*. 2016). While these studies do control for factors such as age and health status, more research may be needed to determine the independence of the effect of anthocyanin consumption on disease risk.

Interventional studies have also demonstrated the health benefits of berries. There are many excellent reviews that describe the health benefits of commonly consumed berries and review the evidence from nutritional clinical trials (Afrin *et al*. 2016; Kalt. 2016; Shi *et al*. 2017; Yang and Kortesniemi. 2015). A meta-analysis of twenty-two randomized controlled trials studying the effects of berry consumption on cardiovascular risk factors revealed that higher berry consumption significantly reduced low density lipoprotein cholesterol levels, systolic blood pressure, fasting glucose, body mass index, and plasma TNFα levels (Huang *et al*. 2016).

There is *in vitro*, *in vivo*, and clinical evidence for the health benefits of lingonberry. *In vitro*, lingonberry polyphenols inhibit LPS-induced IL-1β, IL-6, and TNFα expression in

macrophages (Grace *et al*. 2014; Kylli *et al*. 2011), inhibit carbohydrate degrading enzymes and lipases which may reduce absorption of carbohydrates and lipids *in vivo* (Podsędek *et al*. 2014), inhibit microbial growth (Davidson *et al*. 2014), inhibit neuronal cell death and protect against amyloid-β accumulation (Bhullar and Rupasinghe. 2015b; Hossain *et al*. 2016), activate antioxidant enzyme expression (Bhullar and Rupasinghe. 2015a), and inhibit diabetic processes in skeletal muscle cells, adipocytes and pancreatic cells (Harbilas *et al*. 2009).

Animal models have been used to show the *in vivo* health benefits of lingonberry. Spontaneously hypertensive rats fed lingonberry juice for 8 weeks had improved vascular function (Kivimäki *et al*. 2011), reduced aortic inflammation, reduced plasma biomarkers of inflammation (Kivimäki *et al*. 2012), and reduced systolic blood pressure (Kivimäki *et al*. 2013). Rats fed a high-salt diet and supplemented with lingonberry juice had reduced kidney inflammation (Kivimäki *et al*. 2014). Rats fed a high-fat diet supplemented with a lingonberry extract had increased antioxidant enzyme expression in red blood cells and the liver (Mane *et al*. 2011). Mice fed a high-fat diet supplemented with lingonberries had lower levels of plasma biomarkers of inflammation (Heyman-Lindén *et al*. 2016a) and genome-wide hepatic gene expression profiling revealed that genes associated with inflammatory pathways were downregulated through genome-wide and specific hypermethylation (Heyman-Lindén *et al*. 2016b). In a follow-up study the researchers linked some of the hepatic metabolic improvements in the high-fat diet-fed mice with specific changes in the plasma metabolome following lingonberry consumption (Al Hamimi *et al*. 2017).

There have only been a few clinical trials investigating the health benefits of lingonberry consumption in humans. Consuming lingonberries optimized the post-prandial metabolic

response to sucrose (Törrönen *et al*. 2012) and glucose but not lipids (Linderborg *et al*. 2012) in healthy participants despite the natural sugars present in the berries. The postprandial response to wheat bread was also improved with lingonberry consumption (Törrönen *et al*. 2013). A blinded, randomized, placebo-controlled intervention trial of 72 healthy participants found that consumption of a moderate amount of berries including lingonberry for 8 weeks improved platelet function, blood pressure, and high density lipoprotein levels (Erlund *et al*. 2003).

2.2.4 Absorption, metabolism, and bioavailability of berry compounds

There are several excellent reviews of the fate of polyphenols in the body after consumption. Because berries are particularly rich in anthocyanins this section will focus on the fate of these compounds only. Briefly, metabolism of anthocyanins begins in the oral cavity where microbial enzymes start the deglycosylation process to produce aglycone anthocyanidin compounds from the parent anthocyanins (Lila et al. 2016). Absorption begins in the stomach, which explains the rapid appearance of anthocyanins and anthocyanidins in plasma. Bilitranslocase has been identified as one carrier enzyme responsible for translocating anthocyanins from the stomach lumen to the portal circulation (Lila *et al*. 2016). Metabolism of anthocyanins is minimal in the stomach and the low pH appears to stabilize parent compounds. Over 90% of polyphenols and anthocyanins from blueberry were recovered after simulated gastric digestion whereas intestinal digestion may eliminate almost all parent compounds (Correa-Betanzo *et al*. 2014). Once in the small intestine, digestive enzymes first hydrolyze any remaining sugar moieties to produce aglycones that are more hydrophobic and may pass more

easily into the epithelial cells lining the intestine via passive diffusion. GLUT transporters and intestinal bilitranslocases may assist with intestinal absorption (Lila *et al*. 2016). Next, the large intestine provides a large surface for further anthocyanin absorption. Any remaining anthocyanins may be catabolized by microbial enzymes to smaller phenolic acids which can be either absorbed or excreted (Lila *et al*. 2016).

Parent anthocyanins and anthocyanidins that are absorbed from the stomach, small intestine, and large intestine show up rapidly in plasma. They are quickly taken up by the liver and kidneys where they are methylated, glucuronidated, sulfated, or otherwise conjugated and either excreted or returned to the circulation (Lila et al. 2016; Vanzo et al. 2011). For example, cyanidin-3-glucoside can be taken up by the liver and kidneys less than one minute from appearance in plasma and the methylated product, peonidin-3-glucoside can be detected in plasma, kidneys, and liver 15 seconds later (Vanzo *et al*. 2011). To summarize, parent anthocyanins, deglycosylated anthocyanidins, and pre or post-absorption metabolites including anthocyanin conjugates and small phenolic acids may all be present in plasma and tissues.

Most studies report the bioavailability of anthocyanins as less than 1% of the ingested dose and the maximal plasma concentration of anthocyanins as less than 100 nmol L^{-1} (Kay *et al*. 2017; Lila *et al*. 2016). However, because there is a large body of epidemiological evidence for the health benefits of anthocyanins, either anthocyanins are extremely potent or their bioavailability has been underestimated. Bioavailability may be underestimated due to the inherent instability of anthocyanins during sampling and lack of detection of conjugated anthocyanins (Kay *et al*. 2017). Using isotopically-labelled cyanidin-3-glucoside, Czank et al. (2013) showed that the true bioavailability of anthocyanins is likely much higher. 12% of

ingested cyanidin-3-glucoside was bioavailable and the maximum serum concentration of all metabolites reached 6 µmol L^{-1}, while the maximum serum concentration of just the intact cyanidin-3-glucoside was only 140 nmol L^{-1} (Czank et al. 2013). Following the labelled compound through absorption and metabolism revealed that many the labelled molecules show up in plasma and tissues as small phenolic metabolites such as protocatechuic acid, ferulic acid, vanillic acid, and hippuric acid. These metabolites themselves may be glucuronidated, sulfated, and methylated. The researchers concluded that anthocyanin bioavailability is higher than previously thought and metabolites likely explain the significant health benefits of anthocyanin despite the low perceived bioavailability of the parent compounds (Czank et al. 2013).

Only a handful of studies have examined the bioavailability of polyphenols and anthocyanins following lingonberry consumption. Healthy middle-aged men and women who consumed 160 g of mixed berries including lingonberries for 8 weeks had significantly increased plasma levels of quercetin and several small phenolic acids compared to a control group (Koli *et al*. 2010). Healthy young men and women who consumed 300 g of lingonberries had increased plasma levels of kaempferol and quercetin conjugates (Lehtonen *et al*. 2010). The most abundant metabolites of lingonberry anthocyanins found in plasma and tissue are protocatechuic acid, vanillic acid, hippuric acid, ferulic acid, hydroxybenzoic acid, coumaric acid, and sinapic acid (Koli *et al*. 2010; Lehtonen *et al*. 2013; Nurmi *et al*. 2009). Because lingonberry contains mainly cyanidin glycosides, the most abundant anthocyanins in plasma after lingonberry consumption would be the intact parent cyanidin glycosides and the methylated

forms of these anthocyanins: peonidin-3-glucoside, peonidin-3-galactoside, and peonidin-3-arabinoside (Kay et al. 2017).

2.2.5 Potential mechanisms of the health benefits of berry compounds

The health benefits of berries are often attributed to the polyphenolic compounds they contain. This literature review focused on the potential health benefits of berry polyphenols but it should be noted that berries also contain fibre, healthy fats, vitamins, and minerals and are also low in sugar. The health benefits of replacing part of the diet or supplementing an already healthy diet with berries could be partially explained by these attributes.

It is unlikely that the polyphenols found in berries act as direct antioxidants in tissues and organs of the human body. It is more likely that polyphenols and their metabolites act as signaling molecules in the body (Croft. 2016; Li *et al*. 2017). Some mechanisms of polyphenol bioactivities for which there is mounting evidence include:

1. Improving endothelial function and lowering blood pressure by increasing nitric oxide bioavailability via direct inhibition of NADPH oxidase (Edwards *et al*. 2015; Rodriguez-Mateos *et al*. 2013);
2. Stimulating AMPK activity to switch on catabolic processes and activate endothelial nitric oxide synthase (Li *et al*. 2017; Talagavadi *et al*. 2016);
3. Activating the nuclear factor erythroid 2–related factor (Nrf) 2 signaling through pro-oxidant reactions with Keap1 leading to the expression of antioxidant and detoxification enzymes (Bayele *et al*. 2016; Bhullar and Rupasinghe. 2015a; Cardozo *et al*. 2013);

4. Inhibiting NFκB-mediated pro-inflammatory gene expression through Nrf2-dependent indirect antioxidant effects and Nrf2-independent mechanisms (Lee *et al*. 2014; Ma *et al*. 2015; Nair *et al*. 2014);
5. Inhibiting MAPK signaling leading to decreased expression of pro-apoptotic or pro-inflammatory proteins (Isaak *et al*. 2017b; Vendrame and Klimis-Zacas. 2015);
6. Modulating cholesterol homeostasis to increase the GSH/GSSG ratio and redox status in liver and kidney (Vanzo *et al*. 2013).

This list is not exhaustive and there are likely other ways that polyphenol metabolites affect cell signaling and gene expression. The large range of cellular targets of polyphenols seems fitting considering the broad health benefits associated with polyphenol-rich diets.

2.3 Programmed cell death

2.3.1 Apoptosis

Apoptosis is a form of cell death where the cell dismantles itself and packages its contents into vesicles so that neighbouring cells and tissues are not exposed to pro-inflammatory or damaging effects of cellular debris. The packages can then be cleared by immune cells (Taylor et al. 2008). Apoptosis requires energy and can only proceed so long as the cell has sufficient energy stores or sources to power the proteases and enzymes required for cell dismantling. If energy stores are depleted or the injury is too severe, the cell may die via necrosis, where membrane integrity is lost and the cell's contents spill into surrounding tissue causing a more severe immunogenic response (Dorn. 2013; Taylor *et al*. 2008).

Apoptotic cell death has many triggers but is tightly controlled by multiple signaling pathways and anti-apoptotic proteins. Apoptosis may be activated via extrinsic and intrinsic pathways (Taylor et al. 2008). Oxidative stress, calcium overload, and extensive DNA damage can all trigger the intrinsic pathway of apoptosis which is mediated by the mitochondria. Regardless of the mechanism of mitochondrial stress, the injured, permeable mitochondria release pro-apoptotic proteins from the intermembrane space into the cytosol. Cytosolic cytochrome c initiates the formation of the apoptosome, a structure where pro-caspase 9 can be activated. Caspase-9 is an initiator caspase that activates executioner caspases-3 and 7. Released pro-apoptotic transcription factors can translocate to the nucleus to activate transcription of apoptotic proteins (Taylor *et al*. 2008).

The extrinsic pathway is activated when specific ligands, like tumour necrosis factor (TNF)-α and Fas bind to cell membrane receptors (Xia et al. 2016). The cytokine TNFα can be released from resident immune cells that sense tissue damage and binds the TNF receptor. The Fas ligand is displayed on neighboring injured or apoptotic cells and binds the Fas receptor. Both receptors can activate pro-apoptotic signaling cascades and can activate initiator caspase 8 which activates executioner caspases (Xia *et al*. 2016).

Executioner caspases cleave proteins at specific sites to either promote or abolish their function (Fischer et al. 2003). Targets which are activated by cleavage are pro-apoptotic Bax and Bad proteins, interleukin (IL)-1β and IL-18 cytokines, and pro-apoptotic kinases. Targets which are inactivated by cleavage include anti-apoptotic Bcl proteins, transmembrane cell adhesion molecules which would disrupt cell-cell contacts, cytoskeletal proteins which would induce membrane blebbing and cytoskeletal reorganization, nuclear membrane proteins which

would promote nuclear condensation and disassembly, cell-cycle mediators, and DNA synthesis and repair enzymes. For example, cleavage of actin and gelsolin breaks down the cytoskeleton, lamin cleavage results in nuclear fragmentation, and cleavage of inhibitor of caspase-activated DNase leads to DNA fragmentation (Fischer *et al*. 2003; Taylor *et al*. 2008).

Through the activities of these caspases and other pro-apoptotic proteins, the cell will dismantle itself in a controlled and contained way so that cellular debris is not released to activate apoptosis or inflammation in neighboring cells (Taylor *et al*. 2008). Apoptotic cardiomyocytes condense their nuclei, hydrolyze DNA into short segments, and fragment their nuclei to package the DNA. Other organelles including mitochondria may also be fragmented. A large portion of the proteome is targeted for proteolysis, cytoskeletal structures are dismantled, and the cells become rounded and lose their connections to neighbouring cells and the extracellular matrix (Taylor *et al*. 2008). Finally, the plasma membranes form “blebs” so that the cells’ contents can be packaged into vesicles and these vesicles display specific surface antigens and release chemoattractant molecules so they can be recognized by immune cells and cleared (Taylor *et al*. 2008).

2.3.2 Necroptosis

In response to certain stressors, some cells manifest morphological characteristics that differ from classical apoptosis but still seem to be highly regulated (Vandenabeele et al. 2010). A necroptotic cell may exhibit swollen organelles, translucent cytoplasm, dilatation of the nuclear membrane, chromatin condensation, an increase in cell volume, and plasma membrane disruption. However, necroptosis has been shown to involve cessation of metabolic processes

and loss of energy stores needed for packaging of cell contents as occurs in apoptosis (Dorn. 2013). Therefore, the cell components cannot be packaged into membrane-bound bodies and necroptosis becomes an immunogenic method of cell death (Vandenabeele *et al*. 2010).

Necroptosis, or programmed necrosis, may be initiated by the same receptors involved in the extrinsic pathway of apoptosis, including the Fas receptor, TNF receptor 1, TNF receptor 2, TNF-related apoptosis-inducing ligand receptor 1 and 2, and Toll-like receptors (TLR) (Vandenabeele et al. 2010). Once a death receptor, for example TNF receptor 1, has been activated, several proteins are recruited to the intracellular domain to form a structure named Complex I (Ashkenazi and Salvesen. 2014). Complex I may include TNF receptor-associated death domain (TRADD), receptor-interacting protein 1 (RIP1), cellular inhibitor of apoptosis 1 and 2, and TNF receptor-associated factor 2 and 5. Complex I assembly can signal for survival by activating NFκB or can signal cell death by recruiting NADPH oxidase 1. TNF receptors can also be internalized when bound by its ligand, allowing the formation of the complex II, or the intracellular death-inducing signaling complex (DISC) (Ashkenazi and Salvesen. 2014).

DISC signaling can lead to apoptosis or necroptosis. In both cases, TRADD and Fas-associated protein with death domain (FADD) are recruited, which then recruit pro-caspase-8 (Ashkenazi and Salvesen. 2014). If caspase-8 activity is not inhibited, caspase-8 will activate effector caspases and apoptosis will ensue but the cell will prevent necroptosis through RIP1 and RIP3 cleavage-induced inhibition. If caspase-8 is inhibited, there is no check on RIP1, which will initiate the formation of the necrosome by phosphorylating RIP3. The necrosome consists of RIP1, RIP3, FADD, and caspase-8 and transduces signals throughout the cell leading to the execution of necroptosis (Ashkenazi and Salvesen. 2014; Dickens *et al*. 2012; Zhou *et al*. 2012).

While the initiation stage of necroptosis has been somewhat elucidated, the execution phase is less well characterized (Vandenabeele et al. 2010). It involves mitochondrial fragmentation, unchecked ATP consumption, active ATP depletion through inhibition of mitochondrial adenine nucleotide translocase, reactive oxygen species production, calcium-dependent mitochondrial permeability transition pore, increase in the labile iron pool, lipid peroxidation, and lysosome membrane permeabilization. Because of the cessation of metabolic processes, the cell components cannot be packaged into membrane-bound bodies and necroptosis is immunogenic (Vandenabeele *et al*. 2010; Zhou *et al*. 2012).

2.4 IR injury and oxidative stress in the heart

2.4.1 Pathophysiology of cardiac IR injury

IR injury occurs in the heart when blood flow to the heart muscle is compromised or completely occluded (ischemia) followed by therapeutic restoration of blood flow (reperfusion) (Eltzschig and Eckle. 2011). This can occur during a myocardial infarct which is most often caused by coronary atherosclerosis with thrombus (Burke and Virmani. 2007). This means that a coronary artery, or a vessel that supplies blood to the cardiac tissue, becomes obstructed due to a build-up of plaque in the vessel wall. The plaque deposit can rupture and cause immune cells to form a blood clot at the site of rupture, blocking blood flow through the artery. IR injury could also occur during surgeries requiring cardiopulmonary bypass or because of non-atherosclerotic thrombus (Burke and Virmani. 2007). Ideally, rapid intervention by health care professionals can restore blood flow to the heart muscle either by chemically or surgically removing the blockage.

Cardiomyocyte cell death occurs during both ischemia and reperfusion and the severity of injury to the heart muscle reflects the extent of cardiomyocyte death (Chiong et al. 2011). During ischemia, nutrients and oxygen are not delivered to the muscle tissue, nor are wastes removed. Normally, cardiomyocytes derive 60-90% of their energy needs from aerobic metabolism, mainly through oxidation of fatty acids, but also from oxidation of pyruvate and lactic acid (Burke and Virmani. 2007). When oxygen is not readily available, cardiomyocytes quickly switch to anaerobic glycolysis to produce adenosine triphosphate (ATP), but this method of energy production is not enough to fulfill energy needs for long and generates protons and lactic acid, which lowers the pH of the cell (Burke and Virmani. 2007). The cell attempts to remove protons using the sodium hydrogen exchanger and ATP depletion also impedes sodium-potassium ATPase activity, which together lead to increased intracellular sodium and cell swelling. High intracellular sodium activates the sarcolemmal sodium calcium exchanger which increases cytoplasmic calcium and compromises contractility (Burke and Virmani. 2007). Since the mitochondria are unable to transfer electrons to oxygen for ATP generation, the mitochondria produce free radicals (Sanada *et al*. 2011). To summarize, ischemia causes ATP depletion, intracellular acidosis, increased intracellular sodium and calcium, free radical production, cell swelling, and compromised contractility.

Cell death during reperfusion is usually more pronounced than during ischemia. High intracellular calcium during ischemia activates calpain proteases which degrade cytoskeletal structures, but their activation is inhibited by low pH so their activity increases greatly upon reperfusion, when the intracellular pH is quickly corrected (Garcia-Dorado et al. 2012). The normalized pH also creates a gradient for sodium hydrogen exchange, causing a massive influx

of sodium which forces the cell membrane sodium calcium exchanger to work in reverse, leading to intracellular calcium overload (Garcia-Dorado et al. 2012). Calcium overload can trigger apoptosis by opening the mitochondrial permeability transition pore leading to mitochondrial swelling and release of pro-apoptotic proteins like cytochrome c and Bax. Calcium overload can lead to necrosis if these mitochondrial dysfunctions are sustained or if phospholipases are activated and can also cause arrhythmias due to cell-to-cell electrical uncoupling (Garcia-Dorado *et al*. 2012). During reperfusion, oxygen delivery is restored but the oxygen molecules can combine with accumulated free radicals to form reactive oxygen species (Sanada et al. 2011). Oxidative stress can trigger apoptosis by helping to propagate mitochondrial permeability through membrane lipid peroxidation and by activating NFκB and MAPK signalling cascades (Javadov *et al*. 2014). Calcium overload also activates enzymes like xanthine oxidase which produce more reactive oxygen species (Raedschelders *et al*. 2012). To summarize, reperfusion causes oxidative stress, calcium overload, arrhythmia, and activation of apoptotic and necrotic processes.

Because cardiomyocytes contain so many mitochondria to fulfill the energy requirements of the heart muscle, and because mitochondria are potent mediators of apoptosis, cardiomyocytes are better able to supress apoptosis than other types of cells (Chiong *et al*. 2011). This is reflected in the negligible levels of apoptosis in healthy cardiac tissue and low numbers, less than 1%, of apoptotic cells even in failing hearts. However, because cardiomyocytes have extremely limited capacity for regeneration and repair, even very low levels of apoptosis could significantly affect heart function. An apoptotic rate of 0.1% could

result in a loss of almost 40% of all cardiomyocytes in one year (Chiong *et al*. 2011). Therefore, minimizing cell death is an important aspect to preventing IR-induced cardiac injury.

2.4.2 Oxidative stress in IR-injured cardiomyocytes

Reactive oxygen species were first observed in IR injury in the 1980's using electron spin resonance spectroscopy. Spin trapping experiments then identified superoxide as the parent radical that is generated during reperfusion and leads to the formation of other oxygen radicals (Raedschelders *et al*. 2012). Reactive oxygen species are produced during ischemia and then there is second "burst" of oxygen radical production upon reperfusion (Sanada *et al*. 2011). Important reactive oxygen species produced during IR are superoxide and the hydroxyl radical.

Superoxide is produced when electrons that have leaked from the ATP-depleted mitochondrial electron transport chain combine with oxygen (Braunersreuther and Jaquet. 2012). Superoxide produced at the mitochondria is especially dangerous to mitochondrial membrane lipids, mitochondrial matrix proteins, and mitochondrial DNA because these components are near the source of superoxide. Superoxide can peroxidise membrane lipids like cardiolipin, which constitutes about one fifth of the inner mitochondrial membrane. Cardiolipin is necessary for cytochrome c oxidase activity, which oxidizes cytochrome c so it can scavenge superoxide in the inner membrane space. Inhibition of cytochrome c oxidase increases the reduced form of cytochrome c and superoxide accumulates and can diffuse into the cytosol (Raedschelders *et al*. 2012). Superoxide can also be produced by xanthine oxidase, which predominates over the xanthine dehydrogenase form of the enzyme due to ischemia-induced proteolytic cleavage, and by NADPH oxidase expressed in both cardiomyocytes and infiltrating

neutrophils (Raedschelders *et al*. 2012). High intracellular calcium activates constitutively expressed nitric oxide synthase, and nitric oxide can react with superoxide to produce peroxynitrite, which is extremely toxic and reactive (Braunersreuther and Jaquet. 2012).

Hydroxyl radicals are also very reactive and can be produced through iron-catalyzed reduction and from peroxynitrite decomposition (Raedschelders *et al*. 2012). Free iron, released due to oxidative damage to iron sulfur-containing enzymes or due to lysosomal damage, can react with hydrogen peroxide, produced by superoxide dismutase or monoamine oxidase, to produce hydroxyl radicals. Most hydroxyl radicals, however, are produced through protonation of peroxynitrite which occurs rapidly under acidotic conditions (Raedschelders *et al*. 2012).

Reactive oxygen species can damage membrane bilayers because peroxidised fatty acids can reduce the integrity of the bilayer and propagate the free radical reaction to membrane proteins (Raedschelders *et al*. 2012). Damage to sarcoplasmic reticulum membranes can lead to the formation of arrhythmias and impairment of contractility. Damage to mitochondrial membranes can lead to release and activation of pro-apoptotic proteins. Oxidation reactions can also denature proteins by breaking peptide bonds and modifying functional groups. If contractile proteins are damaged, contractility will be impaired. Reactive oxygen species can react with and activate pro-apoptotic proteins, activate proteolytic enzymes that degrade proteins, and degrade tyrosine residues, compromising tyrosine kinase signaling (Raedschelders *et al*. 2012).

Induction of mitochondrial membrane permeability may be the most detrimental effect of reactive oxygen species generation (Raedschelders *et al*. 2012). Mitochondrial oxidative stress, high intracellular calcium, and physiological pH achieved during reperfusion initiates

mitochondrial permeability transition. Pores form in the inner membrane, inhibiting oxidative phosphorylation and increasing intermembrane pressure, causing the matrix to swell and reducing the integrity of the outer membrane. Peroxidised cardiolipin releases cytochrome c into the cytoplasm which activates pro-apoptotic signaling cascades (Raedschelders *et al*. 2012). Mitochondrial permeability can determine cell fate. If the outer membrane remains intact, the cell may survive. If permeability is sustained but the cell can maintain ATP levels, apoptosis will proceed. If permeability is sustained and ATP stores are depleted, the cell may succumb to necrosis (Eltzschig and Eckle. 2011; Garcia-Dorado *et al*. 2012).

2.4.3 JNK signaling in IR-injured cardiomyocytes

IR can be simulated in primary or immortalized cardiomyocytes using a hypoxic chamber and buffers designed to simulate the extracellular environment (Isaak et al. 2015; Sun et al. 2012). Cell death due to IR injury and the signaling cascades associated with apoptosis have been studied extensively in cultured cardiac cells. Several signaling pathways are known to be activated during IR injury, including the phosphoinositide 3-kinase/Akt/protein kinase (PK) B pathway, the integrin/focal adhesion kinase pathway, the mitogen-activated protein kinase (MAPK) pathway, PKA pathway, PKC pathway, and TNFα/nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway, and all are implicated in cardiomyocyte apoptosis (Xia *et al*. 2016).

MAPK's are serine/threonine kinases that regulate cell growth, differentiation, and survival in response to extracellular signals (Weston and Davis. 2007). There are three main families of MAPK's: extracellular signal-regulated kinase, p38 kinase, and c-Jun N-terminal

kinase (JNK). All three are activated by phosphorylation by upstream MAPK kinases (MAPKK) which are activated by MAPK kinase kinases. This review will focus on the JNK pathway as this thesis tested the hypothesis that berry compounds modulate JNK signaling. JNK1 and 2 are widely expressed but JNK3 is limited to neuronal tissues, testes, and cardiac myocytes. JNK1 and 2 deletions are lethal in embryo (Weston and Davis. 2007). JNK is activated by MAPKK4 and MAPKK7 and both are required for maximal JNK activation. The upstream kinase pathway can be activated by oxidative stress, hypoxia, calcium overload, or cell surface receptors responding to extracellular ligands like cytokines and growth factors (Weston and Davis. 2007; Xia *et al*. 2016).

The regulation, activities, and outcomes of JNK signaling are complicated and at times, seem paradoxical. This is because JNK may promote or protect against disease progression depending on the model, cell type, and JNK isoform and the same biological process in different cell types may have opposite effects on disease progression (Solinas and Becattini. 2017). Using an atherosclerosis model as an example, prolonged JNK activation promotes cell death in both endothelial cells and macrophages, but death of endothelial cells would promote atherosclerosis while macrophage cell death would slow the formation of atherosclerotic lesions (Solinas and Becattini. 2017).

Once activated in stressed cells, JNK phosphorylates a variety of protein targets including c-Jun, activating transcription factor 2, and p53 (Weston and Davis. 2007). Phosphorylated c-Jun forms heterodimeric transcription factors called activator protein (AP)-1 that translocate to the nucleus to activate expression of pro-apoptotic or pro-inflammatory genes (Shaulian and Karin. 2002). For example, the promoters of FasL, p53, vascular cell

adhesion molecule 1, intracellular adhesion molecule 1, E-selectin, monocyte chemoattractant protein (MCP)-1, IL-1β, and IL-6 all contain AP-1 binding sites. AP-1 also has homeostatic roles in cell growth and differentiation and is constitutively expressed for these purposes (Ahmed *et al*. 2009; Shaulian and Karin. 2001; Vukic *et al*. 2009).

Transient JNK activation has been linked to cell survival and stress tolerance while sustained or intense JNK activation usually leads to cell death (Wei *et al*. 2011). This dual action has been demonstrated in several models including IR injury, TNF stimulation, DNA damage, and endoplasmic reticulum stress. For example, inhibiting JNK1 during short periods of cardiac ischemia in mice causes wide-spread cell death but, when ischemia is extended to 20 minutes, JNK1 inhibition reduces infarct size (Wei *et al*. 2011). JNK1 can activate Akt/PKB by phosphorylating threonine residue 450 but can also negatively regulate Akt/PKB by phosphorylating insulin receptor substrate (IRS)-1 serine residue 307, which would reduce Akt/PKB activation. During transient stress, JNK1 seems to phosphorylate both Akt/PKB and IRS-1, and Akt/PKB activity would persist, leading to expression of cell survival-related proteins. During prolonged stress, JNK1 phosphorylates IRS-1 but not Akt/PKB and Akt/PKB activity would be suppressed. Two possible reasons for the dichotomy of effects of JNK activation in IR injury are the depletion of ATP (Lee *et al*. 2017) and the accumulation of intracellular reactive oxygen species (Berdichevsky *et al*. 2010), both of which would alter kinase kinetics during sustained ischemia and provide an interesting link between pro-apoptotic JNK signaling and the metabolic atmosphere of the cell. Varying localization of JNK may also explain the contrasting effects during transient and sustained ischemia, since oxidative stress caused by sustained ischemia

can promote translocation of JNK to the mitochondria where JNK can induce release of pro-apoptotic proteins like Smac into the cytosol (Javadov *et al*. 2014).

2.5 Ischemic acute kidney injury (AKI)

2.5.1 Causes of AKI

AKI can be classified as pre-renal injury, intra-renal injury, or post-renal injury (Sharfuddin and Molitoris. 2011). Pre-renal injury is caused by a decrease in effective blood flow to the kidneys, as would occur with hemorrhage, sepsis, heart failure, surgery and kidney transplant. Intra-renal injury is caused by intrinsic kidney failure associated with damage to the glomeruli, microvasculature, tubules, or interstitium. Post-renal failure is caused by urinary tract obstruction (Sharfuddin and Molitoris. 2011). Pre-renal AKI due to IR is a common post-operative complication of major surgeries such as kidney transplants and coronary artery bypass grafting. Complete cessation of blood flow, called global ischemia, would occur during kidney transplant and some major surgical procedures while compromised blood flow can occur during more common surgeries, heart failure, vascular disease, low blood pressure, low blood volume, and some infections (Sharfuddin and Molitoris. 2011). The kidneys receive up to 25% of all cardiac output and this blood flow is essential to maintaining kidney function (Uchino *et al*. 2005).

AKI is characterized by a rapid decrease in the glomerular filtration rate (GFR) of the kidneys, but the severity of the injury has been defined and classified in many ways (Kellum *et al*. 2002). The most diagnostically relevant definitions are provided by the AKI Network (Bellomo *et al*. 2004) and the Kidney Disease: Improving Global Outcomes Clinical Practice

Guideline (Kellum *et al*. 2012; Levin *et al*. 2013). These systems characterize AKI severity based on increase of serum creatinine, decline in urine output, and the need for renal replacement therapy. Controversies associated with these definitions and their use in clinical practice include over-diagnosis, lack of distinction between "disease" and "pre-disease", methodological issues, biomarker validity, and inability to provide prognoses (Levey *et al*. 2011). Some question the usefulness of the AKIN system in geriatric patients due to lack of predictive power in the context of multiple comorbidities (Chao *et al*. 2012). To this end, research has focused on improved diagnostic tools and biomarker validation, from which neutrophil gelatinase-associated lipocalin (NGAL) has been identified as a putative biomarker (Shemin and Dworkin. 2011) and diagnostic kidney panels have been proposed (Soni *et al*. 2011).

2.5.2 Epidemiology of AKI

AKI continues to be a major form of kidney disease with poor clinical outcomes and high mortality rates (Uchino *et al*. 2005). It is estimated that millions of people worldwide die each year of all forms of AKI. Although there has been a slight decline in mortality associated with AKI, the degree of comorbidity has increased (Waikar *et al*. 2006) and the incidence of AKI has increased in both adults (Xue *et al*. 2006) and children (Andreoli. 2008). Among hospitalized patients, AKI is associated with longer hospital stays, increased mortality and increased likelihood of needing post-hospitalization care (Liangos *et al*. 2006). In a multinational study, mortality among patients admitted to intensive care units with acute renal failure was 60.3%. (Mehrotra *et al*. 2012). Among patients who have received kidney transplants, 11.3% were re-hospitalized with AKI, which was found to be independently associated with risk of transplant

loss and death (Mehrotra *et al*. 2012). Among patients with acute coronary syndromes and those undergoing cardiac surgery, AKI is a common complication that is associated with an increased risk of morbidity and mortality (Marenzi *et al*. 2013; Robert *et al*. 2010), with 20-40% of patients undergoing cardiac surgery developing AKI (Fuhrman and Kellum. 2017). AKI can also complicate recovery of septic and MRSA-infected patients, with up to 40% developing AKI during treatment (Joo *et al*. 2013). Finally, the elderly are at increased risk of AKI due to declining kidney function, comorbidities, and higher exposure to medications and elderly AKI patients often have poorer long-term outcomes (Chronopoulos *et al*. 2010; YIlmaz and Erdem. 2010). There are few treatment options for ischemic AKI besides renal replacement therapy.

2.5.3 Outcomes of ischemic AKI

Renal ischemia causes an imbalance between nutrient and oxygen delivery to the kidney and waste removal from the kidney. ATP pools would be depleted since cells cannot perform oxidative phosphorylation to produce more ATP without constant oxygen delivery (Bonventre and Yang. 2011). Reactive oxygen species would be formed due to uncoupling of the electron transport chain. The resulting oxidative stress, waste accumulation, and ATP depletion can trigger renal cell death via both necrotic and apoptotic pathways and can trigger inflammatory responses (Bonventre and Yang. 2011).

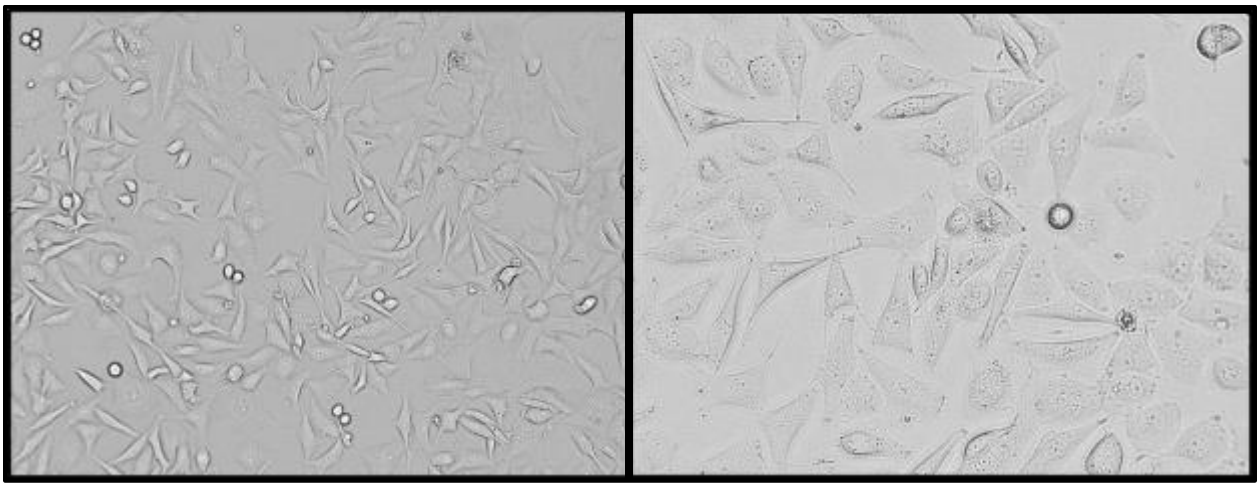
The injured and depleted kidney cannot effectively perform filtration and reabsorption, blood pressure maintenance, or acid-base homeostasis as these are energy-intensive processes (Sharfuddin and Molitoris. 2011). Nitrogen-containing compounds and other waste will rapidly accumulate in the blood, so high blood urea nitrogen and creatinine are good indicators of the

reduced glomerular filtration rate. Urine output will be decreased and damaged tubules may not be able to contain the filtrate, which may “back-leak” into the interstitium and be reabsorbed into blood vessels (Bonventre and Yang. 2011; Sharfuddin and Molitoris. 2011).

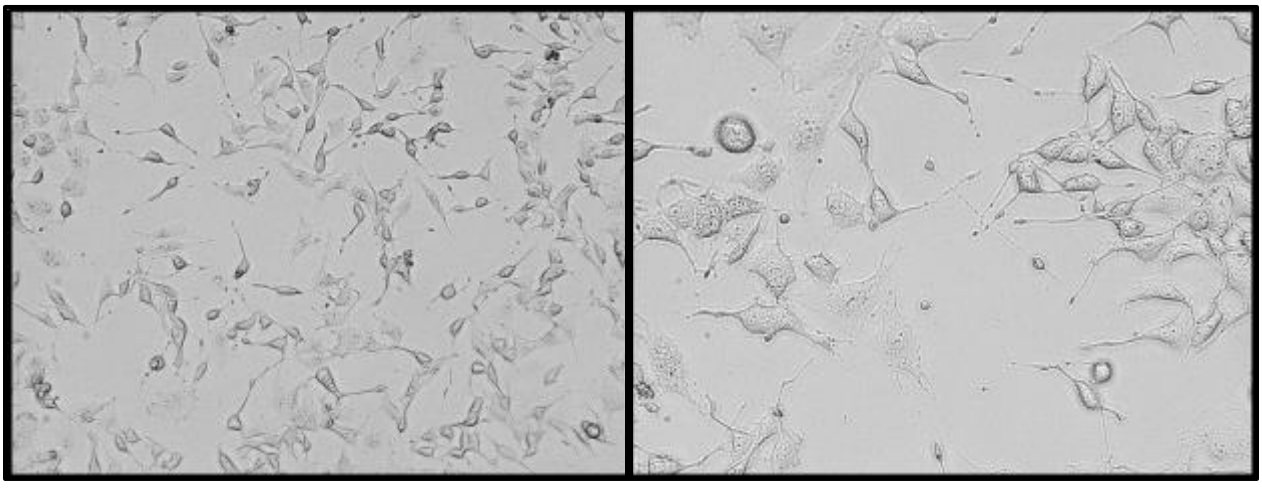
Proximal tubule epithelial cells have the highest metabolic demand of all kidney cells, because ion transport requires a lot of ATP and because they have a limited capacity for anaerobic glycolysis (Sharfuddin and Molitoris. 2011). Their function is also heavily dependent on cytoskeletal arrangement, cell adhesion to the extracellular matrix, and cell polarity. The cell skeleton may be depolymerized during IR to free ATP for other uses, resulting in loss of cell polarity, loss of tight junctions, and even sloughing of tubular epithelial cells. These injuries contribute to an increase in tubular permeability and back-leak of filtrate (Sharfuddin and Molitoris. 2011). Loss of polarity would also impair bidirectional sodium and water transport leading to high sodium concentration in the filtrate and further decline in GFR through glomerular feedback (Bonventre and Yang. 2011; Havasi and Borkan. 2011; Sharfuddin and Molitoris. 2011). Light-microscopy images of cultured HK2 human proximal tubular epithelial cell morphology before and after simulated IR injury are shown in Figure 2.3.

Figure 2.3 Light-microscopy images at 200x (left panels) and 400x (right panels) magnification of cultured HK2 human proximal tubular epithelial cells before (A) and after (B) one hour simulated ischemia using a hypoxic chamber and an ischemic buffer followed by 3 hour reperfusion.

A



B



2.5.4 Mechanisms of cell death in AKI

Historically, necrosis was thought to be the predominant form of cell death in AKI, next apoptosis seemed to be the most relevant cell death pathway, and most recently necroptosis has gained popularity (Molitoris. 2014). Currently, a continuum of cell death morphologies that occur in AKI is recognized but there is debate over which mode of cell death contributes more heavily to renal dysfunction. Proponents of the role of apoptosis in AKI often cite the facts that the low levels of necrosis in injured human renal tissue could not explain the accompanying loss of renal function (Rosen and Heyman. 2001), and that levels of apoptosis seem to correlate with renal dysfunction better than necrosis in animal models (Kelly *et al*. 2001). Necroptosis supporters argue that apoptosis cannot be responsible for the immunogenic cell death observed in many models of AKI (Price and Hodeify. 2012). However, apoptotic and necrotic cells can release or display damage-associated molecular patterns (DAMP) that can be recognized by germline-encoded pattern recognition receptors on cells of the innate immune system, leading to an inflammatory response. Injured renal epithelial cells and endothelial cells themselves can also express and release pro-inflammatory cytokines (Bonventre and Zuk. 2004; Molitoris. 2014). The inflammatory response to ischemic AKI will be reviewed in Section 2.5.4.

The debate over whether necroptosis or apoptosis is more relevant to AKI is clouded because it is unclear which morphological features are specific to each process (Dorn. 2013). Recent investigations have revealed that measuring Bax expression, cytochrome c release, caspase-3 activity, TUNEL, and loss of mitochondrial membrane potential may not distinguish between apoptosis and necroptosis (Dorn. 2013; Galluzzi *et al*. 2012). Cellular injury may or

may not be accompanied by autophagic processes that add another layer of complexity to cellular demise.

2.5.5 Cellular pathophysiology in AKI

Proximal tubular epithelial cells are very susceptible to ischemic insult and loss of these cells via apoptosis would contribute to the pathophysiology of AKI (Bonegio and Lieberthal. 2002). Cytoskeletal derangement seems to be especially dangerous in these cells and ischemia insult leads to loss of cell polarity, loss of brush border microvilli, and loss of tight and adherens junction attachments to neighboring cells and the basement membrane (Sharfuddin and Molitoris. 2011). These phenomena allow backleak of ultrafiltrate into the interstitium and cause cell sloughing, which leads to cellular debris and cast formation in the tubular lumen, ultimately causing a drop in the glomerular filtration rate (Sharfuddin and Molitoris. 2011). Cytokeletal derangement has been shown to precede and possibly induce apoptosis in a rat model of kidney transplantation (warm ischemia followed by cold storage followed by ex vivo reperfusion) and cytoskeletal stabilizers reversed apoptosis measured by TdT-mediated dUTP nick end labeling (TUNEL) and caspase-3 activation (Genescà *et al*. 2006). Cytoskeletal alterations and apoptosis, measured by caspase-3 activity and TUNEL, were inhibited by the peroxynitrite scavenger FeTMPyP in a rat model of IR indicating that reactive oxygen species production may also precede apoptotic cell death. However, the peroxynitrite scavenger also reduced tubular necrosis measured by hematoxylin and eosin (H&E) staining (Viñas *et al*. 2007). Similarly, the superoxide dismutase mimetic MnTMPyP attenuated apoptosis measured by

TUNEL, caspase-3, and pro-apoptotic protein expression and necrosis measured by H&E staining in renal IR (Liang *et al*. 2009) indicating a potential link to oxidative stress.

Wei et al. recently investigated ischemic AKI in global Bak knockout and proximal tubule-specific Bax knockout mice (Wei *et al*. 2013). Bak and Bax are pro-apoptotic proteins that modulate mitochondrial outer membrane permeabilization and subsequent release of apoptotic factors, such as cytochrome c, thereby facilitating apoptosis. The knockout mice were protected from ischemic AKI measured by blood urea nitrogen and serum creatinine, even though tubular necrosis was not reduced, as measured by H&E staining. Apoptosis however, was attenuated as measured by TUNEL and cytochrome c release, indicating that inhibiting apoptosis can protect the kidney from AKI even if necrosis is not inhibited (Wei *et al*. 2013). In human cadaveric renal allografts, IR injury was associated with apoptosis of tubular epithelial cells measured by TUNEL, Bax and Bak activation, and cytochrome c release (Castaneda *et al*. 2003). Mitochondrial outer membrane permeabilization and cytochrome c release appears to be preceded by mitochondrial fragmentation in ischemic AKI in rats, as measured by 2D and 3D electron microscopy. An inhibitor of mitochondrial division, mdivi-1, attenuated the increase in serum creatinine, blood urea nitrogen, proximal tubule necrosis measured by H&E staining, and proximal tubule apoptosis measured by TUNEL (Brooks *et al*. 2009).

In a rat IR model, the selective c-Jun N-terminal kinase (JNK) inhibitor, SP600125, attenuated increases in serum creatinine, blood urea nitrogen, and apoptosis, as measured by JNK activity, c-Jun phosphorylation, Fas ligand expression, and TUNEL (Wang *et al*. 2007), and attenuated increases in tubular injury measured by periodic acid-Schiff staining, lipid peroxidation, expression of pro-inflammatory cytokines, and apoptosis, measured by JNK

activity, c-Jun phosphorylation, and TUNEL (Xu *et al*. 2011). Glycogen synthase kinase (GSK) 3β also mediates apoptosis after ischemic injury and GSK3β inhibition attenuated the increase in Bax and caspase-3 activation induced by renal ischemia in rats, and reduced damage measured by H&E staining (Wang *et al*. 2010). Since MAPK and GSK transduce stress signals to the nucleus and other targets, these pathways are upstream regulators of cellular responses in ischemic AKI (Rana *et al*. 2001).

Caspase activation is considered a hallmark of apoptosis, with both extracellular and intracellular apoptotic pathways resulting in executioner caspase activity. Chatterjee et al. found that a caspase-1 inhibitor reduced serum creatinine, fractional excretion of sodium, serum aspartate aminotransferase, tubular damage measured by H&E staining, ICAM-1 expression, nitrotyrosine formation, myeloperoxidase activity, and malondialdehyde levels following renal IR in rats (Chatterjee *et al*. 2004). A caspase-3 inhibitor reduced serum creatinine, tubular damage, ICAM-1 expression, nitrotyrosine formation, myeloperoxidase activity, and malondialdehyde levels. A pan-caspase inhibitor did not protect against renal injury based on these parameters and TUNEL staining was not performed (Chatterjee *et al*. 2004).

Initial evidence that necroptosis occurs in AKI arose from studies using the specific RIP1 inhibitor, necrostatin-1 (Nec-1) (Linkermann *et al*. 2012). In renal proximal tubular cells, necroptosis can be induced by treating cells with TNFα and cyclohexamide to induce cell death, and a caspase-3 inhibitor to promote caspase-independent programmed cell death. In these conditions, Nec-1 reduces annexin V positivity, indicating that RIP1-dependent necroptosis is occurring. In a mouse model of renal IR, Nec-1 reduced renal damage measured by periodic

acid-Schiff staining, serum creatinine, and blood urea nitrogen, while the caspase-3 inhibitor did not reduce these parameters (Linkermann *et al*. 2012). It is unclear, however, whether Nec-1 inhibits both necroptosis and apoptosis (Price and Hodeify. 2012). Nec-1 also protected against cisplatin injury in human proximal tubule cells. Hoechst fluorescence was induced after cisplatin treatment and Nec-1 and a caspase-3 inhibitor together protected cells from apoptosis better than either treatment on its own, indicating that both necroptosis and apoptosis occur and multi-target therapies may be more useful than single-target therapies (Tristão *et al*. 2012).

2.5.6 Local and systemic inflammatory response in AKI

A large body of evidence indicates that AKI is an inflammatory disease (Molitoris. 2014). The immune response evolved to protect the injured organ and is designed to help the tissue heal. However, if such a response is not resolved in a timely manner or the organ is compromised by additional comorbidities, the inflammation can result in loss of organ function. When the kidney is subjected to ischemic insult, resident cells of the epithelial-endothelial axis are the first to sense the injury and initiate signaling to trigger an immune response (Molitoris. 2014). Renal tubular epithelial cells can produce pro-inflammatory cytokines, including TNFα, IL-6, and IL-1β, and chemokines like MCP-1 (Bonventre and Zuk. 2004; Molitoris. 2014; Thurman. 2007). These factors are released into renal tissue and the systemic circulation. Injured cells can also alert the innate immune system by releasing or displaying DAMP's, including extracellular ATP and DNA. Myeloid cells of the innate immune system use germline-encoded pattern recognition receptors (PRR) to recognize both pathogen-associated molecular patterns and DAMP's released from necrotic cells (Molitoris. 2014). Many classes of PRR's can

activate MAPK and NFκB signaling cascades leading to pro-inflammatory gene expression (Arthur and Ley. 2013).

One well-studied PRR is NLR-family pyrin domain-containing (NLRP) 3. When NLRP3 is activated, apoptosis-associated speck-like protein (ASC) and caspase-1 are recruited to form the inflammasome in which caspase-1 can be activated, allowing processing and secretion of IL-1β and IL-18 (Hutton et al. 2016). Other PRR's activate NFκB, leading to pro-inflammatory gene expression. Several studies have shown an increase in IL-1β and IL-18 in ischemic AKI and caspase-1-deficient mice seem to be resistant to kidney injury, lending indirect evidence for the role of inflammasomes in these settings (Anders and Muruve. 2011; Hutton *et al*. 2016). In mice, NLRP3 expression is upregulated after renal IR and NLRP3-deficient mice are protected from renal dysfunction and inflammation after IR (Iyer *et al*. 2009).

Ischemic AKI is followed by an increase in circulating pro-inflammatory mediators. The most studied inflammatory markers in human AKI and animal renal IR models are MCP-1, IL-1β, and TNFα, which are released from kidney tissue into the circulation following ischemic insult (Thurman. 2007). Keratinocyte-derived chemokine (KC), tissue inhibitor of metalloproteinase (TIMP)-1, and thrombospondin (TSP)-1 are also significantly increased in plasma after AKI (Isaak et al. 2017). These cytokines are needed to attract immune cells like neutrophils and monocytes to the injured tissue, activate immune cells, and initiate repair processes (Thurman. 2007).

MCP-1 stimulates monocyte movement into arterial walls from the blood stream, allowing the access to injured tissue, and higher levels of MCP-1 are found in transplanted kidneys with delayed function than in immediately-functioning kidneys (Grandaliano *et al*. 1997). In rats, AKI induces a significant increase in renal MCP-1 expression, NFκB activity,

monocyte infiltration, and neutrophil infiltration (Sung *et al*. 2002). Oxidative stress can mediate MCP-1 expression, as the SOD antioxidant mimetic TEMPOL was shown to inhibit NFκB activation and subsequent MCP-1 expression (Sung *et al*. 2002).

Intrarenal TNFα is elevated after renal IR injury and resident renal dendritic cells are the predominant source of TNFα in this context (Dong *et al*. 2007). TNFα can also be produced by glomerular mesangial cells and resident macrophages in response to ischemic oxidative stress (via NFκB and p38 activation) and by peripheral monocytes that have infiltrated the kidney early after tissue injury. This inflammatory biomarker contributes to neutrophil infiltration, inflammatory response perpetuation, and tubular cell apoptosis (Dong *et al*. 2007; Donnahoo *et al*. 1999). An increase in circulating TNFα may also be responsible for the extra-renal organ injuries observed after renal IR in rats. Reduced circulating TNFα may slow the progression of the inflammatory response and prevent complications of renal inflammation like endothelial dysfunction and further reduced flow (Sharfuddin and Molitoris. 2011).

IL-1β is a pro-inflammatory cytokine that triggers the production of cytokines like TNFα, IL-6, and MCP-1 in a variety of cell types by binding the IL-1 receptor and inducing intracellular signal transduction via NFκB (Furuichi *et al*. 2006). IL-1α/β-deficient mice were protected against tubular necrosis and renal inflammation during renal IR and IL-1β induced the expression of KC and MCP-1 in renal proximal tubule cells (Furuichi *et al*. 2006).

KC, a neutrophil chemoattractant, is one of the earliest biomarkers of AKI (it is elevated sooner after injury than other biomarkers) and is more specific to ischemic AKI than to other mechanisms of kidney injury (Molls *et al*. 2006). KC is secreted by injured epithelial and endothelial cells and KC antibodies administered before reperfusion in mice decreases

neutrophil infiltration and attenuates the increase in serum creatinine (Miura *et al*. 2001).After renal ischemia in mice, serum levels of KC and MCP-1 were correlated with the number of infiltrated neutrophils and macrophages, respectively, in the kidney at up to 24 hours of reperfusion. Numbers of infiltrated neutrophils and macrophages were both correlated with the area of tubular necrosis (Furuichi *et al*. 2006).

TIMP-1's role in the inflammatory response is to regulate the activity of matrix metalloproteinases (MMP), enzymes that can degrade the extracellular matrix but are also involved in cytokine activation, cell migration, and apoptosis and are expressed during inflammatory responses (Chromek *et al*. 2004). While not well studied in the context of AKI, both MMP-9 and TIMP-1 were shown to be upregulated after ischemia in a model of cerebral IR in rats and the expression of both enzymes was shown to be regulated by ERK. Inhibition of ERK attenuated the expression of both TIMP-1 and MMP-9 and protected against cerebral injury (Maddahi *et al*. 2009). Chromek and colleagues postulated that TIMP-1 may promote inflammation in the kidney by activating granulocytes and protecting them from apoptosis, and higher serum TIMP-1 levels were associated with higher risk of cardiovascular death in patients with acute coronary syndrome (Chromek *et al*. 2004).

TSP-1 is involved in the induction of apoptosis in kidney cells. Its expression is induced rapidly and significantly 3 to 12 hours after ischemia in rats (Thakar *et al*. 2005). Thakar et al. demonstrated that the proximal tubules that were the most injured based on immunocytochemical staining and apoptosis assays were also the tubules with the largest TSP-1 induction. TSP-1 null mice were protected against IR-induced kidney injury (Thakar *et al*. 2005).

A summary of the pathophysiology of ischemic AKI is provided in Table 2.4.

Table 2.4 Pathophysiology of ischemic AKI

Gross pathophysiology	
Reduced GFR	Microvasculopathy
Increased waste products (urea nitrogen and creatinine) in blood Decreased urine output Blood pressure dysregulation Metabolic acidosis	Endothelial cell swelling Vasoconstriction Loss of endothelial barrier Microthrombosis and platelet activation "No reflow" due to congestion in outer medulla or vascular damage
Tubular damage	
Filtrate back-leak into interstitium Loss of brush border and epithelial cell layer polarity Tubular obstruction due to cast formation and cellular debris	
Cellular pathophysiology	
Oxidative stress	Necrosis of tubular epithelial cells
Increased reactive oxygen species formation following uncoupling of electron transport chain Increased protein carbonyls and lipid peroxides Decreased antioxidant enzyme expression and activities	Activation of RIP kinase signaling pathways Increased caspase-8 activity Mitochondrial fragmentation Increased lysosomal membrane permeability Increased reactive oxygen species production
Apoptosis of tubular epithelial cells	Inflammation
Activation of MAPK and p53 signaling pathways Activation of c-Jun and NFκB transcription factors Expression of pro-apoptotic proteins (ex. Bax and Bak) Mitochondrial outer membrane permeabilization and cytochrome c release Increased caspase-3 activity Chromatin condensation and fragmentation Cytoskeletal alterations and fragmentation Cell rounding and loss of cell adhesion Plasma membrane phosphatidylserine exposure and blebbing Shedding of apoptotic bodies	Activation of TLR4, MAPK, and NFκB signaling pathways Increased expression of pro-inflammatory cytokines and chemokines (TNFα, IL-1β, IL-6, IL-10, IL-18, KC, MCP-1, IFNϒ, and others) Increased inflammasome and caspase-1 activity Increased expression of vascular adhesion molecules and selectins Immune cell infiltration and activation Edema Increased blood C-reactive protein reflecting systemic inflammation

2.6

Manuscript I: Targeting inflammation with polyphenol-rich berries

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2.6.1 Abstract

Epidemiological studies and meta-analyses of human clinical intervention trials have provided compelling evidence for the anti-inflammatory effects of dietary polyphenols. These studies have shown that consuming specific polyphenols or a diet rich in polyphenols can modulate the inflammatory responses including pro-inflammatory intracellular signaling, pro-inflammatory gene expression, cytokine secretion, and immune cell activation. Polyphenols seem most effective at alleviating chronic, low-grade inflammation associated with metabolic syndrome and cardiovascular diseases. However, the current body of literature does not adequately address the effects of polyphenols on acute inflammatory events and, while observing a robust range of inflammatory biomarkers and outcomes, does not parse the mechanisms of the anti-inflammatory properties of polyphenols with enough accuracy or resolution. One recurring mechanistic theme is the role of kinases in pro-inflammatory signaling and the regulation of specific kinase activities by polyphenols. Here, we review the role of c-Jun N-terminal kinase (JNK) signaling in acute and chronic inflammation, evaluate the evidence for regulation of JNK by polyphenols, and propose using polyphenol-rich berries to target pro-inflammatory JNK signaling.

2.6.2 Introduction

In a healthy organism, the immune response should rapidly detect pathogens or injured tissues and effectively subdue the pathogen or absorb any dead or dying cells. The response should also include efficient clearing of any pathogenic or cellular debris, and be able to limit itself once all these tasks are complete. If this response is persistently stimulated or is unable to

resolve itself, the organism will be in a chronic state of inflammation, leading to tissue damage and dysfunction, as is the case in inflammatory diseases like metabolic syndrome and cardiovascular disease. Targeting inflammatory processes with pharmaceutical or nutritional therapies can have a significant impact on disease symptoms and outcomes but more research is needed to determine the mechanisms of action and power of effects. This review highlights the role of the JNK signaling pathway in chronic and acute inflammation and proposes nutritional modification using polyphenol rich berries that modulate JNK signaling to regulate inflammation and disease outcomes.

2.6.3 JNK signaling in inflammation

JNK plays an important regulatory role in both acute and chronic inflammation but discerning the regulation, activities, and outcomes of JNK signaling is complicated and at times, seems paradoxical. This is because JNK may promote or protect against disease progression depending on the animal model, cell type, JNK isoform, and the timing of JNK activation. Transient JNK activation has been linked to cell survival and stress tolerance while sustained or intense JNK activation usually leads to apoptotic cell death (Weston and Davis. 2007). Once activated in stressed or cytokine-activated cells, JNK phosphorylates a variety of protein targets including c-Jun and p53. Phosphorylated c-Jun forms heterodimeric transcription factors called AP-1 that translocate to the nucleus and can activate expression of pro-apoptotic or pro-inflammatory genes, including those for MCP-1 and IL-6 (Solinas and Becattini. 2017; Vukic *et al*. 2009). AP-1 also has homeostatic roles in cell growth and differentiation and is constitutively expressed for these purposes. Myeloid cells, including monocytes, macrophages, neutrophils,

and dendritic cells, and lymphoid cells (i.e. T cells and B cells) of the innate immune system use germline-encoded PRR's to recognize both pathogen-associated molecular patterns and molecules released from necrotic cells. Many classes of PRR's can activate MAPK signaling cascades leading to pro-inflammatory gene expression (Arthur and Ley. 2013).

Novel roles for JNK in the inflammatory response include initiating inflammasome formation, propagating inflammatory response by facilitating inflammasome speck formation, triggering the inflammatory response to amyloid-β aggregates, and promoting inflammatory gene expression and immune cell infiltration in response to metabolic oxidative stress (Neumann and Ruland. 2013). Some PRR's oligomerize after sensing stimuli to form a caspase-1-activating scaffold called an inflammasome, made up of the PRR sensor, caspase-1, and adaptor proteins. Once caspase-1 is activated, it can cleave immature pro-inflammatory cytokines like IL-1β and IL-18 into their active forms, perpetuating the inflammatory response. JNK activity is required for activation of some types of inflammasomes (Neumann and Ruland. 2013). For example, the NLR family pyrin domain containing 3 (NLRP3) inflammasome requires aggregation of apoptosis-associated speck-like protein (ASC) into large insoluble cytoplasmic filaments called ASC specks where caspase-1 can be activated. Pharmacological inhibition of JNK reduced secretion of interleukins after stimulation of inflammasomes which require ASC to function, indicating that ASC might require phosphorylation at specific sites to form aggregates (Neumann and Ruland. 2013). ASC specks have also been reported to be released from dying cells, leading to cleavage of extracellular pro-IL-1β and activation of caspase-1 in macrophages that internalize the specks (Guo *et al*. 2016). Through this mechanism, inflammation may be

propagated from cell to cell and specks can build up at sites of inflammation, which has interesting consequences for the role of JNK in chronic inflammatory diseases.

In cerebral inflammation associated with Alzheimer's disease, c-Jun activation by JNK may accelerate disease progression through increased AP-1-mediated expression of MCP-1 (Vukic *et al*. 2009). The link between obesity, insulin resistance, and cardiovascular disease is evident because metabolic oxidative stress can activate JNK, leading to increased expression of pro-inflammatory proteins (Joseph *et al*. 2016). In obesity, hypertrophic adipocytes increase secretion of cytokines and chemokines like MCP-1, causing infiltration and activation of macrophages in adipose tissue. These macrophages produce pro-inflammatory proteins including TNFα, IL-6, and MCP-1 which enter the circulation. Circulating TNFα may promote atherosclerosis by inducing MCP-1 expression in the vascular endothelium leading to immune cell infiltration and progression of atherosclerotic lesions. JNK inhibitor peptides were shown to inhibit MCP-1 expression in cultured endothelial cells and in vivo vascular tissue (Ahmed *et al*. 2009; Vukic *et al*. 2009). Sustained JNK activation has been implicated in insulin resistance by direct inhibition of IRS-1 via phosphorylation. Aside from direct regulation of IRS-1, JNK signaling also drives metabolic inflammation in adipose, muscle, and liver tissue which can exacerbate insulin resistance (Solinas and Becattini. 2017). Myeloid-specific JNK deficiency using the Lyz2-Cre+ $JNK1^{LoxP/LoxP}$, $JNK2^{LoxP/LoxP}$ system has been shown to protect against the development of insulin resistance in response to a high-fat diet by regulating inflammatory processes (Han *et al*. 2013).

2.6.4 Anti-inflammatory effects of whole berries

Several types of berries have been shown to produce anti-inflammatory effects using animal models of chronic inflammatory diseases (Shi *et al*. 2017). However, most human clinical trials using berries focus on short term inflammatory processes like the postprandial response. Blueberries may have anti-inflammatory effects in complications of metabolic syndrome. Obese Zucker rats that had 2% of their diet replaced with dried blueberry had better management of glucose tolerance, blood pressure, and renin-angiotensin than control obese Zucker rats without blueberry supplementation. They also had improved renal function, renal blood flow, and glomerular morphology and lower oxidative stress in the kidney. The improved kidney health induced by the blueberry diet could be attributed to modulation of TLR-mediated MAPK signaling. The blueberry-fed rats had attenuated TLR4 gene and protein expression and decreased extracellular signal-regulated kinase and p38 kinase phosphorylation in the renal cortex. Modulation of the TLR4-MAPK signaling pathway corresponded to decreased renal IL-1β and IL-18 expression. The effects of the blueberry diet on renal JNK phosphorylation was not reported in this study (Nair *et al*. 2014). Obese Zucker rats that had 8% of their diet replaced with dried blueberry had lower plasma IL-6, TNFα, and C-reactive protein (CRP) and less pro-inflammatory gene expression in adipose tissue and liver (Shi *et al*. 2017). High fat diet-fed mice supplemented with 10% wild blueberry powder had reduced plasma pro-inflammatory biomarkers, including MCP-1 and IL-1β (Shi *et al*. 2017).

Lingonberries may have anti-inflammatory effects in cardiovascular disease and metabolic syndrome. Spontaneously hypertensive rats given lingonberry juice instead of tap water for 8 weeks had lower aortic cyclooxygenase-2, MCP-1, and P-selectin expression

indicating an anti-inflammatory and anti-atherothrombotic effect (Kivimäki *et al*. 2012). High fat diet fed mice that had 20% of their diet replaced with lingonberry (dietary fat was maintained) had significantly less pro-inflammatory gene expression in the liver analyzed by genome-wide microarray profiling. Epigenome-wide DNA methylation analysis revealed that lingonberry consumption caused genome-wide hypermethylation and specific hypermethylation of a gene regulating inflammatory pathways (Heyman-Lindén *et al*. 2016b). Using these results, pathway analysis revealed that the likely targets of lingonberry compounds in the liver were NFκB, signal transducer and activator of transcription 3, or mechanistic target of rapamycin which would be upstream regulators of the affected genes (Heyman-Lindén *et al*. 2016b). The study concluded that the anti-inflammatory effects of lingonberry in the liver were due to epigenetic modification of pro-inflammatory genes and inhibition of upstream regulators of the pro-inflammatory response.

2.6.5 Lingonberry juice consumption prior to ischemic AKI reduces the inflammatory response by modulating JNK signaling

Our lab investigated the benefits of supplementing daily diet with lingonberry juice when rats were later exposed to AKI (Chapter 5; Isaak *et al*. 2017b). The novel findings of this study were: 1) Daily lingonberry juice consumption for three weeks protected the rat kidney from acute ischemic injury and significantly improved kidney function demonstrated by attenuated increases in serum creatinine and neutrophil gelatinase-associated lipocalin compared to animals fed a sugar-matched polyphenol-free beverage; 2) Lingonberry juice consumption inhibited JNK phosphorylation and activity (measured by c-Jun phosphorylation)

in the injured kidney; 3) Lingonberry juice consumption attenuated the renal inflammatory response by inhibiting the release of pro-inflammatory cytokines into the circulation; and 4) Anthocyanins likely contribute to the bioactivities of lingonberry juice *in vivo* since physiologically-relevant doses of lingonberry anthocyanins protected renal proximal tubule epithelial cells from IR-induced apoptosis *in vitro* by inhibiting JNK phosphorylation and activity and MCP-1 expression.

These results suggested that intake of lingonberry juice could effectively attenuate IR injury in the kidney and modulate stress-activated signaling pathways leading to a decrease in inflammatory response. The effects of lingonberry anthocyanins were also tested in proximal tubule cells, which were used to demonstrate the link between JNK activation and inflammatory gene expression. The decreased MCP-1 expression in proximal tubule cells treated with either lingonberry anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, or cyanidin-3-arabinoside) or a specific JNK inhibitor, SP600125, shows that this preventative dietary approach may target the initial cellular response to injury, rather than downstream amplification of the inflammatory response, and therefore could effectively modulate multiple components of the concerted cellular responses to IR injury (Chapter 5; Isaak *et al*. 2017b). These results are in accordance with other AKI studies that showed that the JNK inhibitor SP600125 reduced renal MCP-1 expression following IR injury in rats, indicating that stress-activated JNK signaling is linked to the subsequent inflammatory response (De Borst *et al*. 2009). De Borst also showed that the extent of JNK activation correlates with interstitial macrophage accumulation in kidney biopsies from patients with renal disease, indicating that inflammatory outcomes are modulated by JNK signaling. MCP-1, TNFα, IL-6, and IL-1β are

released first by injured proximal tubular epithelial cells and cause the endothelium to increase expression of adhesion molecules to facilitate infiltration of leukocytes. These leukocytes (neutrophils and macrophages) are the first immune cells to accumulate in the epithelium after AKI. Resident dendritic cells in the kidney also sense damage and release TNFα and interferons to attract leukocytes to the injured tissue. The processes induced by cytokines in the endothelium can lead to congestion, vasoconstriction, and platelet aggregation so dampening the inflammatory response seems to protect the kidney from ischemic AKI (Molitoris. 2014). Since the types of surgeries that cause ischemic AKI, including kidney transplant and coronary artery bypass grafting, are often preceded by lengthy waiting periods, a pre-treatment approach using functional foods may be appropriate.

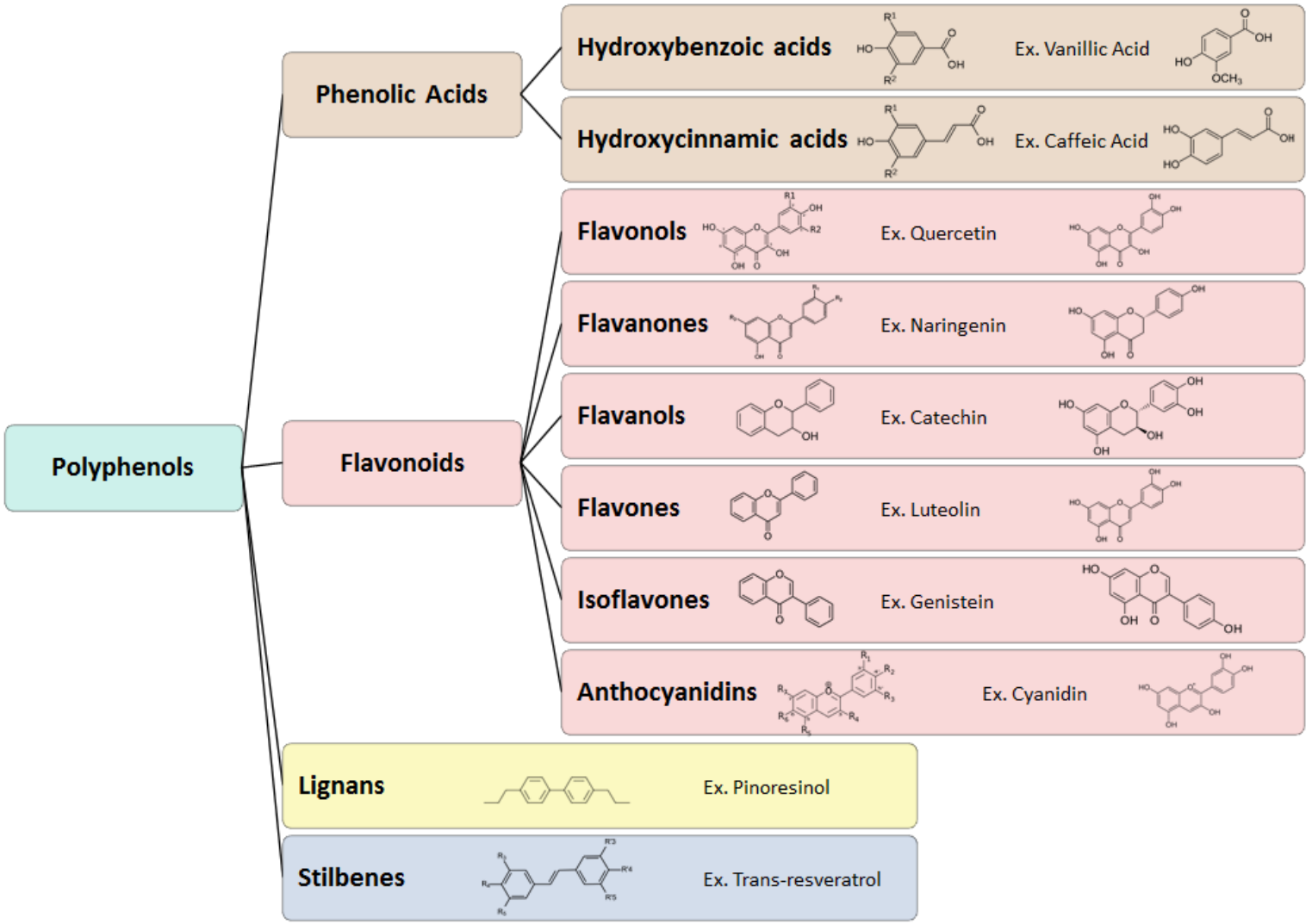
2.6.6 Chemistry and anti-inflammatory effects of polyphenols

Berries, especially lingonberries, are rich in polyphenols, which are compounds that contain phenol units, or aromatic rings containing phenolic hydroxyl groups, that allow them to act as antioxidants (Jimenez-Garcia *et al*. 2013). This means they can accept an electron from or donate a hydrogen atom to a reactive species while remaining as a stable entity. The radical form of the polyphenol, which now has an unpaired electron, can "share" or delocalize the unpaired electron throughout the phenol ring. This delocalization allows the polyphenol to be a more stable or longer-lived radical than other molecules, ending the chain of reactions. In this way, antioxidants could end a chain of free radical reactions before cellular structures like DNA, membranes, or proteins are damaged. There are several classes of polyphenols: 1) phenolic acids, 2) stilbenes, 3) lignans, and 4) flavonoids (Figure 2.4) Polyphenols are generally water-

soluble and are often conjugated to sugars or organic acids. They are secondary metabolites formed in plants to provide pigmentation to berries and flowers, to combat microbial infections, to act as signaling molecules, and to protect against UV damage. Berries contain all classes of polyphenols, but are especially rich in anthocyanins (the glycosylated form of anthocyanidins), flavonols, flavanols, and phenolic acids (Jimenez-Garcia *et al*. 2013).

Despite their high *in vitro* antioxidant capacity, due to rapid metabolism upon ingestion, low bioavailability of intact polyphenols, and the varying chemical environments encountered after absorption, it is unlikely that direct radical scavenging is responsible for the reported health benefits of polyphenols in humans. Some studies have shown that polyphenols can decrease biomarkers of oxidative stress, like F_2-isoprostanes or lipid peroxides, in stressed organisms but there is little evidence for direct antioxidant activity leading to health outcomes. Theories about how polyphenols produce health benefits include acting as signaling molecules to enhance nitric oxide production which would improve endothelial function and lower blood pressure, inhibiting NADPH oxidase which would indirectly reduce oxidative stress, and enhancing expression or activity of antioxidant enzymes by forming intracellular electrophiles upon metabolism which would activate the Nrf2 transcription factor (Croft. 2016). Inhibiting the pro-inflammatory response by modulating JNK signaling could be another mechanism by which polyphenols exert their protective effects. It is also important to consider the bioactivities of polyphenol metabolites when studying the anti-inflammatory effects of polyphenols. While it's true that intact polyphenol bioavailability is low, pre- and post-absorption metabolism gives rise to small phenolic acids and other metabolites that may carry out the bioactive effects attributed to the parent molecule (Vendrame and Klimis-Zacas. 2015).

Figure 2.4 Classification and examples of polyphenol compounds.



Epidemiological studies have shown an inverse association between higher polyphenol intake and risk of chronic inflammatory diseases including type 2 diabetes, cardiovascular disease, Parkinson's disease, and cancer (Medina-Remón *et al*. 2017; Wang *et al*. 2014b). One of the most common ways to analyze whole organism inflammation is to measure plasma CRP levels. Other studies may measure cytokines and chemokines like TNFα, IL-6, IL-1β, and MCP-1, adipocyte-derived cytokines like adiponectin or leptin, or immune cell adhesion molecules as biomarkers of inflammation (Medina-Remón *et al*. 2017). In a cohort of 2375 adults over age 60, higher anthocyanin and flavonol intake was inversely associated with an inflammation score calculated based on twelve inflammatory biomarkers, which may explain the reduced risk of chronic diseases in those who consume more polyphenol-rich foods (Cassidy *et al*. 2015). Intervention studies that included patients living with obesity or metabolic syndrome have shown inconsistent results with regards to effects of berry consumption on chronic inflammatory biomarkers. These inconsistencies may be explained by varying lengths of interventions, different doses of berries, food matrices in which the berries were included, and inclusion of relatively healthy individuals with low base-line inflammation (Shi *et al*. 2017). The only polyphenol-rich intervention that has produced consistent anti-inflammatory effects in chronic disease states seems to be red wine (Joseph *et al*. 2016). There are already excellent reviews on the broad health benefits of polyphenols (Amiot *et al*. 2016; Joseph *et al*. 2016) so this review will only highlight a few studies that investigated the effects of polyphenols and berries on specific inflammatory processes or outcomes with MAPK involvement.

2.6.7 Evidence for polyphenols modulating inflammation through JNK signaling

The anti-inflammatory and anti-atherosclerotic properties of red wine polyphenols may be due to modulation of AP-1 activity. *p*-coumaric acid, quercetin, and resveratrol from red wine inhibited the gene and surface expression of adhesion molecules intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in human umbilical vein endothelial cells. These compounds also inhibited lipopolysaccharide-induced MCP-1 and macrophage colony-stimulating factor expression in conjunction with reduced AP-1 activation (Calabriso *et al*. 2016). Resveratrol, quercetin, and hydroxycinnamic acids like *p*-coumaric acid are also present in many berries, though at differing proportions and concentrations than red wine, and *p*-coumaric acid is also formed *in vivo* from anthocyanin metabolism so circulating levels after berry consumption may be higher than what could be directly absorbed.

Water-soluble polyphenols are metabolized post-absorption in the liver and kidneys. Thus, bioactive metabolite concentrations may be higher in these tissues. Proanthocyanidin supplementation for four weeks improved hepatic insulin signaling and suppressed hepatic inflammation in diabetic ob/ob mice. Both outcomes may be attributed to inhibition of JNK signaling, since JNK inhibition may ameliorate insulin sensitivity by reduced phosphorylation of IRS-1 and reduced AP-1 activation and subsequent pro-inflammatory gene expression. The mice supplemented with proanthocyanidins had reduced hepatic JNK phosphorylation as compared to control mice (Ogura *et al*. 2016).

Polyphenols may support oral health by reducing inflammation, which is a key element of periodontitis. Cranberry proanthocyanidins reduced IL-1β-induced c-Jun phosphorylation and inhibited IL-6 production in human gingival epithelial cells. Nuclear accumulation of

phosphorylated c-Jun was significantly reduced but no decrease in JNK activation was detected. The authors concluded that cranberry proanthocyanidins may inhibit nuclear translocation or nuclear activity of transcription factors rather than upstream kinases (Tipton *et al*. 2014).

Cisplatin chemotherapy is a widely used first-line treatment for epithelial cancers of ovarian, testicular, cervical, neck, and lung cancers. However, the adverse effects of this treatment include apoptosis and necrosis of renal cortical tubular epithelial cells where the drug is excreted (Malik *et al*. 2016). Rats treated with cisplatin after ten days of consuming epigallocatechin gallate, a flavanol, had better kidney function, lower levels of the oxidative stress marker malondialdehyde, increased superoxide dismutase and catalase activities, lower serum TNFα and IL-6 levels, and less focal inflammation and necrosis of the tubules. The treatment may have induced these protections by inhibiting MAPK phosphorylation (Malik *et al*. 2016).

Modulation of JNK signaling may also underlie the protective effects of polyphenols during acute inflammation. Proanthocyanidins, anthocyanins, and lignans, have all been shown to suppress the inflammatory response in LPS-stimulated macrophages by inhibiting MAPK signaling. In all cases, JNK phosphorylation and activity were reduced in cultured macrophages when stimulated with LPS following pretreatment with polyphenols and JNK inhibition resulted in reduced TNFα secretion and reduced nitric oxide production (Jo *et al*. 2015; Limtrakul *et al*. 2016). Anthocyanins were also effective at reducing lipopolysaccharide-induced lung injury in mice by inhibiting JNK activation and subsequent pro-inflammatory gene expression (Ma *et al*. 2015).

Oxidative stress and neuroinflammation occur during aging and can cause cognitive impairment. In a model of cognitive impairment in rats, anthocyanin supplementation inhibited JNK phosphorylation and activity in the brain cortex. Neuroinflammation was concurrently reduced, measured by inducible nitric oxide synthase and TNFα expression in the hippocampus and cortex. Clinically relevant outcomes including spatial learning and memory were also improved in the anthocyanin-supplemented group (Rehman *et al*. 2017).

2.6.8 Considerations for targeting chronic and acute inflammation using polyphenol-rich berries

Three major considerations are required when investigating the effects of berry polyphenols on JNK-mediated inflammation.

1. Have you selected an appropriate dose of your polyphenol or food product?
2. Have you considered the post-absorption metabolism of your polyphenol or food product?
3. Will your selected measurements adequately and reliably capture the inflammatory response?

While there are certain limitations to the currently available methods for estimating polyphenol bioavailability, most studies conclude that plasma levels of intact polyphenols generally do not exceed 100 ng mL^{-1} and for many polyphenols, the bioavailability is closer to 10 ng mL^{-1} (De Ferrars *et al*. 2014). Selected doses should be more in line with those that could be achieved in blood or tissue through dietary modification such as daily consumption of a moderate quantity of berries. Doses of as low as 10 ng mL^{-1} or 20 nmol L^{-1} of anthocyanins have been shown to

have significant effects on apoptosis in cardiac cells demonstrating that physiologically relevant doses can produce meaningful effects in cells (Isaak *et al*. 2017a; Isaak *et al*. 2015). For *in vivo* models where an animal diet is supplemented with pure polyphenols or a berry product, doses that could be achieved through reasonable dietary modification should be pursued. Unless the expressed goal is to promote a high-dose supplement, for example resveratrol supplements have been tested in clinical trials for cardiovascular benefits, more conservative doses should be selected.

Especially for mechanistic studies using cultured cells, high doses of intact polyphenols are often used. If the polyphenol of interest does not produce positive results at physiological doses, it may be beneficial to look at the post-absorption metabolites formed in various tissues. For example, high levels of anthocyanins are generally not seen in kidney tissue because they are rapidly metabolized to small phenolic acids or methylated derivatives. There are many metabolomics studies published for a variety of polyphenols that highlight the plasma, tissue, and urine metabolites formed from the parent compound. Testing the bioactivities of *in vivo* metabolites like small phenolic acids has the benefit of often being less expensive than using pure complex polyphenols, as well as being more physiologically relevant. Furthermore, there may be synergistic effects between compounds that are needed for the realization of physiological effects. While it may be impossible to dissect out the action of each, studying the mechanisms of action of the compounds, either individually or in combination, is essential to map out the potential health benefits.

Study design is critically important for animal studies and clinical trials. Selection of a broad and robust panel of inflammatory biomarkers, consideration of the health status of

participants or disease severity of the animal model, usage of multiple sampling times, determination of best practices for analysis of phosphorylated proteins and unstable biomarkers in plasma and tissue, inclusion of groups consuming different amounts of berries, and inclusion or exclusion of food matrices, will yield more useful and reproducible results. These considerations must also be disclosed for publication.

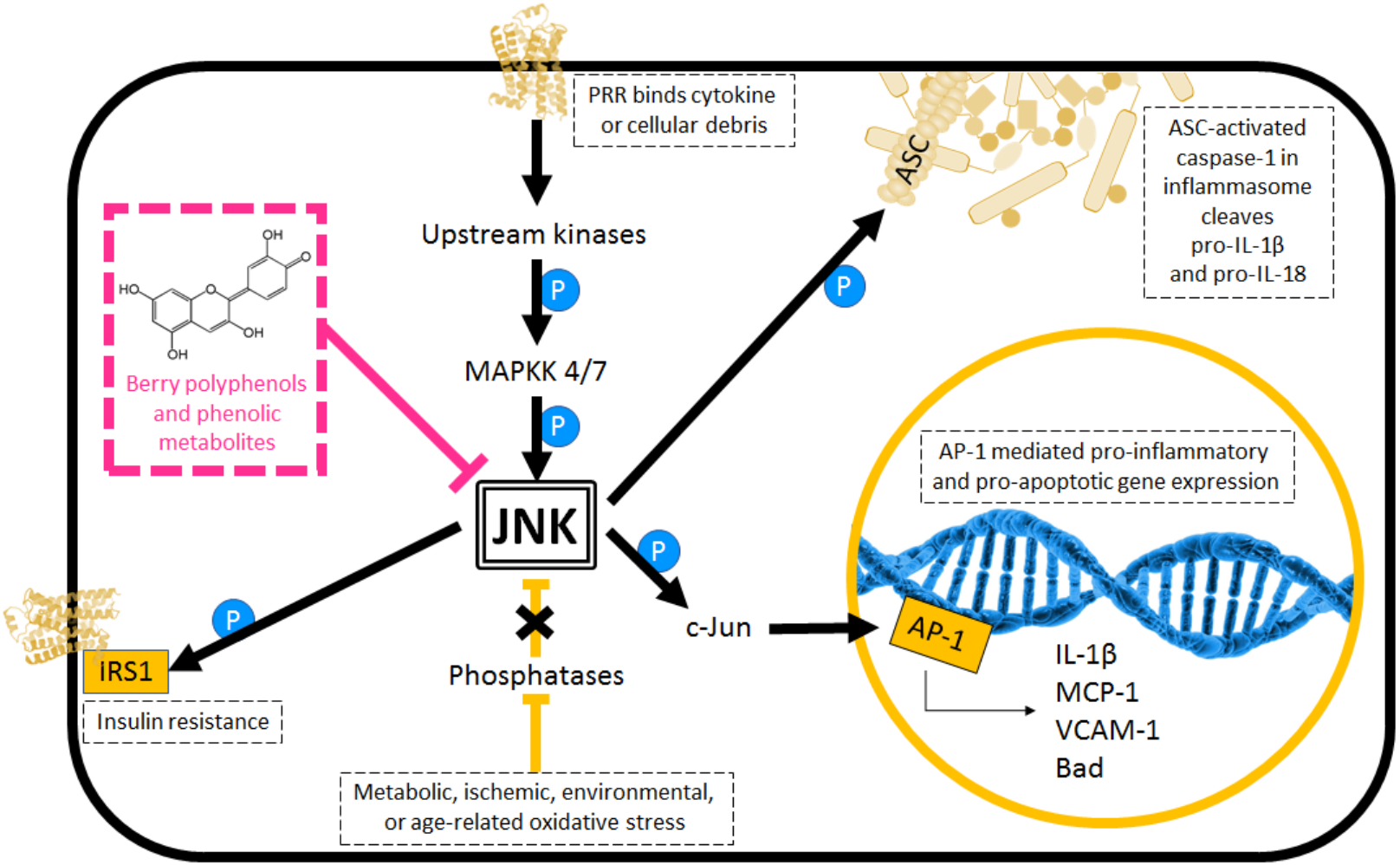
2.6.9 Conclusion

While clinical trials have produced inconsistent results regarding the anti-inflammatory effects of berries, there is strong mechanistic evidence that berry compounds may inhibit the inflammatory response by targeting the JNK signaling pathway. JNK inhibition by bioactive compounds found in berries has been demonstrated in several cell lines *in vitro* as well as in healthy and disease model organisms *in vivo*. The downstream target of JNK, c-Jun, forms a transcription factor complex that is known to promote expression of pro-inflammatory proteins, which may explain the anti-inflammatory effects of JNK inhibition. Additionally, attenuating JNK activity may reduce other aspects of the inflammatory response including inflammasome activation, insulin resistance, and metabolic dysfunction (Figure 2.5). However, the other roles of JNK and the redundancy built into the inflammatory response necessitate further study to fully understand the role of JNK in chronic and acute inflammation. The epidemiological evidence for the health benefits of polyphenol-rich berries is strong and the interventional and biological evidence is strengthening each year. Consumption of polyphenol-rich berries, especially wild Northern berries like blueberry and lingonberry that contain a variety and abundance of polyphenols, may constitute a safe, non-invasive, inexpensive

method for targeting inflammation associated with metabolic syndrome, cardiovascular disease, kidney injury, neurodegeneration, and other chronic disease states.

Figure 2.5 Berry polyphenols may modulate JNK-mediated inflammatory processes.

JNK can be activated by the MAPK signaling cascade triggered by cytokine or damage-associated molecular pattern binding a pattern recognition receptor (PRR). Upstream kinases associated with the PRR phosphorylate mitogen activated kinase kinase (MAPKK) 4 and 7 which specifically target JNK. Alternatively, oxidative stress can also activate JNK by inhibiting phosphatases that would normally inactivate JNK. Once activated by phosphorylation, JNK phosphorylates downstream targets such as c-Jun, which translocates to the nucleus to form part of the activator protein (AP)-1 transcription factor. AP-1 binding sites exist in the promoter regions of pro-apoptotic proteins, including Bad, and pro-inflammatory proteins, including many cytokines. Expression of monocyte chemoattractant protein (MCP-1) and interleukin (IL)-1β would perpetuate the inflammatory response by promoting immune cell infiltration. Inhibition of JNK using a specific inhibitor or low doses of anthocyanin polyphenols has been shown to abolish MCP-1 expression. JNK may also be required for the function of specific cytokines by activating apoptosis-associated speck-like protein filaments in the inflammasome by phosphorylation, leading to activation of caspase-1 which cleaves pro-cytokines into their active form. Polyphenols may either directly inhibit JNK or indirectly target upstream signaling components to dampen the inflammatory response in a variety of tissues and disease states.


PRR binds cytokine or cellular debris
Upstream kinases
P
MAPKK 4/7
P
JNK
P
P
P
Berry polyphenols and phenolic metabolites
HO
OH
OH
OH
O
IRS1
Insulin resistance
Phosphatases
Metabolic, ischemic, environmental, or age-related oxidative stress
c-Jun
ASC
ASC-activated caspase-1 in inflammasome cleaves pro-IL-1β and pro-IL-18
AP-1 mediated pro-inflammatory and pro-apoptotic gene expression
AP-1
IL-1β
MCP-1
VCAM-1
Bad

Chapter 3

Manuscript II: Manitoba lingonberry (Vaccinium vitis-idaea) bioactivities in ischemia-reperfusion injury

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3.1 Abstract

Evidence for the efficacy of dietary interventions in protecting against cardiovascular disease has grown significantly, with flavonoids and anthocyanins receiving special attention. Lingonberry (*Vaccinium vitis-idaea* L.) is a good source of these compounds, and this study examined the protective effects of wild lingonberry found in Manitoba, Canada, against IR injury. Manitoba lingonberry contained 3793 ± 27 mg gallic acid equivalents (GAE), 120,501 ± 7651 μmol trolox equivalents, and 575 ± 20 mg cyanidin-3-glucoside equivalents per 100 g dry weight, which correspond with high total phenolic content, antioxidant activity, and anthocyanin content, respectively. A complete methanolic extract and both anthocyanin-rich and phenolic-rich fractions inhibited apoptosis in H9c2 cells during simulated IR. Lingonberry extract and fractions significantly inhibited several markers of apoptosis induced by IR, including nuclei condensation, caspase-3 activation, and MAPK signaling. These results provide the first analysis of Manitoba lingonberry and highlight the mechanistic importance of dietary berry compounds for cardiovascular health.

3.2 Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is a woody, evergreen dwarf shrub that grows in northern temperate, boreal, and subarctic climates. Historically, it has been a popular fruit crop in Scandinavia but there is increasing interest in North America, where it grows in several Canadian provinces and the Northern United States. There are two subspecies of lingonberries: the larger lowland race, *V. vitis-idaea* ssp. *vitis-idaea* (L.) Britton which is Eurasian in

distribution, and the dwarf arctic-montane race, *V. vitis-idaea* ssp. *minus* (Lodd) Hult. that spreads over Iceland, Greenland, North America, northern Asia and Scandinavia. While lingonberries collected from Manitoba and Newfoundland are in the *minus* subspecies, European lingonberries are ssp. *vitis-idaea* type (Hulten. 1949). Lingonberries are a versatile health food, containing dietary fibre, vitamin C, vitamin E, and a range of polyphenolic compounds (Ek *et al*. 2006).

Lingonberry contains 0.3 g of polyunsaturated fatty acids per 100 g fresh weight (FW) (Bere. 2007), up to 160 mg of plant sterols per 100 g dry weight (DW) (Szakiel *et al*. 2012), and up to 15 mg quercetin per 100 g FW which is enough to raise serum quercetin levels (Erlund *et al*. 2003; Häkkinen *et al*. 1999). It also contains 50-130 mg anthocyanins per 100 g FW (Bakowska-Barczak *et al*. 2007; Lee and Finn. 2012), 150-180 mg proanthocyanidins per 100 g FW (Kylli *et al*. 2011), and up to 580 µg resveratrol per 100 g DW, which is only slightly less than the amount found in Canadian table grapes (Rimando *et al*. 2004). The health-promoting effects of these compounds are well-documented in the literature (Del Rio *et al*. 2010). Reports have confirmed that lingonberries have higher antioxidant activity than most commonly consumed berries, including blueberries, cranberries, raspberries, and strawberries (Bakowska-Barczak *et al*. 2007; Zheng and Wang. 2003). Anthocyanins give the berries their bright red colour, and several studies have aimed to characterize the phenolic profile of this fruit. Early studies indicated that lingonberry contained mainly cyanidin-3-galactoside, with minor amounts of other cyanidin glycosides, catechins, and conjugates of quercetin and kaempferol (Ek *et al*. 2006; Zheng and Wang. 2003). More recent work has confirmed the presence of delphinidin

and peonidin glucosides, delphinidin and malvidin galactosides, and large amounts of proanthocyanidins (Bakowska-Barczak *et al*. 2007; Kylli *et al*. 2011; Lee and Finn. 2012).

A few studies have looked specifically at the *in vivo* health effects of lingonberry. Mane et al. found that a food grade lingonberry extract was able to significantly reduce total oxidant status and improve antioxidant defenses in red blood cells and the liver of rats fed a diet that induces oxidative stress (Mane *et al*. 2011). Kivimaki et al. showed that an 8-week treatment with lingonberry juice improved endothelium-dependent vasodilation and lowered blood pressure in spontaneously hypertensive rats, even though cranberry juice and black currant juice did not produce the same effects (Kivimäki *et al*. 2011; Kivimäki *et al*. 2013). Consumption of whole lingonberries or lingonberry nectar was shown to improve the postprandial metabolic response to sucrose in healthy women (Törrönen *et al*. 2012). Epidemiological studies have associated anthocyanin-rich diets with decreased total mortality risk, cardiovascular disease risk, and cancer risk (Zamora-Ros *et al*. 2011).

Cardiovascular disease is the second leading cause of death in Canada (Statistics Canada. 2015). It is characterized by oxidative stress and apoptosis at every stage of pathogenesis and apoptosis is now recognized as an integral process during the spread of atherosclerotic plaques to neighboring healthy tissue (Kavurma *et al*. 2005). Oxidative stress and apoptosis are even more apparent in vascular tissue following an acute IR event, such as a myocardial infarct after a lesion ruptures in a coronary artery (Dorweiler *et al*. 2007; Powers *et al*. 2007). The lack of waste removal and uncoupling of the electron transport chain during ischemia, combined with the oxidative burst during reperfusion, lead to large amounts of reactive oxygen species being produced in cells. Oxidative stress can trigger apoptosis by

activating the mitochondrial pathway, caspases, and MAPK signaling (Kumar *et al*. 2002). High levels of oxidative stress and apoptosis therefore characterize IR-induced myocardial damage. We hypothesized that antioxidant and anthocyanin-rich Manitoba lingonberry would have a protective effect against apoptosis induced by IR.

In this study, we aimed to extract the phenolic and anthocyanic compounds found in wild lingonberry clones collected from Newfoundland and Labrador and in a composite of two wild populations of Manitoba lingonberries and to examine the anti-apoptotic effects of Manitoba lingonberry extracts by measuring caspase-3 activation, nuclear condensation, and MAPK signaling in H9c2 myoblasts undergoing simulated IR injury.

3.3 Materials and Methods

3.3.1 Chemicals and materials

Optima-grade methanol, acetonitrile, HPLC-grade phosphoric acid, and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) from Wako Chemicals (Richmond, VA) and all other chemicals of at least ACS reagent grade, including the selective JNK inhibitor SP600125, were acquired from Sigma-Aldrich (St. Louis, MO). Reference compounds cyanidin-3-glucoside (95%) and cyanidin-3-galactoside (95%) were purchased from Chromadex (Irvine, CA) while cyanidin-3-arabinoside chloride (>95%) was from Cerilliant Corp. (Round Rock, TX).

Plastic pots were purchased from East-Chem Inc. (Mount Pearl, NL), Acrodisc® syringe filters and BioTrace Nitrocellulose Transfer Membrane were acquired from Pall Corp. (Ann Arbor, MI), SepPak tC18 columns were acquired from Waters Corp. (Milford, MA), and SensoLyte Homogeneous AFC Caspase-3/7 Assay Kits were acquired from Anaspec (Freemont,

CA), Cleaved poly-ADP ribose polymerase (PARP) antibody (rat specific) was purchased from Cell Signaling (Danvers, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and western immunoblotting equipment and reagents were purchased from BioRad Laboratories (Hercules, CA). Personal protective equipment and laboratory safety guidelines were implemented for all experiments.

3.3.2 Apparatus and software

A Milli-Q Synthesis A10 water purification system was acquired from Millipore Corp. (Billerica, MA), and the SpectraMax M5 microplate reader and SoftMax Pro (version 6.2) software were acquired from Molecular Devices (Sunnyvale, CA), ACQUITY UPLC equipment and Empower™ 3 Software were acquired from Waters Corp. (Milford, MA), the AlphaInnotech FluorChemQ system were purchased from Fisher Scientific (Fair Lawn, NJ). A Billups-Rothenberg modular incubator chamber was purchased from Stem Cell Technologies (Vancouver, BC) and a Sector Imager 2400 and electrochemiluminescent assay kits were purchased from Meso Scale Discovery (Rockville, MD).

3.3.3 Berry collection

Eastern Canada and European berries

Five wild lingonberry clones, designated as L3, L10, L16, L20, and L23 collected from Newfoundland and Labrador, Canada and the European (Polish) cultivar Mesovia (L24) have been grown and maintained at the Atlantic Cool Climate Crop Research Centre, St. John's, NL, Canada for over 10 years. Each clone represents a single plant selected from the wild based on

vigour, berry colour, size and yield per plant and apparent freedom from disease and insects. GPS locations of the collection sites are shown in Table 3.1. All genotypes were grown in a greenhouse in plastic pots (25 cm in diameter and 18 cm deep, equivalent to 6 L) containing 2 peat:1 perlite (v/v), under natural light conditions at a maximum photosynthetic photon flux of 90 µmol m^{-2} s^{-1} at 20 ± 2 °C and 85% relative humidity. Fully ripe berries were collected and were shipped to our laboratory in Winnipeg, Manitoba, Canada for analysis and stored at -80°C until use.

Table 3.1 GPS locations for collection sites of wild lingonberry clones from Newfoundland and Labrador (NL) and harvest sites of wild lingonberries (not clones) from Manitoba (MB) and Saskatchewan (SK).

Clone	Province	Community	Latitude (N)	Longitude(W)
L3	NL	Colinet	47°13'	53°32'
L10	NL	L'anse aux Meadows	51°36'	55°32'
L16	NL	Logy Bay	47°37'	52°40'
L20	NL	Gunner's Cove	51°31'	55°27'
L23	NL	Lords Cove/Pump Cove	46°52'	55°40'
Not Clones	MB	Sherridon Lake	55°7'	101°5'
Not Clones	SK	Deschambault Lake	54°55'	103°21'

Manitoba berries

Berries from two wild populations were hand-picked by local berry pickers at Deschambault Lake, Saskatchewan and Sherridon Lake, Manitoba in September 2011 and immediately frozen. GPS locations of harvest sites are shown in Table 3.1.

3.3.4 Extraction

Frozen berries were ground roughly in a coffee grinder, freeze-dried, and ground to a fine powder with mortar and pestle. Methanol, ethanol, water, acetonitrile, acetone, and ethyl acetate were tested in aqueous and acidified solutions for their ability to extract phenolic compounds from lyophilized lingonberry. Acidified methanol was selected as the extraction solvent due to its ability to extract high amounts of phenolic compounds and anthocyanins, while also being a compatible solvent for other analysis, such as HPLC. Extraction conditions were optimized by testing several temperatures and sonication times to maximize total phenolic and anthocyanin extraction (data not shown). 0.5 g of lyophilized berry powder was extracted twice in 6 mL methanol (2% formic acid) with 15 minutes sonication at 37°C followed by centrifugation for 15 minutes at 3000*g*. The supernatants from the two extractions were combined and filtered with a 0.2 µm syringe filter and stored at -20°C. Methanol was then removed by rotary evaporation and remaining extract was brought back up to the original volume with ultrapure water so it could be loaded onto the SepPak columns.

3.3.5 Separation of anthocyanin-rich and phenolic-rich fractions

The filtered aqueous Manitoba lingonberry extract was named the “Complete Extract” (CE). 8 mL were removed and separated into two fractions, one named the “Anthocyanin-rich Fraction” (ARF) and the other named the “Phenolic-Rich Fraction” (PRF), using a protocol modified from Rodriguez-Saona and Wrolstad (Rodriguez-Saona and Wrolstad. 2005). Briefly, a SepPak tC18 column (plus long cartridge, 900 mg silica per cartridge, 37-55 µm particle size) was conditioned using methanol (0.5% formic acid), washed with ultrapure water (0.5% formic acid), loaded with 8 mL CE, and washed again with ultrapure water (0.5% formic acid) to remove sugars and acids. 10 mL ethyl acetate was used to collect polyphenolic compounds (PRF) and 5 mL methanol (0.5% formic acid) was used to collect anthocyanins (ARF). The ethyl acetate and acidified methanol fractions were collected in separate flasks and solvents were removed by rotary evaporation to near dryness. Both fractions were resuspended in methanol (0.2% formic acid) to 1/5 the original loaded volume. Both fractions were filtered with a 0.2 µm syringe filter and stored at 4°C until analysis. Fractions were standardized based on ORAC and total anthocyanin content.

3.3.6 Determination of total phenolics

Total phenolic contents of the complete extract and fractions were determined colourimetrically using Folin–Ciocalteu (FC) reagent, following a protocol modified from Ainsworth and Gillespie (Ainsworth and Gillespie. 2007). Briefly, 40 µL of 25% F-C reagent and 20 µL sample were added to a 96-well plate. After 5 minutes incubation, 140 µL of an aqueous 700 mM sodium carbonate solution was added to each well. This was followed by 2 hours

incubation at room temperature and the developed colour was measured at 765 nm using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software. All samples and standards were tested in triplicate. Results were expressed as mg GAE per 100 g dry weight of lyophilized berries using gallic acid as a standard.

3.3.7 ORAC assay

The ORAC of all samples were determined fluorescently using AAPH as a peroxyl generator to oxidize fluorescein, following a method modified from Gillespie, Chae, and Ainsworth (Gillespie *et al*. 2007). Briefly, 25 µL sample or standard was incubated with 75 µL of a 0.2 µM fluorescein solution for 10 minutes with agitation at 37°C. After incubation, 100 µL of a 37 mM AAPH solution was added to each well. Loss of fluorescence intensity was measured kinetically at 1.5-minute intervals over 60 minutes using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software. All samples and standards were tested in triplicate. Results were expressed as µmol Trolox equivalents (TE) per 100 g dry weight of lyophilized berries using Trolox as a standard.

3.3.8 Determination of total anthocyanins

The total anthocyanins of the complete extract and fractions were determined using the pH differential method from Lee, Durst, and Wrolstad (Lee *et al*. 2005). Briefly, samples were diluted in each of 0.025M potassium chloride (pH 1.0) and 0.4M sodium acetate (pH 4.5) at an appropriate dilution factor. Within 15 minutes, absorption was measured at both 520 nm and

700 nm using a SpectraMax M5 microplate reader. Data was analyzed using SoftMax Pro (version 6.2) software. Anthocyanin concentration was calculated as follows:

$$\text{mg cyanidin-3-glucoside equivalents/L sample} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times PL}$$

where $A = (A_{520}-A_{700})pH1.0-(A_{520}-A_{700})pH4.5$; MW = 449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor; PL = pathlength in cm; ε = 26,900 molar extinction coefficient; and10^3 = conversion factor from g to mg. All samples were tested in quadruplicate and results were expressed as mg cyanidin-3-glucoside equivalents per 100 g dry weight of lyophilized berries.

3.3.9 UPLC conditions and analysis

Chromatography was performed using a Waters ACQUITY UPLC system. For each sample and standard, 1 µL was injected onto a 2.1 x 100 mm, 1.7 µm ACQUITY UPLC BEH C18 column with the flow rate set at 0.6 mL min^{-1}. The column temperature was maintained at 42°C using an ACQUITY UPLC Column Manager and spectral data (210-600 nm) was collected with an ACQUITY UPLC photodiode array (PDA) eλ Detector. The mobile phases used were A, 0.3% phosphoric acid, 5% methanol in 18.2 MΩ·cm water and B, acetonitrile. The gradient condition was 0 min, 1% B; 0.5 min, 1% B; 2.25 min, 8% B; 3 min, 9% B; 4.5 min, 12% B; 5 min, 15% B; 6.5 min, 16% B; 10 min, 30% B; 11 min, 95% B; 11.65 min, 95% B; 11.7 min, 1% B; 15 min, 1% B. The system was controlled and data was collected and analyzed using Empower™ 3 Software. Peak identification was achieved by comparing retention time and spectral data with those of standards listed in the chemicals and materials section.

3.3.10 Cell culture and cytotoxicity assay

H9c2 rat myoblasts were purchased from American Type Culture Collection (Manassas, VA). H9c2 cells were cultured in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. To assess cytotoxicity of the lingonberry samples, cells were seeded into black 96-well plates (1.0×10^4 cells per well). After allowing cells to adhere, media was aspirated and replaced with media supplemented with berry samples. After 24 hours, alamarBlue cell viability reagent was added to each well and plates were incubated for 2 hours at 37°C. Fluorescence was measured (excitation = 570 nm, emission = 585 nm) using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software. All samples and controls were tested in quadruplicate and results were expressed as percentage of control (control = 100%).

3.3.11 Simulated IR

After 24 hour pretreatment with berry samples to allow for changes in gene expression, media was aspirated and cells were washed twice with phosphate-buffered saline (PBS) and IR was simulated using the method established in our previous studies (Sun *et al*. 2012). Media was replaced with ischemic buffer (137 mM sodium chloride 15.8 mM potassium chloride, 0.49 mM magnesium chloride, 0.9 mM calcium chloride, 4 mM HEPES, 10 mM deoxyglucose, 20 mM sodium lactate, 1 mM sodium dithionate, pH 6.4) and cells were placed in a modular incubator chamber. Oxygen was removed by flushing the chamber with a mixture of 95% N_2 and 5% CO_2 gas for 5 minutes at 25 L per minute. The chamber was sealed and incubated for 1 hour at 37°C to simulate ischemia. After 1 hour, ischemic buffer was aspirated, cells were

washed twice with PBS, and cells were incubated in media for 30 minutes under normoxic conditions to simulate reperfusion. For treatment groups, both ischemic buffer and reperfusion media were supplemented with berry extract, fractions or anthocyanins. For control cells (no IR), media was aspirated, cells were washed twice with PBS, fresh media was added, and cells were incubated for 90 minutes.

3.3.12 Determination of apoptosis

Hoechst Stain

Cells were seeded into 6-well plates (1.2×10^5 cells per well) and incubated in cell culture media (control and IR groups) or cell culture media pretreated with berry samples (IR treatment groups) for 24 hours. Similarly, in place of the berry samples, the selective JNK inhibitor SP600125 was also tested at 5 and 10 µM. IR was simulated while control plates were incubated for 90 minutes. After 1 hour ischemia and 10 minutes reperfusion, 7 µL of Hoechst 33258 was added to each well and plates were returned to the incubator for the final 20 minutes of reperfusion. Cells were then fixed with 10% formalin and viewed at 200x magnification with an Olympus IX81 microscope equipped with X-CITE Fluorescence Illumination Series 120Q. Each sample was tested on 3 wells and 34 random fields were photographed per well, for a total of 102 fields per treatment. Photos were analyzed using ImageJ software (Abràmoff *et al*. 2004) to count the numbers of apoptotic nuclei and non-apoptotic nuclei based on pre-set parameters for size and brightness of nuclei. Results were expressed as percentage of nuclei that were apoptotic compared to the IR group (IR = 100%).

Caspase-3 activity assay

Caspase-3 assays were performed using SensoLyte Homogeneous AFC Caspase-3/7 Assay Kits. Cells were seeded into 100 mm plates (4 x 10^5 cells per well) and incubated in cell culture media (control and IR groups) or cell culture media pretreated with berry samples (IR treatment groups) for 24 hours. IR was simulated while control plates were incubated for 90 minutes. Cells were washed twice with ice cold PBS, covered with 1 mL ice cold lysis buffer, and scraped into microfuge tubes. Tubes were shaken for 30 minutes at 4°C, centrifuged for 10 minutes at 2500 g at 4°C, and the supernatant was collected. Protein was determined using a Bradford protein assay (Bradford. 1976). Supernatant and caspase-3 tetrapeptide substrate (DEVD) solution was added to each well of a black 96-well, using lysis buffer for blank wells. The plate was shaken at 100-200 rpm for 30-60 minutes at room temperature, fluorescent intensity was measured (excitation = 380 nm, emission = 500 nm) using a SpectraMax M5 microplate reader, and data was analyzed using SoftMax Pro (version 6.2) software. All samples were tested in quadruplicate and results were expressed as percentage of the IR group (IR = 100%).

Assays for total and phosphorylated JNK and c-Jun

Cells were seeded into 100 mm plates (4 x 10^5 cells per well) and incubated in cell culture media (control and IR groups) or cell culture media pretreated with berry samples or anthocyanins (IR treatment groups) for 24 hours. IR was simulated while control plates were incubated for 90 minutes. Control cells, IR cells, and IR cells treated with berry samples or pure anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-arabinoside at 0.1 μg/mL, 1 μg/mL, and 5 μg/mL) were collected and frozen as whole cells at -80°C until use. On

the day of the assay, cells were lysed, supernatants were collected, and assays were performed according to Meso Scale Discovery protocols. One set of electrochemiluminescent multiplex microplates were spotted with JNK and phospho-JNK antibodies and one set of microplates were spotted with c-Jun and phospho-c-Jun antibodies. Briefly, multiplex microplates were blocked with blocking solution, washed 3 times, incubated with cell lysates, washed another 3 times, incubated with detection antibodies, and analyzed using a Sector Imager 2400. Samples were tested in triplicate and % phosphoprotein was calculated as [(Phospho-signal/Total signal) x 100]. Protein was determined using a Bradford protein assay (Bradford. 1976). Results were expressed as percentage of IR group (IR = 100%).

Western immunoblotting analysis

Cells were seeded into 100 mm plates (4 x 10^5 cells per well) and incubated in cell culture media (control and IR groups) or cell culture media pretreated with berry samples (IR treatment groups) for 24 hours. IR was simulated while control plates were incubated for 90 minutes. Cleaved PARP levels were measured in control cells, IR cells, and IR cells treated with lingonberry samples using western immunoblotting analysis. Whole cell lysate proteins (40 µg) were separated by electrophoresis on an 8% SDS-polyacrylamide gel (Laemmli. 1970), transferred to a nitrocellulose membrane (Pall BioTrace, pore size 0.2 µm) (Towbin *et al*. 1979), and probed with rabbit polyclonal anti-cleaved PARP antibody (1:1000). Subsequent to incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000), bands were visualized using enhanced chemiluminescence reagent (Millipore Corp.,

Billerica, MA) and the Alpha Innotech FluorChem Q imaging system. β-Actin was used as an internal control for equal loading of samples in the gel.

3.3.13 Statistical analysis

Data are presented as means ± SE of at least three independent experiments. Results were analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. *P* values less than 0.05 were considered statistically significant. For all data, groups specified by the same letter are not significantly different. All statistical analysis was performed using PASW Statistics 18 software (SPSS, New York, NY).

3.4 Results

3.4.1 Antioxidant capacity and anthocyanin content of lingonberry extract and fractions

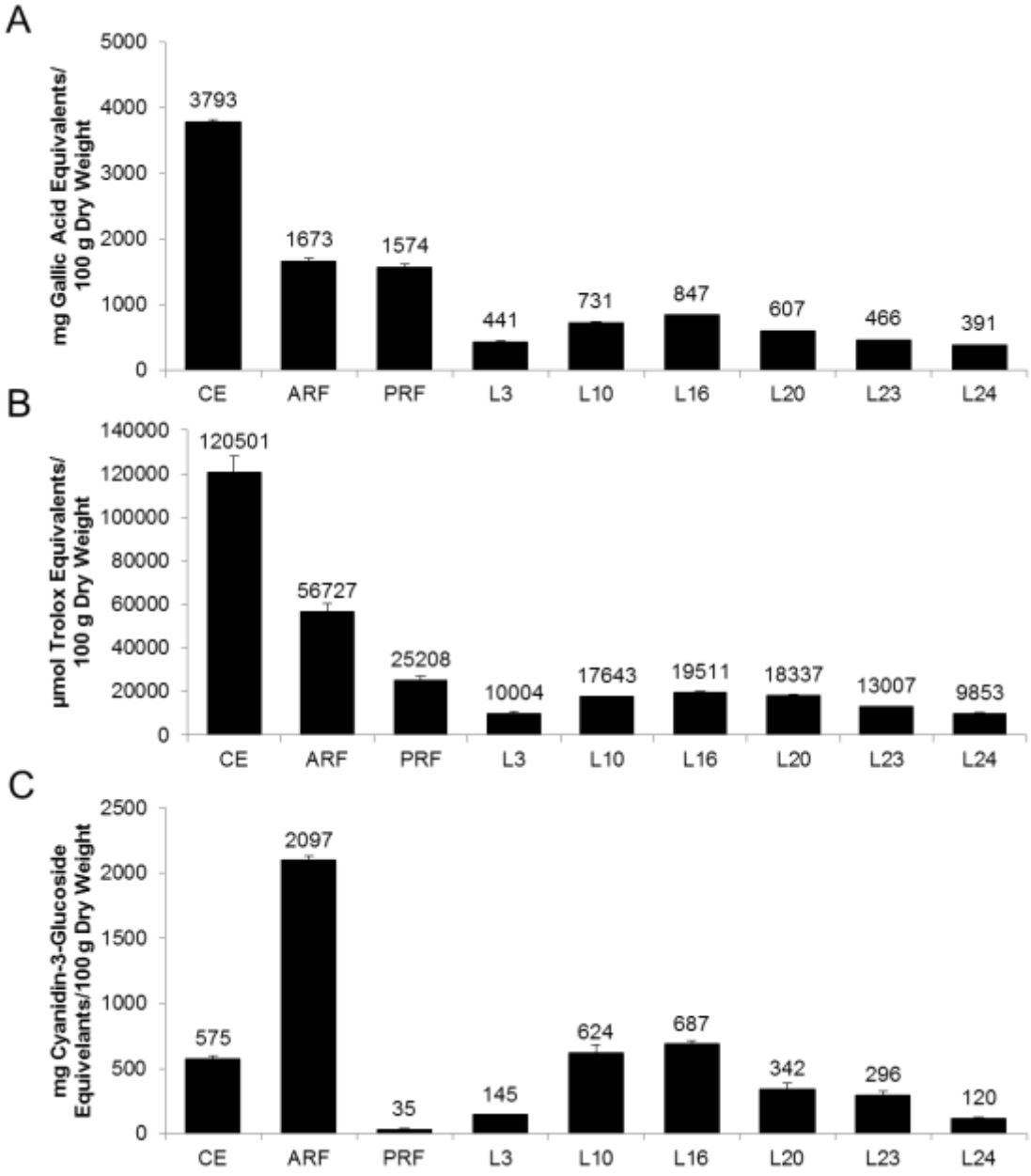
In Figure 3.1, the antioxidant capacity (Figure 3.1A) and total phenolic content (Figure 3.1B) of the five Eastern Canada lingonberry clones, the European cultivar, CE, ARF, and PRF, and anthocyanin content (Figure 3.1C) of CE, ARF, and PRF are shown; all values are shown per 100 g dry weight. The undiluted CE contained 240 µg cyanidin-3-glucoside equivalents and 1580 µg GAE per mL, the undiluted ARF contained 874 µg cyanidin-3-glucoside equivalents per mL, and the undiluted PRF contained 656 µg GAE per mL. Based on these results, for subsequent experiments 4 concentrations of each sample were used, corresponding to 1:100, 1:500, 1:1000, and 1:2000 dilutions with cell culture media.

Overall, based on the methods used in this study, the Manitoba lingonberry had high antioxidant capacity. It should be noted that the antioxidant capacities of ARF and PRF add up

to only two thirds of the antioxidant capacity of CE. This may be because small phenolic compounds like gallic acid are washed out of the column during the initial wash steps of the fractionation process or due to synergistic actions of some polyphenols. These compounds likely account for a significant proportion of the antioxidant capacity of CE.

Figure 3.1 Antioxidant capacity of lingonberry extracts.

Phenolic content in mg GAE per liter (A), antioxidant capacity in µmol Trolox equivalents per liter (B), and anthocyanin content in mg cyanidin-3-glucoside equivalents per liter (C) of lingonberry genotypes measured by total phenolics assay, oxygen radical absorbance assay, and total anthocyanins assay respectively.



3.4.2 UPLC profiles of extract and fractions

UPLC chromatograms detected at 525 nm showed that lingonberry extract contained 3 main anthocyanin peaks. We identified the smallest peak as cyanidin-3-glucoside, the next-smallest peak as cyanidin-3-arabinoside, and the largest peak as cyanidin-3-galactoside by comparing retention times and spectral maximums with available standards (Figure 3.2A). The structures of these anthocyanins are shown in Figure 3.3. We confirmed the effectiveness of our fractionation technique by observing the chromatograms detected at 525 and max plots of the CE, ARF, and PRF. As expected, the chromatogram of ARF was devoid of peaks other than the three anthocyanin peaks, while the PRF chromatogram contained many peaks identified as phenolic compounds but did not contain any of the three anthocyanin peaks (Figure 3.2B).

Figure 3.2 UPLC chromatograms of lingonberry extracts.

Compounds in the complete extract were detected at 525 nm (A) and compounds in the complete extract (CE, red), phenolic-rich fraction (PRF, blue), and anthocyanin-rich fraction (ARF, black) were detected at full scanning range (B). In (B), ARF contains only the 3 anthocyanin peaks, anthocyanin peaks are absent from PRF, and CE contains all peaks present in ARF and PRF.

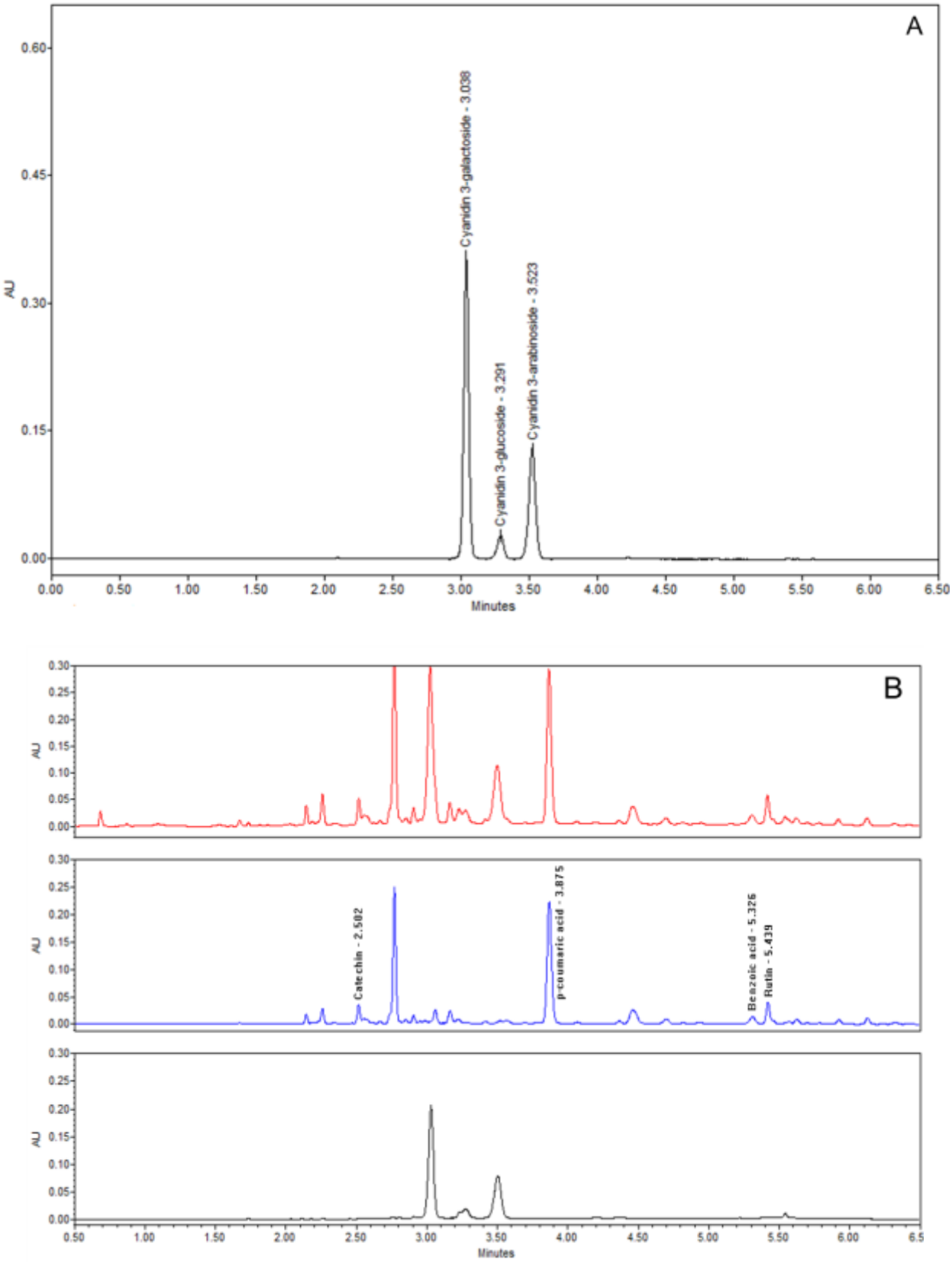
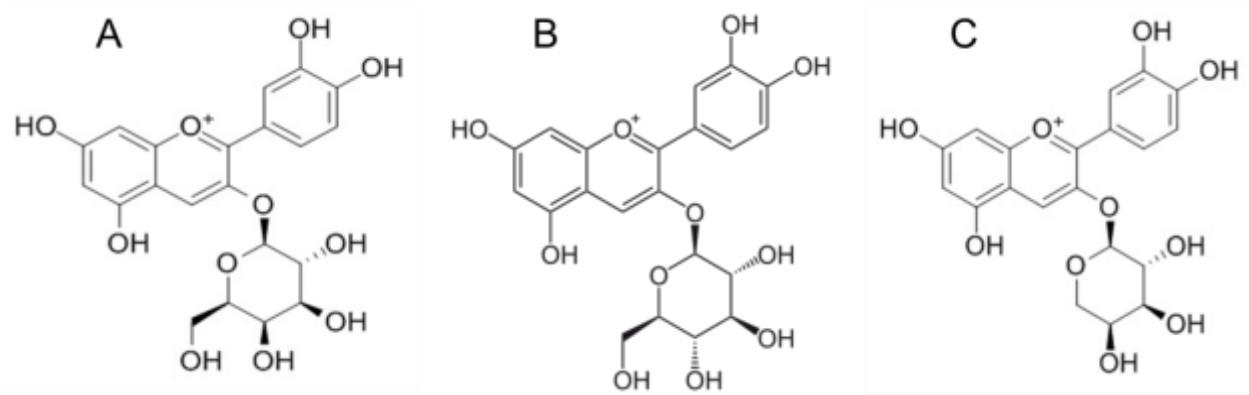

A
Cyanidin 3-galactoside - 3.038
Cyanidin 3-glucoside - 3.291
Cyanidin 3-arabinoside - 3.523
AU
Minutes
B
Catechin - 2.502
p-coumaric acid - 3.875
Benzoic acid - 5.326
Rutin - 5.439
AU
Minutes

Figure 3.3 Structures of the major anthocyanins found in lingonberry: cyanidin-3-galactoside (A), cyanidin-3-glucoside (B), and cyanidin-3-arabinoside (C).



3.4.3 Characterization of apoptosis and cytoprotection

Inhibition of nuclei condensation

All samples were tested at the high concentrations for cytotoxicity prior to subsequent experiments. None of the concentrations tested affected cell viability, as indicated in Figure 3.4. Our model of IR induced a 25-fold increase in nuclei condensation (an indicator of apoptosis) compared to H9c2 cells not exposed to IR (Figure 3.5A and Figure 3.5B). Addition of CE (Figure 3.5C), ARF (Figure 3.5D), and PRF (Figure 3.5E) to the cell culture medium prevented IR-induced nuclei condensation as measured by the Hoechst stain. The ratio of condensed to normal nuclei in the IR group without treatment was set to 100% and CE, ARF, and PRF treatment groups and controls were compared to this ratio (Figure 3.6). For Manitoba lingonberry, even at the lowest concentrations tested, all three samples inhibited nuclei condensation by at least 50% when

compared to the IR group. Each sample exhibited a dose-dependent inhibition of nuclei condensation, which is indicative of apoptosis.

Figure 3.4 Effect of lingonberry on cell viability. *Cell viability of H9c2 cells treated with lingonberry extracts as measured fluorescently using alamarBlue cell viability reagent. None of the treatments reduced cell viability.*

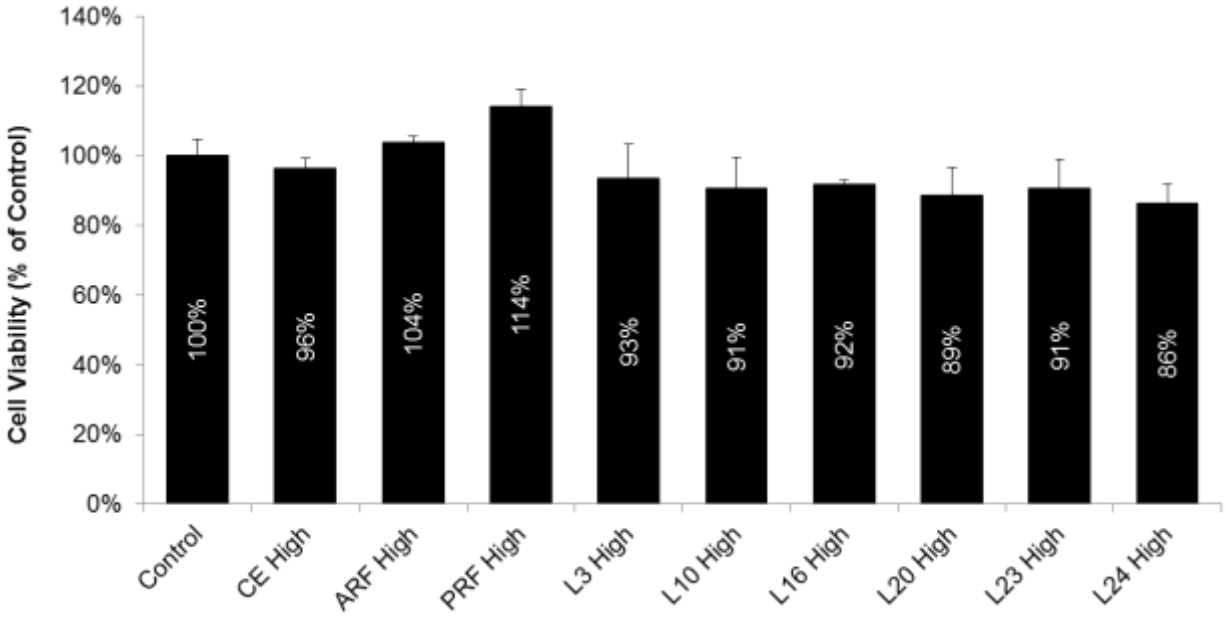
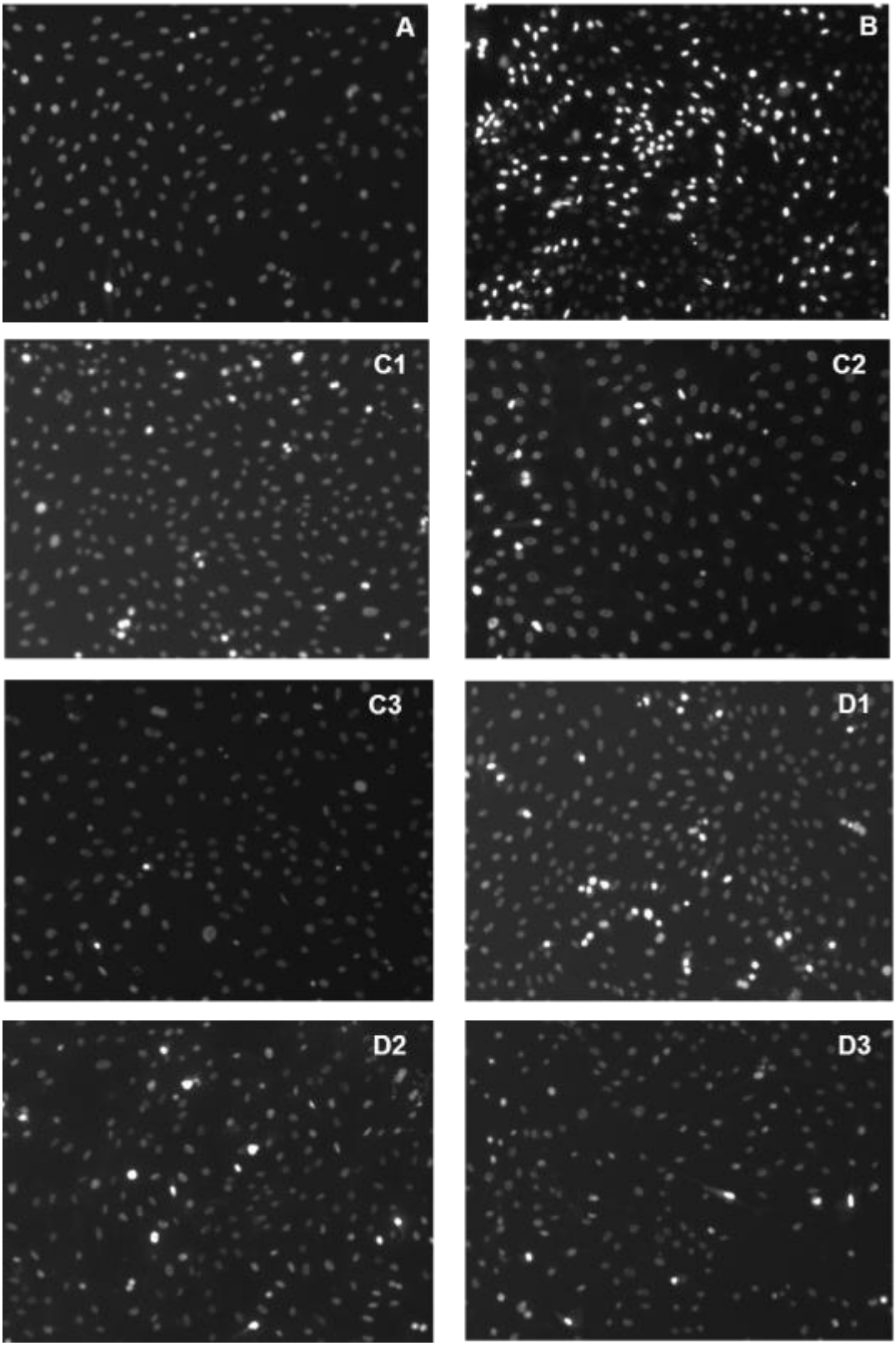


Figure 3.5 Representative fluorescence microscopy images of the effect of lingonberry on chromatin condensation.

Hoechst staining of H9c2 cells A) under normoxic conditions, B) after 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR), C) after 1 hour simulated ischemia and 30 minutes simulated reperfusion with CE treatment at 3 concentrations (1 = very low, 2 = low, 3 = medium), D) after 1 hour simulated ischemia and 30 minutes simulated reperfusion with ARF treatment at 3 concentrations, and E) after 1 hour simulated ischemia and 30 minutes simulated reperfusion with PRF treatment at 3 concentrations (1, 2, 3).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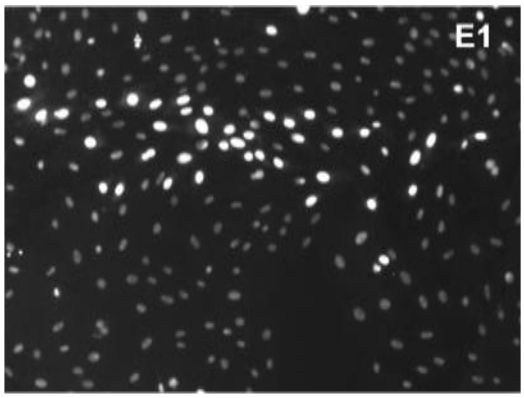
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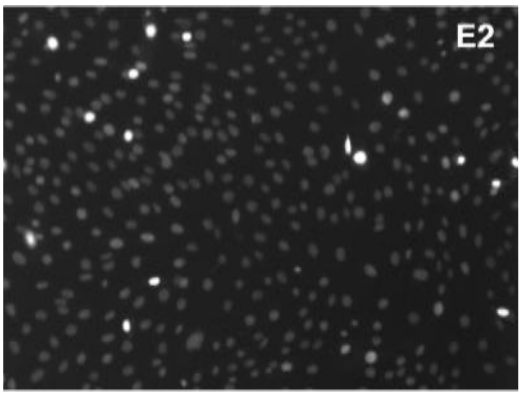
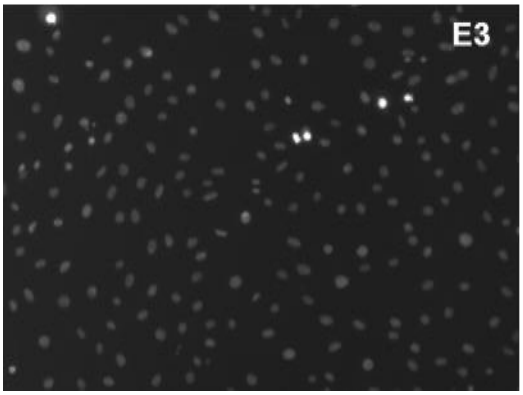
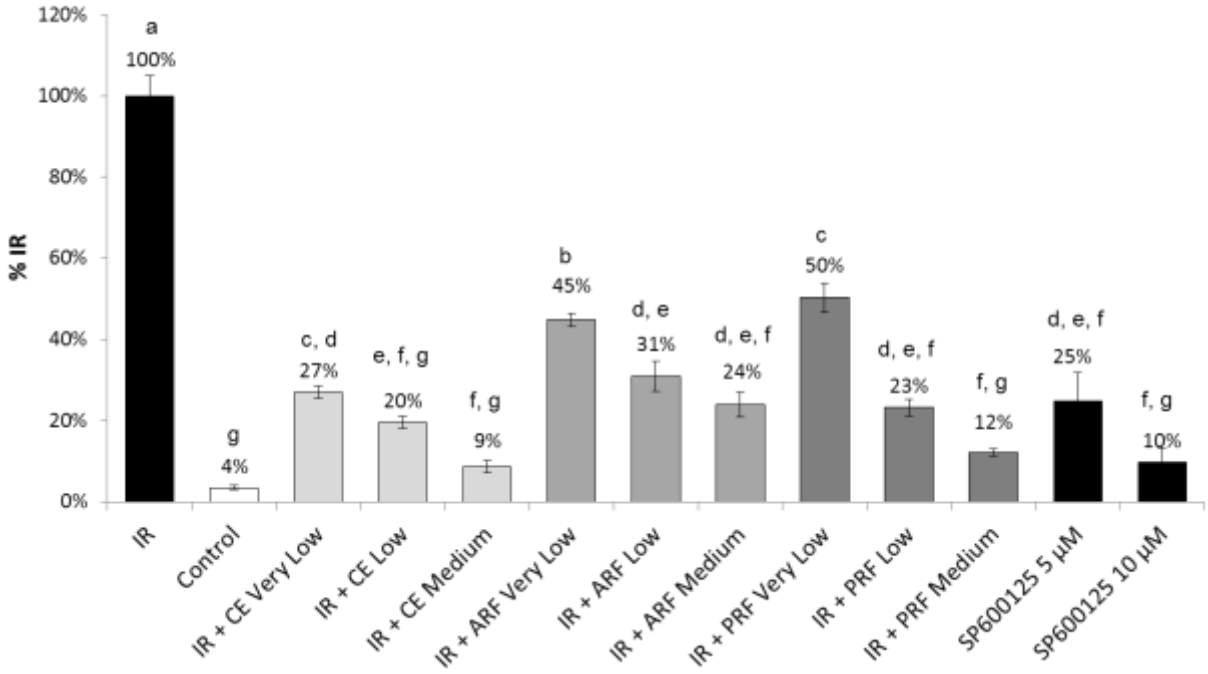

E2


E3

The selective JNK inhibitor SP600125 was also tested for its ability to inhibit nuclei condensation in H9c2 cells in order to demonstrate the role of JNK in the apoptotic mechanism induced by our model of IR (Figure 3.6). SP600125 was able to inhibit nuclei condensation by 75% at a concentration of 5 μM, indicating that JNK activation plays a role in apoptosis in this setting.

Figure 3.6 Quantification of effect of lingonberry on chromatin condensation.

Effect of CE, ARF, and PRF (medium dose = 1:500 dilution in cell culture media, low dose = 1:1000 dilution, very low dose = 1:2000 dilution) on ratio of condensed to normal nuclei as measured by Hoechst stain in control cells and cells subjected to 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR), with IR set to 100%. Results are expressed as the mean ± SEM (n= 3). Groups specified by the same letter are not different at $P < 0.05$.



Inhibition of caspase-3 activity and cleaved PARP expression

In our model, IR induced more than a 2-fold increase in caspase-3 activity compared to control cells. The effects of all lingonberry samples on caspase-3 activity are shown in Figure 3.7. In the case of Manitoba Lingonberry, CE exhibited stronger inhibition of caspase-3 activity than PRF and ARF, and PRF was slightly more effective than ARF at inhibiting caspase-3 activity. All three samples significantly inhibited caspase-3 activation. IR induced a 2-fold increase in levels of cleaved PARP, a target of caspase-3 in IR-injured cells, and CE prevented this increase (Figure 3.8).

Figure 3.7 Effect of lingonberry on caspase-3 activation.

Effect of CE, ARF, and PRF (medium dose = 1:500 dilution in cell culture media, low dose = 1:1000 dilution) on caspase-3 activity in control cells and cells subjected to 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR), with IR set to 100%. Enzyme activity was measured fluorescently and results are expressed as the mean ± SEM (n= 3). Groups specified by the same letter are not significantly different at P < 0.05.

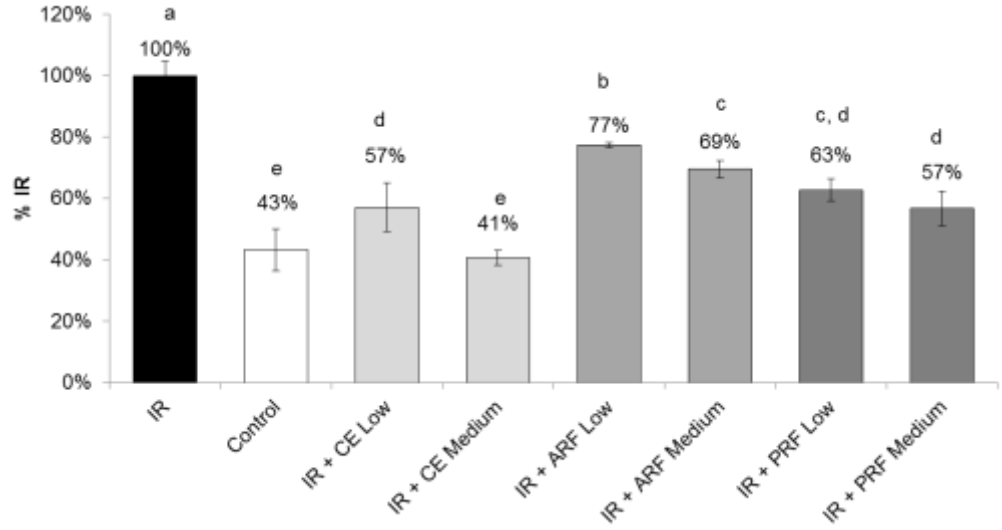
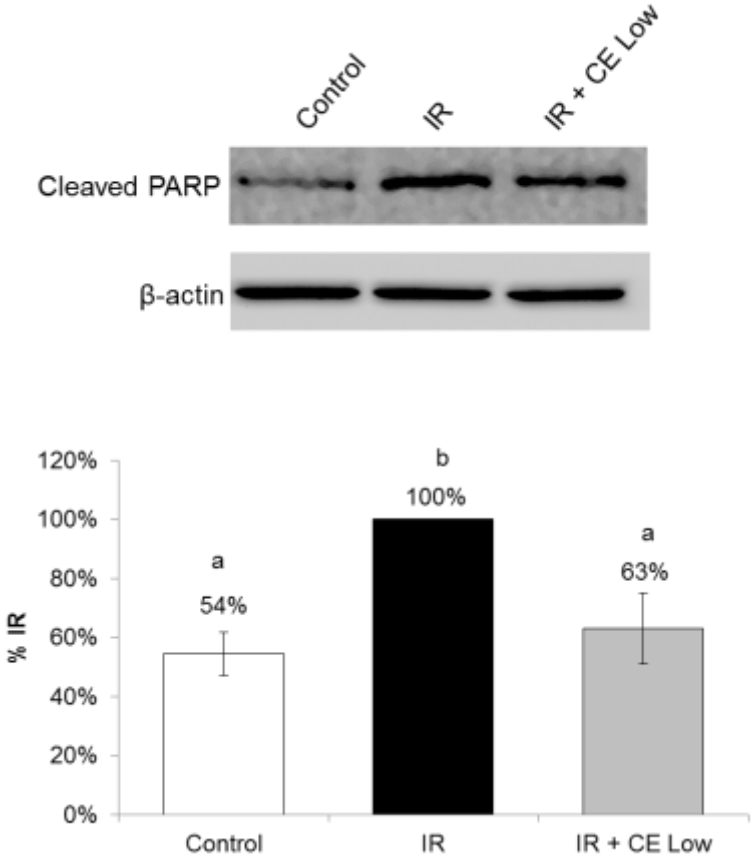


Figure 3.8 Effect of lingonberry on PARP cleavage.

Effect of CE (medium dose = 1:500 dilution in cell culture media) on levels of cleaved PARP in cells subjected to 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR) as compared to untreated normoxic cells and untreated IR cells. Protein was determined by Western immunoblotting analysis (40 µg protein). Results are expressed as the mean ± SEM (n= 3). Groups specified by the same letter are not significantly different at P < 0.05.

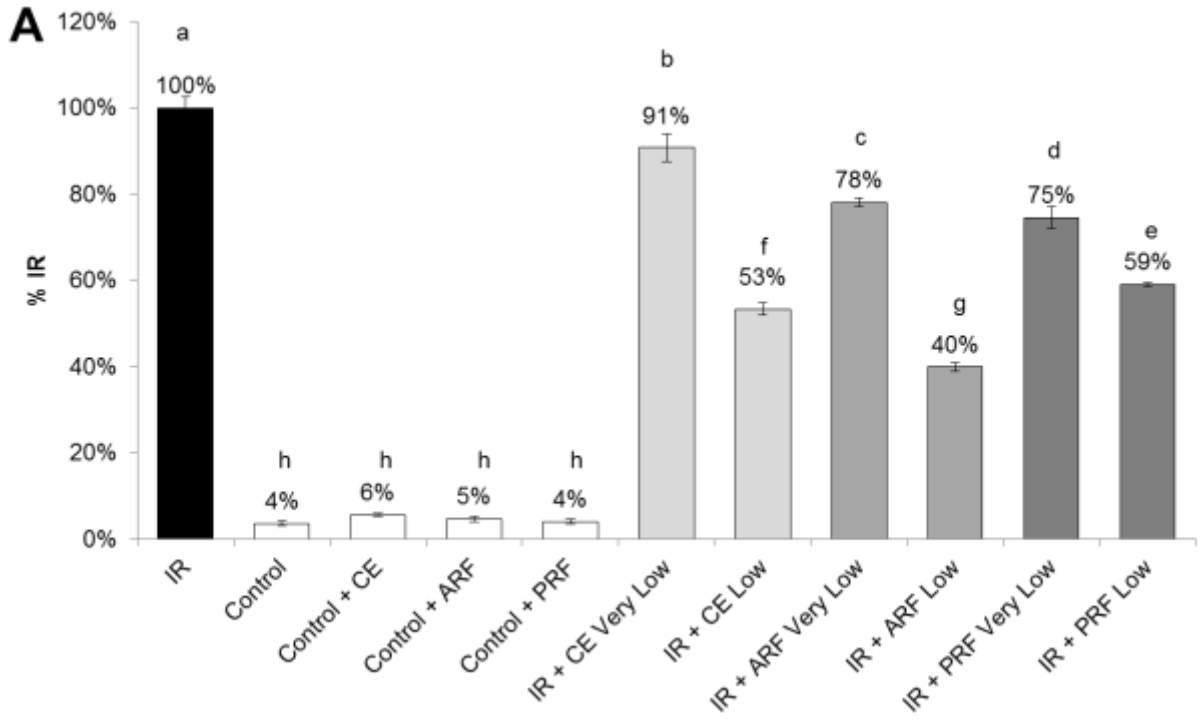


Inhibition of JNK activity

Using electrochemiluminescent multiplex assays, it was determined that IR induced a 20-fold increase in phosphorylated JNK and a 7-fold increase in c-Jun phosphorylation. Treatment with CE, ARF, and PRF had no effect on JNK or c-Jun phosphorylation in control cells. The effects of treatment with CE, ARF, and PRF on JNK phosphorylation are shown in Figure 3.9A and the effects on c-Jun phosphorylation are shown in Figure 3.9B. All three samples significantly inhibited JNK activity in a concentration-dependent manner. ARF provided the strongest inhibitory effect on IR-induced JNK activation.

Figure 3.9 Effect of lingonberry on JNK and c-Jun activation.

Effect of CE, ARF, and PRF (low dose = 1:1000 dilution in cell culture media, very low dose = 1:2000 dilution) on JNK activation (A) and c-Jun phosphorylation (B) in control cells and cells subjected to 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR), with IR set to 100%. Percent protein phosphorylation was measured electrochemiluminescently with Meso Scale Discovery multiplex assay kits. Results are expressed as the mean ± SEM (n= 3). Groups specified by the same letter are not significantly different at $P < 0.05$.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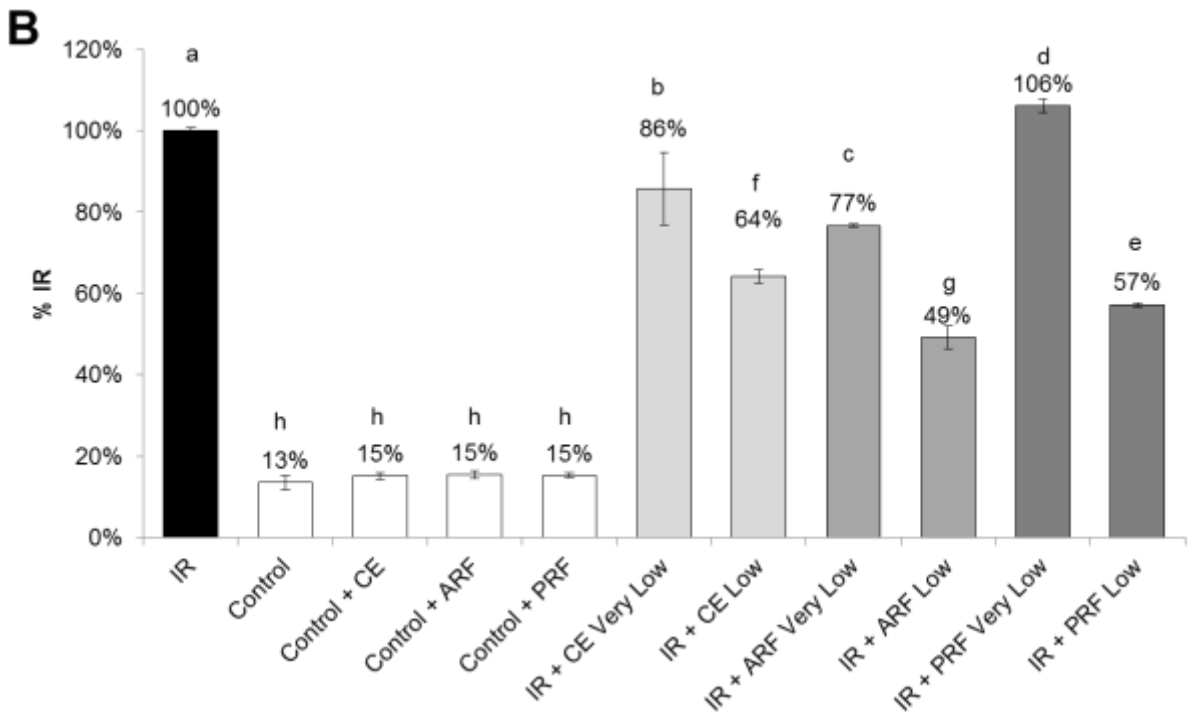
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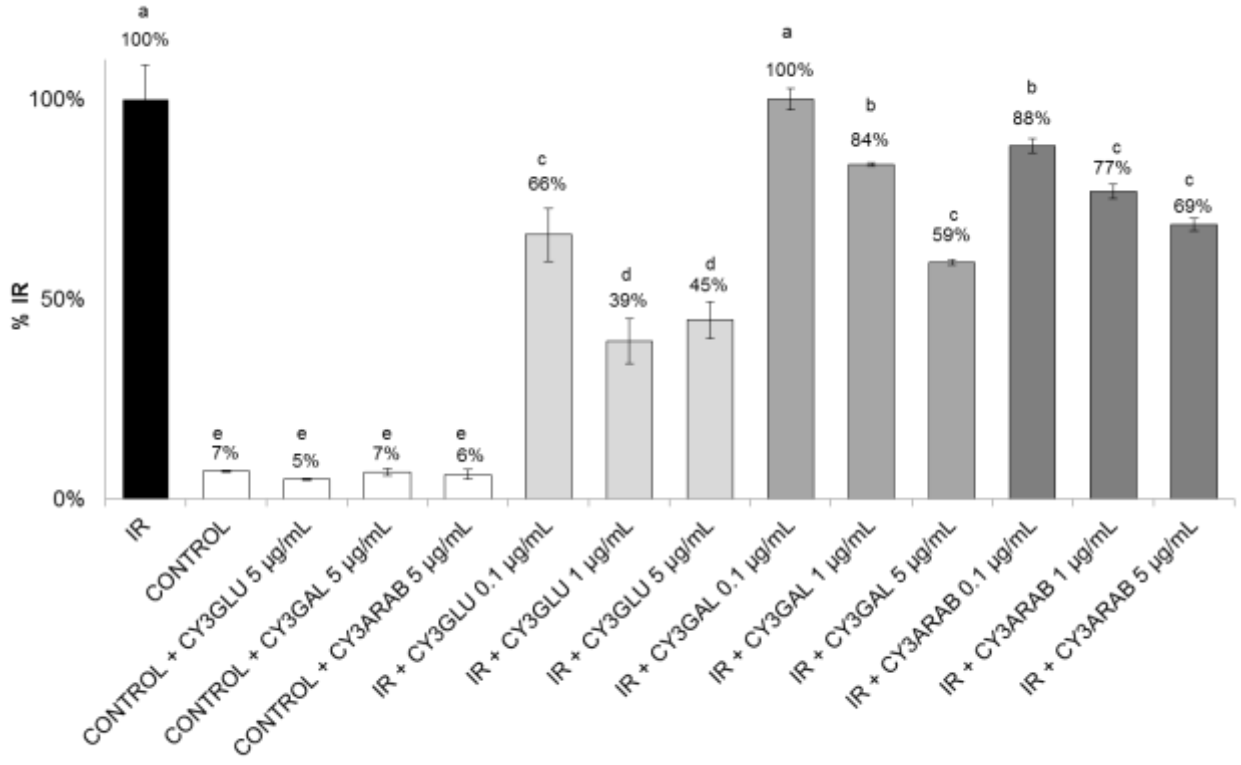
IR + PRF Low

The effects of treatment with cyanidin-3-glucoside, cyanidin-3-galactoside, and cyanidin-3-arabinoside on JNK phosphorylation are shown in Figure 3.10. All three anthocyanins showed an inhibitory effect on JNK phosphorylation, an indication of their protective effect against IR-induced JNK activation. Cyanidin-3-glucoside provided the highest level of protection but all three anthocyanins effectively protected against JNK activation at physiologically relevant doses.

Figure 3.10 Effect of lingonberry anthocyanins on JNK activation.

Effects of individual anthocyanins cyanidin-3-glucoside (CY3GLU), cyanidin-3-galactoside (CY3GAL) and cyanidin-3-arabinoside (CY3ARAB) at 5, 1, and 0.1 µg/mL on JNK activation in control cells and cells subjected to 1 hour simulated ischemia and 30 minutes simulated reperfusion (IR), with IR set to 100%. Percent protein phosphorylation was measured electrochemiluminescently with Meso Scale Discovery multiplex assay kits. Results are expressed as the mean ± SEM (n= 3). Groups specified by the same letter are not significantly different at P < 0.05.



3.5 Discussion

The novel findings of this study are 1) Manitoba lingonberry, analyzed for the first time in the scientific literature, has high *in vitro* antioxidant capacity and contains large quantities of anthocyanins including 3 main anthocyanins in agreement with European varieties, and 2) the significant protection against nuclear condensation, caspase-3 activation, and JNK phosphorylation provided by Manitoba lingonberry samples and pure anthocyanins in a model of IR in H9c2 cells.

The antioxidant capacity of the Manitoba lingonberry CE was higher than many reported ORAC values for commonly consumed berries (Bakowska-Barczak *et al*. 2007; Zheng and Wang. 2003) and the ORAC values of Eastern Canada and European lingonberry. However, from this study, it is not possible to compare the antioxidant activity among different genotypes and the Manitoba populations. The Manitoba lingonberries consist of two populations containing a number of genotypes. They were collected from the wild in 2011 but the Newfoundland clones and the cultivar Mesovia were being grown in a greenhouse under controlled conditions for more than 10 years. There is evidence that berries grown in colder temperatures and at higher latitudes produce more anthocyanins and polyphenolic compounds (Uleberg *et al*. 2012). It is possible that the colder climate of Manitoba may be responsible for the increase in antioxidant capacity in Manitoba lingonberry. Anthocyanin and total phenolic content were strongly correlated (Pearson correlation coefficient = 0.97, data not shown) for the Newfoundland and European clones grown in house, however the ratio of anthocyanin content to total phenolic content in wild Manitoba lingonberry was much higher than the lingonberries grown in house

lower due to the exceptionally high levels of total phenolics. Further research will investigate whether the change in ratio is due to genetic variability or growing conditions.

The identified anthocyanin peaks are in agreement with results from studies on North American and European lingonberry (Ek *et al*. 2006; Lee and Finn. 2012). Brown et al. confirmed that wild lingonberry from British Colombia, Canada contained 300 mg anthocyanins per 100 g dry weight and identified cyanidin-3-glucoside, cyanidin-3-galactoside, and cyanidin-3-arabinoside as the three major anthocyanins (Brown *et al*. 2012). Using the results for the ORAC, total phenolics, and total anthocyanins assays of CE, ARF, and PRF, the doses used for the subsequent cell studies were selected based on a literature review of other cellular studies on lingonberry as well as reported plasma concentrations of anthocyanins and antioxidants achieved following oral dosing (McGhie and Walton. 2007; Wang *et al*. 2005). Future studies will need to standardize berry cultivation and collection to account for these differences and also test the year-to-year variability of wild lingonberry extracts to confirm the exceptionally high anthocyanin and antioxidant content of Manitoba lingonberry.

IR injury in H9c2 cells is well-described in the literature. In these cells, IR has been shown to induce JNK and ERK activation (Becatti *et al*. 2012; Borchi *et al*. 2009; Fiorillo *et al*. 2008; Pechtelidou *et al*. 2008; Xu *et al*. 2010; Yin *et al*. 2013), nuclei condensation (Sun *et al*. 2012), caspase-3 activation (Fiorillo *et al*. 2008), and pro-apoptotic protein activation (Becatti *et al*. 2012; Pechtelidou *et al*. 2008; Yin *et al*. 2013). Because reactive oxygen species accumulate rapidly during reperfusion, polyphenolic compounds with high *in vitro* antioxidant capacity have been hypothesized as potential therapeutic treatments for IR injury. Indeed, the polyphenols tyrosol (Sun *et al*. 2012), curcumin (Fiorillo *et al*. 2008), danshensu (Yin *et al*. 2013), resveratrol

(Becatti *et al*. 2012), and others have been shown to be protective in cardiomyocyte IR injury. Polyphenols may act as direct intracellular antioxidants, since intracellular reactive oxygen species accumulation is generally inhibited with these treatments. An NADPH oxidase inhibitor has been shown to simultaneously reduce intracellular reactive oxygen species and JNK phosphorylation in H9c2 cells (Borchi *et al*. 2009), so perhaps antioxidants may inhibit apoptosis by reducing reactive oxygen species production. Recently, Bhullar and Rupasinghe demonstrated that lingonberry extracts could protect fibroblasts against peroxyl radical-induced stress by scavenging reactive oxygen species and activating the Nrf2 pathway, indicating that lingonberry polyphenols may be potent intracellular antioxidants (Bhullar and Rupasinghe. 2015a).

Several lines of evidence in this study demonstrated the anti-apoptotic effects of Manitoba lingonberry compounds. First, our results indicated that Manitoba lingonberry was efficient at preventing nuclei condensation in a model of IR. Nuclei condensation is an indicator of apoptosis because as the cell dismantles its nucleus and fragments its DNA, nuclei become small and stain brightly with Hoechst 33528. Second, Manitoba lingonberry also inhibited caspase-3 activation significantly in this model and reduced levels of cleaved PARP. Caspase-3, an executioner caspase, has numerous cellular targets, the cleavage of which is essential for apoptosis, including PARP (Chowdhury *et al*. 2008). The fact that Manitoba lingonberry treatment inhibited nuclei condensation and caspase-3 activation simultaneously suggested that apoptosis, rather than necrosis, was the primary mode of cell death in our model of IR. Third, we observed that Manitoba lingonberry inhibited JNK and c-Jun phosphorylation concurrently with inhibiting apoptosis. We used the selective JNK inhibitor, SP600125 (Bennett

et al. 2001) to show the relevance of this MAPK signaling pathway in our model. JNK inhibition was associated with a marked decrease in apoptosis, indicating that JNK activation is relevant to apoptosis in this setting. Importantly, pure anthocyanins also inhibited JNK activation in IR-injured H9c2 cells. These results are significant since several reports have confirmed the presence of pure anthocyanins in plasma and physiologically relevant doses were used. However, future studies are needed to examine the bioactivities of lingonberry metabolites since these compounds certainly have bioactivities themselves and may be formed in cell culture studies (Nurmi *et al*. 2009; Vitaglione *et al*. 2007). MAPK's include JNK and extracellular signal-regulated kinase and are involved in critical signaling pathways needed for cell proliferation, differentiation, and apoptosis. In the MAPK signaling cascades, many different signals may converge on JNK at once and the balance between the pro- and anti-apoptotic signals will decide whether JNK will be activated or not. Once activated via phosphorylation, JNK may translocate to the nucleus, where it phosphorylates specific pro-apoptotic transcription factors, or to the mitochondria, where it modulates the activity of mitochondrial proteins by phosphorylation. An important nuclear target of JNK is c-Jun, a component of the AP-1 pro-apoptotic transcription factor complex. JNK inhibitors have been shown to be protective against apoptosis in several cell types *in vitro* and a JNK inhibitor decreased myocardial apoptosis and reduced infarct size after myocardial IR in rats (Ferrandi *et al*. 2004).

In conclusion, our study provides the first analysis of Manitoba lingonberry and demonstrates the protective effects of lingonberry in a model of IR injury, which is a clinically relevant phenomenon in many diseases. Lingonberry fruits can be safely consumed at low-cost and, due to their high content of various bioactive compounds, may constitute part of a healthy

diet. Future research will allow further mechanistic insight into the role of lingonberry phytochemicals in human health.

3.6 Acknowledgement

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Chapter 4

Manuscript III: Lingonberry anthocyanins protect cardiac cells from oxidative stress-induced apoptosis

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4.1 Abstract

Lingonberry grown in Northern Manitoba, Canada contains exceptionally high levels of anthocyanins and other polyphenols. Previous studies from our lab have shown that lingonberry anthocyanins can protect H9c2 cells from IR injury and anthocyanin-rich diets have been shown to be associated with decreased cardiovascular disease and mortality. Oxidative stress can impair function and trigger apoptosis in cardiomyocytes. This study investigated the protective effects of physiologically relevant doses of lingonberry extracts and pure anthocyanins against hydrogen peroxide-induced cell death. Apoptosis and necrosis were detected in H9c2 cells after hydrogen peroxide treatment via flow cytometry using FLICA 660 caspase 3/7 combined with YO-PRO-1 and then confirmed with Hoechst staining and fluorescence microscopy. Each of the three major anthocyanins found in lingonberry, cyanidin-3-galactoside, cyandin-3-glucoside, and cyanidin-3-arabinoside, was protective against hydrogen peroxide-induced apoptosis in H9c2 cells at 10 ng mL^{-1} (20 nmol L^{-1}) and restored the number of viable cells to match the control group. A combination of the three anthocyanins was also protective and a lingonberry extract tested at three concentrations produced a dose-dependent protective effect. Lingonberry anthocyanins protected cardiac cells from oxidative stress-induced apoptosis and may have cardioprotective effects as a dietary modification.

4.2 Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is an evergreen shrub that grows in northern climates across Europe and North America. The berries contain very high levels of

anthocyanins, which are the red pigments that give all berries their bright red, blue, or purple colours Penhallegon 2006. The three main anthocyanins in lingonberry are cyanidin-3-galactoside (58% of all anthocyanins), cyanidin-3-arabinoside (35%), and cyanidin-3-glucoside (7%) (Dudonné *et al*. 2015; Isaak *et al*. 2015; Lee and Finn. 2012). In addition to containing more anthocyanins per gram of fresh weight than other commonly consumed berries, lingonberry also contains other polyphenols, including quercetin glycosides, kaempferol glycosides, resveratrol, phenolic acids and proanthocyanidins (Bakowska-Barczak *et al*. 2007; Grace *et al*. 2014; Kalt *et al*. 2008; Rimando *et al*. 2004; Zheng and Wang. 2003).

While polyphenols and anthocyanins can act as direct antioxidants *in vitro*, the *in vivo* mechanisms of many of their beneficial effects are still unclear (Croft. 2016; Loffredo *et al*. 2017). Anthocyanins and their metabolites likely act by modulating the gene expression or activity levels of pro-oxidant and antioxidant enzymes in cells (Bhullar and Rupasinghe. 2015a) or by mediating cell signaling pathways (Vendrame and Klimis-Zacas. 2015). Anthocyanins have been shown to improve the ratio of reduced and oxidized glutathione in tissue and decrease uric acid levels in plasma indicating they produce an "antioxidant effect" without directly scavenging free radicals (Alvarez-Suarez *et al*. 2014; Kuntz *et al*. 2014; Vanzo *et al*. 2013). These indirect antioxidant activities may be beneficial against cellular oxidative stress (Ahmed and Wilson Tang. 2012; Louis *et al*. 2014), which plays an important role in cardiovascular diseases by promoting programmed cell death (Dey *et al*. 2016; Martín-Fernández and Gredilla. 2016). This study utilized hydrogen peroxide treatment as a model of oxidative stress. There are several potential sources of hydrogen peroxide in cells, including xanthine oxidase, NADPH

oxidase, and superoxide dismutase, and each source may play a role in different cardiac diseases (Förstermann. 2008; Harvey and Grieve. 2012).

Polyphenol-rich diets have been shown to protect against cardiovascular pathologies associated with oxidative stress, such as myocardial infarct and coronary artery disease, and anthocyanin-rich diets specifically have been shown to be associated with decreased cardiovascular disease and mortality (Cassidy *et al*. 2013; McCullough *et al*. 2012; Quiñones *et al*. 2013). However, pharmacokinetic analyses of anthocyanin metabolism have shown that after eating an anthocyanin-rich meal or consuming pure anthocyanins, the intestinal absorption rate is low (McGhie and Walton. 2007). Intact anthocyanins generally do not exceed 100 nmol/L in plasma because they are rapidly taken up by the liver and kidneys where they are metabolized via methylation, glucuronidation, and degradation for excretion in feces or urine (De Ferrars *et al*. 2014; Vanzo *et al*. 2011; Vanzo *et al*. 2013). This study investigated the effects of lingonberry extracts and pure anthocyanins on hydrogen peroxide-induced apoptosis in H9c2 rat cardiomyoblasts at a physiological dose, 10 ng mL^{-1} or 20 nmol L^{-1}, which is within the reported rates of bioavailability for anthocyanins.

4.3 Methods

4.3.1 Sample preparation

Ultrapure water from a Milli-Q Synthesis A10 water purification system (Millipore Corp., Billerica, MA, USA) was used for all experiments. Unless otherwise stated, all chemicals of at least ACS grade were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ripe fruits from

wild lingonberry plants were hand-picked and immediately frozen at -20°C by a commercial berry picker on public land at Sherridon, Manitoba, Canada (55° 7’ N; 101° 5’ W). Frozen berries were lyophilized and ground to a fine powder. 0.5 g of lyophilized berry powder was extracted twice in 6 mL Optima-grade methanol /2% formic acid with 15 minutes sonication at 37°C followed by centrifugation for 15 minutes at 3000 x *g*. The supernatants from the two extractions were combined, filtered with a 0.2 µm syringe filter (Pall, Port Washington, NY, USA), and stored at -20°C. Methanol was then removed by rotary evaporation and the remaining extract was brought back up to the original volume with ultrapure water so it could be used for cell culture studies, which produced only minimal losses in antioxidant activity (data not shown).

4.3.2 Antioxidant capacity assays

These assays were used for standardizing the lingonberry extracts and ensuring stability following storage, rather than for assessing quality of the sample or predicting *in vitro* bioactivity. Total phenolic content of the lingonberry extract was determined colourimetrically as previously described (Isaak *et al*. 2015) by combining the FC reagent and the sample in a sodium carbonate buffer. Results were expressed as mg GAE per 100 g dry weight or per 100 g fresh weight using gallic acid as a standard. The ORAC of the extract was determined fluorimetrically as previously described (Isaak *et al*. 2015) using 2,2'-azobis(2-amidinopropane) dihydrochloride (Wako Chemicals, Richmond, VA, USA) as a peroxyl generator. Decline in fluorescence of fluorescein was measured kinetically for 60 minutes so the area under the curve could be calculated for each sample. Results were expressed as µmol TE per 100 g dry

weight or per 100 g fresh weight using Trolox as a standard. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging capacity was determined colourimetrically by mixing 7 mM ABTS with 5 mM potassium persulfate to generate blue radical cations, then adding sample to reduce the radicals back to the colourless form (Re *et al*. 1999).Results were expressed as µmol Trolox equivalents per 100 g dry weight or per 100 g fresh weight using Trolox as a standard. The FRAP of the extract was determined colourimetrically by mixing 10 mM 2,4,6-Tris(2 pyridyl)-s-triazine with 20 mM ferric chloride in acetate buffer (300 mM, pH 3.6) and then adding sample (Benzie and Strain. 1996). As antioxidants in the sample reduce ferric to ferrous iron, blue colour develops. Results were expressed as µmol Trolox equivalents per 100 g dry weight or per 100 g fresh weight using Trolox as a standard. All samples and standards were tested in quadruplicate. Developed colour or fluorescent intensity was measured using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software (Molecular Devices, Sunnyvale, CA, USA). Dry weight to fresh weight conversions were based on the moisture content of Manitoba lingonberries which was determined experimentally to be 85% (data not shown).

4.3.3 Cell culture

H9c2 rat cardiomyoblasts (CRL-1446; American Type Culture Collection, Manassas, VA, USA) were maintained in high glucose (4500 mg/L) DMEM (Hyclone, GE Life Sciences, Mississauga, ON, Canada) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% oxygen/5% carbon dioxide. Cells were subcultured when they reached 80-90% confluency.

4.3.4 Induction of apoptosis

Forty-eight hours prior to experiments, cells were seeded into 100 mm dishes for flow cytometry or 6-well plates for fluorescence microscopy. 24 hours prior to experiments, media was aspirated and replaced with fresh media for controls or media supplemented with lingonberry extract or anthocyanins. The lingonberry extract was tested at high (H, 1:500), medium (M, 1:1000), and low (L, 1:2000) dilutions. Three anthocyanins were previously reported (Isaak *et al*. 2015) to be found in Manitoba lingonberry: cyanidin-3-galactoside (95% purity) and cyanidn-3-glucoside (95% purity), which were purchased from Chromadex (Irvine, CA, USA), and cyanidin-3-arabinoside chloride (>95%) which was purchased from Cerilliant Corp. (Round Rock, TX, USA). Lingonberry anthocyanins were tested at 10 ng mL^{-1} (20 nmol L^{-1} for cyanidn-3-glucoside and cyanidin-3-galactoside, 22 nmol L^{-1} for cyanidin-3-arabinoside). A combination of the three main lingonberry anthocyanins was tested using the ratio found in Manitoba lingonberry (58% cyanidin-3-galactoside, 7% cyanidn-3-glucoside, and 35% cyanidin-3-arabinoside) at 10 ng mL^{-1}. After 24 hour pretreatment, cells were treated with 600 µmol L^{-1} hydrogen peroxide for two hours to induce apoptosis. This is the optimal concentration of hydrogen peroxide to induce apoptosis following experimental testing at 100, 200, 300, 400, 600, and 800 µmol L^{-1} hydrogen peroxide in accordance with the most commonly used concentrations found in the literature (data not shown).

4.3.5 Cell labeling and flow cytometry

After treatment with hydrogen peroxide, cells were washed with warm PBS and collected with 0.25% trypsin-EDTA. Cells were counted using a Countess Cell Counter (Life

Technologies, Carlsbad, CA, USA) and 10^6 cells were added to each polystyrene round-bottom tube. After centrifugation at 300 x *g* for 5 minutes, the supernatant was discarded to remove traces of trypsin and the cell pellet was resuspended in 600 µL DMEM. 10 µL FLICA 660-DEVD-FMK (ImmunoChemistry Technologies, Bloomington, MN, USA) was added and cells were incubated at 37°C for 60 minutes with agitation every 20 minutes. 1% bovine serum albumin (BSA) in PBS was then added to each tube to sequester any excess FLICA reagent. After centrifugation at 300 x *g* for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in 1 mL 1% BSA in PBS. 3 µL YO-PRO-1 (Molecular Probes, Eugene, OR, USA) was added and cells were incubated on ice for 20 minutes prior to flow cytometry analysis.

Labeled cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with laser excitation tuned to 488 nm and 633 nm. Forward versus side scatter histograms were used to identify cardiomyocytes. FLICA 660 excites at 660 nm, emits at 690 nm, and covalently binds active caspase 3 and caspase 7. YO-PRO-1 excites at 491 nm, emits at 509 nm, and detects necrotic and late apoptotic cells because viable cells are impermeant to the compound. The fluorescence signals were separated with the standard dichroic long pass filters provided with the instrument and detected through 530/30 nm (FITC) and 712/21 nm (Alexa-700) bandpass filters. Untreated and hydrogen-peroxide treated cells without FLICA reagent or YO-PRO-1 were used as unstained controls. FLICA labeled cells without YO-PRO-1 and YO-PRO-1 labeled cells without FLICA were used to adjust colour compensation. Each treatment or control was tested on three different days and 50,000 events were recorded for each experiment. Data was analyzed using FACSDIVA software (version 8.0.1; BD Biosciences, San Jose, CA, USA).

4.3.6 Fluorescence microscopy

To detect chromatin condensation, Hoechst 33258, which excites at 352 nm and emits at 461 nm, was used. After treatment with hydrogen peroxide, cells were incubated with 5 µg mL^{-1} Hoechst 33258 for 20 minutes, washed twice with PBS, fixed with 10% formalin, and viewed at 200x magnification with an Olympus IX81 microscope (Olympus, Richmond Hill, ON, Canada) equipped with X-CITE Fluorescence Illumination Series 120Q light source (Excelitas, Waltham, MA, USA). Photos of three independent experiments were analyzed using ImageJ software to count the numbers of apoptotic nuclei (bright) and non-apoptotic (dark) nuclei (Abràmoff *et al*. 2004). A minimum of 10,000 cells were counted for each treatment group.

4.3.7 Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test using PASW Statistics 18 (SPSS, New York, NY, USA). *p* values less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 *In vitro* antioxidant capacity of lingonberry extract

Table 4.1 shows the antioxidant capacity of the lingonberry extract based on four antioxidant assays. The antioxidant activity of the lingonberry extract is likely due to the combined action of polyphenols, procyanidins, and small phenolic acids. The total phenolics assay measures the total reducing capacity of the sample, or the ability of the sample to donate electrons to the FC reagent used in the assay. The ORAC assay measures antioxidant capacity

against peroxyl radicals specifically, or the ability of the sample to protect the fluorescein reagent from degradation by peroxyl radicals. The FRAP assay measures the ferric reducing capacity of the sample, or the ability of the sample to donate electrons to ferric ions. The ABTS assay measures the ability of the sample to scavenge ABTS radicals. These results are provided to demonstrate the high *in vitro* antioxidant capacity of lingonberry, in accordance with our previous results (Isaak *et al*. 2015) and reinforced using additional antioxidant assays.

Table 4.1 In vitro antioxidant capacity of lingonberry extract using four different antioxidant assays.

	per 100 g DW	**per 100 g FW**
Total phenolic content	3844 ± 433	577 ± 65
ORAC	100393 ± 4974	15059 ± 746
ABTS scavenging capacity	21556 ± 1627	3233 ± 244
FRAP	35114 ± 1851	5267 ± 278

Total phenolic content is expressed as mg GAE per 100 g dry or fresh weight of berries. ORAC, ABTS scavenging capacity, and FRAP results are expressed as µmol TE per 100 g DW or FW of berries. Results are shown as mean ± standard deviation and all samples were tested in quadruplicate.

4.4.2 Effects of lingonberry extract and anthocyanins on hydrogen peroxide-induced apoptosis

The two stains used, FLICA 660 and YO-PRO-1, allowed for differentiation between viable, early apoptotic, late apoptotic, and necrotic cells. Viable cells are FLICA 660 and YO-PRO-1 negative, as FLICA 660 is cell-permeant but caspase-3 and 7 would be active only at basal levels and healthy cells would be impermeant to YO-PRO-1. Early apoptotic cells are FLICA 660 positive but YO-PRO-1 negative, as caspases-3 and 7 would be strongly activated during the onset of oxidative stress-induced apoptosis, but the cell would still have an intact membrane that excludes YO-PRO-1. Late apoptotic cells are FLICA 660 and YO-PRO-1 positive, due to the activated caspase activities and loss of cell membrane integrity. Necrotic cells would be YO-PRO-1 positive and FLICA 660 negative, as caspases are no longer active.

After hydrogen peroxide treatment, there was a significant decrease in the number of viable cells, with a concurrent increase in the number of late apoptotic cells, compared to the untreated group (Figure 4.1A and Figure 4.1B). We tested the three major anthocyanins found in lingonberry at 10 ng mL^{-1} (20 nmol L^{-1} for cyanidin-3-galactoside and cyanidin-3-glucoside; 22 nmol L^{-1} for cyanidin-3-arabinoside) and found them to be protective against hydrogen peroxide-induced apoptosis in rat cardiomyoblasts (Figure 4.1C-E). The number of necrotic, late apoptotic, and early apoptotic cells after treatment with each anthocyanin were not significantly different from the control cells, demonstrating a protective effect against hydrogen peroxide-induced cell death. A combination of the three anthocyanins was also protective (Figure 4.1F). There were no significant differences between the effects of each anthocyanin or combination treatment, indicating that the glycoside does not appear to affect bioactivity of

the anthocyanin in cardiac cells. A lingonberry extract tested at three concentrations produced a dose-dependent effect (Figure 4.1G-I). A summary of the effects of each treatment on progression from viability to necrosis is shown in Figure 4.2.

Figure 4.1 Effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced apoptosis.

Scatterplots of (A) untreated cells, (B) cells treated with hydrogen peroxide, and cells treated with hydrogen peroxide after pretreatment with (C) cyanidin-3-galactoside at 10 ng mL^{-1}, (D) cyanidine-3-glucoside at 10 ng mL^{-1}, (E) cyanidin-3-arabinoside at 10 ng mL^{-1}, (F) anthocyanin combo at 10 ng mL^{-1}, (G) lingonberry extract 1:2000, (H) lingonberry extract 1:1000, or (I) lingonberry extract 1:500. Changes in FLICA-660 and YO-PRO-1 fluorescence were detected using flow cytometry.

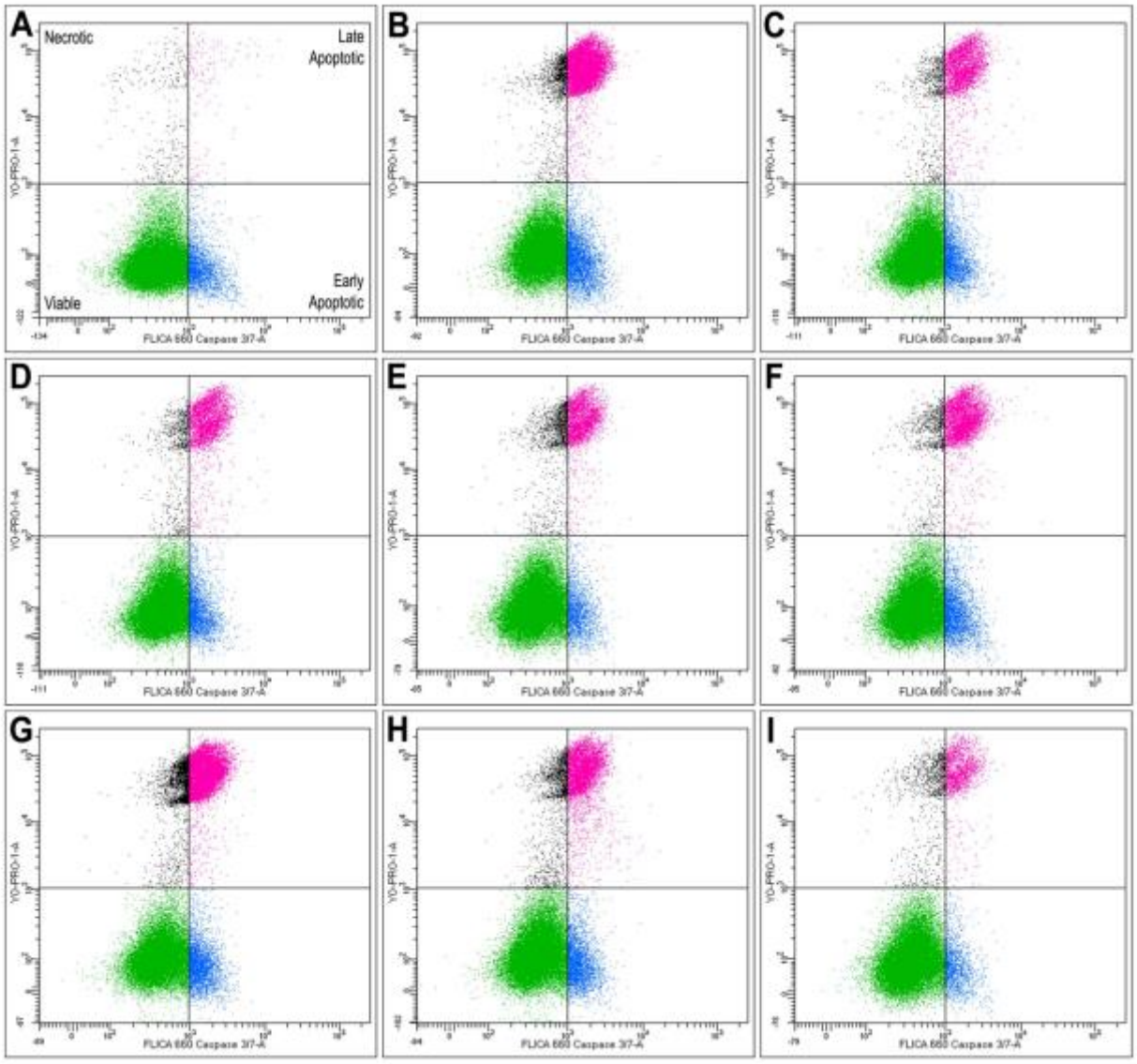
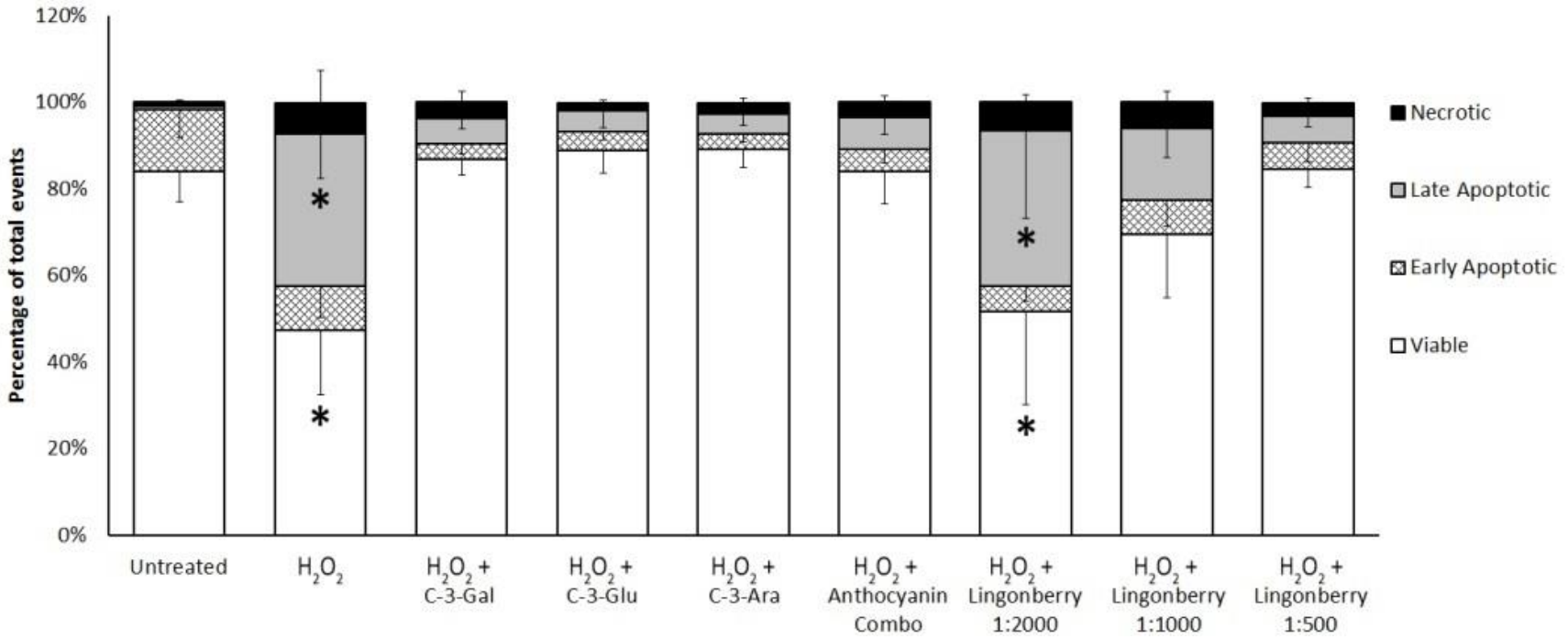

A
Necrotic
Late Apoptotic
Viable
Early Apoptotic
B
C
D
E
F
G
H
I
YO-PRO-1-A
FLICA 660 Caspase 3/7-A

Figure 4.2 Summary of effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced apoptosis.

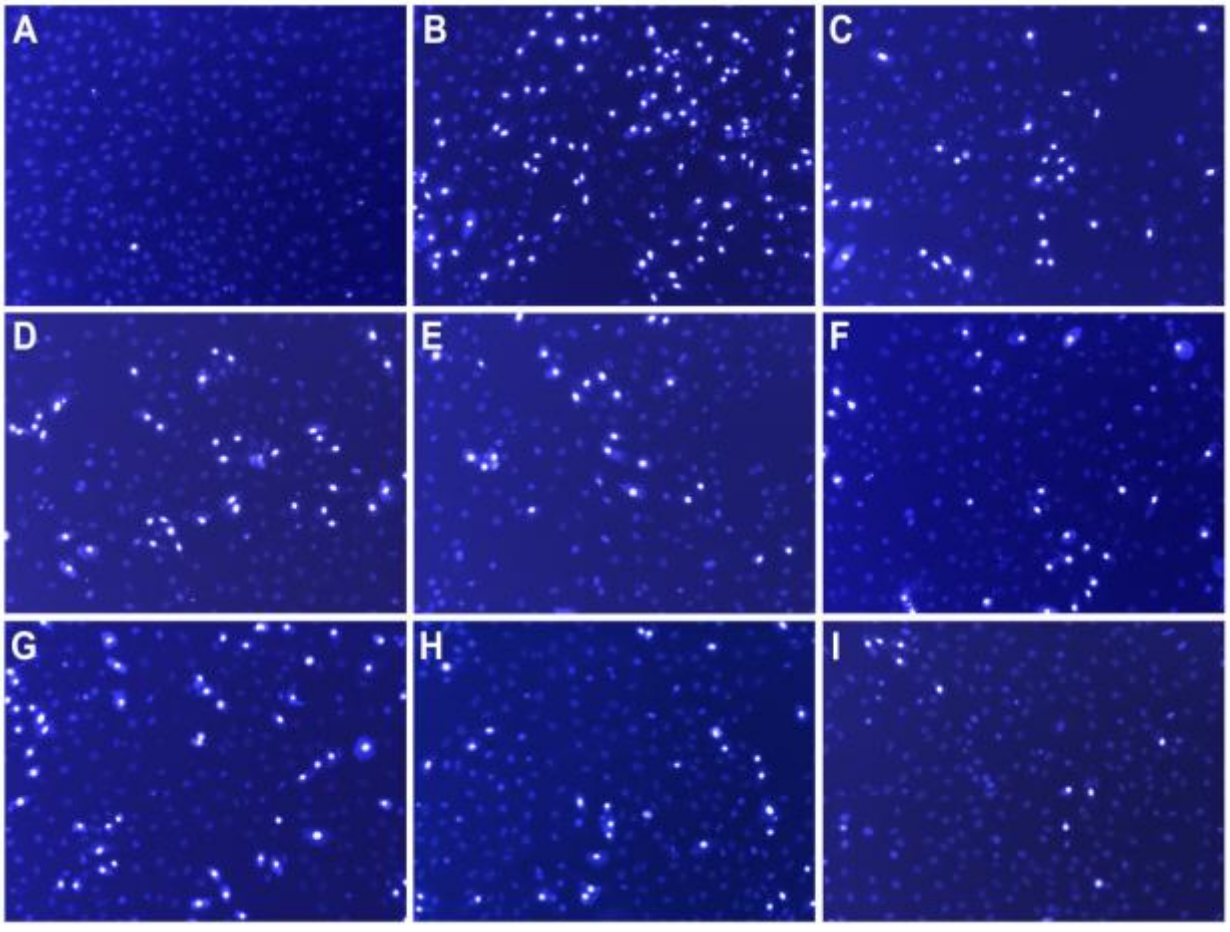
*Progression of cells from viable to necrotic was detected by flow cytometry after hydrogen peroxide treatment with cyanidin-3-galactoside (C-3-Gal), cyanidin-3-glucoside (C-3-Glu), cyanidin-3-arabinoside (C-3-Ara), an anthocyanin combo, or a lingonberry extract. Percentages of cells in each stage are shown as mean ± standard deviation of three independent experiments. * denotes $p<0.05$ compared to the untreated group.*



This study provided evidence for the anti-apoptotic effects of lingonberry anthocyanins. Caspase-3 activity, a strong indicator of apoptosis, was significantly increased after hydrogen peroxide treatment, and treatment with lingonberry anthocyanins at 10 ng mL^{-1} significantly protected against caspase-3 activation measured by flow cytometry after labeling cells with caspase 3/7-specific FLICA 660. Apoptosis was also visualized using Hoechst 33258 to detect chromatin condensation and representative images are shown in Figure 4.3. The hydrogen peroxide-treated group contained more brightly stained nuclei containing condensed chromatin indicative of apoptosis than the untreated group (Figure 4.3A and 4.3B). Anthocyanin-treated groups contained fewer brightly stained nuclei (Figure 4.3C-F), demonstrating a protective effect against apoptosis. A lingonberry extract tested at three concentrations produced a dose-dependent effect (Figure 4.3G-I).

Figure 4.3 Fluorescence microscopy images of H9c2 cells stained with Hoechst 33258 to detect chromatin condensation.

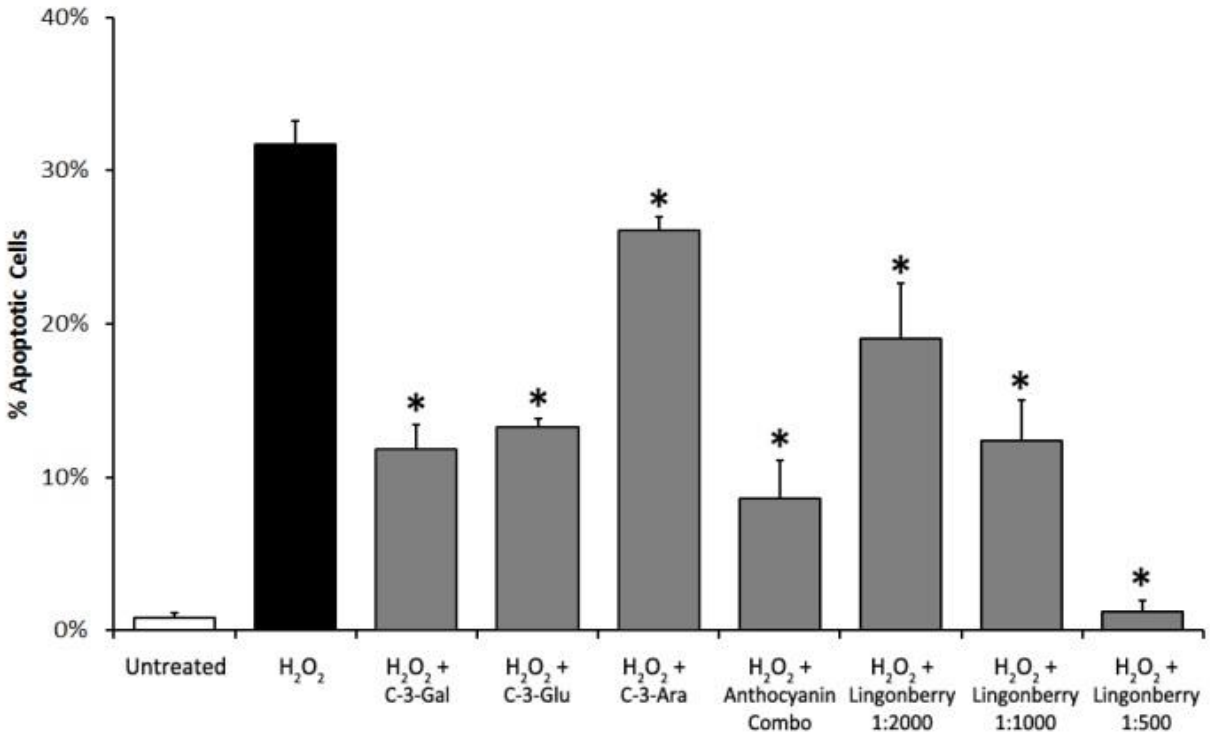
Representative images of (A) untreated cells, (B) cells treated with hydrogen peroxide, and cells treated with hydrogen peroxide after pre-treatment with (C) cyanidin-3-galactoside at 10 ng mL^{-1}, (D) cyanidin-3-glucoside at 10 ng mL^{-1}, (E) cyanidin-3-arabinoside at 10 ng mL^{-1}, (F) anthocyanin combo at 10 ng mL^{-1}, (G) lingonberry extract 1:2000, (H) lingonberry extract 1:1000, or (I) lingonberry extract 1:500 stained with Hoechst 33258 to detect chromatin condensation and viewed under fluorescence microscope.



Chromatin condensation was quantified by counting a minimum of 10,000 nuclei per treatment group from three independent experiments and the effects of anthocyanin and extract treatment on hydrogen peroxide-induced nuclei condensation are shown in Figure 4.4.

Figure 4.4 Effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced chromatin condensation.

*Percentages of apoptotic nuclei out of total nuclei after hydrogen peroxide treatment with cyanidin-3-galactoside (C-3-Gal), cyanidin-3-glucoside (C-3-Glu), cyanidin-3-arabinoside (C-3-Ara), an anthocyanin combo, or a lingonberry extract are shown as mean ± standard deviation of three independent experiments. * denotes p<0.05 compared to the hydrogen peroxide-treated group.*



4.5 Discussion

The novel findings of this study are 1) lingonberry anthocyanins could protect cardiac cells from apoptotic cell death measured by caspase-3 activation, loss of membrane permeability, and chromatin condensation and 2) only physiologically relevant (20 nmol L^{-1}) doses of anthocyanins are needed to significantly inhibit programmed cell death and increase the number of viable cells after oxidative insult.

Oxidative stress is a hallmark of cardiovascular disease and constitutes a major mechanism of many cardiovascular pathophysiologies (Dey *et al*. 2016; Martín-Fernández and Gredilla. 2016). Hydrogen peroxide was used to induce oxidative stress in H9c2 cardiac myoblast cells, a model which has been extensively studied in this cell line and in adult cardiomyocytes. Reactive oxygen species can trigger many components of the apoptotic machinery, including activating MAPK's like JNK and extracellular signal-regulated kinase, activating caspases and their downstream effectors, and increasing expression of pro-apoptotic mitochondrial factors like Bcl and Bax. Oxidative stress in cardiomyocytes can occur during an acute IR event, such as a myocardial infarct, when free radicals formed as a result of impaired waste removal and uncoupling of the mitochondrial electron transport chain combine with oxygen at the onset of reperfusion (Dorweiler *et al*. 2007; Powers *et al*. 2007). Leakage of electrons from the electron transport chain may be more pronounced in cardiomyocytes, where there is a particularly high abundance of mitochondria due to the high-energy demands of the heart muscle. When reactive oxygen species form but are not adequately metabolized, they can cause dysfunction in organelles like the mitochondria and sarcoplasmic reticulum. These organelles play major roles in calcium release and uptake, so dysfunction at this level can

lead to the formation of arrhythmias and impairment of contractility, exacerbating heart failure (Tse *et al*. 2016). Oxidative stress in cardiomyocytes also occurs due to environmental exposures such as air pollutants and alcohol, and after drug exposures like chemotherapy (Cosselman *et al*. 2015; Wang *et al*. 2013b). If oxidative stress progresses to cardiomyocyte apoptosis, heart function can be significantly compromised, and cell death may be an important component of heart failure even if only a small number of cells undergo apoptosis, as cardiomyocytes cannot be regenerated (Chiong *et al*. 2011). Reducing cardiomyocyte cell death is therefore an important step to preserving heart function following reactive oxygen species exposure.

Antioxidant compounds have been shown to protect against oxidative stress-induced apoptosis in this *in vitro* model via several mechanisms including induction of autophagy, inhibition of mitochondrial dysfunction, and activation of antioxidant enzymes (Angeloni *et al*. 2007; Kim *et al*. 2014; Lei *et al*. 2015; Lv and Zhou. 2012; Movahed *et al*. 2012; Park *et al*. 2003; Yang *et al*. 2012). Turner et al. demonstrated that JNK inhibition was protective against oxidative stress-induced apoptosis in H9c2 cells (Turner *et al*. 1998), and our previous study showed that the lingonberry extract significantly inhibited JNK activation and reduced apoptosis in a model of IR injury in H9c2 cells (Isaak *et al*. 2015). However, this is the first study to test doses of anthocyanins that are in line with reported bioavailable concentrations.

A potential limitation of this study is that only intact anthocyanins were tested for biological effects, while *in vivo*, the beneficial effects of bioavailable anthocyanins after lingonberry consumption would also be bolstered by the presence of secondary metabolites formed after anthocyanin absorption. These metabolites, including small phenolic acids and glucuronidated or methylated metabolites (Fernandes *et al*. 2014), may have cardioprotective

effects of their own. Thus, future studies are warranted to examine the combined beneficial effects of intact anthocyanins and metabolites in animal models of cardiovascular disease. Another limitation may be that neonatal cardiomyoblasts were used rather than primary adult cardiomyocytes. These cells have been used extensively to model cardiac injuries and have been shown to respond similarly to adult cardiomyocytes in models of cardiac cell death, but future studies will need to test the cardioprotective effects of anthocyanins in more robust models *in vivo*.

Dietary modifications that increase the consumption of anthocyanin and antioxidant-rich berries like lingonberry may be beneficial in disease states hallmarked by oxidative stress (Louis *et al*. 2014). Using four antioxidant capacity assays, this study demonstrated the high antioxidant capacity of lingonberry, which is in agreement with the literature showing that lingonberry contains exceptionally high levels of anthocyanins and has higher antioxidant activity than other commonly consumed berries (Bakowska-Barczak *et al*. 2007; Grace *et al*. 2014; Isaak *et al*. 2015; Mane *et al*. 2011; Zheng and Wang. 2003). The study also showed that lingonberry anthocyanins could protect cardiac cells from apoptotic cell death measured by caspase-3 activation, loss of membrane permeability, and chromatin condensation. The results of this study are especially novel because the dosages used are within the reported ranges of bioavailability, or plasma concentration, of anthocyanins indicating that lingonberry anthocyanins may have cardioprotective effects when used as a dietary modification.

4.6 Acknowledgement

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Chapter 5

Manuscript IV: Supplementing diet with Manitoba lingonberry juice reduces kidney ischemia-reperfusion injury

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5.1 Abstract

BACKGROUND: Lingonberry (*Vaccinium vitis-idaea* L.) contains high levels of anthocyanins which are bioavailable in the kidney and may be protective against IR-induced AKI. This study investigated the effects of lingonberry juice on IR-induced stress-activated signaling pathway and inflammatory response in the kidney.

RESULTS: Sprague-Dawley rats subjected to kidney IR had significantly impaired kidney function, with increased activation of the JNK signaling pathway and increased inflammatory response, measured using a multiplex panel containing an extensive array of inflammatory biomarkers. In rats fed 1 mL of lingonberry juice daily for 3 weeks prior to IR, kidney function was protected and attenuation of inflammatory response and JNK signaling was reflected in the reduction of the measured biomarkers. *In vitro* results in cultured HK-2 cells confirmed that lingonberry anthocyanins reduced JNK signaling and inflammatory gene expression after IR.

CONCLUSION: This study shows, for the first time, that daily supplementation with lingonberry juice may protect against loss of kidney function induced by IR injury by modulating JNK signaling and inhibiting the subsequent inflammatory response.

5.2 Introduction

Lingonberry (*Vaccinium vitis-idaea* L.), an evergreen dwarf shrub native to northern climates in Europe and North America, is known to contain more antioxidants than other commonly consumed berries (Bakowska-Barczak *et al*. 2007; Zheng and Wang. 2003) due to the presence of various polyphenols including anthocyanins, quercetin, resveratrol, phenolic acids,

and proanthocyanidins (Häkkinen *et al*. 1999; Jungfer *et al*. 2012; Rimando *et al*. 2004). Anthocyanins, which are the red pigments of the berries, are of particular interest as lingonberry contains more anthocyanins per gram of fresh weight than other commonly consumed berries and anthocyanin metabolites are detected in plasma at up to micromolar concentrations following oral consumption of berries in rats (Kamiloglu *et al*. 2015; McGhie and Walton. 2007). Many of the health benefits of anthocyanins are likely due to the actions of these metabolites of which the pharmacokinetics and elimination kinetics have been elucidated using ^{13}C-labelled cyanidin-3-glucoside (De Ferrars *et al*. 2014). Lingonberry may have protective effects in the kidney in particular, because it has been reported that 1) anthocyanins and their metabolites are water-soluble and therefore are excreted in urine (Lehtonen *et al*. 2009; Lehtonen *et al*. 2010) and 2) studies have shown rapid accumulation and metabolism of anthocyanins in the kidney when injected into rats (Vanzo *et al*. 2011). Metabonomic investigation has revealed that even small doses of anthocyanins can cause major changes in the rat kidney metabolic profile (Vanzo *et al*. 2013).

AKI due to IR is one of the most prevalent postoperative complications and it is estimated that millions of people worldwide die each year of all forms of AKI (Hobson *et al*. 2015; Mehrotra *et al*. 2012; Murugan and Kellum. 2011; Rewa and Bagshaw. 2014). Complete cessation of blood flow can occur during kidney transplant and some major surgical procedures while compromised blood flow can occur during more common surgeries, heart failure, vascular disease, low blood pressure, low blood volume, and some infections (Sharfuddin and Molitoris. 2011). When kidney hypo-perfusion or ischemia occurs, there is an imbalance between nutrient and oxygen delivery to the kidney and waste removal from the kidney. The

resulting intracellular ATP depletion coupled with reactive oxygen species generation during reperfusion can trigger cell death via both necrotic and apoptotic pathways (Sharfuddin and Molitoris. 2011). Mitogen-activated protein kinases, such as JNK, are important components of stress-activated signaling pathways that can cause activation of pro-apoptotic and pro-inflammatory transcription factors (Au-Yeung *et al*. 2001). Specifically, JNK may play an important role in eliciting renal inflammation following IR injury, further exacerbating tissue injury (De Borst *et al*. 2009). As a result of these processes, AKI due to IR can cause a rapid decline in kidney function, which can complicate recovery from renal or cardiac surgeries. The high rate of mortality and morbidity associated with AKI may be due to the fact that there are few treatment options for ischemic AKI besides renal replacement therapy (Murugan and Kellum. 2011; Negi and Shigematsu. 2012); hence, there is interest in alternative approaches to mitigate such complications.

In animal models of renal IR, intra-peritoneal injections of antioxidant compounds such as quercetin, resveratrol, vitamin C, TEMPOL, and tyrosol prior to renal IR reduce kidney damage (Inal *et al*. 2002; Knight *et al*. 2012; Korkmaz and Kolankaya. 2009; Sener *et al*. 2006; Wang *et al*. 2013a). However, injections of high doses of phytochemicals directly before surgery may not be applicable to humans due to regulatory, financial, and ethical considerations. Lingonberry contains a large amount of anthocyanins, which may be beneficial during IR injury (Isaak *et al*. 2015). Lingonberry consumption as a preventative approach to alleviating kidney damage during transplant or major surgery would be easily translatable to the human diet, and may have the benefit of providing health benefits beyond kidney protection including

cardiovascular and metabolic effects (Davidson *et al*. 2014; Erlund *et al*. 2003; Jennings *et al*. 2012; Linderborg *et al*. 2012; Törrönen *et al*. 2012).

This study investigated, for the first time, the protective effects of consumption of wild Manitoba (Canada) lingonberry juice on kidney damage, JNK signaling pathways, and a comprehensive array of biomarkers of systemic and renal inflammation caused by renal IR injury in Sprague-Dawley rats.

5.3 Materials and Methods

5.3.1 Materials and Safety

Unless otherwise indicated, all chemicals of at least ACS reagent grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). For all experiments, water was purified using a Milli-Q Synthesis A10 ultrapure water purification system (Millipore Corp., Billerica, MA, USA). Personal protective equipment and laboratory safety guidelines were implemented for all experiments.

5.3.2 Berry harvest and juice preparation

Ripe fruits from wild lingonberry plants were hand-picked and immediately frozen at -20°C by a commercial berry picker on public land at Sherridon, Manitoba, Canada (55° 7’ N; 101° 5’ W) in September 2011. Authorization was not required for these activities as there is no prohibition to harvest wild berries including lingonberries. To the best of our knowledge, there is no involvement of endangered or protected species.

Thawed berries were processed in a commercial centrifugal juicer (Kenwood, Mississauga, ON, Canada) and juice was collected into chilled bottles. One part pure juice was

diluted with two or four parts distilled water to achieve two commercially relevant lingonberry juices based on the percentage of juice indicated on the labels of commercial juices, designated "33% LBJ" and "20% LBJ" for future reference. The juices were clarified with a Nalgene vacuum filtration system (0.2 µm pore size; Sigma-Aldrich, St. Louis, MO, USA) and sweetened with sucrose (4% w/v) to ensure palatability for the animal study, as the juice is tart. The sweetened juices were pasteurized by heating to 85°C for five minutes, poured into pre-sanitized hot glass jars, inverted for five minutes to pasteurize the lids, and cooled to 4°C for storage.

For comparison of the anthocyanin and antioxidant contents, a 100% cranberry juice and a 100% tart cherry juice from a commercial brand that specializes in organic "not from concentrate" juices were purchased from a local store and diluted to 33% for use.

5.3.3 Determination of total phenolics, ORAC, and total anthocyanins in lingonberry juice

These assays were performed as previously described (Isaak *et al*. 2015) and results were used for standardizing batches of juice and ensuring stability following storage, rather than for assessing quality of the product or predicting *in vitro* bioactivity. Total phenolic content was determined colourimetrically by combining the sample with FC reagent in a sodium carbonate buffer (Ainsworth and Gillespie. 2007). Results were expressed as mg GAE L^{-1} juice using gallic acid as a standard. The ORAC of the juice was determined fluorimetrically using AAPH (Wako Chemicals, Richmond, VA, USA) as a peroxyl generator to oxidize fluorescein. Because ORAC measures peroxyl radical absorbance of antioxidants at 37°C in a pH 7.4 buffer, the assay provides more physiological relevance than other *in vitro* antioxidant capacity assays. Decline in fluorescence was measured kinetically over 60 minutes so the area under the curve

could be calculated for each sample (Gillespie *et al*. 2007). Results were expressed as µmol TE L^{-1} juice using Trolox as a standard. Total anthocyanins were determined using the pH differential method, which is highly correlated with HPLC methods for quantification of anthocyanins in juice samples (Lee *et al*. 2008), by diluting sample in each of potassium chloride solution (pH 1.0) and sodium acetate solution (pH 4.5) and measuring the absorbance of each dilution at 520 nm and 700 nm. Results were expressed as mg cyanidin-3-glucoside equivalents L^{-1} juice using the formula described by Lee, Durst, and Wrolstad (Lee *et al*. 2005). All samples and standards were tested in quadruplicate. Developed colour or fluorescent intensity was measured using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software (Molecular Devices, Sunnyvale, CA, USA).

5.3.4 UPLC analysis

Chromatography was performed using a Waters ACQUITY ultra performance liquid chromatography (UPLC) system managed by Empower™ 3 Software (Waters Corp., Milford, MA, USA). For each sample and standard, 1 µL was injected onto a 2.1 x 100 mm, 1.7 µm ACQUITY UPLC BEH C18 column (Waters Corp., Milford, MA, USA) with the flow rate set at 0.6 mL min^{-1}. The column temperature was maintained at 42°C using an ACQUITY UPLC Column Manager and spectral data (525 nm) was collected with an ACQUITY UPLC Photodiode Array eλ Detector. The mobile phases used were A: 0.3% HPLC-grade phosphoric acid, 5% Optima-grade methanol (Fisher Scientific, Fair Lawn, NJ, USA) in Milli-Q water and B: acetonitrile. The gradient condition was 0 min, 1% B; 0.5 min, 1% B; 2.25 min, 8% B; 3 min, 9% B; 4.5 min, 12% B; 5 min, 15% B; 6.5 min, 16% B; 10 min, 30% B; 11 min, 95% B; 11.65 min, 95% B; 11.7 min, 1% B; 15

min, 1% B. Peaks were identified by comparing retention time and spectral data with those of the reference compounds (purity >95%) cyanidin-3-glucoside (Chromadex, Irvine, CA, USA), cyanidin-3-galactoside (Chromadex, Irvine, CA, USA) and cyanidin-3-arabinoside (Cerilliant Corp., Round Rock, TX, USA).

5.3.5 Experimental design

This protocol (#13-019) was approved by the University of Manitoba Animal Care Committee, in agreement with the Canadian Council on Animal Care and Use of Experimental Animals. All surgical procedures were performed under isoflurane gas anesthesia and all efforts were made to minimize the animals' suffering. Male Sprague-Dawley rats (101-125g) were obtained from the University of Manitoba Central Animal Care Services. Animals were housed two per cage in a temperature- and humidity-controlled room with a 12-h dark/12-h light cycle. Water and Prolab RMH 3000 diet (LabDiet, St. Louis, MO, USA) were provided *ad libitum* throughout the study. Animals were divided into five groups of four rats each: "Sham", "Sham + 33% LBJ", "IR", "IR + 33% LBJ", and IR + 20% LBJ". Two groups (Sham and IR) were fed 1 mL 4% sucrose solution (sugar water) via syringe daily for three weeks, following the alternative oral dosing method described by Atcha et al. (Atcha *et al*. 2010). Two groups (Sham + 33% LBJ and IR + 33% LBJ) were fed 1 mL 33% LBJ via syringe daily for three weeks. One group (IR + 20% LBJ) was fed 1 mL 20% LBJ via syringe daily for 3 weeks. A daily intake of 1 mL of juice for an adult rat corresponds to approximately 1 cup for an 80-kg human. For juice feeding, each animal was placed in a clean cage so they could drink from the syringe without competition and they consumed the juice voluntarily from the syringe after one training day. The sucrose-matched

control was selected over a tap-water control to correct for any effects due to the increased sugar intake, while still respecting animal ethics guidelines for minimizing animal numbers.

5.3.6 Renal IR surgery

IR surgery was performed as previously described (Wang *et al*. 2013a; Wang *et al*. 2014a). Briefly, rats were given analgesic, anesthetized with 3% isoflurane/oxygen gas and kept on a heat pad with the rectal temperature maintained at 37°C throughout the experimental procedure. When the animals reached Stage III anesthesia, maintenance 1-2% isoflurane/oxygen gas was administered and ischemia was induced by clamping the left renal vascular pedicle with a non-traumatic vascular clamp through a midline abdominal incision. After 45 minutes, the clamp was removed and a right nephrectomy was performed. All animals recovered from anesthesia and were allowed access to food and water ad libitum while being observed for signs of distress or major discomfort. Animals were euthanized after 6 hours of reperfusion with no adverse events. Sham-operated rats were subjected to the same surgical procedure without inducing IR. Blood samples were drawn from the abdominal aorta prior to sacrifice and collected into vacutainers containing 0.2 mg mL^{-1} heparin as anticoagulant. Plasma was separated by centrifugation of blood at 3,000 x *g* for 20 minutes at 4°C. After sacrifice, kidneys were collected and bisected in ice-cold potassium phosphate buffer (pH 7.4).

5.3.7 Simulated IR in proximal tubule kidney cells

HK-2 human kidney cortex proximal tubule cells (CRL-2190; American Type Culture Collection, Manassas, VA, USA) were cultured in keratinocyte serum-free media supplemented

with 5 ng mL^{-1} human recombinant epidermal growth factor and 50 ng mL^{-1} bovine pituitary extract (Life Technologies, Carlsbad, CA, USA) at 37°C in 95% oxygen/5% carbon dioxide. HK-2 cells were seeded into 6-well plates at 2 x 10^5 cells per well 48 hours prior to IR and were pre-treated with pure anthocyanins in fresh supplemented media at 10 ng mL^{-1} (cyanidin-3-galactoside, cyanidin-3-glucoside, or cyanidin-3-arabinoside) for 24 hours prior to IR. An anthocyanin combination (58 ng yanidin-3-galactoside + 7 ng cyanidin-3-glucoside + 35 ng cyanidin-3-arabinoside mL^{-1}) using the ratio found in Manitoba lingonberry (58% cyanidin-3-galactoside, 7% cyanidin-3-glucoside, and 35% cyanidin-3-arabinoside based on UPLC analysis) and the selective JNK inhibitor SP600125 were also tested. To simulate ischemia, media was replaced with ischemic buffer (137 mM sodium chloride 15.8 mM potassium chloride, 0.49 mM magnesium chloride, 0.9 mM calcium chloride, 4 mM HEPES, 10 mM deoxyglucose, 20 mM sodium lactate, 1 mM sodium dithionate, pH 6.4), cells were placed in a Billups-Rothenberg modular incubator chamber (Stem Cell Technologies, Vancouver, BC, Canada), and oxygen was removed by flushing the chamber with a mixture of 95% N_2 and 5% CO_2 gas for 5 minutes at 25 L min^{-1}, as previously described (Isaak *et al*. 2015; Wang *et al*. 2014a; Wu *et al*. 2010). After ischemia, cells were washed with Hank's Balanced Salt Solution (Life Technologies, Carlsbad, CA, USA) and subsequently incubated in media under normoxic conditions to simulate reperfusion.

5.3.8 Protein and pro-inflammatory marker analysis in plasma, kidney tissue, and HK-2 cells

Creatinine levels in plasma were measured using the Cobas C111 Analyzer (Roche, Laval, QC, Canada). A Sector Imager 2400 and multiplex assay kits were purchased from MesoScale

Discovery (Rockville, MD, USA). Proteins were extracted from HK-2 cells using gentle agitation and kidney tissue using a hand-held homogenizer (VWR, Radnor, PA, USA) on ice using lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and serine/threonine phosphatase inhibitor cocktail and tyrosine phosphatase inhibitor cocktail (MesoScale Discovery, Rockville, MD, USA) where necessary. Lysates were centrifuged at 10,000 x g for 10 minutes at 4°C and protein was quantified using the Pierce BCA Protein Assay Kit (Life Technologies, Carlsbad, CA). The Rat Pro-inflammatory Panel VPLEX Kit was used to measure interferon (IFN) γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, KC, and TNFα in plasma and kidney tissue. The Inflammation Panel 1 (rat) Kit was used to measure NGAL, TSP-1, TIMP-1, and MCP-1 in plasma. The Phospho/Total JNK Lysate Kit and Phospho-c-Jun lysate Kit were used to measure phosphorylated (Thr183/Tyr185) and total JNK, and phosphorylated c-Jun, respectively. Data was analyzed using MesoScale Discovery Workbench Software and results were expressed as a percentage of the IR group which was set to 100%.

5.3.9 Measurement of mRNA expression

RNA was extracted from HK-2 cells and kidney tissue using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and the quality and quantity of RNA was assessed using the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA). cDNA was synthesized from 500 ng of total RNA using the Superscript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). Amplification was performed using the StepOne Plus Real-Time Polymerase Chain Reaction (PCR) System (Applied Biosystems, Foster City, CA, USA). The

reaction mixture contained 100 ng cDNA, 1X iTaq Universal SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA), 300 nM primer mix, and RNase-free water for a total reaction mixture of 20 µL. The following primers were used: MCP-1 (GenBank accession number NM_002982.3) forward 5'-TCGCTCAGCCAGATGCAATCAATG-3' and reverse 5'-AGTTTGGGTTTGCTTGTCCAGGTG-3', and hypoxanthine-guanine phosphoribosyltransferase (GenBank accession number NM_000194.2) forward 5'-GCTATAAATTCTTTGCTGACCTGCTG-3' and reverse 5'-AATTACTTTTATGTCCCCTGTTGACTG-3'. The PCR programme consisted of an initial incubation of 50°C for 10 minutes, followed by denaturation at 95°C for 1 minute, and then 40 cycles of 95°C for 30 seconds and 60°C for 15 seconds. PCR product specificity was validated by melt curve analysis. All samples were tested in triplicate, data was analyzed using the comparative C_t method with gene expression level normalized to that of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase, and results were expressed as a percentage of the IR group which was set to 100%.

5.3.10 Statistical Analysis

Data are presented as mean ± standard error. Results were analyzed using one-way ANOVA and Student-Newman-Keuls multiple comparison test (PASW Statistics 18, SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

5.4 Results

5.4.1 Analysis of two commercially relevant juice products

Manitoban lingonberry juices contained high amounts of anthocyanins and antioxidants (Table 5.1). The major anthocyanin present in the juice was identified as cyanidin-3-galactoside and the next two largest anthocyanin peaks were identified as cyanidin-3-glucoside and cyanidin-3-arabinoside by comparing UPLC retention times and spectral maximums with reference standards. A UPLC chromatogram showing the three anthocyanin peaks in lingonberry juice is shown in Figure 5.1.

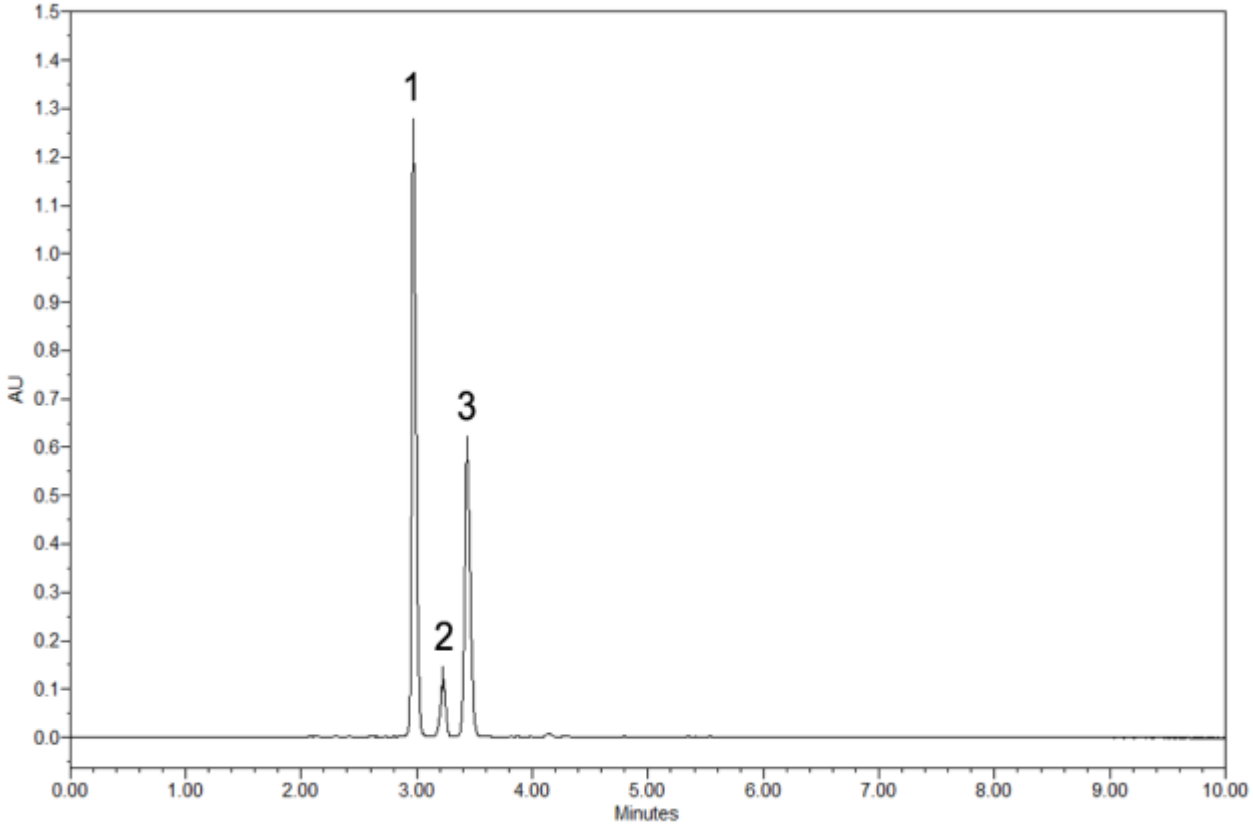
Pure cranberry and tart cherry commercial juices were purchased and diluted to 33% for comparison. Once diluted to 33% juice, the cranberry juice and tart cherry juice contained 815 and 731 mg GAE L^{-1} respectively, indicating the antioxidant content of the lingonberry juice (998 mg GAE L^{-1}) was comparable to commercial juices.

Table 5.1 Total anthocyanins, total phenolics, and ORAC values of 33% and 20% LBJ.

Sample	Total Anthocyanins	Total Phenolics	ORAC
33% LBJ	198 ± 3	998 ± 8	32,171 ± 521
20% LBJ	118 ± 1	624 ± 9	18,442 ± 256

Total anthocyanins are measured as mg cyanidin-3-glucoside equivalents L^{-1}, total phenolics are measured as mg GAE L^{-1}, and ORAC is measured as µmol TE L^{-1}. Results are expressed as mean ± SEM (n=4).

Figure 5.1 UPLC chromatogram of 33% lingonberry juice detected at 525 nm with three major peaks: 1, cyanidin-3-galactoside; 2, cyanidin-3-glucoside; 3, cyanidin-3-arabinoside.



5.4.2 Effect of lingonberry juice consumption on body weight and kidney function after renal IR

Rats were fed with sucrose (Sham, IR), 20% lingonberry juice or 33% lingonberry juice and their body weights are shown in Table 5.2. Weights were first recorded after the seven-day acclimatization period. There were no statistically significant differences ($p<0.05$) in weight gain between groups over the 21-day study period.

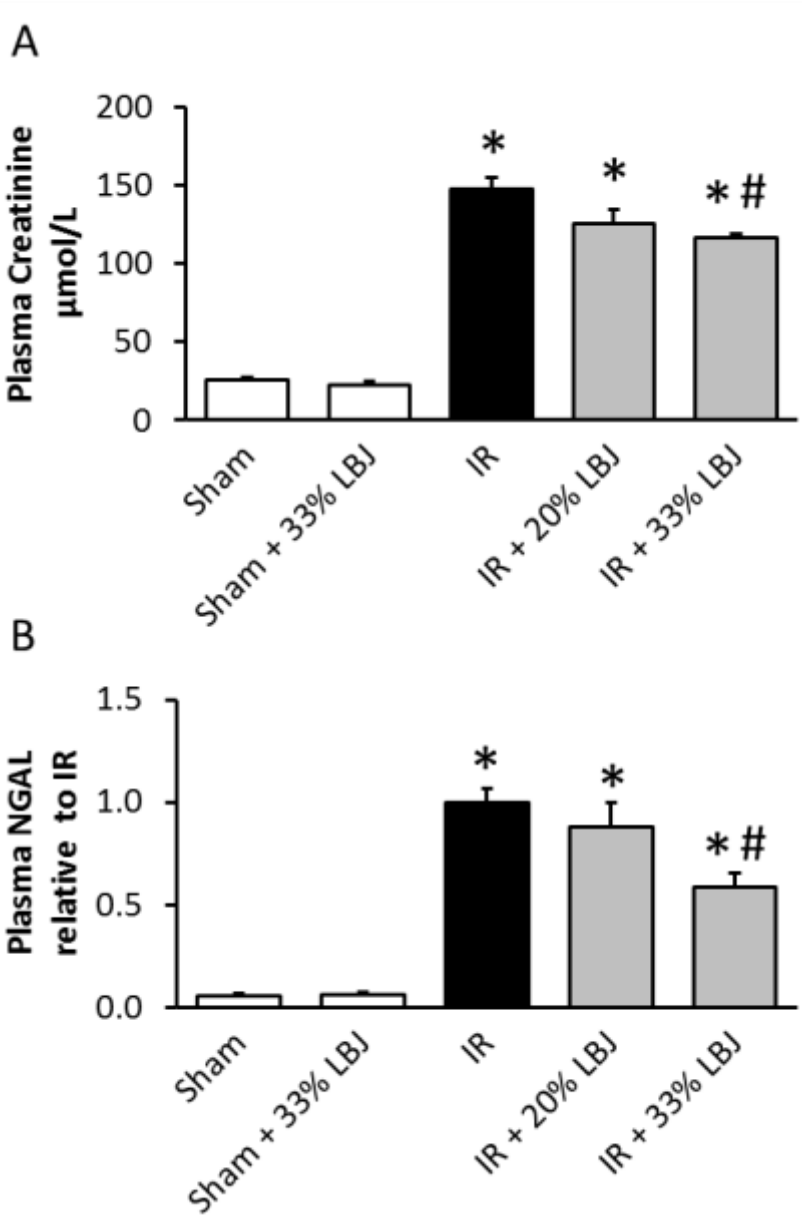
Plasma creatinine and NGAL were used as indicators of kidney function after IR. Renal IR induced a 6-fold increase in plasma creatinine and a 20-fold increase in plasma NGAL, indicating impaired kidney function. The increases in creatinine and NGAL were effectively attenuated by the 33% lingonberry juice (creatinine by 25%, NGAL by 40 %) but not by the 20% lingonberry juice (Figure 5.2), when compared to the IR group.

Table 5.2 Body weight gains throughout study.

Treatment	Starting body weight (g)	Final body weight (g)
Sham	205 ± 8	366 ± 34
Sham + 33% LBJ	204 ± 12	355 ± 32
IR	212 ± 7	363 ± 10
IR + 20% LBJ	204 ± 10	362 ± 17
IR + 33% LBJ	198 ± 11	345 ± 16

Body weights are expressed as mean ± SD, n = 4.

Figure 5.2 Effect of lingonberry juice consumption on plasma creatinine and plasma NGAL levels after IR injury. *Plasma creatinine levels (A) and plasma NGAL levels (B) were assessed after sham operation (Sham), 45 minute ischemia and 6 hour reperfusion (IR), or IR following lingonberry juice consumption (IR + 20% LBJ, IR+ 33% LBJ). * indicates p < 0.05 when compared to the sham group; # indicates p < 0.05 when compared to the IR group.*



5.4.3 Effects of lingonberry consumption on JNK signaling after renal IR

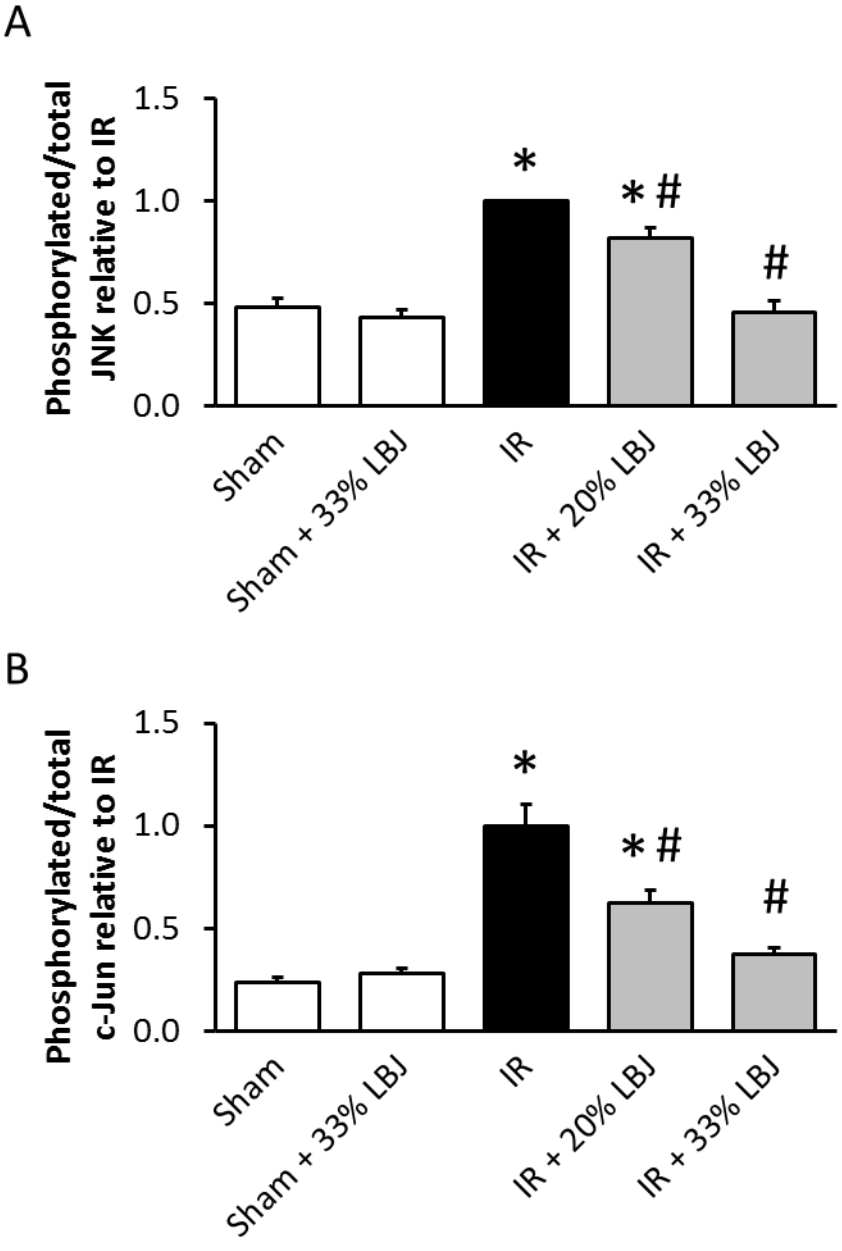
Total and phosphorylated JNK, as well as the downstream effector molecule of JNK, transcription factor c-Jun, were detected in kidney tissue. Renal IR induced two-fold increases in JNK phosphorylation in kidney tissue and this increase was significantly attenuated by 50% in rats that had consumed the 33% LBJ (Figure 5.3A). The model also induced a 4-fold increase in c-Jun phosphorylation in kidney tissue indicating increased kinase activity and both juices significantly inhibited c-Jun activation. 33% LBJ consumption reduced phosphorylated c-Jun levels to that of the sham-operated animals (Figure 5.3B). These results suggested that lingonberry juice consumption could attenuate IR-induced activation of JNK in the kidney.

5.4.4 Effect of lingonberry consumption on IR-induced inflammatory response in rats

The effects of IR and lingonberry juice consumption on inflammatory biomarkers after 6 hours reperfusion were examined. Figure 5.4 shows that renal IR induced significant increases in plasma MCP-1 (4-fold), KC (10-fold), TNFα (2.5-fold), TIMP-1 (20-fold), and TSP-1 (25%). Consumption of the 33% LBJ reduced MCP-1 by 30%, KC by 50%, TNFα by 40%, TIMP-1 by 30%, and TSP-1 by 30% (Figure 5.4). Although not shown, plasma IL-6 and IL-1β were both elevated by 25% after IR but juice consumption did not attenuate these increases. IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-13 were unchanged in plasma after 6 hours of reperfusion.

In kidney tissue (Figure 5.5), IR induced significant increases in KC (16-fold), IL-1β (2-fold), TNFα (2-fold), MCP-1 (14-fold), TIMP-1 (10-fold), TSP-1 (14-fold), and IL-6 (7-fold) but lingonberry juice consumption significantly attenuated the tissue increases in only KC (by 40%)

Figure 5.3 Effect of lingonberry juice consumption on phosphorylated JNK and c-Jun levels in kidney tissue after IR injury. *Total and phospho-JNK (A) and c-Jun (B) levels were assessed after operation (Sham), 45 minute ischemia and 6 hour reperfusion (IR), or IR following lingonberry juice consumption (IR + 20% LBJ, IR+ 33% LBJ). * indicates p < 0.05 when compared to the sham group; # indicates p < 0.05 when compared to the IR group.*



and IL-1β (by 25%) compared to the IR group. IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-13 were unchanged in kidney tissue after 6 hours of reperfusion.

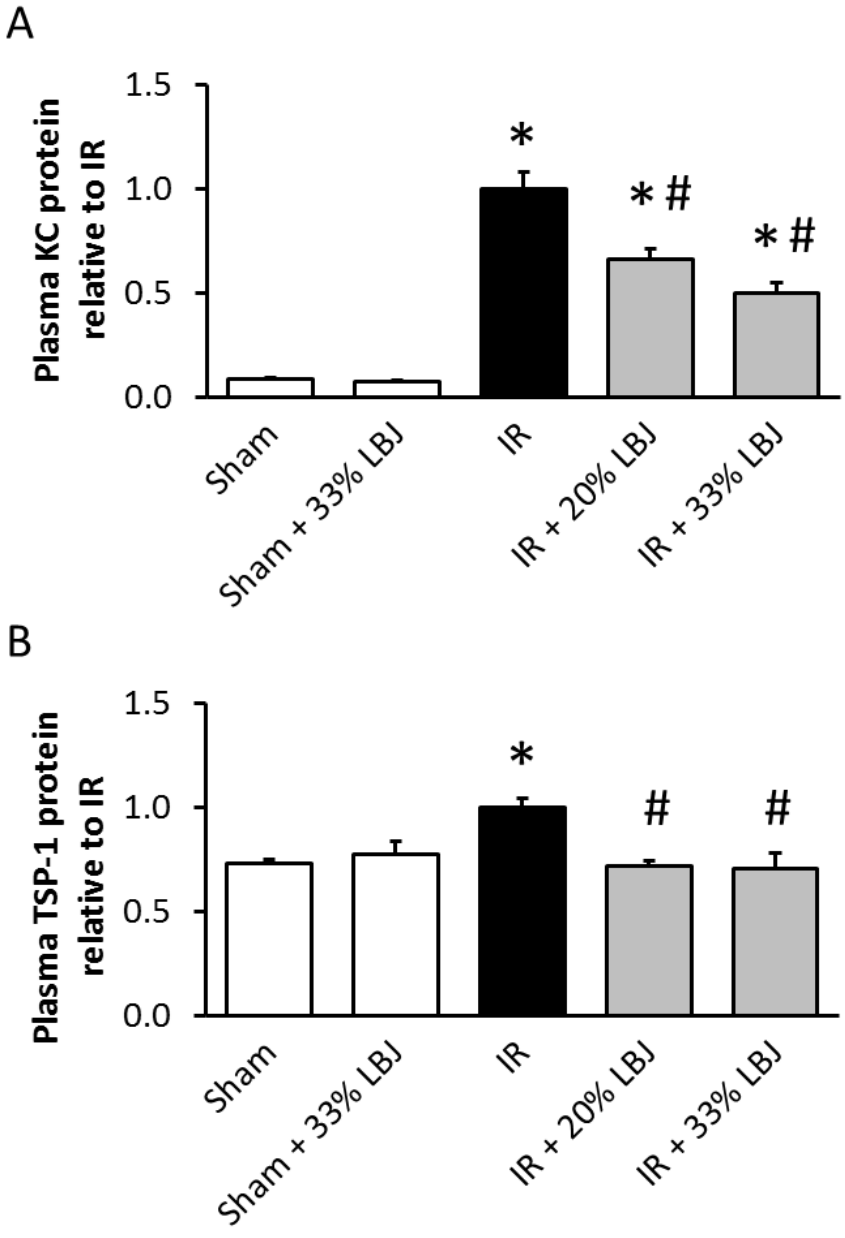
The mRNA expression of renal MCP-1, a hallmark of inflammation, increased 10-fold after IR but juice consumption prevented any significant increase in MCP-1 expression (Figure 5.6); i.e. the MCP-1 expression in the lingonberry-fed group was not significantly different from the sham group.

5.4.5 Effect of lingonberry anthocyanins on JNK signaling and MCP-1 expression in cultured renal proximal tubular cells

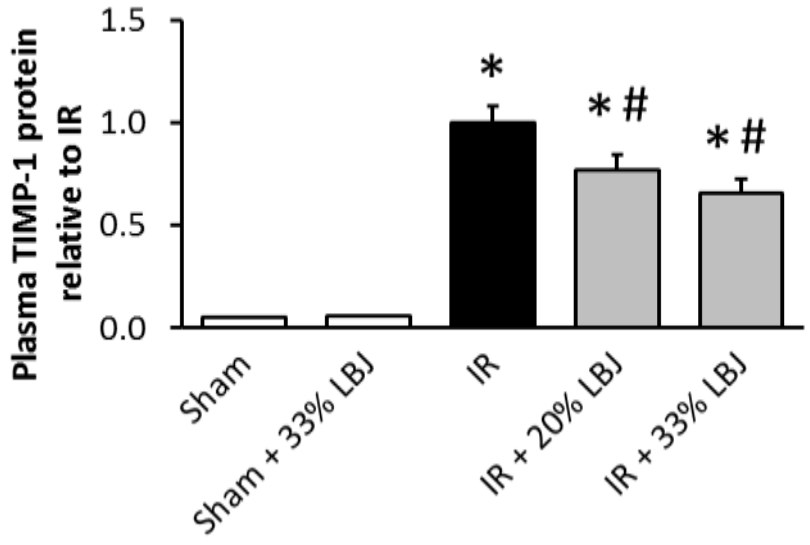
To investigate the potential mechanisms by which lingonberry inhibits the inflammatory response, the link between JNK and MCP-1 signaling following IR was further examined in HK-2 human kidney proximal tubular cells. IR injury increased the ratio of phosphorylated to total JNK 3-fold and the level of phosphorylated c-Jun 7-fold. Physiological doses of individual anthocyanins found in lingonberry juice as well as the lingonberry anthocyanin combination treatment reduced activation of JNK and c-Jun (Figure 5.7).

The JNK inhibitor, SP600125, significantly reduced c-Jun phosphorylation. IR injury increased the expression of MCP-1 mRNA 8-fold. Individual and combined anthocyanin treatments, as well as treatment with SP600125 significantly attenuated MCP-1 mRNA expression (Figure 5.8).

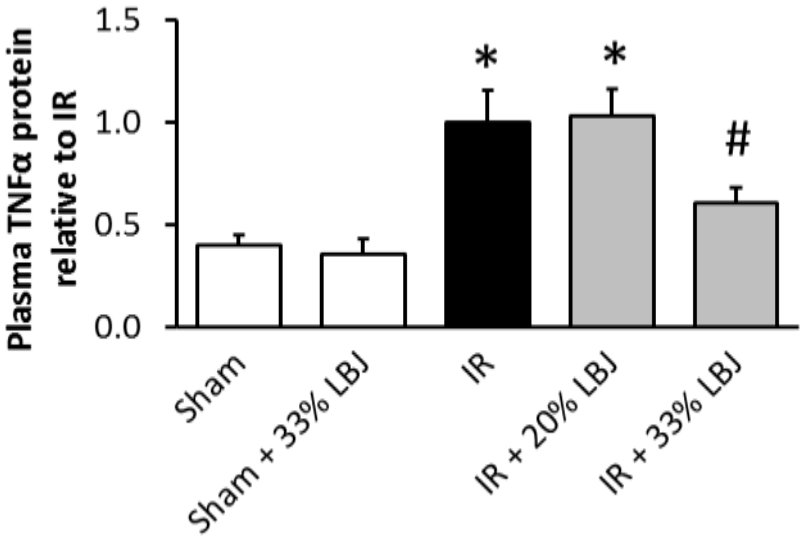
Figure 5.4 Effect of lingonberry juice consumption on inflammatory mediators in plasma after IR injury. *Plasma KC (A), TSP-1 (B), TIMP-1 (C), TNFα (D), and MCP-1 (E) were assessed after sham operation (Sham), 45 minute ischemia and 6 hour reperfusion (IR), and IR following lingonberry juice consumption (IR + 20% LBJ, IR+ 33% LBJ). * indicates p < 0.05 when compared to the sham group; # indicates p < 0.05 when compared to the IR group.*



C


Plasma TIMP-1 protein relative to IR
1.5
1.0
0.5
0.0
*
*#
*#
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

D


Plasma TNFα protein relative to IR
1.5
1.0
0.5
0.0
*
*
#
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

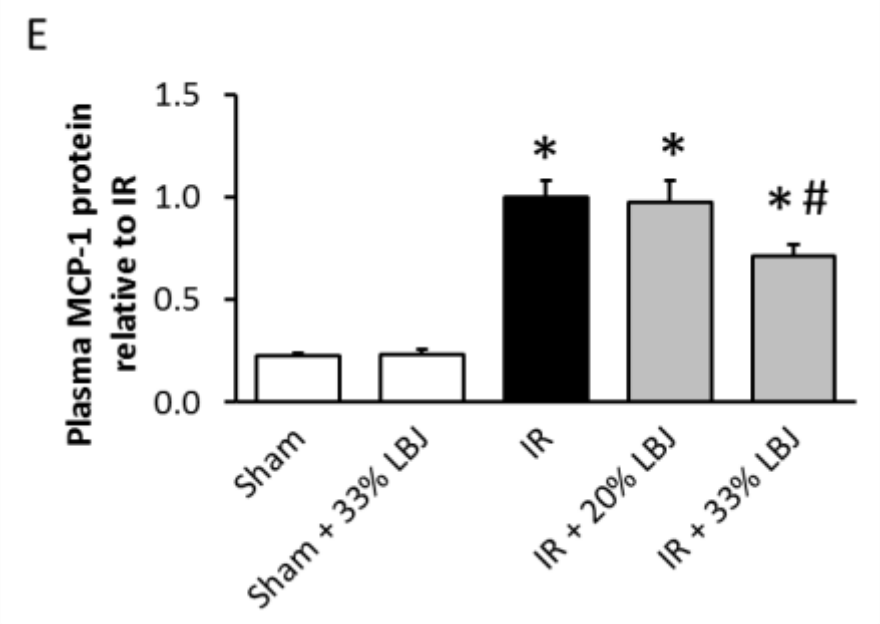
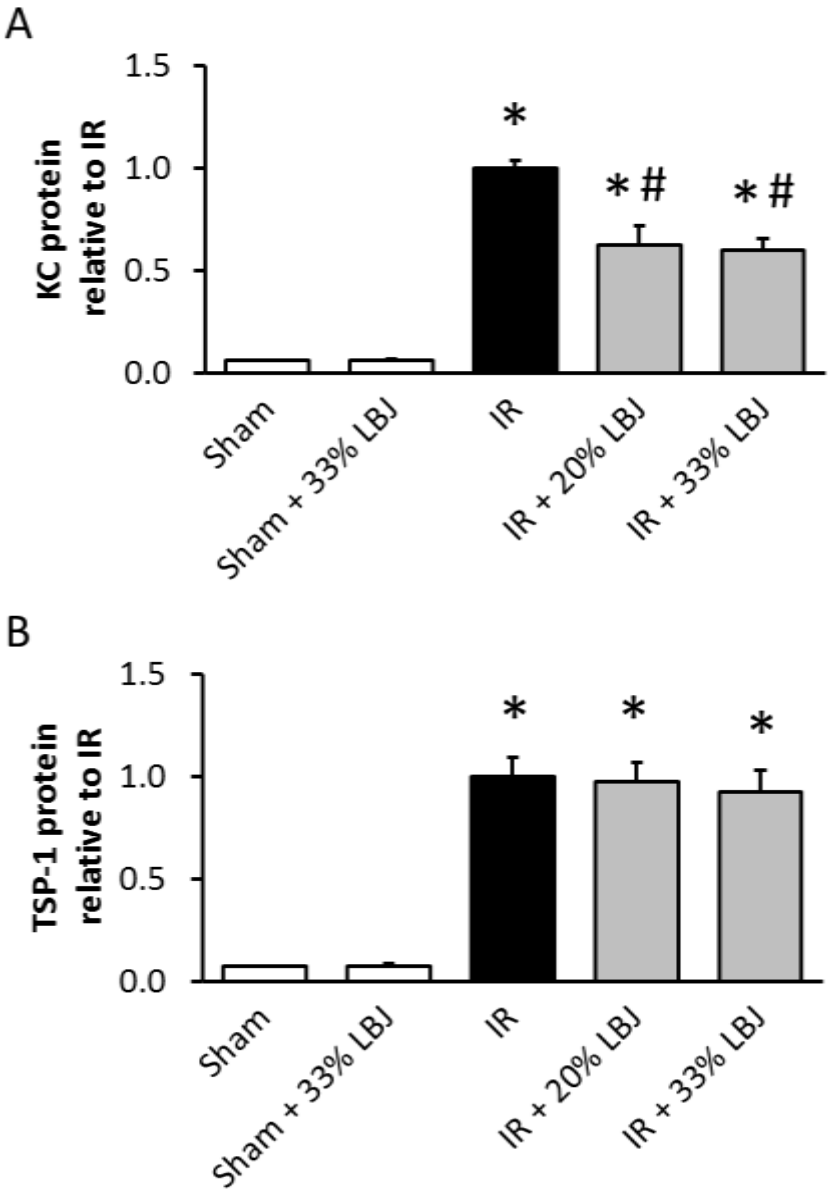
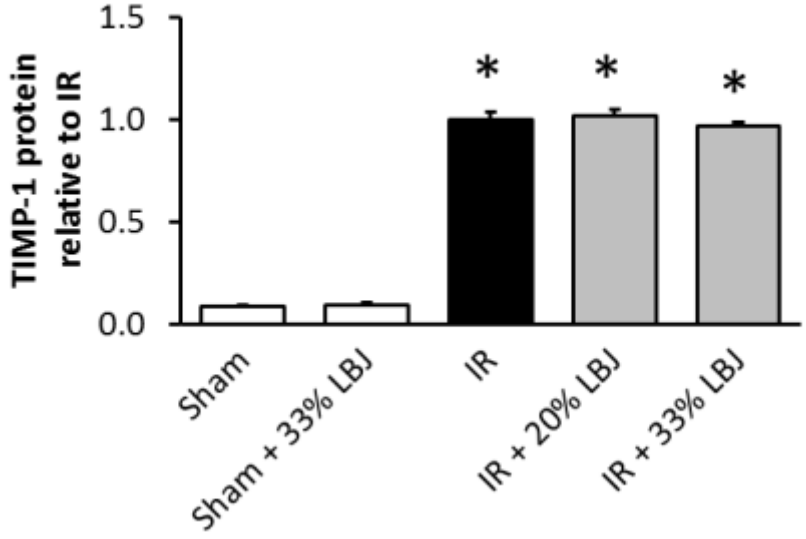

E
Plasma MCP-1 protein relative to IR
1.5
1.0
0.5
0.0
*
*
*#
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

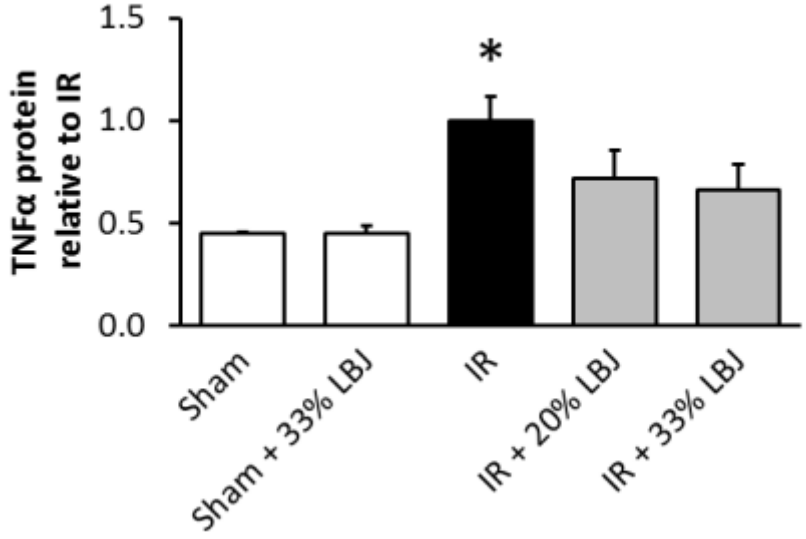
Figure 5.5 Effect of lingonberry juice consumption on inflammatory mediators in the kidney after IR injury. *Kidney KC (A), TSP-1 (B), TIMP-1 (C), TNFα (D), MCP-1 (E), IL-1β (F), and IL-6 (G) were assessed after sham operation (Sham), 45 minute ischemia and 6 hour reperfusion (IR), and IR following lingonberry juice consumption (IR + 20% LBJ, IR+ 33% LBJ). * indicates p < 0.05 when compared to the sham group; # indicates p < 0.05 when compared to the IR group.*

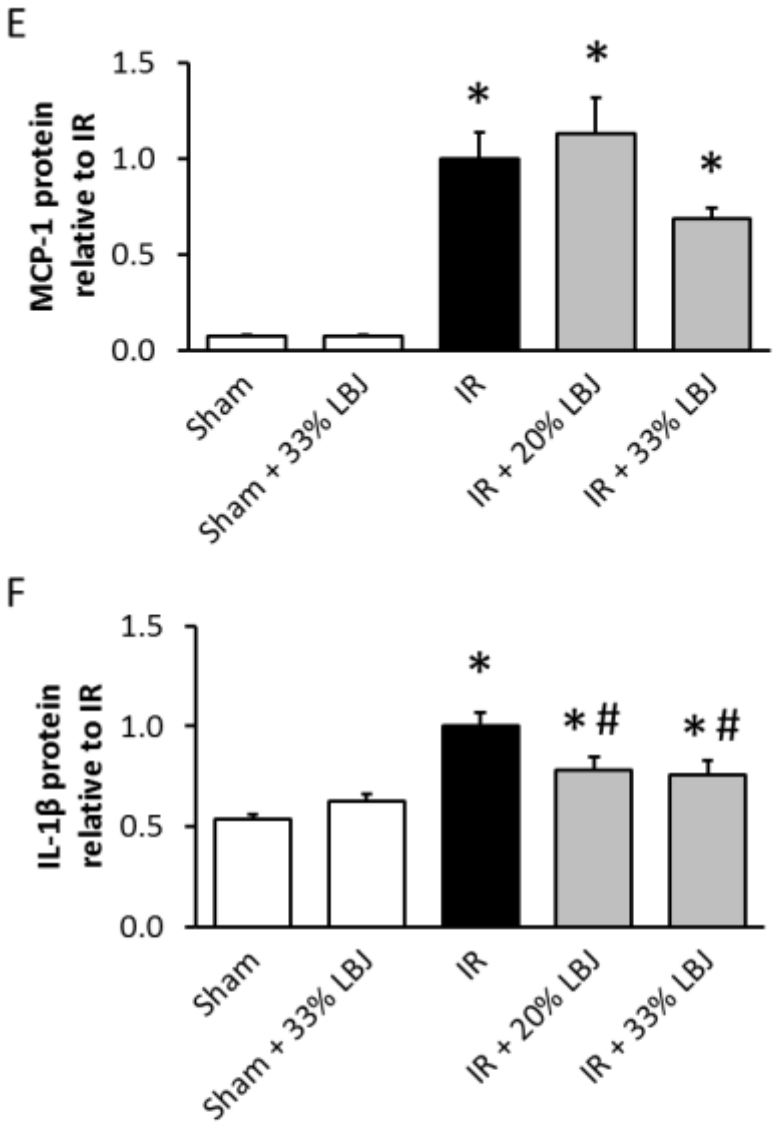


C


TIMP-1 protein relative to IR
1.5
1.0
0.5
0.0
*
*
*
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

D


TNFα protein relative to IR
1.5
1.0
0.5
0.0
*
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ


E
MCP-1 protein relative to IR
1.5
1.0
0.5
0.0
*
*
*
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ
F
IL-1β protein relative to IR
1.5
1.0
0.5
0.0
*
*#
*#
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

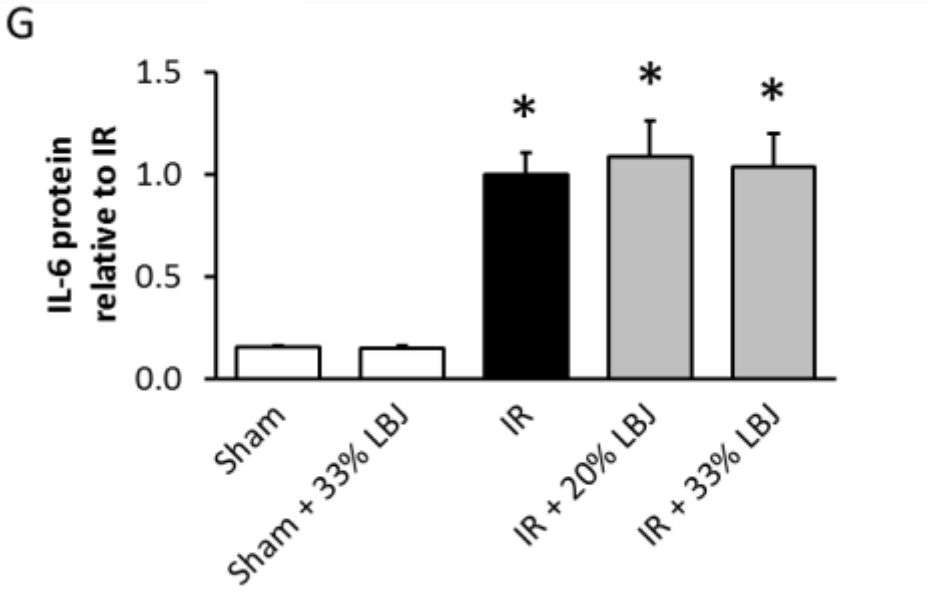

G
IL-6 protein relative to IR
1.5
1.0
0.5
0.0
*
*
*
Sham
Sham + 33% LBJ
IR
IR + 20% LBJ
IR + 33% LBJ

Figure 5.6 Effect of lingonberry juice consumption on renal MCP-1 mRNA expression after IR injury. *MCP-1 mRNA was measured using real-time PCR analysis after sham operation (Sham), 45 minute ischemia and 6 hour reperfusion (IR), and IR following lingonberry juice consumption (IR + 20% LBJ, IR+ 33% LBJ). * indicates $p < 0.05$ when compared to the sham group; # indicates $p < 0.05$ when compared to the IR group.*

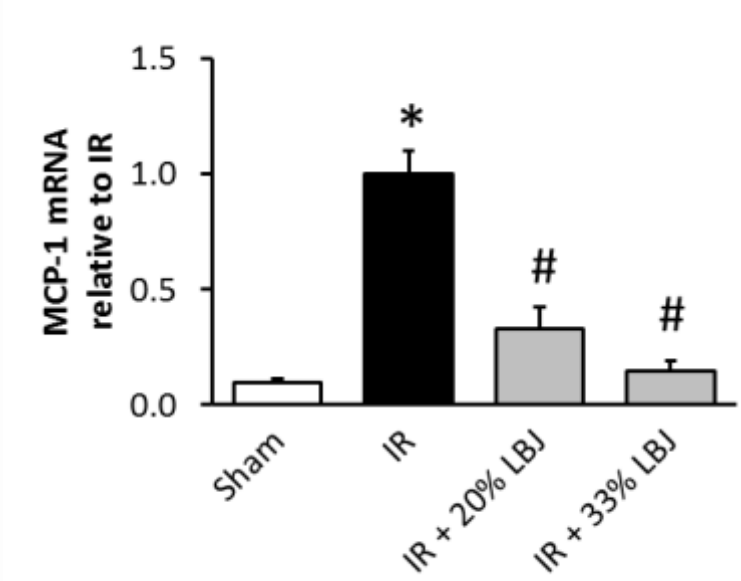


Figure 5.7 Effect of anthocyanins on phosphorylation of JNK and c-Jun in cultured renal proximal tubular cells. *Total and phosphorylated JNK (A) and c-Jun (B) were assessed after HK-2 cells were subjected to normoxic conditions (Control), 1 hour ischemia and 3 hour reperfusion (IR), and IR following pre-treatment with cyanidin-3-galactoside (C-3-Gal) at 10 ng mL^{-1}, cyanidin-3-glucoside (C-3-Glu) at 10 ng mL^{-1}, cyanidin-3-arabinoside (C-3-Ara) at 10 ng mL^{-1}, anthocyanin combo at 100 ng mL^{-1}) or JNK inhibitor SP600125 at 5 µM. * indicates p < 0.05 when compared to the sham group; # indicates p < 0.05 when compared to the IR group.*

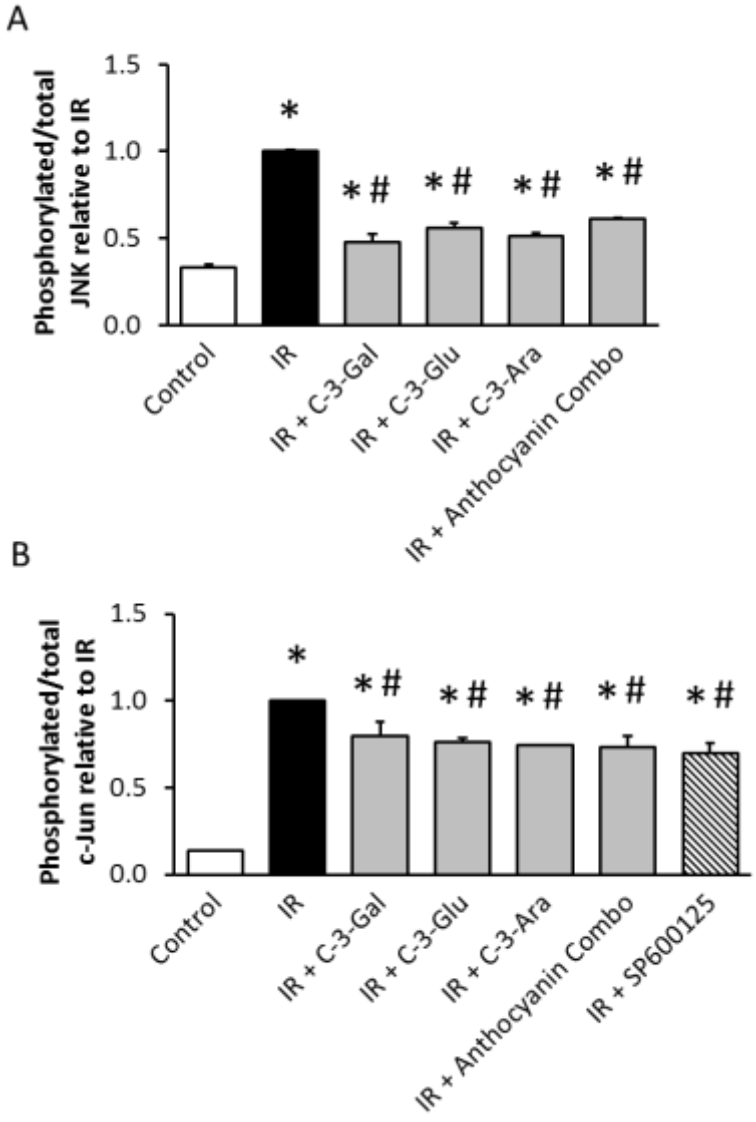
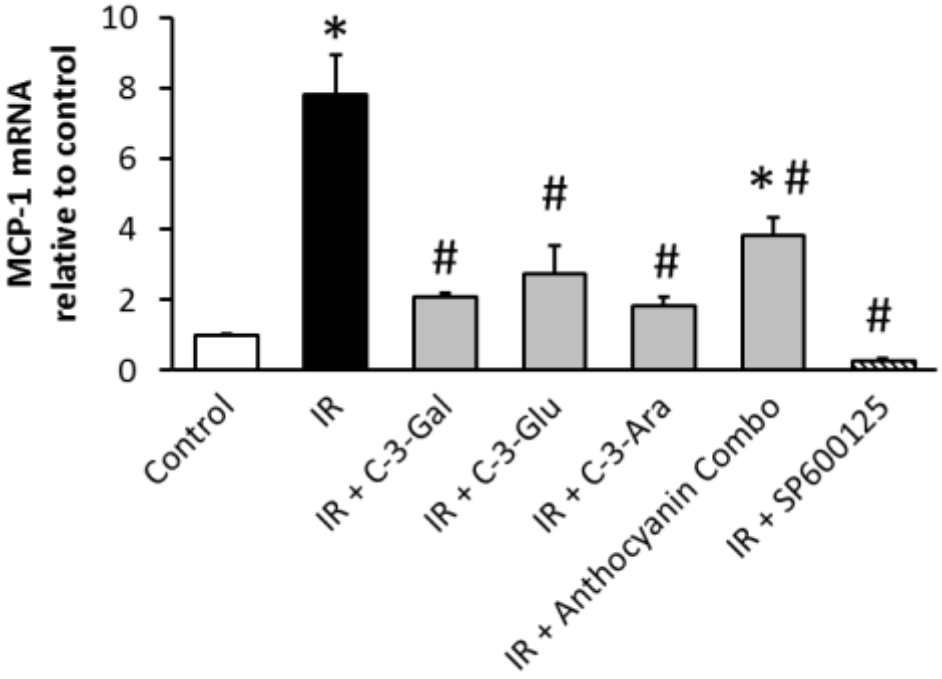


Figure 5.8 Effect of anthocyanins and JNK inhibitor SP600125 on MCP-1 expression in cultured renal proximal tubular cells. *MCP-1 mRNA was quantified by real-time PCR analysis after HK-2 cells were subjected to normoxic conditions (Control), 2 hour ischemia and 6 hour reperfusion (IR), and IR following pre-treatment with cyanidin-3-galactoside (C-3-Gal) at 10 ng mL^{-1}, cyanidin-3-glucoside (C-3-Glu) at 10 ng mL^{-1}, cyanidin-3-arabinoside (C-3-Ara) at 10 ng mL^{-1}, anthocyanin combo at 100 ng mL^{-1}) or JNK inhibitor SP600125 at 5 µM. * indicates $p < 0.05$ when compared to the sham group; # indicates $p < 0.05$ when compared to the IR group.*



5.5 Discussion

The results reported in this study show that long-term intake of lingonberry juice can effectively attenuate the IR-induced stress-activated signaling pathway leading to a decrease in inflammatory response. These effects may contribute to its overall protective effects against IR injury. Lingonberry juice consumption for three weeks prior to the IR event in rats significantly reduced JNK and c-Jun activation in the injured kidney and reduced an extensive array inflammatory biomarkers in plasma and kidney tissue, measured using a validated, multiplex panel. Upon further investigation in cultured proximal tubule cells, the activation of JNK signaling was linked to subsequent inflammatory sequelae. In these cells, anthocyanin treatment inhibited JNK activity and MCP-1 expression.

Activation of JNK can result in apoptotic cell death and loss of kidney function. The activation of JNK via phosphorylation by upstream stress-activated kinases results in increased phosphorylation of the transcription factor c-Jun, which accumulates in the nucleus and activates transcription of both pro-apoptotic and anti-apoptotic proteins. Our previous results, which agreed with other published results, showed that the activation of JNK could lead to apoptotic cell death and subsequent loss of function due to organ failure (De Borst *et al*. 2009; Sun *et al*. 2012; Xu *et al*. 2010). When the activation of JNK was prevented in the kidneys of rats fed lingonberry juice, kidney function was improved as indicated by significant reduction in plasma creatinine and NGAL. While creatinine is the most commonly used indicator of kidney function, NGAL is an alternative clinical biomarker that allows for earlier diagnosis of AKI and can be used to grade the severity of kidney injury (Haase *et al*. 2011; Paragas *et al*. 2011). JNK and c-Jun activation were alleviated after IR in rats fed lingonberry, indicating reduced cellular

stress in the kidney. Our results agree with those of Xu and colleagues (Xu *et al*. 2011) who have shown a protective role of JNK on renal function in rats through intraperitoneal administration of the specific JNK inhibitor, SP600125.

Reducing inflammation following ischemic AKI has been the focus of many experimental therapies but remains an elusive goal in clinical AKI patients (Lutz *et al*. 2010; Snoeijs *et al*. 2010; Thurman. 2007). In this study, we showed, for the first time, that the consumption of lingonberry juice attenuated both the renal and systemic inflammatory response following ischemic kidney injury. The release of important mediators of immune cell infiltration (MCP-1, KC, and TNFα) into the circulation was reduced, which might slow the progression of the inflammatory response and prevent complications of renal inflammation such as endothelial dysfunction. Reduced stimulus for monocyte and neutrophil attraction to the kidney has been correlated with reduced area of tubular necrosis and overall kidney injury (Sung *et al*. 2002). Inflammatory cytokines in the kidney were also lower after lingonberry consumption. Of note, inhibiting IL-1β expression in the kidney may modulate multiple components of the inflammatory cascade, as it triggers the production of cytokines like TNFα, IL-6, and MCP-1 in other cells by binding the IL-1 receptor and inducing intracellular signal transduction via NFκB (Furuichi *et al*. 2006). With reduced triggers of inflammation in the kidney, there will be fewer circulating pro-inflammatory cytokines and fewer cells will be lost to apoptosis triggered by death-receptor signaling (Molitoris. 2014), allowing quicker recovery of function. Our results demonstrated that the supplementation of lingonberry juice to rats prior to IR injury may play an important role in achieving this effect.

Interestingly, JNK activity and MCP-1 expression were concurrently reduced in the kidney. De Borst *et al.* showed that the JNK inhibitor SP600125 could reduce MCP-1 expression following IR injury in rats, indicating that stress-activated JNK signaling is linked to the subsequent inflammatory response (De Borst *et al*. 2009). The immune response evolved to protect the organ and is designed to help the tissue heal, however, if such a response is not resolved in a timely manner or the organ is compromised by additional comorbidities, the inflammation can result in loss of organ function. When the kidney is subjected to ischemic insult, resident cells of the epithelial-endothelial axis are the first to sense the injury and initiate signaling to trigger an immune response (Molitoris. 2014). Dampening the inflammatory response by targeting the initiating signaling cascades in the cells that detect injury may be protective to the organ. Using HK-2 proximal tubule epithelial cells to model these initiator cells, we demonstrated that IR induced significant activation of JNK and c-Jun and increased MCP-1 expression, and pure anthocyanins at physiologically relevant doses (10 ng mL^{-1}, equivalent to 20 nmol L^{-1} for cyanidin-3-glucoside and cyanidin-galactoside and 22 nmol for cyanidin-3-arabinoside) attenuated each of these effects. The specificity of this linkage is established by the inclusion of the JNK inhibitor SP600125 in the study. Thus, these results obtained from the HK-2 cells corroborate and extend our findings from the animal experiment. Nonetheless, it should be noted that anthocyanins are metabolized by deglycosylation and phase II conjugation after absorption (Kamiloglu *et al*. 2015) and the circulating metabolites could be different from those tested in the cell culture experiment. Future studies will be necessary to address the activities of these metabolites.

Further investigation of preventative approaches to alleviating ischemic AKI using a moderate intake of anthocyanin-rich lingonberry juice is warranted, as many surgeries that lead to AKI, such as coronary artery bypass grafting and kidney transplant (Panek *et al*. 2016; Pickering *et al*. 2015), can be accompanied by wait times that would allow for pretreatment (up to weeks for coronary artery bypass and up to years for kidney transplant) (Reese *et al*. 2015; Sobolev *et al*. 2012). This approach may be valuable since it is non-invasive and only requires a small change in dietary pattern.

5.6 Conclusion

The novel findings of this study are that a physiologically relevant daily intake of anthocyanin and antioxidant-rich lingonberry juice could improve kidney function induced by IR injury by modulating JNK signaling in the kidney and inhibiting the local and systemic inflammatory response. The effects of lingonberry anthocyanins in proximal tubule cells and the demonstrated link between JNK activation and inflammatory gene expression show that this preventative dietary approach may target the initial cellular (kinase) response to injury and therefore is able to effectively change multiple components of the concerted cellular responses to IR injury. Using validated, multiplex biomarker arrays combined with animal and cell culture models, this is the first study to show that modifying the diet to include lingonberry juice could improve outcomes after an AKI.

5.7 Acknowledgement

Cara Isaak is supported in part by a Mindel and Tom Olenick Research Studentship in Medicine, a BMO Financial Group Research Scholarship for Excellence, a studentship from the Manitoba Health Research Council, and by the estate of Margurite Jermaine Gerome (deceased). We thank Alphonsus Utioh for his help with acquiring lingonberries and Jiancheng Qi for his guidance in preparing the lingonberry juice. Funding was provided by Agriculture and Agri-Food Canada Project ID J-000071 and J-001297.

Chapter 6

Conclusions and Recommendations

6.1 Novel findings

The novel findings of manuscripts II, III, and IV are:

1) Wild Manitoba lingonberry has high *in vitro* antioxidant activity measured by four different antioxidant assays and also contains large amounts of anthocyanins.
2) The three main anthocyanins in Manitoba lingonberry are cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-arabinoside and they are found in similar proportions to European cultivars.
3) Lingonberry extracts and anthocyanins protect rat cardiomyoblasts from IR-induced apoptosis by inhibiting chromatin condensation, caspase-3 activation, JNK phosphorylation, and c-Jun activation.
4) Lingonberry anthocyanins protect rat cardiomyblasts from oxidative stress-induced apoptosis by inhibiting caspase-3 activation, loss of membrane permeability, and chromatin condensation.
5) Only physiological doses (10 ng mL^{-1} or 20 nmol L^{-1}) of anthocyanins are needed to protect heart cells from oxidative-stress induced apoptotic cell death.
6) Daily lingonberry juice consumption for three weeks protects the rat kidney from acute ischemic injury and attenuates increases in serum creatinine.
7) Lingonberry juice consumption modulates MAPK signaling in the kidney during AKI.
8) Lingonberry juice consumption decreases the inflammatory response in the kidney and release of pro-inflammatory proteins into the circulation after renal IR.
9) In cultured proximal tubule epithelial cells, lingonberry anthocyanins inhibit IR-induced JNK activation and subsequent pro-inflammatory gene expression.

6.2 Conclusion

These studies provide evidence that lingonberry compounds are protective against IR and oxidative stress-induced apoptosis in cultured cardiomyoblasts and against IR-induced apoptosis in kidney cells and that daily consumption of lingonberry juice protects the kidney from ischemic injury. Lingonberry compounds were shown to inhibit JNK activity in each model used, providing a unifying mechanism for cellular protection by reducing pro-apoptotic and pro-inflammatory processes (Figure 2.5). Manuscript II was the first study to demonstrate that doses of anthocyanins within the reported ranges of bioavailability could protect cells from apoptotic cell death. These studies also provided the first characterization and highlighted the unique properties of Manitoba lingonberry. Since lingonberry grows abundantly and sustainably in Northern Manitoba, research concerning the berry's health benefits could help promote a seasonal berry product from the Northern regions of the province.

6.3 Recommendations for future lingonberry research

Some interesting questions are raised by these studies that require further exploration and experimentation. First, how do lingonberry anthocyanins alter JNK activation kinetics? Lingonberry compounds were shown to target JNK activation during simulated IR in H9c2 cardiomyoblasts, oxidative stress in H9c2 cardiomyoblasts, simulated IR in HK2 proximal tubule epithelial cells, and renal AKI in Sprague-Dawley rats. In healthy cells and tissues, JNK phosphorylation was largely unchanged. As discussed in review manuscript IV, some anthocyanins with specifically placed hydroxyl groups may be capable of direct enzyme

interaction at the ATP binding site. However, pharmacological doses used in pull-down assays and enzyme kinetic assays may not reflect the physiological doses found *in vivo.* Phosphatases are inhibited by oxidative stress, for example during hypoxia, so bolstering their activity by minimizing oxidative stress could also explain JNK inhibition by anthocyanins. Anthocyanins have been shown to inhibit NADPH oxidase in a variety of cell types and tissues which would directly reduce oxidative stress. Alternatively, intracellular metabolism of anthocyanins produces small phenolic electrophiles that can activate the Nrf2 transcription factor which would enhance expression and activity of antioxidant enzymes, indirectly reducing oxidative stress. Manuscripts I through IV provide evidence that JNK inhibition may be one mechanism of the health benefits on lingonberry compounds, however the way in which these compounds target JNK requires further investigation.

Second, how does JNK modulate the inflammatory response in AKI? More research is needed regarding the link between JNK activation and the inflammatory response in this setting. Figure 2.5 of this thesis showed a model of the link between JNK inhibition by berry compounds and reduced inflammatory response; however the exact nature of the JNK inhibition requires further study. In our study, a large panel of inflammatory biomarkers was elevated in the kidney and in the circulation after AKI, but not all biomarkers were affected by lingonberry consumption. No studies have been performed examining the effects of JNK inhibition on each of these biomarkers and their expression may be controlled by JNK-activated transcription factors or their secretion or activation may be controlled by JNK-mediated inflammasome activity. Alternatively, the inhibitory effects of lingonberry on some of these biomarkers may have been observed if additional time-points of ischemia and reperfusion were

tested in the rat model of AKI, or if expression of the biomarkers was measured in specific regions or structures of the kidney. The mechanism, timing, and location of JNK regulation of the inflammatory response in AKI requires further research.

Third, will lingonberry juice consumption provide health benefits for humans? So far, lingonberry-based nutritional interventions have only examined effects on the postprandial inflammatory response. Chronic inflammation, cardiovascular risk factors, and kidney function would be interesting avenues of investigation for a clinical trial using lingonberry juice or whole berries. In addition, only healthy people have been used in these intervention trials and effects of lingonberry on inflammatory processes in patients with chronic diseases may be more pronounced.

Fourth, what is the basis for the higher antioxidant capacity, phenolic content, and anthocyanin content in Manitoba lingonberry compared to European or Newfoundland cultivars? Further research will be needed to determine whether the change in ratio is due to genetic variability or growing conditions. If Manitoba lingonberry has a superior biochemical profile over other varieties there may be more impetus for commercializing this Northern fruit crop. A combination of genetic, biochemistry, and metabolomics techniques will be required to determine the basis for Manitoba lingonberry's high polyphenol content, including mass spectrometry identification of the bioactive compounds in lingonberry. Studies regarding the differences in properties and health benefits of different varieties of lingonberry are already underway in our laboratory. If wild lingonberries are shown to offer increased health benefits, year-to-year changes in growing conditions and their effects on berry quality will need to be addressed.

Fifth, how does each of the components of lingonberry or other berries contribute to overall health benefits? While this body of research focused on anthocyanins, berries contain a wide range of other polyphenolic compounds in addition to dietary fibre, healthy fats, vitamins, and minerals. While this study used a juice product which would mainly contain the water-soluble portion and exclude seeds and pulp, other components in the whole berries may affect the absorption and metabolism of bioactive compunds and subsequently the health benefits of lingonberry. In addition, metabolites of lingonberry polyphenols (including the methylated forms of anthocyanins) may act as the effectors of any health benefits but it is not known right now which of these metabolites are biologically active. Further research is needed to test the protective effects of individual berry components and metabolites as compared to whole berries or juices. Synergistic effects of polyphenols and metabolites should also be investigated if purified compounds are to be pursued as therapeutic interventions.

These opportunities for future research will be important for Canadian-grown lingonberry to gain acceptance as a functional food with proven health benefits. Mechanistic knowledge of the effects of lingonberry anthocyanins on JNK enzyme kinetics and pro-inflammatory gene expression may point to the specific compounds responsible for the health benefits of anthocyanin-rich berries and will provide substantial evidence for these benefits. Finally, wild Canadian lingonberry has not been used in a nutritional clinical trial so health benefits of these specific berries cannot yet be extrapolated to humans. Understanding of the genetic and botanical basis for the polyphenolic content of lingonberry grown across Canada will allow for agricultural development and the promotion of value-added lingonberry products.

Chapter 7

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