APPLE POMACE POLYPHENOLS AND THEIR EFFECT ON THE PROLIFERATION OF HUMAN EPITHELIAL COLORECTAL ADENOCARCINOMA CELLS (HT-29)

by

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A Thesis
presented to
The University of Guelph

In partial fulfillment of the requirements
For the degree of
Master of Science
in
Animal Biosciences

Guelph, Ontario, Canada

ABSTRACT

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In an effort to eventually produce a human health promoting, polyphenol-enriched dairy product for human consumption by apple pomace (AP) inclusion in the diet of a dairy cow, two preliminary studies were conducted. Initially, 8 commonly found polyphenols, in locally-sourced AP, were quantified via UPLC/MS and total polyphenol content was determined by the Fast Blue BB salt method. To determine the effect of the rumen environment on the recovery of polyphenols originally found in AP, the sample was subjected to 24hr rumen digestion in an *in vitro* incubator and the quantities of the polyphenols in the digesta were analyzed by UPLC/MS. The second study evaluated the effect of the aglycone (quercetin) of the most adundant polyphenol in this sample of AP, quercetin-3-glucoside, on the proliferation of human epithelial colorectal adenocarcinoma cells (HT-29). Their proliferation was quantified by use of a cell-protein binding dye, sulforhodamine B.

ACKNOWLEDGEMENTS

I consider myself very lucky to have had such an amazing group of people supporting me throughout the last two years. I would like to offer my sincerest gratitude to my thesis advisor Dr. Vern Osborne for his guidance and patience throughout this process. I am also very grateful for my advisory committee, consisting of Drs. Brian McBride, John Cant, and Tom Wright, who continuously provided me with their support and recommendations. The financial generosity of Dr. McBride and Osborne made my life as a student much less stressful and allowed me to focus on the completion of my thesis.

I would like to thank Drs. Milena Corredig and Praveen Saxena at the Canadian Research Institute for Food Safety and Gosling Research Institute for Plant Preservation, respectively, for the use of their laboratories and supplies. I would also like to thank Abhishek Chattopadhyay, Justina Zhang, Drs. Mukund Shukla and Elena Arranz for their continued advice throughout the learning process of antioxidant and cell assay development. Ron Piett of A&L Laboratories and Lauren Erland deserve tremendous gratitude for performing *in vitro* digestion on apple pomace samples and the UPLC analysis, respectively. I am grateful for the funding provided by the Ontario Centres for Excellence, the Natural Sciences and Engineering Research Council of Canada, and the Dairy Farmers of Ontario, which made this project possible.

Lastly, I would like to thank my parents, partner and the rest of my family and friends for their encouragement during these challenging past two years.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

AP	Apple pomace
CA	Chlorogenic acid
CPT	Camptothecin
CPT-11	Irinotecan
CRC	Colorectal cancer
DM	Dry matter
DW	Dry weight
E	Epicatechin
ECG	Epicatechin-3-gallate
EGCG	Epigallocatechin gallate
FG	Flavonoid glycoside(s)
FR	Free radical
FW	Fresh weight
GAE	Gallic acid equivalent
GOF	Gain of function
IC_{50}	Concentration which inhibits 50% of cell growth
P	Phenol(s)
PCA	*p*-coumaric acid
PN	Phloridzin
PP	Polyphenol(s)
Q	Quercetin
QG	Quercetin glycoside(s)
Q3G	Quercetin-3-glucoside
Q3GL	Quercetin-3-galactoside
ROS	Reactive oxygen species
SRB	Sulforhodamine B assay
SN-38	7-ethyl-10-hydroxycamptothecin
TOPO-I	Topoisomerase I
UPLC/MS	Ultra-high performance liquid chromatography/mass spectrometry

CHAPTER ONE

GENERAL INTRODUCTION

Over the past decade, a great deal of research has been carried out to develop a broadened understanding of the relationship between diet and health. As a result, it has been shown that consumption of certain plant-derived compounds, such as polyphenols (PP), can have a disease preventative effect on the subject. Increased consumer interest in these health protective effects has resulted in a large expansion of the functional foods and nutraceuticals market. Although a number of polyphenol-enhanced functional food products exist in today's market, the dairy industry is under-represented in this sector. Being nature's vehicle-delivery system, milk represents an ideal carrier for ingested bioactive compounds that may confer a benefit to human health.

Of the thousands of PP found in plants, flavonoids are the most ubiquitously represented and possess the strongest evidence for their health-promoting properties. Due to their abundance in apple pomace (AP), a largely wasted but highly produced co-product of the juice industry, AP presents a sustainable resource for natural PP fortification of dairy products. Since ingested PP have been documented in the milk of lactating dairy cows and goats, and numerous studies have characterized AP as a by-product feed for dairy cows, this study is, in part, focused on evaluating the rumen stability of AP PP. In an effort to meet the demands of consumers seeking sustainable, naturally produced health foods, we also examined the effect of AP PP on the proliferation of human colorectal cancer cells *in vitro*.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Introduction

Cancer is the second leading cause of death worldwide with approximately 14.1 million new cases diagnosed and 8.2 million cancer deaths occurring worldwide in 2012 (Araujo et al. 2011; Torre et al. 2015). For this reason, over the last few decades, there is an overwhelming amount of scientific research that has focused on finding the causing, and preventative, factors of cancer. Although the process of carcinogenesis is much more complex than a cause-and-effect model, a comprehensive international review in 1997 concluded that about one-third of all cancers in Western societies are attributed to environmental factors, such as diet and lack of physical activity (Glade 1999). Experimental and epidemiological research reviewed by the panel of experts confirmed that consumption of fruit and vegetables decreases the risk of cancers of the mouth, pharynx, larynx, esophagus, lung, stomach, colon, rectum, pancreas, breast, and bladder.

Among the macro- and micronutrients found in fruit and vegetables, edible plant matter contains other health-promoting compounds, which are largely phenolic in nature and possess powerful antioxidant properties (Manach et al. 2004). The realization of the direct relationship between diet and health has fueled a large scientific interest in the health-affective properties of plant polyphenols. Although evidence from human clinical trials is currently lacking, numerous epidemiological, *in vitro* and animal trials have

shown the antioxidant, anti-carcinogenic, anti-inflammatory, (Bravo et al. 1998; Middleton et al. 2000; Manach et al. 2004; Scalbert et al. 2005) neuroprotective, (Soobrattee et al. 2006) anti-allergic, anti-diarrheal, anti-ulcer, antibiotic, (Manach et al. 2004; Howells et al. 2007) anti-lipidemic, vasorelaxing (Signorelli and Ghidoni 2005) and anti-thrombotic (Bravo et al. 1998; Middleton et al. 2000; Nijveldt et al. 2001; Manach et al. 2004; Scalbert et al. 2005) properties of phenols.

The health properties of PP are largely thought to be associated with their ability to scavenge and stabilize free radicals formed during cellular respiration, reactive oxygen species (ROS) (Middleton et al. 2000). ROS are naturally occurring in the human body, but if left unchecked, they can disrupt cell-signaling pathways and affect gene expression by modifying the redox status of the cell and affecting protein conformation and function, including, but not limited to, transcription factors, cell cycle regulators, and tumor suppressors. Because of this, they are thought to be an important risk factor in the development of inflammation, cardiovascular disease, cancer, diabetes, and Alzheimer's disease (Middleton et al. 2000).

In studies of anti-cancer properties of PP, they exerted antioxidant, antiproliferative (Kuntz et al. 1999), anti-inflammatory effects and induced cell cycle arrest , apoptosis and inhibition of angiogenesis and metastasis in cancer cells (Manson 2003; Araujo et al. 2011). Flavonoids, a class of phenylbenzo-pyrone polyphenols with an assortment of structures based on a common 3-ring nucleus, have in particular shown significant anti-tumor properties (Kuntz et al. 1999; Wenzel et al. 2000; Middleton et al.

2000). Kuntz et al (1999) tested 36 flavonoids, 30 of which demonstrated significant anti-proliferative activity in the absence of cell cytotoxicity in human colon cancer cell lines (Kuntz et al. 1999). The concentration needed to kill 50% of cells in 24h (IC_{50}) ranged from 39.7±2.3μM – 203.6 ±15.5μM, depending on the flavonoid and cell type. In nude mice with xenografted human colorectal cancer tumors, flavonoid quercetin significantly decreased tumour volume after a 6-week treatment period by 35-45% compared to the control group (Kim et al. 2010).

AP, the solid residue (skin, pulp, and seeds) from the apple juice and cider industry, is rich source of flavonoids, such as quercetin and phloretin (Rana et al. 2014). AP is a widely produced and largely wasted co-product, with the 2008-2009 world production amounting to approximately 17 400 910-20 881 092 tons and Canada contributing approximately 113 840-136 610 tons to this estimate (Dhillon et al. 2013). Its high moisture content (70-75%) presents a problem for affordable transportation and supplies a perfect breeding ground for human disease vectors and bacteria that produce greenhouse gas (ex. methane) and noxious smells through fermentation. Only a small fraction of this co-product is currently used as animal feed or added to soil as fertilizer, with most of it being composted or deposited in a landfill and contributing to greenhouse gas emissions as well as pollution of groundwater (Dhillon et al. 2013).

AP has previously successfully been incorporated into the diets of dairy cows and is thought to be a highly palatable and high soluble carbohydrate feed (Pirmohammadi et al. 2006; Ghoreishi and Pirmohammadi, Rasoul; Yansari 2007; Abdollahzadeh et al.

2010). Along with potential health benefits to the animals themselves, evidence of phenolic transfer to the milk of lactating dairy cows and goats has sparked an interest in the production of PP-rich animal products for human health (Jordan et al. 2010; Soberon et al. 2012). This study was designed to examine the PP content of locally produced AP, the effects of *in vitro* rumen digestion on its PP content and the anti-cancer properties of the core structure of its most abundant flavonoid.

2.2 Colorectal cancer epidemiology, pathogenesis, risk factors, and prevention

Colorectal cancer (CRC) is the third (men) and second (women) most common type of cancer worldwide, with approximately 1.4 million cases diagnosed and 693 900 deaths in 2012 (Torre et al. 2015). Although CRC can be hereditary or sporadic, dietary factors are responsible for 70-90% of cases (Shannon et al. 1996; Glade 1999; Ahmed 2004). Risk factors for CRC include high consumption of lipid and calorie-rich diet (especially fats of animal origin) (Hamer et al. 2008), red and processed meat (Cross and Sinha 2004), and ethanol (Payne 1990), as well as smoking, presence of obesity, diabetes, (Khaw et al. 2004) and chronic inflammation (García-Lafuente et al. 2009; Torre et al. 2015). Preventative factors include a diet rich in fruits and vegetables (Hamer et al. 2008), fiber (Mariadason et al. 2001), mineral water (Shannon et al. 1996), red wine (Scalbert et al. 2005), green tea (Yang et al. 2000; Scalbert et al. 2005; Yang et al. 2011a) use of non-steroidal anti inflammatory drugs, (NSAIDS) (and other cyclooxygenase (COX) inhibitors) (Ahmed 2004; Half and Arber 2009) estrogen replacement therapy, and moderate to vigorous exercise (Ahmed 2004; Jemal et al. 2008).

In the process of carcinogenesis, multiple stages exist where the potential for prevention or intervention by chemopreventive agents, including PP, can occur. According to Fearon & Vogelstein (1990), in order for a normal cell to become cancerous it must obtain certain characteristics: (1) self sufficiency in growth signaling and limitless replicative potential, (2) become unresponsive to anti-proliferative signals, (3) evade apoptosis, (4) induce and sustain angiogenesis, and (5) acquire the ability to invade and metastasize (Fearon and Vogelstein 1990). Several connected signaling cascades are known to play an important role in this process: MAPK pathways involved in communication between the cell surface and nucleus, transcription factors involved in mRNA production, cell cycle factors involved in regulation of cell proliferation, and factors involved in regulation of apoptosis (Manson 2003).

Mitogen-activated protein kinases (MAPK) belong to a large family of serine-threonine kinases and consist of three major subfamilies: the extracellular-signal-regulated kinases (ERK MAPK, Ras/Raf1/MEK/ERK), the c-Jun N-terminal or stress activated protein kinases (JNK or SAPK) and MAPK14 (p38α) (Fang and Richardson 2005). Several key growth factors and proto-oncogene products transduce signals through the ERK MAPK pathway, making it an essential for regulation of cell proliferation and intestinal epithelial differentiation and its aberrant signaling most pertinent in the development of CRC (Troppmair et al. 1994; Sebolt-Leopold 2000). Also often deregulated in cancers, the JNK and p38 MAPK pathways, which are activated by environmental and genotoxic stress signals, play key roles in inflammation and tissue homeostasis through control of cell proliferation, differentiation, survival and migration

(Chen et al. 1996). The role of JNK and p38 pathways in carcinogenesis is not as well elucidated as the role of ERK MAPK (Wagner and Nebreda 2009). In normal differentiating rat intestinal epithelial cells (IEC-6), downregulation of ERK 1/2 phosphorylation was observed (Lemieux et al. 2011). Additionally, expression of activated MEK1 in IEC-6 cells blocked Cdx3 (transcription factor)-dependent differentiation, demonstrated by a significant reduction in the number of microvilli on the apical surface and the lack of induction of general enterocyte marker genes (Lemieux et al. 2011).

A mutation in the Raf proto-oncogene, giving rise to oncogenic B-Raf or BRAF tyrosine kinase is found in ~4-15% of CRCs, including the tumour that gave rise to the HT-29 cell line (Davies et al. 2002; Bardelli and Siena 2010; Cantwell-Dorris et al. 2011). This activating mutation increases signaling through ERK/MAPK, which results in activation of transcription factor genes involved in anti-apoptotic behavior (Sebolt-Leopold and Herrera 2004). Raf activation of the JNK/SAPK pathway also leads to inactivation of p53 tumour suppressor protein, and its ERK-independent activation of pro-survival transcription factor NF-κβ induces a wide array of other anti-apoptotic functions (Baumann et al. 2000). Additionally, HT-29 cells, and an array of other human tumours, contain missense mutations in the gene encoding for tumour suppressor p53, which results in a mutated protein with an altered activity. Since p53 is an important factor in regulation of stress-induced pathways affecting DNA repair, cell-cycle progression, and apoptosis, its altered function plays an important role in carcinogenesis (Gobert et al. 1999).

Another mutation affecting the ERK MAPK pathway, is that of Ras genes (KRAS oncogene), which is found in 35-45% of CRC tumours (Bardelli and Siena 2010). Ras are a family of tyrosine kinases that activate Raf in the MAPK ERK pathway and PI3 kinase in the PI3K/Akt(PKB)/mTOR pathway (Figure *2.1*) (Davies et al. 2002). This pathway is involved in diverse cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis (Vivanco and Sawyers 2002; Engelman et al. 2006). Mutations of PI3K subunit α (p110 α)-encoding gene, PIK3CA, and loss of its downstream tumor suppressor protein's (PTEN) expression occur in 10-18% and 19-42% of CRC, respectively (Bardelli and Siena 2010). Phosphatase and tensin homologue (PTEN) is the most important negative regulator of the PI3K signaling pathway, and its loss therefore results in unrestrained signaling through PKB/Akt and suppression of multiple pro-apoptotic, cell cycle inhibiting and tumor suppressor genes (Manson 2003; Liu et al. 2009).

As a result of the mutations occurring in CRC, NF-κβ, PI3K, PKB/Akt, Ras and Raf are usually overexpressed, while PTEN is under-expressed or absent in these tumours (Figure *2.1*) (Manson 2003). The effects of the alterations in the network connecting these signaling cascades results in overexpression of anti-apoptotic and cell proliferative factors, and/or the under-expression of pro-apoptotic and tumour suppressing factors. Together, these effects play an important role in pathogenesis, progression, and oncogenic behavior of human CRC (Sug et al. 2004). For those reasons, these factors present valuable targets in cancer prevention and treatment, and their activity is often used to assess drug efficacy (Sebolt-Leopold 2000; Sebolt-Leopold and Herrera 2004;

Liu et al. 2009; Moschetta et al. 2014).

People who eat five servings of fruit and vegetables per day are reported to be approximately 50% less likely to develop cancer (Ren et al. 2003; Surh 2003; Yang et al. 2011b). Evidence from various *in vitro* and *in vivo* studies suggests that this is at least partially attributable to intake of high concentrations of polyphenols, particularly flavonoids, which have shown potent chemoprotective properties by modification of common signaling pathways involved in carcinogenesis (Figure *2.1*) (Kuntz et al. 1999; Wenzel et al. 2000; Manson 2003; Van Der Woude et al. 2003; Barth, Stephan Cloudy apple juice decreases DNA damage et al. 2005). Evidence from epidemiological studies is not as straightforward, with numerous studies reporting positive results for the anti-CRC effect of flavonoids (Theodoratou et al. 2007; Bobe et al. 2008; Simons et al. 2009; Kyle et al. 2010) and others reporting no effect (Lin et al. 2006; Sun et al. 2006; Nimptsch et al. 2016). The need exists for carefully executed clinical trials. Research into the chemo- preventative and –therapeutic mechanisms of flavonoids are still being elucidated in the scientific community, but it is known that they protect cells from oxidative stress and modulate various key elements in cellular signal transduction pathways involved in the pathogenesis of CRC and other cancers (Manson 2003).

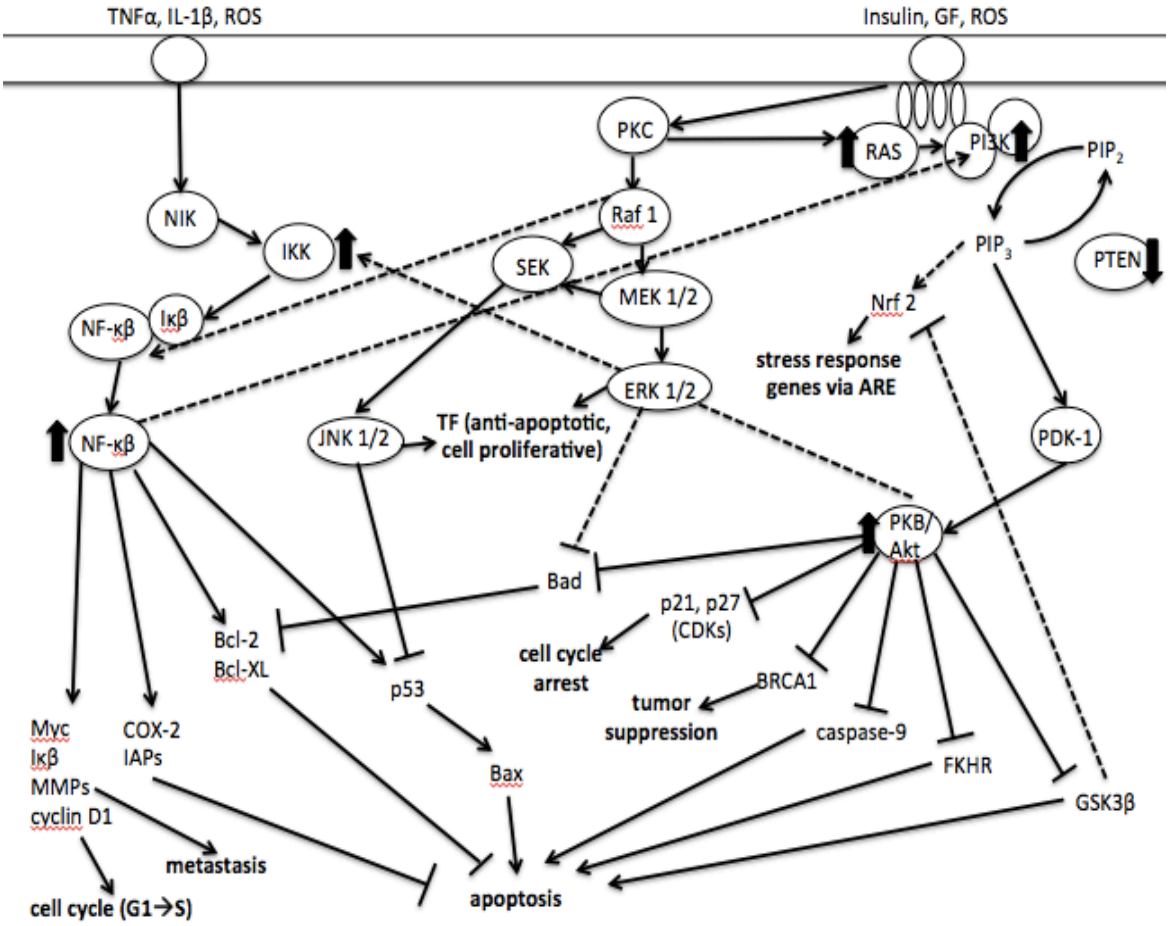

Figure 2.1 Common pathways that are deregulated in cancer. TNFα – tumour necrosis factor α; IL-1β – interleukin 1β; ROS – radical oxygen species; NIK – NF-κβ inducing kinase; IKK - Iκβ kinase; NF-κβ – nuclear factor κβ; MMPs – matrix metalloproteases; COX-1 – cyclooxygenase-1; IAPs – inhibitors of apoptosis; Bcl– B cell lymphoma; JNK – c-Jun N-terminal kinases; TF – transcription factor; PKC – protein kinase C; Bad – Bcl-2 associated death promoter; BRCA1 – breast cancer 1; FKHR – forkhead transcription factor; GSK3β – glycogen synthase kinase 3β; GF – growth factor; PI3K - phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP_2 - phosphatidylinositol (4,5)-bisphosphate; PIP_3 - phosphatidylinositol (3,4,5)-trisphosphate; PDK-1 - 3-phosphoinositide dependent protein kinase-1; PKB/Akt – protein kinase B; PTEN – phosphatase and tensin homolog.
↑/↓- overexpressed/under-expressed or defective in cancer cells, respectively. Adapted from Manson, 2003. (Manson 2003).

2.3 HT-29 Cell Line

The use of *in vitro* cell cultures increases practicality and reproducibility of experiments by reducing variability between subjects compared to human *in vivo* methods. Additionally, when studying anti-cancer properties of dietary components, *in vivo* studies are impractical, as they can take years to complete (i.e. epidemiological studies), may not always prove a cause and effect relationship, and can be confounded by many variables. Shortcomings of *in vitro* studies include limited extrapolation to *in vivo* effects.

HT-29 is a pluripotent and moderately differentiated primary colon cancer cell line that has been widely used in anti-proliferative studies. Under normal glucose supply (~0.6μmol/h/mg of protein) and serum containing medium, these cells contain more than 95% undifferentiated cells. During growth and post-confluence, HT-29 cells stack and form a non-polarized multilayer. Although they lack characteristics of epithelial intestinal cells, they are able to differentiate depending on the addition of inducers of differentiation to the medium (Atcc 2012).

This cell line possesses two main point mutations. The first is a Glu to Val substitution at residue 600 in the gene encoding for Braf, a member of the Raf family of serine-threonine protein kinases. The mutation, called BRAF V600E, results in a 500-fold increase in Braf's protein kinase domain, and therefore sustained activation of the MEK1/2 to ERK ½ MAPK pathway (Cantwell-Dorris et al. 2011). Activation of this

signaling cascade remains in the absence of any extracellular stimuli, allowing the cell to become self-sufficient in growth signals. Erhardt (1999) showed that overexpression of BRAF and therefore MEK indirectly inhibits cytochrome c-induced (mitochondrial) apoptosis (Erhardt et al. 1999). This mutation therefore results in alterations in a range of physiologic and tumour-promoting processes, such as, proliferation, self-renewal, senescence, apoptosis, invasion and metastasis (Cantwell-Dorris et al. 2011). The second missense mutation (His 273) is found in the gene encoding for tumour suppressor p53, which results in a base change from Arg to His at residue 273 (Shao et al. 1997; Rad et al. 2013). This mutation is considered a gain of [new oncogenic] function (GOF) mutation, resulting in a mutant p53 (mut-p53), which is constitutively associated with topoisomerase I (TOPO-I). In contrast to cells containing wild-type p53 (wt-p53), whose association with topoisomerase I is transient and highly regulated, mut-p53 cells, such as HT-29, contain high nuclear levels of p53 associated with TOPO-I, which results in its non-regulated, continuous activation (Gobert et al. 1999). Other target genes that may be affected by expression of a mut-p53 protein include: p21 (arrests cell cycle in G1 and G2), 14-3-3σ and GADD45 (cell cycle arrest in G2), ferrodoxin reductase (FDXR) (potential mediator in mitochondria-mediated apoptosis) and Bax and MCG10 (mediate apoptosis), as they are normally activated in response to DNA damage by wt-p53 (Liu and Chen 2002).

2.4 Flavonoids: Structure, chemical nature, and food sources

Oxygen, although essential to life, can produce potentially health-damaging compounds, such as reactive oxygen species (ROS), by transfer of unpaired electrons during its reactions with biological substrates. Additional ROS in the human body can result from exposure to their environmental sources, such as cigarette smoke, alcohol, chronic infection and UV radiation (Mena et al. 2009). Strong evidence linking ROS to the pathogenesis of many degenerative diseases in humans has resulted in interest in the mitigation of ROS through diet and lifestyle. Their overabundance in the body, either through failed clearance or overproduction, results in oxidative stress by free radicals that are damaging to important cell components, such as DNA, RNA, lipids, proteins, carbohydrates, and enzymes. Eventually, as a result of insufficiency to repair ROS-caused damage to genes and products of important cell signaling factors, carcinogenesis and development of other degenerative diseases can take place (Craft et al. 2012). Phenolic compounds found in plants can reduce oxidative stress through scavenging of free radicals and chelating of pro-oxidative metal ions. They are therefore an important exogenous antioxidant source in the human diet (Middleton et al. 2000).

Any compound with a hydroxyl substituted aromatic ring is considered a phenol and its polymer, a polyphenol (PP) (Craft et al. 2012). More than 8000 PP structures are currently known, and they are divided into several classes based on structure: phenolic acids, flavonoids, lignans, and stilbenes. These molecules are secondary metabolites of plants that are generally involved in defense against UV radiation or microbial attack

(Manach 2004). Flavonoids, the largest and most abundant class (over 4000 structures), are phenylbenzo-pyrones with an assortment of structures based on a common 3-ring nucleus (Figure *2.2*) (Middleton et al. 2000). They are further divided into several subclasses based on variations in the C- ring: flavones, flavonols, flavanones, flavanols, dihydrochalcones, proanthocyanidins/condensed tannins and anthocyanidins (Middleton et al. 2000; Manach et al. 2004). Flavonoids, particularly quercetin (Q), are potent antioxidants known to inhibit free-radical (FR) producing enzymes, such as xanthine oxidase and protein kinase C (PKC), among many others. Additionally, FR-producing metals, such as Fe and Cu, are successfully chelated by flavonoids. They are also able to thermodynamically reduce highly oxidizing free radicals, such as superoxide and hydroxyl, by hydrogen atom donation (Pietta 2000). Middleton et al (2000) provide an excellent summary of important flavonoid structural characteristics that make them exceptional antioxidants.

Flavonols are the most ubiquitously represented subclass in edible plants and usually occur as hydrophilic glycosides, attached to a sugar moiety (Manach et al. 2004). Quercetin (Figure *2.3*), the most consumed flavonol in the human diet (~10mg/day in typical Western diet) (Bohm et al. 1998), is abundant in its glycoside form in onions and apples, attached to a variety of sugar residues, depending on plant and variety (Scalbert and Williamson 2000; Ramos 2007). Their biosynthesis is stimulated by light, so these flavonols tend to accumulate in the outer and aerial tissues of plants.

Based on data collected from the Zupthen Elderly cohort study in the Netherlands, daily flavonoid intake in adults averages 23mg and surpasses that of vitamin E and β-

carotene (Hertog et al. 1994). On the contrary, results from a USA study report much higher intakes of 1000mg/day (Kühnau 1976). Undeniably, the intake and main food sources in diet can vary depending on geographical region and food availability. For instance, the Mediterranean diet, containing an abundance of olive oil, citrus fruits and greens is richer in flavonoids than most average Western diets (Ramos 2007). Nevertheless, the main flavonoid sources in the human diet, irrespective of geographical region, are tea, apples, and onions, with one apple providing approximately 400mg flavonoids (Middleton et al. 2000; Scalbert and Williamson 2000).

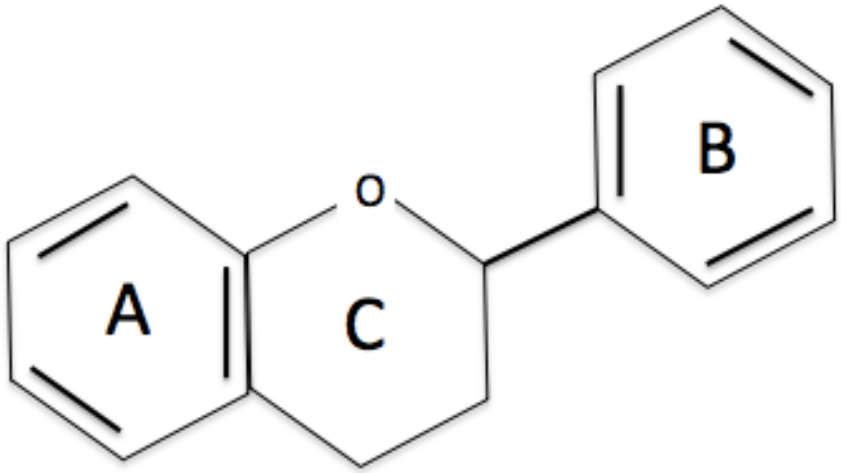

Figure 2.2 General flavonoid structure. Adapted from Ross and Kasum (2002).

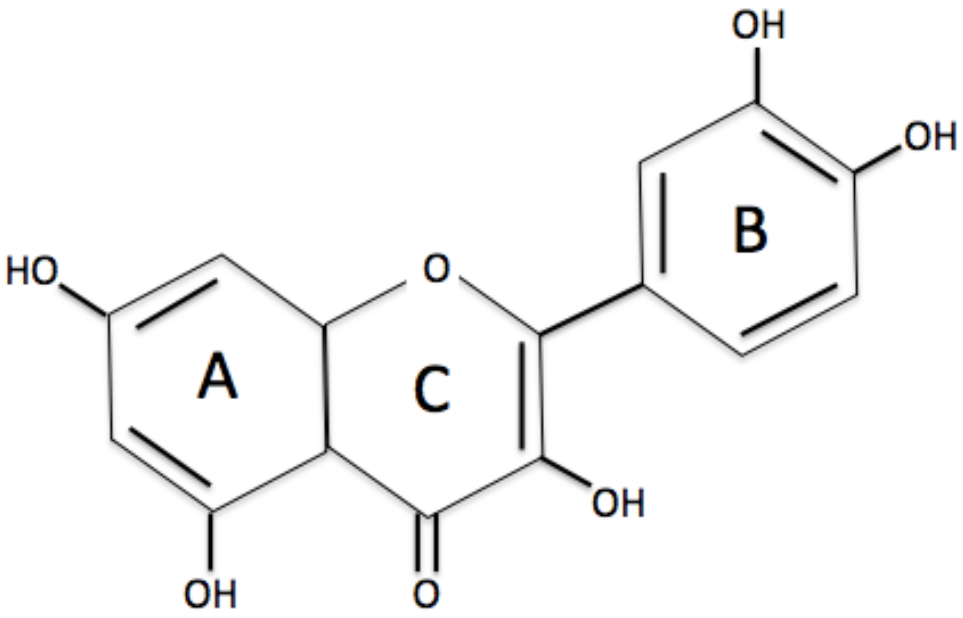

Figure 2.3 Structure of quercetin. Adapted from D'Andrea (2015).

2.5 Apple polyphenols and apple pomace

The apple fruit tree, being cultivated worldwide, is the most widely grown species in its genus. World apple production (2008-2009) was approximately 69 603 440 tons, with Canada contributing 455 361 tons to this estimate (Dhillon et al. 2013). Apples are an important source of phenolic compounds in the human diet, accounting for 22% of fruit phenolics consumed in the US (Lotito and Frei 2004). They contain and abundance of PP, including flavonoids (flavanols, flavonols, proanthocyanidins, dihydrochalcones, and anthocyanidins), and phenolic acids (hydroxycinnamates; particularly chlorogenic acid). Apple PP content is influenced by a number of factors, including variety (Van der Sluis et al. 2001; Wolfe et al. 2003), environmental factors such as sunlight exposure and fertilization, and is negatively influenced by length of storage (Golding et al. 2001;

Lattanzio et al. 2001). Concentrations of major phenolic compounds in 8 cultivars of apple, determined by Vrhovsek et al (2004), are found in Table *2.1*. Additionally, studies have shown that the flesh and peel of an apple differ in their phenolic concentration and antioxidant activity, with the peel being higher in both than the flesh (Escarpa and Gonzalez 1998; Eberhardt et al. 2000; Wolfe et al. 2003). This is largely due to the stimulating effect of UV radiation on flavonoid synthesis in apple peel, where these compounds are exclusively found (Price et al. 1995).

Table 2.1 Concentration of major phenolic compounds in 8 cultivars (Golden Delicious, Red Delicious, Granny Smith, Morgenduft, Fuji, Braeburn, Renetta, and Royal Gala) of apple, *Malus domestica* (adapted from (Vrhovsek et al. 2004)).
*FA-flavanols; FO-flavonols; A-anthocyanins; H-hydroxycinnamates; D-dihydrochalcones; nd-not detectable; sd-standard deviation

	FA	FO	A	H	D
	mean ± sd (mg/100g fresh weight)				
Renatta	203.3±26.5	3.4±1.1	nd	38.4±8.3	15.5±2.7
Red Delicious	112.4±17.0	5.9±3.0	2.5±1.2	9.8±1.2	4.4±0.9
Granny Smith	105.4±9.2	4.9±1.7	nd	4.5±0.7	2.0±0.4
Morgenduft	99.6±5.7	5.4±1.6	3.7±2.8	18.9±3.2	2.5±0.5
Golden Delicious	70.8±8.7	7.1±2.0	nd	10.7±1.9	2.8±0.5
Royal Gala	67.8±14.9	5.5±2.5	1.0±0.4	11.5±1.3	1.9±0.3
Braeburn	58.9±nd	8.3±nd	0.5±nd	6.4±nd	2.3±nd
Fuji	52.2±16.9	4.8±2.0	0.4±0.1	13.4±4.6	2.0±0.4

Four apple varieties, Rome Beauty, Idared, Cortland, and Golden Delicious, were analyzed for flesh, peel and flesh + peel phenolic content, composition and antioxidant

activity (Wolfe et al. 2003). In all of the varieties studied, the total phenolic and flavonoid contents and antioxidant activity were highest in the peel, followed by flesh + peel, and flesh ($p<0.05$). In the same study, the apple peel extract of all varieties were shown to more effectively inhibit the *in vitro* growth of HepG2 human liver cancer cells than either flesh or flesh + peel extracts. The IC_{50} values, depending on apple variety, ranged from 12.4±0.4 – 20.2±0.7mg/mL for apple peel compared to 26.5±0.3-125.1±58.8 and 103.9±16.5- 155.3±11.7mg/mL for flesh + peel and flesh, respectively. The antioxidant and anti-proliferative activities were also highly correlated for flesh + peel extracts ($R^2 = 0.94$, $p<0.05$), illustrating that the antioxidant activity or polyphenol content is likely responsible for the apples' anti-cancer properties *in vitro* (Wolfe et al. 2003).

Leontowicz et al (2003) also found significantly higher total PP and flavonoid content, as well as FR scavenging activity in the extract of apple and pear peels compared to their flesh counterparts ($p<0.05$). A 5mg/mL dose of apple peel extract quenched 98.0% of DPPH radical within 10min, while the same concentration of apple flesh extract only quenched 54.4% (Leontowicz et al. 2003). This study confirmed high correlation between the total polyphenol content of the extracts and their FR scavenging ability (R^2=0.9207, 0.9350, and 0.9453; three different assays). Leontowicz et al found potent *in vivo* antioxidant activity of an apple and pear extract-supplemented to a high cholesterol diet (10%) fed to rats, compared to control rats (1% cholesterol). Apple and pear peels and to a lesser extent, their flesh, were shown to hinder the increase in plasma lipids and liver cholesterol in rats fed a high cholesterol diet. Apple peel had the most biological

activity, lowering lipid levels by: 21.6-35.5% compared to control rats. The plasma antioxidant activity (determined *in vitro* by its ferric-reducing power) of apple and pear peel-fed rats was also significantly less negatively affected by high cholesterol intake when compared to the control group ($p<0.05$), with apple peel being more protective than pear ($p<0.05$). These beneficial effects of apple and pear extracts *in vivo* are largely attributed to their free radical scavenging abilities (Aprikian et al. 2002; Leontowicz et al. 2003).

With regard to effects on human CRC, Eberhardt et al, (2000) tested the anti-proliferative properties of extract from apples with or without skin on human Caco-2 cells. At 50mg/mL of apple extract for 96hr, inhibition of proliferation was 43±1% and 29±4% for apples with skin and without skin, respectively (Eberhardt et al. 2000). Gathering the above evidence, it seems that the antioxidant activity (*in vitro* and *in vivo*), polyphenol content, and *in vitro* anti-proliferative effects of apples is dependent on the part of the apple used (flesh or peel).

Given the health-promoting properties of apple peel and flesh, apple pomace (AP), a co-product of the apple juice and cider industry, is unnecessarily wasted. AP is heavily produced in Canada (113 840-136 610 tons in 2009) and contains skin/flesh (95%), seeds (2-4%) and stems (1%), with an overall water content of 70-75%. Its high moisture content makes affordable transportation difficult and supplies a breeding ground for human disease vectors and bacteria that produce greenhouse gas (ex. methane) and noxious smells through fermentation. Most of this product is presently composted or

deposited into landfills, contributing to greenhouse gas emissions and groundwater pollution. Only a small percentage of AP produced is used as animal feed or fuel for boilers (Dhillon et al. 2013).

AP contains an abundance of the same PP found in apples, with the total concentration widely ranging from 2410-7240 mg/kg DW (Lu and Foo 1997; Schieber et al. 2003). The PP which have previously been isolated from AP are shown in Table *2.2*. Its total polyphenol content, which is largely assayed by the Folin-Ciocalteu reagent and expressed in gallic acid equivalents (GAE) in literature, ranges from 578-1612mg GAE/100g DW (Lu and Yeap Foo 2000; Soares et al. 2008; Wijngaard et al. 2009; Wijngaard and Brunton 2010; Dhillon et al. 2013). The PP content (TPC) and antioxidant activity (AA) of AP depends on the starting ingredient's (the apple) TPC, as well as methods used for processing them into juice or cider. The quantities of select PP found in AP by three different studies are summarized in Table *2.3*.

Table 2.2 Polyphenols previously isolated from apple pomace. Adapted from Lu and Foo, 1997, 2000; Schieber et al., 2002, 2003; Sanchez-Rabaneda et al., 2004; Guyot et al., 2007; Cetkovic et al., 2008; Bhushan et al., 2008; Suarez et al., 2010, and Rana et al., 2014.

Apple pomace polyphenols	
Catechin	Quercetin-3-rhamnoside
Epicatechin	Quercetin-3-arabinopyranoside
p-Coumaric acid	Quercetin-3-arabinofuranoside
p-Coumaroylquinic acid	Quercetin-3-pentoside
Caffeic acid-glucoside	Sinapic acid-glucoside
Chlorogenic acid	Apigenin
Caffeoylquinic acid	Chrysoeriol
Cyanidin-3-glucoside	Eriodictyol-hexoside
Dicaffeoylquinic acid	Eriodictyol
Ferulic acid	Hesperidin-pentoside
3-Hydroxyphloridzin	Luteolin
Kaempferol-glucoside	Luteolin-7-glucoside
Procyanidin B2	Luteolin-7-galactoside
Phloridzin	Naringenin
Phloretin	Naringenin-7-rutinoside
Phloretin xyloglucoside	Naringenin-hexoside
Quercetin	Naringenin-glucuronide
Quercetin-3-diglucoside	Naringenin-7-neohesperidoside
Cyanidin-3-glucoside	Naringenin-7-glucoside
Quercetin-3-rutinoside	Protocatechuic acid
Quercetin-3-galactoside	Quercetin-pento-hexoside
Quercetin-3-glucoside	Rhamnetin
Quercetin-3-xylanoside	Rhamnetin-3-glucoside
Quercetin-hexoside	Salicylic acid

Table 2.3. Apple pomace polyphenol quantities determined in other studies by solvent extraction (Lu and Foo 1997; Suárez et al. 2010; Rana et al. 2014).
nd – not detected; na – not analyzed; * total of epicatechin, caffeic acid, Q-glucoside and chlorogenic acid; A – acetone; M – methanol; E - ethanol

	Lu & Foo, 1997	Suarez et al., 2010		Rana et al., 2014		
Drying method	Freeze	Oven		Oven		
Solvent Used	A	A	M	A	E	M
Phenol			*mg/kg DM*			
Catechin	na	nd	nd	na	na	na
Epicatechin	640	88	88	*	*	*
Caffeic acid	280	22	20	*	*	*
3-hydroxyphloridzin	270	na	na	na	na	na
Chlorogenic acid	na	166	171	*	*	*
Phloretin-2'-xyloglucoside	170	171	170	na	na	na
Phloretin	na	na	na	1100	1200	900
Phloridzin (phloretin glucoside)	1420	380	362	956	820	620
Quercetin-3-galactoside	1610	254	213	na	na	na
Procyanidin B2	na	75	66	na	na	na
Quercetin-3-glucoside	870	123	103	*	*	*
Quercetin-3-xyloside	530	74	60	na	na	na
Quercetin-3-arabinoside	980	185	147	na	na	na
Quercetin-3-rhamnoside	470	132	106	na	na	na
Quercetin	na	na	na	4000	3600	1400
Coumaric acid	na	na	na	*	*	*
Protocatechuic acid	na	134	118	na	na	na
*Others	na	na	na	31	21	nd

In whole apple tissue, cell walls, PP, and polyphenoloxidase (PPO), an enzyme which oxidizes PP, are separately stored. During processing, cells are ruptured, causing these elements to come into contact and forming AP. PPO starts oxidizing PP, while PP, especially polymerized procyanidins, associate with cell wall constituents (polysaccharides) (Renard et al. 2001). Renard et al (2001) noted that apple varieties rich in highly polymerized procyanidins give rise to juice low in PP content, meaning that most of the procyanidins remain associated with cell wall material in the pomace (Candrawinata et al. 2013). After processing apple cortexes for juice, most of the antioxidants were found to remain in the AP (58.3±3.2 %) (Guyot et al. 2003). However, differences in production methods, such as the addition of pectolytic enzymes to pulp to increase pressability (pulp enzyming), may have a negative effect on PP retention in AP compared to straight pressing of apples. In apple juice produced by pulp enzyming, over 80% of the quercetin glycosides (QG) present in apples remained in the AP (Van Der Sluis et al. 1997), while straight pressing resulted in 93% AP retention of QG (Van der Sluis et al. 2002). Further proof of high PP retention (especially QG) in AP comes from results obtained by Van der Sluis et al (2004), where the PP content of apple juice was improved by the addition of an alcoholic extraction of PP from pomace. The AP extract was added to the juice, resulting in a 1.4 (chlorogenic acid) to 9-fold (QG) increase in PP content and 5-fold increase in antioxidant activity compared to conventional production (Van Der Sluis et al. 2004).

Considering the significant sequestration of original apple PP in apple pomace, it seems that AP is a valuable, but wasted co-product worldwide. Recent research

supporting the health-promoting properties of apple-derived PP as well as consumer interest in foods that provide health benefits demand that AP be meaningfully utilized. The potential of phenolic transfer from feed to milk in the dairy cow presents a route for developing naturally-enhanced milk with antioxidant benefits by feed inclusion of AP (Soberon et al. 2012; Santos et al. 2014).

2.6 Flavonoid effects on cancer

Exposure to carcinogens or ROS causes DNA damage, which can transform a normal cell to an initiated one or aid in the full development of metastatic cancer from already transformed cells. Blocking agents, such as endogenous antioxidant enzymes and exogenous PP, can block initiation in normal cells and prevent further DNA damage in transformed cells by scavenging ROS (Manson 2003). In addition to their antioxidant properties, flavonoids have particularly been shown to modulate several regulators of pathways that are often altered in cancer. A few of these factors include: protein kinases, epidermal growth factor receptors (EGFRs), platelet derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs) and cyclin-dependent kinases (CDKs) (Singh and Agarwal 2006). For this reason, flavonoids have become a popular topic in anti-cancer drug research.

Flavanols, such as epigallocatechin gallate (EGCG) and (-)-epicatechin-3-gallate (ECG), can, in addition to scavenging ROS themselves, also influence antioxidant enzyme-ROS scavenging and carcinogen metabolism through induction of antioxidant response element (ARE) found in the promoter region of several drug metabolizing and

antioxidant enzymes (Kong et al. 2001). By activating the upstream MAPK pathways (ERK, JNK, p38), these PP induce binding of transcription factor, Nrf2, to ARE and the transcription of its downstream antioxidant enzyme genes (Chen et al. 2000). Mice with a disruption in the Nrf2 gene are deficient in the induction of antioxidant enzymes by phenolic antioxidants and synthetic chemopreventatives (McMahon et al. 2001). Additionally, apple proanthocyanidins, largely polymers composed of (-)-epicatechin, were shown to inhibit cell growth and induce apoptosis by arresting SW620 (CRC) cells in the G_2/M phase of the cell cycle and activating their caspase-3, respectively (Gosse et al. 2005). Six-week ingestion of the apple procyanidin fraction (equivalent to two apples per day in humans) reduced the total number of premalignant hyperproliferative crypts in rat models of human CRC carcinogenesis by 50% (Gosse et al. 2005).

In most studies, flavonoids have also been shown to selectively inhibit cell proliferation and its associated tumorigenic factors in cancer cells but not normal ones. EGCG inhibited activation of PI3K, NF-κβ and AP-1 in Ras-mutated mouse epidermal cells (Chung et al. 1999; Nomura et al. 2001) and selectively inhibited cell proliferation (at 10-100μM) in human epidermoid carcinoma cells but not in normal epidermal keratinocytes. In the same study, 10-80μM of EGCG caused G0/G1 phase arrest in the cell cycle in the cancer cells only. Inhibited NF-κβ expression, however, was seen at doses of ≥20μM and ≥40μM in cancer and normal cells, respectively (Ahmad et al. 2000).

Quercetin, a flavonol with powerful antioxidant properties abundant in apple skin and AP, has been extensively studied as an anti-cancer agent (Sak 2013). *In vitro,* it has

shown to inhibit growth of tumor cells (Van Der Woude et al. 2003; Kim et al. 2005; Kim et al. 2010), can cause undifferentiated cancer cells to differentiate (van Erk et al. 2005), and is not cytotoxic (assessed up to 150μM)(Kuntz et al. 1999). In a few studies, Q induced a biphasic response on CRC cell proliferation, moderately inhibiting and occasionally increasing it at low concentrations (20-40μM, 0.5-5μM), but significantly decreasing it at high concentrations (>80 μM, >10μM)(Van Der Woude et al. 2003; van Erk et al. 2005). The differences in these concentrations are most likely attributable to the addition of ascorbic acid to stabilize Q medium in the study by van der Woude et al, which may decrease its antioxidant and proapoptotic effect (Olson et al. 2008). Stabilization of Q by ascorbic acid in Dulbecco's Modified Eagle's Medium (DMEM) reduced the accumulation of Q products to which a part of its apoptotic activity is thought to be attributed to (Olson et al. 2008). Q's biphasic effect on cell growth may also be explained by its concentration-dependent balance between antioxidant and prooxidant activity *in vitro,* as well as by regulatory over-correction by synthetic control systems to low levels of a growth-inhibiting challenge. Additionally, it is thought that Q may target different phases of the cell cycle depending on its concentration, by blocking S phase and G_1 at low and high concentrations, respectively (Van Der Woude et al. 2003; Araujo et al. 2011).

In human CRC cell lines, HCT15 and CO115 harboring KRAS and BRAF mutations, respectively, Q and its analogue, luteolin, displayed significant antiproliferative and pro-apoptotic effects in a dose-dependent manner (Xavier et al. 2009). After 48hr of exposure, the antiproliferative effect was seen with 20μM and 5-

15μM of quercetin in HCT15 and CO115 cells, respectively (p<0.001-0.05). Pro-apoptotic effects were seen at 48hr with 10-20μM and 12-15μM of Q in HCT15 and CO115 cells, respectively (p<0.001). Both compounds significantly decreased phosphorylation of ERK in HCT15 cells (15μM of luteolin, p<0.001, and 20μM of quercetin, p<0.05) and PKB/Akt in CO115 cells (15μM of Q and 12μM of luteolin, p<0.001). The BRAF mutation in CO115 cells overrode the compounds' inhibition of KRAS on the ERK MAPK, but not on the PI3K pathway (Xavier et al. 2009). In another study by Kim et al (2005), quercetin decreased the number of viable HT-29 cells dose-dependently, with an 81±3% decrease after incubation with 100μM for 96hr. Cell proliferation was significantly decreased at 50-100μM (48hr) and 25-200μM (96hr). DNA oligonucleosomal laddering, a hallmark of apoptosis was seen after 72hr incubation with 50μM Q. Pro-apoptotic Bax levels were unchanged by treatment, while its anti-apoptotic inhibitor Bcl-2 was decreased significantly, along with active phosphorylated Akt/PKB. Additionally, 24hr treatment of 50μM Q significantly decreased the tyrosine residue phosphorylation and activation of EGFR/Erb B type I receptor kinases. EGFR is a known activator of Akt/PKB, a key mediator of cell survival and suppression of apoptosis in a variety of cell types. The inhibition of this pathway, which is often upregulated in CRC, seems to be involved in Q's anti-CRC effects *in vitro*, through its inactivation of EGFR. In another study by Kim et al (2010), ≥50μM and 100μM of quercetin significantly (p<0.05) decreased cell viability after 48 and 24hr, respectively (Kim et al. 2010). Significant chromatin condensation, indicative of apoptosis, was seen after 48hr exposure to 100μM quercetin, while flow cytometry revealed that ≥50μM for 24hr induced cell cycle arrest in the G_1 phase. Additionally, after 24hr exposure to

100μM Q, levels of Bax, CDK inhibitors (p53 and p21), and phosphorylated levels of serine-threonine kinase AMP-activated protein kinase (AMPK) increased, while Bcl-2 decreased. AMPK activation influences apoptosis in several cancer types by signaling pathways such as COX-2, Akt/PKB, and mTOR. The results of AMPK activation include: the activation of caspases, inhibition of molecules related to growth and proliferation, and up-regulation of CDK inhibitors p53 and p21, which cause cell cycle arrest in the G1 phase (Manson 2003; Kim et al. 2010). A summary of PP effects on several signaling cascades involved in carcinogenesis is shown in Figure *2.4*.

The *in vitro* properties of flavonoids have also been documented *in vivo.* In nude mice with subcutaneous tumours developed from transplanted HT-29 cells, Q significantly decreased tumour volume after a 6-week treatment period. In mice receiving daily treatment of 50 or 100mg Q/kg body mass, tumour volume was reduced by 35 and 45%, respectively, when compared to the control group (0 mg Q)($p<0.05$) (Kim et al. 2010). Several other studies have confirmed anti-CRC properties of flavonoids in rat models of carcinogenesis (Li et al. 2000; Fukushima et al. 2001; Kohno et al. 2002; Tao et al. 2002). Epidemiological evidence of anti-cancer properties of flavonoids, on the other hand, is not as convincing, as numerous studies have provided protective evidence against all(Knekt et al. 1997), lung(Stefani et al. 1999; Feskanich et al. 2000; Arts et al. 2001), and GIT cancers(Garcia-Closas et al. 1999), while others have found no association (Goldbohm et al. 1995; Hertog et al. 1995).

Although significant evidence of anti-carcinogenic properties of flavonoids exists

in cell culture studies and animal models of carcinogenesis, there is a need for carefully executed human clinical trials to better understand their health effects in humans *in vivo.*

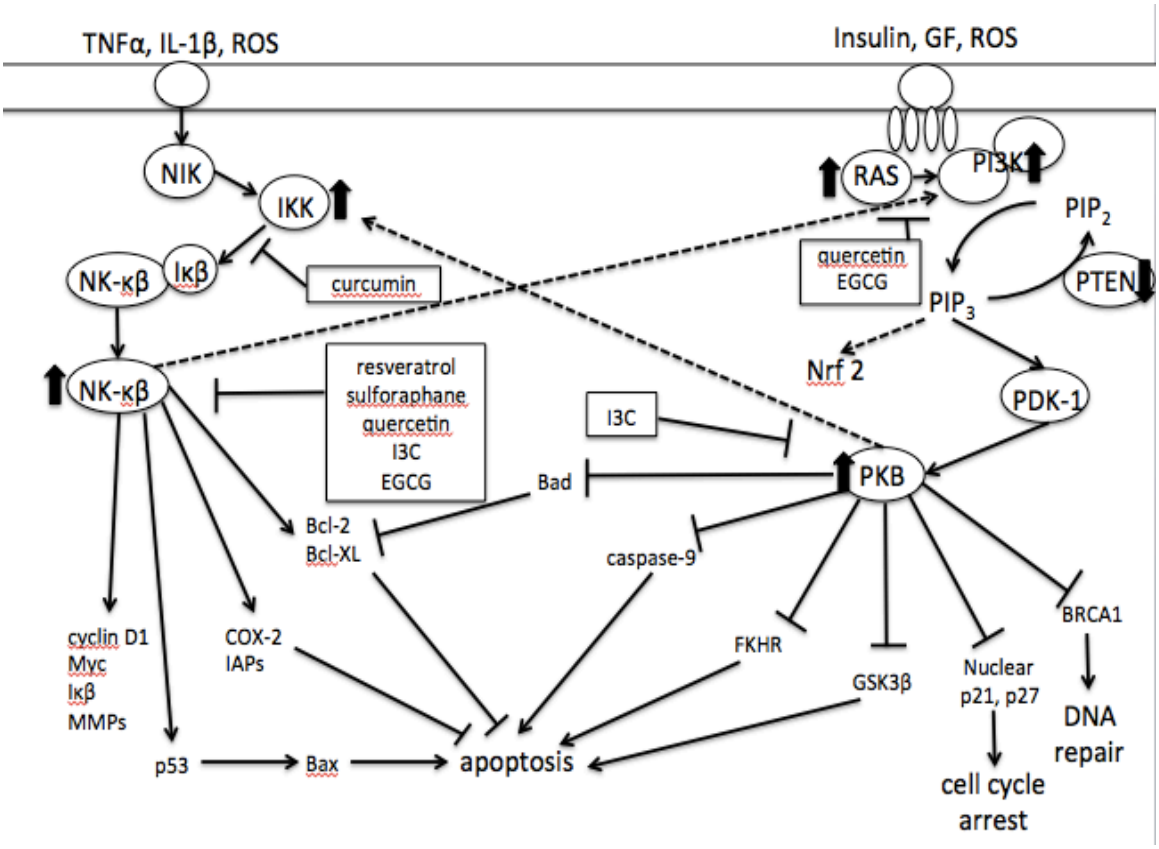

Figure 2.4 Communication between several signaling cascades involved in regulation of cell proliferation, apoptosis, differentiation and metastasis and intervention by common anti-cancer PP. TNFα – tumour necrosis factor α; IL-1β – interleukin 1β; ROS – radical oxygen species; NIK – NF-κβ inducing kinase; IKK - Iκβ kinase; NF-κβ – nuclear factor κβ; MMPs – matrix metalloproteases; COX-1 – cyclooxygenase-1; IAPs – inhibitors of apoptosis; Bcl– B cell lymphoma; JNK – c-Jun N-terminal kinases; TF – transcription factor; PKC – protein kinase C; Bad – Bcl-2 associated death promoter; BRCA1 – breast cancer 1; FKHR – forkhead transcription factor; GSK3β – glycogen synthase kinase 3β; GF – growth factor; PI3K - phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP_2 - phosphatidylinositol (4,5)-bisphosphate; PIP_3 - phosphatidylinositol (3,4,5)-trisphosphate; PDK-1 - 3-phosphoinositide dependent protein kinase-1; PKB/Akt – protein kinase B; PTEN – phosphatase and tensin homolog; I3C – indole-3-carbinol; EGCG – epigallocatechin gallate;
↑/↓- overexpressed/under-expressed or defective in cancer cells, respectively. Adapted from Manson, 2003.

2.7 Flavonoid metabolism and bioavailability

The absorption, metabolism and bioavailability of polyphenols is influenced by their structure and co-consumed foods, as well as the anatomical, physiological and biochemical characteristics of the species and individual consuming them (Wiczkowski et al. 2008). Contradictory evidence on anti-cancer properties of PP between *in vitro* and animal, and epidemiological studies may be due to their low absorption in the gastrointestinal tract, extensive biotransformation within the gut, and largely undetermined accumulation in target tissues *in vivo* (Del Rio et al. 2013).

Due to their low bioavailability, research has focused on targeted delivery of bioactive compounds, such as flavonoids, through methods such as encapsulation in food-derived biopolymers (Livney 2010; Wang et al. 2013; Kimpel and Schmitt 2015; Vieira da Silva et al. 2016). Milk proteins with amphiphillic structure, such as caseins and whey, represent natural vehicles for bioactive delivery as they evolved to deliver essential micronutrients (Ca and PO_4), building blocks (amino acids), and immune system components (immunoglobulins) from mother to the newborn. Their wide availability, high nutrient content, generally regarded as safe (GRAS) status, and desirable sensory properties have driven the food and drug industry's use of them as naturally-derived delivery-systems for bioactive compounds and drugs (Livney 2010). In turn, flavonoids, due to their covalent (protein amine/amide groups and quinones formed from flavonoid oxidation) and non-covalent (hydrogen bonding and hydrophobic interactions) binding with proteins, are suitable bioactives for protein-facilitated delivery (Bordenave et al.

2014). BSA, a whey protein found in milk, represents a colon-specific vehicle, as nanospheres composed of it were found to specifically release the encapsulated drug in *in vitro* silumated intestinal fluid (pH 7.6) (Luppi et al. 2008). The barrier formed by milk proteins complexed with apple PP has been shown to provide physical stability during thermal processing and allow PP to retain their antioxidant activity (Wegrzyn et al. 2008). Fang et al, (2011) found that Q aglycone was successfully bound to BSA nanoparticles by hydrophobic and hydrogen binding within the BSA core, which did not have a significant effect on its antioxidant activity (Fang et al. 2011). Curcumin, the active anti-cancer constituent of turmeric (*Curcuma longa*), complexed with casein micelles, actually exhibited higher anti-proliferative effects on human cervical cancer (HeLa) cells (Sahu et al. 2008), while EGCG retained majority its anti-CRC activity present in free EGCG *in vitro* (Haratifar et al. 2014).

Evidence of retained antioxidant and anti-cancer activity of PP complexed with milk proteins, as well as that of PP transfer from diet to milk in lactating cows and goats, may therefore allow for development of a natural targeted delivery system for PP through diet inclusion of high-PP ingredients, such as AP, to lactating dairy cows. In order to accomplish this, the possible effects of cow digestion, absorption and metabolism on the activity of AP PP must be elucidated.

Flavonoids are generally regarded as xenobiotics in the body and their transport rates across the intestinal membrane are therefore highly correlated with their degree of hydrophobicity. However, with the exception of catechins, most flavonoids in botanic

sources exist as glycosides that are too hydrophilic to passively diffuse across the intestinal membrane in their native form (Prasain and Barnes 2007). Flavonoid metabolism has only been limitedly explored in ruminants (Berger et al. 2012; Gohlke et al. 2013; Berger et al. 2015) but has been widely studied *in vitro* and in numerous animal and human models (Hollman and Katan 1997; Hollman et al. 1997; Day et al. 2003; Lesser et al. 2004; Wein and Wolffram 2013; Wiczkowski et al. 2014). After ingestion in monogastrics, the mastication, and churning of the oral cavity and stomach, respectively, free flavonoids from the food matrix. β-glucosidase produced by the bacteria in the oral cavity cleaves glycosidic bonds of some flavonoid monoglycosides at this time, but majority of this action occurs in the small intestine (Velderrain-Rodríguez et al. 2014). Absorption of some phenolic acids may occur in the stomach, but flavonol glycosides (FG) and anthocyanidins remain stable during *in vitro* gastric digestion and reach the small intestine intact (Bermudez-Soto et al. 2007). Aglycones, that possibly survive gastric digestion and make it to the duodenum, can be absorbed through passive diffusion, due to their small structure and hydrophobic nature. FG, on the other hand, are absorbed through two mechanisms. First, lactase-phlorizin hydrolase (LPH), a brush border enzyme, cleaves the sugar moiety, freeing the hydrophobic aglycone which then crosses the cell membrane via passive diffusion. Second, some glycosides seem to be transported across the cell membrane via sodium-dependent glucose transporter (SGLT1), after which their sugar moiety is cleaved by cytosolic β-glucosidase. A large portion of ingested polymeric flavonoids, such proanthocyanidins (polymers of flavanols) are not absorbed in the small intestine and reach the colon intact where they are broken down into aglycones, phenolic acids and other non-phenolic products by microbial

enzymatic cleavage and oxidation (Ou and Gu 2014). These products can then either be absorbed into systemic circulation, subject to conjugative metabolism, exert their bioactions on colonic cells, or be excreted out in the feces (Velderrain-Rodríguez et al. 2014). Majority of these actions are also assumed to take place in the ruminant GIT, but the rumen environment must be taken into consideration, as it may affect the delivery of certain PP into the latter part of the GIT.

Rumen effects on PP have been limitedly explored, mainly *in vitro*. Berger et al (2015) assessed the *in vitro* degradation of Q (aglycone) supplemented at 0 or 100 μmol/L Q equivalents/L to a concentrate and grass hay (50:50) substrate and found that majority of Q disappearance was attributed to microbial activity, rather than spontaneous decomposition. The authors concluded this as Q showed a fast exponential decrease within the first 8hr of incubation only in the presence of an active rumen inoculum. Along with the fast disappearance of Q, concentration of its metabolites, 3,4-dihydroxyphenylacetic acid (3,4-DHAA) and 4-methylcatechol (4-MC) rapidly increased to maximum values within 8hr. These findings are in agreement with other *in vitro* batch culture studies involving rumen and monogastric colonic microflora (Cheng et al. 1969; Rechner et al. 2004; Cermak and Breves 2006; Lu et al. 2013; Ulbrich et al. 2015).

As for rumen digestion of flavonoids containing sugar moieties, conclusions must be drawn from *in vitro* studies of human fecal slurries. The sugar moiety and the type of glycosidic binding seem to affect microbial degradation of these PP, as β-glycosidic, β-rhamnosidic and ring fission activities differ between species. Species, such as

Bacteroids sp., *Eubacterium* sp., *Enterococcus* sp., are known to possess high β-glucosidase and β-rhamnosidase activities, which allows them to cleave flavonoid glycosides, such as QG, releasing Q aglycone (Ulbrich et al. 2015). Absorption of the aglycone then likely takes place through passive diffusion. Unabsorbed aglycones, can on the other hand, be further degraded by C-ring fission into products such as phenolic acids and phloroglucinol (PG). Absorption or further degradation into butyrate and CO_2 may then occur (Ou and Gu 2014).

Before passage to the bloodstream, absorbed aglycones undergo a degree of metabolism in the intestinal endothelium, forming sulfate, glucuronide, and/or methylated metabolites through action of sulfotranferases (SULTs), uridine-5'-diphosphate glucuronosyltranferases (UGT), and catechol-*O*-methyltransferases (COMTs), respectively (Del Rio et al. 2013). Aglycones produced in the colon, however, only undergo methylation and glucuronidation, suggesting that sulfation in the small intestine is performed by the SULTs in its wall and not the colon or the liver (Mullen et al. 2006; Lu et al. 2010). The relative proportions of conjugate structure in plasma seems to be species-specific (Berger et al. 2012). Efflux of some metabolites into the small intestinal lumen (and therefore feces) may occur and is largely due to the action of members of the adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein (Del Rio et al. 2013).

Once the metabolites of the absorbed PP reach the liver, they are subject to further conjugative metabolism. Some metabolites formed may be recycled back to the small

intestine through bile excretion and enterohepatic recirculation, later being excreted though the feces, while others may enter systemic circulation to peripheral tissues. The route of excretion depends on metabolite structure and chemistry, as large, extensively conjugated metabolites are more likely to be eliminated in the bile and feces, while small conjugates, such as monosulfates, are preferentially excreted in the urine (Manach 2004).

The physiological effects of flavonoids are dependent on sufficient bioavailability, defined as the ratio of an orally administered substances and the amount which is absorbed and available for physiologic activity or storage (D'Andrea 2015). Studies of bioavailabiity in humans have shown that concentrations of intact flavonoids in plasma rarely exceed 1uM regardless of the amount ingested, with the peak concentrations occurring 1-2 hours after ingestion (Scalbert and Williamson 2000). Plasma levels rapidly decrease after small intestinal absorption due to an elimination half-life of 1-2 hours. In ruminants, the bioavailability of Q aglycone was shown to be about 10 times lower. In a study determining bioavailability of Q aglycone and its glucorhamnoside rutin in non-lactating cows, 0, 10 or 50mg quercetin/kg of BW was administered intraruminally (Berger et al. 2012). Administration of 50mg/kg BW resulted in peak plasma levels of total flavonols (aglycone and conjugated metabolites) of 0.09 and 1.0uM at 30.0 and 42.0min post-infusion, for quercetin and rutin, respectively. The short time required to reach peak plasma concentrations indicates that rumen absorption of flavonoids may occur. Regardless of the dose applied (10 or 50mg/kg BW), the plasma concentrations of quercetin and its metabolites were more than 10-fold higher after administration of rutin than quercetin aglycone, with those of rutin being

comparable to human plasma Q levels. The relative bioavailability of total flavonols from rutin compared to quercetin was 767.3%. This finding is in contrast to results obtained in monograstric species, where bioavailability of the two is reversed. The authors concluded this was likely due to rapid and extensive degradation of Q aglycone and protection of Q from microbial degradation in its glucorhamnoside form in the rumen. Additionally, Q aglycone and rutin are known to be absorbed in the small and large intestine, respectively, in monogastrics, compared to much earlier absorption in ruminants (Berger et al. 2012).

Significant accumulation in peripheral tissues is not well elucidated in ruminants, but numerous studies have found conjugated metabolites of PP in brain, endothelial cells, heart, kidney, liver, pancreas, prostate, uterus, ovary, mammary glands, testes, bladder, bone, and skin tissues in humans, pigs and rodents (Manach 2004; Talavéra et al. 2004; de Boer et al. 2005; Gladine et al. 2007). Although low serum levels have been noted in dairy cows (Borsy 2011; Berger et al. 2012), evidence of PP transfer from diet to milk in dairy cows and goats has been described (Jordan et al. 2010; Soberon et al. 2012). Since their mechanism of tissue uptake is not well understood, and some studies have shown that plasma concentrations are not necessarily correlated with concentrations in peripheral tissues, these serum levels may not necessarily be a good measure of tissue accumulation. For example, equol concentrations (main isoflavone metabolite) in women ingesting isoflavones were found to be higher in breast tissue than serum (Maubach et al. 2003). With this in mind, it is important to note that evidence exists that conjugates produced by metabolism still retain about half of their original antioxidant activity

(Manach et al. 1998) and posses many biological properties, *ex vivo* and *in vitro,* originally described for their parent compounds (Williamson et al. 2005). It is therefore possible that metabolites secreted in the milk of dairy cows consuming a high-PP diet may be effectively delivered into target sites in humans consuming the milk to provide the observed health benefits.

2.8 Conclusion

Consumer interest in foods with health-protective properties has grown drastically in recent years due to the rise of degenerative diseases, such as cancer. Awareness of the beneficial properties of polyphenols, which have shown promise as anti-cancer agents by modulating cell communication pathways involved in carcinogenesis, has grown in the general public. Due to the abundance of polyphenols that are active against a variety of cancer cells and animal models of cancer in apples, apple pomace, a largely wasted co-product of the apple juice and cider industry, could be used as an environmentally-beneficial source of anti-cancer agents.

In order to increase their generally low bioavailability in humans, the potential exists for development of a naturally-derived protein-protected source of polyphenols for human consumption through the inclusion of apple pomace in the lactating dairy cow diet. Observed polyphenol transfer to milk of lactating dairy cows and goats may therefore mean that milk proteins fulfill their natural purpose as nutrient-delivery systems, even with metabolites of ingested polyphenols. In an effort to evaluate the

feasibility of producing a naturally-derived polyphenol-enriched milk/milk product, the effect of *in vitro* rumen digestion on the degradation of common apple pomace polyphenols from Ontario AP was determined in this study. Additionally, anti-proliferative effect of the aglycone of the most abundant polyphenol in AP, quercetin-3-glucoside, on human colorectal cancer cells was evaluated *in vitro.*

CHAPTER THREE

RECOVERY RATES AND ANTIOXIDANT ACTIVITY OF APPLE POMACE POLYPHENOLS AFTER *IN VITRO* RUMEN INCUBATION

3.1 Abstract

Apple juice and cider production results in the generation of a large amount of apple pomace (AP). This co-product is a rich source of bioactive phenolic compounds that could be used in the production of value-added products. The phenolic content of methanolic extract of cider apple pomace and its digesta after 24hr incubation in an *in vitro* rumen incubator was evaluated in this study. The total of the 8 phenols analyzed was 1562 ± 355.2 and 5.58 ± 0.85 mg/kg DM for non-digested AP and digested samples, respectively. The individual content of phenolics found in non-digested AP on a dry matter basis per kg was: quercetin-3-glucoside (1283 ± 196.6 mg/kg), quercetin-3-galactoside (216.1 ± 167.90mg/kg), epicatechin (5.43 ± 2.52 mg/kg), phloridzin (52.95 ± 24.70mg/kg), and quercetin (3.99 ± 2.66 mg/kg). The digested sample's individual phenolic contents were: quercetin-3-glucoside (2.32 ± 0.58 mg/kg), quercetin-3-galactoside (1.33 ± 0.06mg/kg), epicatechin (0.48 ± 0.03 mg/kg), coumaric acid (0.29 ± 0.21mg/kg), and phloridzin (1.25 ± 0.35 mg/kg). Recovery rates, expressed as percent of unchanged phenol recovered in the digesta compared to the non-digested AP, were: quercetin-3-glucoside (0.18 ± 0.02%), quercetin-3-galactoside (0.87 ± 0.52%), epicatechin (9.90 ± 3.43%), and phloridzin (2.72 ± 1.30%).

3.2 Introduction

The potential health effects of plant secondary metabolites are an increasingly popular topic in today's scientific literature. This is largely due to increased understanding of the relationship between diet and health and the resulting growth in demand for natural products that confer health benefits. Leftover fruit residue from the apple juice industry is an environmental burden but it is also an excellent source of polyphenolic compounds (PP) that may confer health-promoting benefits.

Apple pomace, a co-product of the apple juice and cider industry, is heavily produced in Canada (113 840-136 610 tons in 2009) and contains 70-75% water. On a DM basis, it contains skin/flesh (95%), seeds (2-4%) and stems (1%) (Dhillon et al. 2013). Its high moisture content makes affordable transportation difficult and supplies a breeding ground for human disease vectors and bacteria that produce greenhouse gases (ex. methane) and noxious smells through fermentation. Most of this product is presently composted or deposited into landfills, contributing to greenhouse gas emissions and groundwater pollution, while only a small percentage is used as animal feed or fuel for boilers (Dhillon et al. 2013). It contains a high phenolic content and is therefore capable of being used for the production of value-added products or nutraceuticals for human health.

AP contains an abundance of the same PP found in apples, with the total concentration widely ranging from 2410-7240 mg/kg DW (Lu and Foo 1997; Schieber et al. 2003). Apple skin, and therefore AP, is particularly abundant in flavonoids, a major

class of PP ubiquitously found in plants and known for its powerful antioxidant properties. AP is most abundant in quercetin (Q), its glycosides, and dihydrochalcone glucosides such as phloridzin (Lu and Foo 1997; Schieber et al. 2003; Suárez et al. 2010; Rana et al. 2014). In Gala AP investigated by Lu and Foo (1997), quercetin glycosides, accounted for more than half of the total PP content (4240mg/kg out of 7240mg/kg DW). The two most prominent PP were quercetin-3-galactoside (1610mg/kg DW) and phloridzin (1420mg/kg DW). After quantifying individual QG in AP, Schieber et al (2002) found that the most abundant was Q-3-rhamnoside, followed by Q-3-galactoside>Q-3-arabinofuranoside>Q-3-xyloside>Q-3-glucoside (Schieber et al. 2002). Total QG quantified in this study was 886.7mg/kg DW. On the contrary, results from Rana et al., 2014 reported Q [aglycone] to be the most abundant, followed by phloretin (Rana et al. 2014). The discrepancy between the results of these studies is multifactorial. The differences largely stem from the differences in phenols analyzed, solvents used for extraction, apple variety, as well as drying method.

Given the recent consumer demand for natural foods that confer a health benefit, much research has been devoted to understanding the relationship between dietary phenolics from plants and their effect on chronic conditions, such as cancer and cardiovascular disease. As a result, a wide range of activities has been attributed to PP, including: antioxidant, anticarcinogenic, anti-inflammatory, anti-allergic, anti-diarrheal, antibiotic, anti-ulcer, antilipidemic, antithrombotic, vaso-relaxing, and neuro-protecting (Waddell 1999; Wang et al. 1999; Martínez et al. 2003; Rusak et al. 2005; Lee et al. 2006; Aalinkeel et al. 2008; Touil et al. 2009; Avci et al. 2011). Much of this bioactivity

is attributed to the powerful anti-oxidant properties of PP, as both cancer and CV disease are thought to be mainly the result of oxidative stress and DNA damage (Middleton et al. 2000). The antioxidant property of apple skin has been shown though its radical scavenging ability *in vitro* and ability to increase the antioxidant capacity of plasma *in vivo* in lab rats (Leontowicz et al. 2003; Wolfe et al. 2003). Numerous epidemiological studies have found that consumption of flavonoid-rich foods is associated with a decrease in incidence of certain cancers and cardiovascular disease, although positive evidence is stronger for CV disease (Ross and Kasum 2002; Boyer and Liu 2004; Del Rio et al. 2013). In cell signaling studies, these molecules have been shown to provide protection against cancer and CV disease by stimulating the immune system, scavenging free radicals, modulating the cell cycle, modifying gene expression, and inducing apoptosis (Kuo 1996; Kuntz et al. 1999; Martínez et al. 2003; Wang et al. 2004; Xavier et al. 2009; Yang and Liu 2009; Linsalata et al. 2010; Angst et al. 2013). Due to their health-promoting properties, documented transfer to plasma in ruminants (Borsy 2011; Soberon et al. 2012) and in some cases milk (Crozier et al. 2009; Jordan et al. 2010; Soberon et al. 2012), an interest has sparked in the possible production of naturally enhanced animal products, such as polyphenol-containing milk, which confer a benefit to human health.

With regards to rumen metabolism of PP, ingested and ruminally infused ferulic acid (a phenolic acid) was detected in dairy cow plasma 15min after intake in non-lactating dairy cows (Soberon et al. 2012). Berger et al, (2012) also confirmed ruminal absorption of PP, as Q and its rumen metabolites peaked in plasma only 30 and 42min after ingestion of quercetin and rutin, respectively (Berger et al. 2012). Additionally, milk and plasma

of dairy cows ingesting a diet containing grape pomace had significantly higher antioxidant capacity than those ingesting a non-supplemented diet (Santos et al. 2014). Evidence of transfer of bioactives to the goat mammary gland came from a study which found that PP from a rosemary containing diet fed to does had a transfer rate of 0.1-6.3% in the plasma of suckling kids (Jordan et al. 2010). In dairy cows, ruminally infused ferulic acid (bolus of 150g) appeared in milk at 1.5µg/mL 6hr after ingestion. Although rumen and human fecal slurry inoculums have been shown to rapidly metabolize flavonoids, in some cases, the metabolism actually increased their bioactivity by cleaving sugar residues that would normally not be cleavable by the organism's native enzymes (ex. rhamnose) (Ou and Gu 2014). Additionally, aglycone metabolites, such as phenolic acids produced by ring fission and their conjugated metabolites have been found to possess beneficial properties *in vitro* (Manach et al. 1998; Morand et al. 1998; Williamson et al. 2005; Wiczkowski et al. 2014). Therefore, the possibility exists that PP metabolites secreted in the milk of a dairy cow ingesting a diet high in PP may retain their human-health promoting properties.

In order to examine the effect of the rumen environment on the extent of microbial degradation of AP PP, we examined the recovery rates of 8 commonly found AP PP in rumen digesta after 24hr *in vitro* rumen incubation.

3.3 Materials and Methods

3.3.1 Apple Pomace

Two samples of AP produced by cold-pressing of cider apples (S1A- 25% Royal Gala, 25% Northern Spy, 25% Cortland, and 25% Ambrosia and S2A – 25% Cortland, 25% Empire, 25% Gala, and 25% Ida Red) were collected from a local producer in Norfolk County, Ontario, Canada. The pomace was kept at -20°C and out of direct sunlight until analyzed. Due to availability issues, S1A was used in the determination of total phenol content by Fast Blue BB assay, and S2A was used in the determination of *in vitro* (24 and 48hr) NDFd in an ANKOM incubator. Both samples were analyzed for feed nutritional composition.

Two samples of AP (S1B and S2B) produced from a mixture of Red Delicious, MacIntosh, Empire and Gala apples (% composition unknown) by a cloth hydraulic pressing system was collected from a local producer in Waterloo Region, Ontario, Canada. The pomace was kept at -20°C and out of direct sunlight until analyzed. It was then dried in an industrial drying oven for ~24hr to 6.09% moisture. The dried AP was finely ground using a mortar and pestle and stored out of the light at room temperature until extraction. S2B was used for the polyphenol recovery rate study and both samples were analyzed for nutritional composition.

3.3.2 Chemicals and reagents

All reagents used were HPLC grade. Methanol, 4-Benzoylamino-2,5 diethoxybenzenediazonium chloride hemi(zinc chloride) salt (Fast Blue BB Salt), 3,4,5-Trihydroxybenzoic acid (gallic acid), quercetin, quercetin 3-β-D-glucoside, quercetin-3-β-D-galactoside, chlorogenic acid, (-)-epicatechin, caffeic acid, coumaric acid, phloridzin, and phloretin were all purchased from Sigma (Oakville, Canada).

3.3.3. In vitro fermentation for phenolic recovery

The ANKOM Daisy II Incubator (ANKOM Technology, Macedon, New York, USA) used in this study employs filter bag technology, which encapsulates samples to prevent filtration errors. It allows for determination of *in vitro* true digestibility (IVTD), which is a simulation of rumen digestion. Rumen fluid is collected from high-producing, ruminally-fistulated dairy cows consuming a typical total mixed ration (TMR) diet. Feed samples are then placed in filter bags and incubated under anaerobic conditions with rumen inoculum and buffer in glass jars at 39.5°C for a set time-point, usually 24-48hr.

Samples of dried AP (S2B) were subjected to a 24hr digestion in an ANKOM Daisy Incubator. The digestion was performed as per ANKOM Method 3 by A&L Labs (London, Ontario, Canada) (ANKOM 2005). The ANKOM filter bags were pre-rinsed in acetone for 3-5 minutes and completely air-dried to remove a surfactant that inhibits microbial digestion. Each filter bag was weighed and its weight recorded. 3 samples of AP were labelled S1, S2, and S3, oven dried at 60°C for 24hr and split into halves. One half of each sample was used for digestion and the other was used for UPLC analysis of

the non-digested sample. For the rumen fermentation, 0.25g of oven dried and ground AP was weighed directly into each filter bag. This was repeated 25 times for each jar (4 jars total) for each sample, and the digesta were pooled post-treatment.

Buffer solutions (A&B) were prepared for each jar and sample. For buffer A, 10g/L monopotassium phosphate, 0.5g/L hydrated magnesium sulfate, 0.5g/L sodium chloride, 0.1g/L calcium chloride dihydrate, and 0.5g/L urea (reagent grade) were combined. Buffer B comprised of 15g/L sodium carbonate and 1g/L sodium sulfide nonahydrate. Buffer solutions were warmed to 39°C and combined in a 1:5 ratio (B:A). pH was adjusted to 6.8 and 1600mL of the buffer mixture solution was added to each jar. The digestion jars including the samples and buffer solution were placed into the incubator and allowed to equilibrate temperature for 30min.

Rumen inoculum from two high-producing cows in mid lactation was collected into two 1L warm thermos bottles (39°C). Two fistfuls of the fibrous mat from the rumen were also collected and the inoculums and mat were purged in a blender to assure a representative microbial population for the *in vitro* fermentation. The blended digesta were filtered through four layers of cheesecloth into a 5L flask (pre-heated 39°C). The transfer of inoculum was done under CO_2 purging. 400ml of inoculum was added to each jar containing the buffer solution and samples and the jar was purged with CO_2 for 30 seconds and the lid was secured. The jars were then incubated for 24hr. At completion of incubation, the jars were removed and fluid drained. The bags were rinsed under cold water until the water ran clear. The digested samples were oven dried for 24hr at 60°C

and the contents inside the bags were gently scraped into polypropylene tubes for extract preparation. They were extracted as mentioned below in section *3.3.4.*

3.3.4. Extract Preparation

AP PP were extracted with 80% methanol with a modified method by Singh et al (2014). Fresh AP was laid out to dry in a drying oven at 60°C for ~48hr to ~6% moisture. 24hr digested AP was dried at the same temperature for 24hr to ~3% moisture. A 15mL polypropylene centrifuge tube was filled with finely ground oven dried AP and digested AP and sample weights were recorded. Samples were suspended in 80% methanol as a 1:10 ratio of sample (w:v) and placed in an ultrasonic bath for 1 hour at 25°C. Samples were centrifuged at 2000 rpm for 10 min and the supernatant was carefully collected and filtered through a 0.2µm PVDF syringe filter. Prepared extracts were covered with aluminum foil and stored in the freezer (-20°C) until further analysis.

3.3.5 Nutritional analysis of apple pomace

Wet chemical analysis was performed on all 4 samples by A&L Labs (London, Ontario, Canada) to determine: moisture, dry matter (DM), crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), Ca, Cu, P, K, S, Mg, Zn, Fe, Mn, Na, starch, crude fat (CF), and ash. Nonfibrous carbohydrates (NFC), total digestible nutrients (TDN), net energy for lactation (NEL), net energy for gain (NEG), net energy for maintenance (NEM) were calculated as per Pioneer Hi-Bred International, (1990). Values are reported as mean ± SD of the two sources (Table *2.1*). S2A was additionally subjected to 24 and 48hr *in vitro* digestion in an ANKOM incubator to obtain values for

NDF digestibility (NDFd) 24 and 48hr, as well as lignin, and relative feed value (RFV) by use of ANKOM Method 3.

3.3.6 Determination of total phenolic content by Fast Blue BB Method

Total phenolic content was estimated using a modified Fast Blue BB (FBBB) method developed by Singh et al*,* 2014. Standards of gallic acid were prepared by serial dilution in deionized water (ddH_2O) (0, 31.3, 62.5, 125, 250, 500, 1000µg/mL). 200µL of sample in 80% methanol or gallic acid standard was added to a well of a 96 well flat-bottom microplate in quadruplicate, with one replicate serving as the blank. 20µL of 0.1% Fast Blue BB was added to each standard and sample well and the plate was mixed manually for 30-60 seconds. 20µL of 5% NaOH was then added to each sample and standard well. 5% NaOH was previously determined to be the optimal concentration, resulting in no sample precipitation. 40µL of ddH_2O was added to the blank wells. The plate was covered and left in the dark for 90 min at room temperature. Absorbance was measured at 420 nm using a microplate reader (Synergy H1 hybrid reader, BioTek Inc).

3.3.7 Determination of polyphenolic content by UPLC

Method validation and analysis of sample extracts was developed in Dr. Saxena's lab at the Gosling Research Institute for Plant Preservation (University of Guelph, Guelph, Ontario). Samples of extract (5 µL) were injected onto a Waters Acquity classic ultra-performance liquid chromatography system with single quadropole mass spectrometry detector (Waters QDa) on a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) and separated using a binary gradient in which mobile phase A was 0.1

% formic acid in water (Waters, Canada) and B was 0.1 % formic acid in acetonitrile (LC grade, Fisher Scientific, Canada). A 4.5 min gradient from 95% to 20% A was used to separate components. Flow rate was 0.5 mL/min and column temperature was 30 °C. Analytes were quantified in negative mode (ESI-) using a cone voltage of 15V, probe temperature of 600°, and capillary voltage of 0.8kV. Quercetin, quercetin-3-glucoside, quercetin-3-galactoside, phloridizin, caffeic acid, *p*-coumaric acid, chlorogenic acid, and epicatechin were monitored at m/z of 317, 463, 463, 435, 179, 163, 353 and 289, respectively, and quantified through interpolation of standard curves in GraphPad Prism v.7 (GraphPad Software, San Diego, California, USA).

3.4 Calculations and Statistics

3.4.1 Quantification of total phenolic content by Fast Blue BB

Total polyphenol content was determined using the gallic acid standard curve determined using the linear fit in GraphPad Prism v.7 (R^2> 0.99), and expressed as gallic acid equivalents (mg GAE/100g DW).

3.4.2 Recovery rate of polyphenols via UPLC

Polyphenols in non-digested and digested samples were quantified by interpolation of standard curves in GraphPad Prism, (R^2>0.99). Recovery rate of PP expressed as % recovery, was calculated using equation 1

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% recovery = [digested (mg/kg DW)/non-digested (mg/kg DW)] x 100% *Eq. 1*

3.5 Results and Discussion

3.5.1 Nutritional analysis and in vitro digestibility of apple pomace

The nutritional analysis of AP, expressed as mean ± standard deviation of the two sources, is shown in Table *3.1*. Source A and B only significantly differ in their Fe content (A = 134.1 μg/g DM; B = 34.47 μg/g DM) ($p = 0.018$), but according to the NRC (2001) values for AP, both fall within the range reported (185 ± 190μg/g DW) (NRC 2001). The wide range of values reported for Fe content of AP, in the NRC and in our samples, may be due to high susceptibility of apples to iron chlorosis (deficiency), which is largely due to low Fe availability in calcerous, high pH soils (López-Millán et al. 2001). The rest of the values, averaged over the two sources, coincide well with reported values for AP, mainly with respect to it being a low CP (4.40 ± 0.40 %DW), and a useful soluble carbohydrate source (NFC of 52.6 ± 3.2 %DM) (Table *3.1*). Taking into account the variances of the values determined by NRC (2001) and our study, DM, CP and ash content fall within the NRC range reported (NRC 2001). The EE, NDF, and ADF NRC values, as well as those of Abdollahzadeh (2010) are slightly higher than in our samples.

Table 3.1 Comparison of wet chemical analysis of apple pomace by NRC (2001), Abdollahzadeh et al, 2010, and current study.
A-producer A; B-producer B; SD – standard deviation; nd – not determined
* Micronutrient and additional analysis shown below table; all except Fe are represented as average of A&B ± SD.

Nutrient (units)	NRC (2001)	Abdollahzadeh et al, 2010	A&B
		Mean ± SD	
DM (% as is)	35.9 ± 29.4	30.7 ± nd	20.0 ± 4.0
NE_L (MCal/kg)	1.12 ± nd	nd	1.61 ± 0.01
NE_M (MCal/kg)	1.18 ± nd	nd	1.75 ± 0.01
NE_G (MCal/kg)	0.62 ± nd	nd	1.03 ± 0.01
NFC (%DM)	32.2 ± 16	nd	52.6 ± 3.2
CP (%DM)	7.7 ± 3.8	5.6 ± nd	4.40 ± 0.4
CF (%DM)	5.0 ± 1.9	4.7 ± nd	2.30 ± 0.6
NDF (%DM)	52.5 ± 9.5	45.3 ± nd	31.5 ± 2.8
ADF (%DM)	43.2 ± 6.6	38.0 ± nd	23.8 ± 0.2
Ash (%DM)	2.6 ± 1.1	nd	3.0 ± 2.8

*Ca - 0.12±0.03; P - 0.12±0.02; K- 0.82±0.11; S – 0.05±0.005; Mg – 0.06±0.01; Na - 0.01±0.005 (% DM); Cu – 5.85±0.72; Zn – 8.14±4.99; Mn – 6.68±1.68 (µg/g DW)
*Fe – A = 134.1±3.62, B = 34.47±16.94 (µg/g DW)
*Lignin – 4.41%DM, RFV – 264 (sample S2A)

3.5.2 Polyphenol content in non-digested apple pomace

The total content of the 8 polyphenols quantified by UPLC/MS in the non-digested sample (S2B) was 1562 ± 355.2mg/kg DW. The non-digested AP PP content is below the range reported for total PP in literature (2410-7240mg/kg DW). However, differences exist in the number of PP quantified between studies, so instead, when the total of the 8 PP currently analyzed is calculated for other studies, 1562mg/kg DW falls within the lower end of the new range of 957-4956mg/kg DW (Lu and Foo 1997; Suárez et al. 2010; Rana et al. 2014). Individual polyphenol content ranged between 3.99-1283

in the non-digested sample (Figure *3.1*). The order of abundance for the quantified polyphenols in the non-digested sample was: quercetin-3-glucoside (Q3G) > quercetin-3-galactoside(Q3GL) > phloridzin(P) > epicatechin(E) > quercetin(Q). Caffeic acid (CF), coumaric acid (PCA), and chlorogenic acid (CA) levels were below their lower detection limits (Table *3.2*). This is in agreement with others, who also report either flavonol glycosides (Q3G or Q3GL) (Lu and Foo 1997; Wijngaard and Brunton 2010; Rana et al. 2014) or P (Suárez et al. 2010) as the most abundant PP in AP.

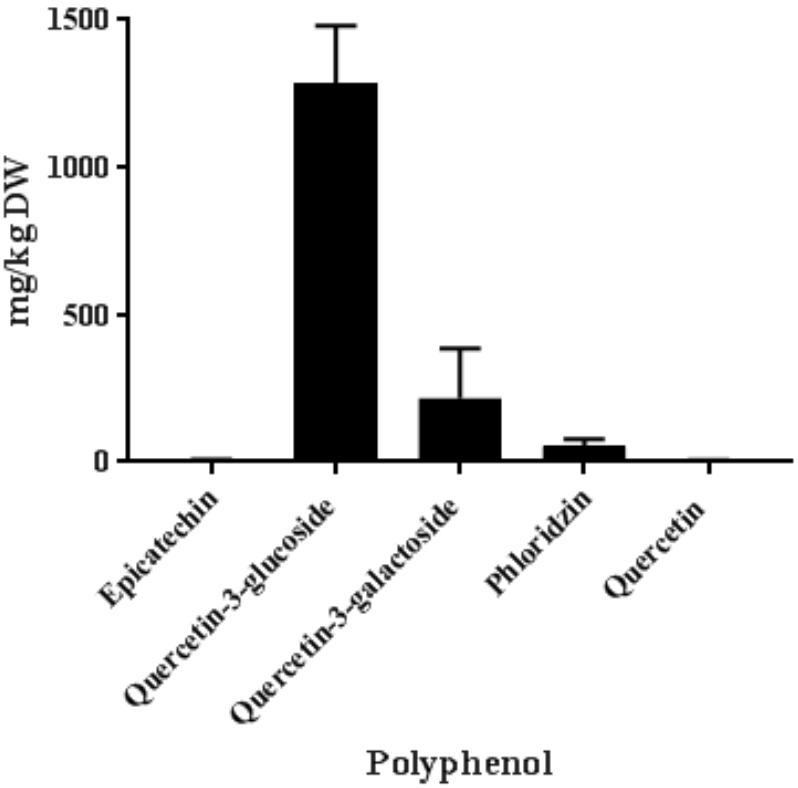

Figure 3.1 Polyphenol content (mg/kg dry weight (DW) of apple pomace (AP)) of non-digested AP determined by UPLC/MS (n=3).

The standardized method used to determine total phenolic content (TPC) of plant material is the Folin-Ciocalteau (FC) assay. The alkaline environment in which the assay is carried out in results in the transfer of electrons from phenolic compounds and other reducing substances, such as ascorbic acid, sugars, sulphites and aromatic amino acids to the reagent which contains phosphomolybdic/ phosphotungstic acid complexes (Singleton et al. 1965). This results in the formation of a blue colour that absorbs light at 750-765nm. Because this method measures the "total phenolics" through the reducing capacity of the plant extracts, it is actually more applicable as a measure of reducing or antioxidant activity. FBBB is a more phenol-specific method developed by Medina et al. (2011) and does not suffer the same fate as FC, as it uses a Fast Blue BB diazonium salt, whose diazonium group specifically couples with reactive hydroxyl groups to form stable azo-complexes which can be measured at 420nm (Medina 2011).

The TPC of the AP (S1A) analyzed by FBBB was 399.6±7.6 mg GAE/100g AP DW. The inter- and intra-day variation, measured as CV%, was <15% and <10%, respectively. The value determined for TPC is also within the lower range of that which is reported in literature as determined by the FC assay (63-1612 mg GAE/100g DW) (Wijngaard et al. 2009; Bai et al. 2010; Wijngaard and Brunton 2010; Dhillon et al. 2013). This may be due to the overestimation of TPC by FC in other studies, owing to the interference from non-phenolic compounds in AP, such as sugars (Gülçin 2012). In fact, sugar rich fruits, such as prunes and jujubes (Chinese dates), were found to have significantly higher FC values than those reported by FBBB (Lester et al. 2012). This is opposite to the finding with strawberries, which have approximately one quarter of sugar

content of prunes and jujubes, and one half of the content of raw apples (Canada 2016).

Possible factors for the differences in the quantities of individual PP between studies noted include: apple variety (genetics) (Soares et al. 2008), sunlight exposure/geographical region (increased FG with increased UV) (Awad et al. 2001c), growing conditions (N fertilization shown to decrease flavonoids) (Awad et al. 2001a), pomace production methods (ex. pulp enzyming shown to significantly decrease phloridzin and catechins by ~50%) (Van der Sluis et al. 2002), and fruit maturity at harvest (PP peak in early growth) (Awad et al. 2001b). Without elaborate details regarding most of these factors for our samples and those of other studies, it is difficult to discern which factors affected our results.

In order to quantify polyphenols in a plant sample, a phenol extract must first be prepared. There are many methods of extraction, but solid-liquid solvent extraction is one of the most accessible, affordable and routinely used. The choice of solvent depends upon the sample matrix, nature of the material and chemical nature of the compound(s) to be extracted. For extraction of AP PP, mixtures of methanol, acetone, ethyl acetate, and ethanol with water, or simply water at different compositions and temperature ranges have been reported (Lu and Foo 1997; Schieber et al. 2003; Suárez et al. 2010; Wijngaard and Brunton 2010; Reis et al. 2012; Rana et al. 2014). Concentrations of ≥80% methanol in water proved to be a significantly better solvent than 100% acetone for the HPLC–detected extraction of Q and its glycosides from AP according to Rupasinghe et al, (2011). However, literature results regarding this topic are inconclusive, as other studies

oppose this trend (Suárez et al. 2010; Wijngaard and Brunton 2010). Suarez et al (2010) showed better extraction of flavonols with 70% acetone than 80% methanol. The discrepancies between these studies are likely due to the complex effects on polarity in varying mixtures of these solvents with water, coupled with differences in sample composition.

Solvent selection is likely partially responsible for the lower amount of total PP analyzed in our AP, as Suarez et al (2010) and Rana et al (2014) have shown that acetone (Suárez et al. 2010) and acetone and ethanol (Rana et al. 2014) aqueous solutions may be more effective at extracting PP from AP than methanol. The effect of solvent on PP extraction may also, in turn, be dependent on environmental and genetic apple factors that determine PP quantities, their degree of polymerization, solubility, and interaction with other components of the plant matrix. Additionally, Schieber et al (2003) noted a decrease in flavanols (ex. epicatechin) with oven drying of AP, which could be one of the reasons for the much lower quantity of epicatechin in ours (5.430mg/kg DW) and Suarez et al's (88mg/kg DW) sample compared to the freeze dried AP analyzed by Lu and Foo (1997) (640mg/kg DW).

Polyphenol content, especially flavonoid glycosides contained in the skin, are largely positively influenced by the exposure to UV radiation. However, geographical region and therefore the exposure to sunlight are an unlikely explanation for the large variation in TPC reported in literature. This is due to the observation that cider apples from Ireland (Wijngaard and Brunton 2010) had FC values comparable to ones from

Spain and Australia, where the average monthly sunshine hours are greater (Suárez et al. 2010; Candrawinata et al. 2014; World Tourism Organization 2014). Additionally, based on greater average sunshine hours per month, as determined by the World Tourism Organization (2014), Canadian apples would be expected to have higher TPC than Irish ones. However, the FC values in the Irish apples are ~3.5 times higher than the values obtained by our FBBB analysis (Wijngaard and Brunton 2010). Additionally, no obvious association between geographical region, average monthly sunshine hours, and FG content determined by HPLC/UPLC exists in Suarez et al (2010) (Spain), Lu & Foo (1997) (New Zealand), Rana et al (2014) (India) and our study compared with each other. Geographical factors are therefore an unlikely explanation, and the differences in chromatographic quantities determined are likely to stem from the apple and solvent factors noted above, as well as possible effects of oven drying. As for differences in TPC, they are likely due to overestimation of values by FC in other studies, as well as the differences in apple factors and solvent extraction methods.

3.5.3 Effects of digestion on apple pomace polyphenols

The total content of the 8 polyphenols quantified by UPLC/MS in the digested sample was 5.58 ± 0.85 mg/kg DW, with a very low total recovery rate of 0.36 ± 0.03%. The order of abundance for digested samples was: quercetin-3-glucoside>quercetin-3-galactoside>phloridzin>epicatechin>coumaric acid (Figure *3.2*). Quercetin, chlorogenic and caffeic acids were below their detection limits (Table *3.2*). The recovery rates in the digested sample were: quercetin-3-glucoside (0.18 ± 0.02%), quercetin-3-galactoside (0.87 ± 0.52%), epicatechin (9.90 ± 3.43%), and phloridzin (2.72 ± 1.30%) (Figure *3.3*).

These numbers suggest extensive transformation by either the rumen environment (ex. pH) or microbes. In one of the only studies that examined *in vitro* rumen metabolites of PP, Berger et al, 2015, concluded that microbial metabolism, and not spontaneous degradation of Q, was the primary reason for its exponential disappearance from the digesta. This was due to the lack of significant disappearance of Q from digesta of an inactive rumen inoculum compared to the one containing active microbes (Berger et al. 2015). Q disappearance was associated with the appearance of its phenolic acid metabolites, due to microbial ring fission of the C ring. Ring fission of the aglycone produces phloroglucinol (PG), which then undergoes degradation to butyrate and acetate, as well as CO_2 (Rechner et al. 2004; Ulbrich et al. 2015). These findings are in agreement with other *in vitro* experiments involving cultures of rumen and monogastric colonic microflora (Cheng et al. 1969; Rechner et al. 2004; Cermak and Breves 2006; Lu et al. 2013; Ulbrich et al. 2015). The drastic decrease in Q3G and Q3GL and the absence of Q from 24hr digested AP in our study is therefore likely due to extensive deglycosylation [of FG's] and microbial ring fission [of original Q in AP, and Q produced from deglycosylation of FG's] by the rumen microbes into phenolic acids and further metabolites. The 24hr incubation period for the present study was originally selected, as it is representative of forage rumen retention time in high producing dairy cows. Given the results of this study and those obtained by Berger et al (2015), a time course of shorter incubation periods may have been beneficial to better elucidate the stability of Q and other AP PP in the rumen environment.

Although having better recovery than Q and its glycosides, phloridzin, a

dihydrochalcone glucoside, still had a low recovery of only 2.72 ± 1.30%. Extensive microbial degradation is also likely responsible here, as neohesperidin dihydrochalcone, a synthetic derivative of hesperidin (main flavonoid in bitter oranges), was completely metabolized within 22hr by human fecal slurries *in vitro* (Braune et al. 2005). Its disappearance from the incubation medium coincided with the appearance of deglycosylated intermediates, such as hesperetin dihydrochalcone 4'-D-glucoside (due to rhamnosidase activity) and subsequently its aglycone (due to glucosidase activity). Similarly to QG, the aglycone was then further cleaved by ring fission to a phenolic acid and PG, subsequently undergoing rapid degradation to butyrate and acetate by *Eubacterium* sp. (Braune et al. 2005). *Eubacterium ramulus* was responsible for glucosidic activity, while both *Clostridium orbiscindens* and *E. ramulus* possessed hydrolase activity for ring fission. *Bacteroides* sp. has been reported to have rhamnosidase activity required for conversion of neohesperidin dihydrochalcone to hesperetin dihydrochalcone 4'-D-glucoside (Bokkenheuser et al. 1987). All three of the above-mentioned microbe species have been previously isolated from the rumen of cattle (Bryant 1959).

The appearance on *p*-coumaric acid in the digesta may be a result of microbial degradation of anthocyanins. They are a subgroup of flavonoids, existing as several different glycosides in AP, one being cyanidin-3-glucoside (cyanidin-3G). Inoculation and batch-culture fermentation of a mixture of several anthocyanins: malvidin-3G, delphidin-3G, peonidin-3G, peunidin-3G, and cyanidin-3G with human fecal slurries for 24hr resulted in complete metabolism to gallic, syringic and *p*-coumaric acids (Hidalgo et

al. 2012). A similar study by Keppler et al, (2005), confirmed the deglycosylation and degradation of monoglucosides and diglucosides of anthocyanins by colonic microbiota. They reported that cyanidin-3-rutinoside was deglycosylated to its glucoside and then converted to phenolic acids, which were further metabolized, likely to volatile FA's (Keppler and Humpf 2005).

Epicatechin (E), although not overly abundant in AP, had the highest recovery of all PP analyzed (9.90 ± 3.43%). No known studies have analyzed the effects of *in vitro* rumen incubation on E recovery, but *in vitro* microbial experiments using human fecal slurries point to similar microbial degradation of E by C and A ring fission by *Eubacterium* sp. (Stoupi et al. 2010). The higher recovery rate of E is, therefore, less likely to be a result of higher stability in the rumen, but more so due to its derivation from degradation of another PP. Procyanidin B2 (PB2), a proanthocyanidin (condensed tannin) is a dimer of E which is abundant in AP (~219mg/kg DW) (Bai et al. 2010). Baba et al (2002) detected procyanidin B2 and E in rat plasma and urine after administration of pure PB2, suggesting that a portion of the dimer was degraded into E (Baba et al. 2002). These findings were confirmed in human fecal slurries of PB2, where 10% of the dimer was converted into E, alternatively undergoing C-ring cleavage and A-ring oxidation to produce phenolic acids and non-phenolic metabolites such as oxaloacetate, succinate, butyrate and CO_2 (Stoupi et al. 2010). The cleavage of the interflavan bond between the E units was likely due to large intestinal microbiota as procyanidin dimers and trimers are thought to be highly stable during human gastric and duodenal digestion and reach the colon intact (Serra et al. 2010). Although a small

amount of PB2 was degraded to E, the authors note that this route may assume greater importance based on the microflora population (Stoupi et al. 2010). Additionally, the rate of PB2 degradation was twice as fast as that of E, so that if the same rates of degradation are assumed in the rumen, it is possible that accumulation of E in the present study would have resulted from net production due to unequal rates of production and degradation.

Results from this study point to extensive microbial metabolism of AP PP into simpler phenols and likely non-phenolic compounds, which is dependent on their sugar moieties, the type of glycosidic binding present and the individual organism's microbiome, which itself determines glucosidase, rhamnosidase, and ring fission activities (Ulbrich et al. 2015). However, evidence from *in vitro* studies suggests that microbial and conjugated metabolites of PP may still contribute to their beneficial properties *in vivo*, making the production of a naturally PP-enhanced animal product for human consumption feasible (Manach et al. 1998; Morand et al. 1998; Williamson et al. 2005; Ou and Gu 2014; Wiczkowski et al. 2014).

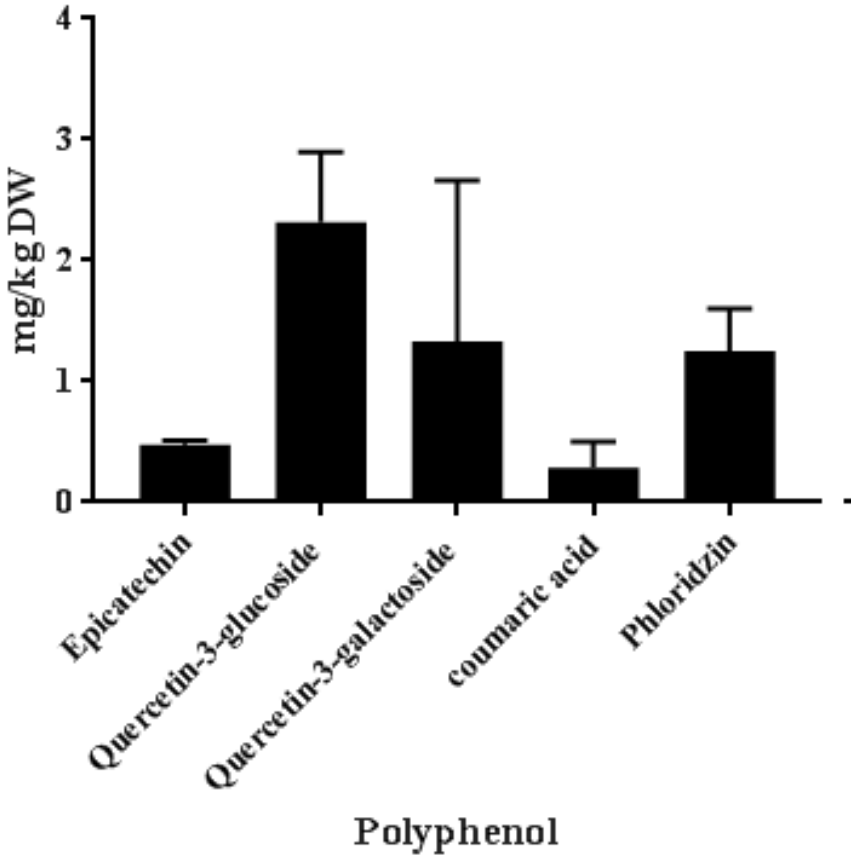

Figure 3.2 Polyphenol content (mg/kg dry weight (DW) apple pomace (AP)) of 24hr rumen-digested AP determined by UPLC/MS. (n=3)

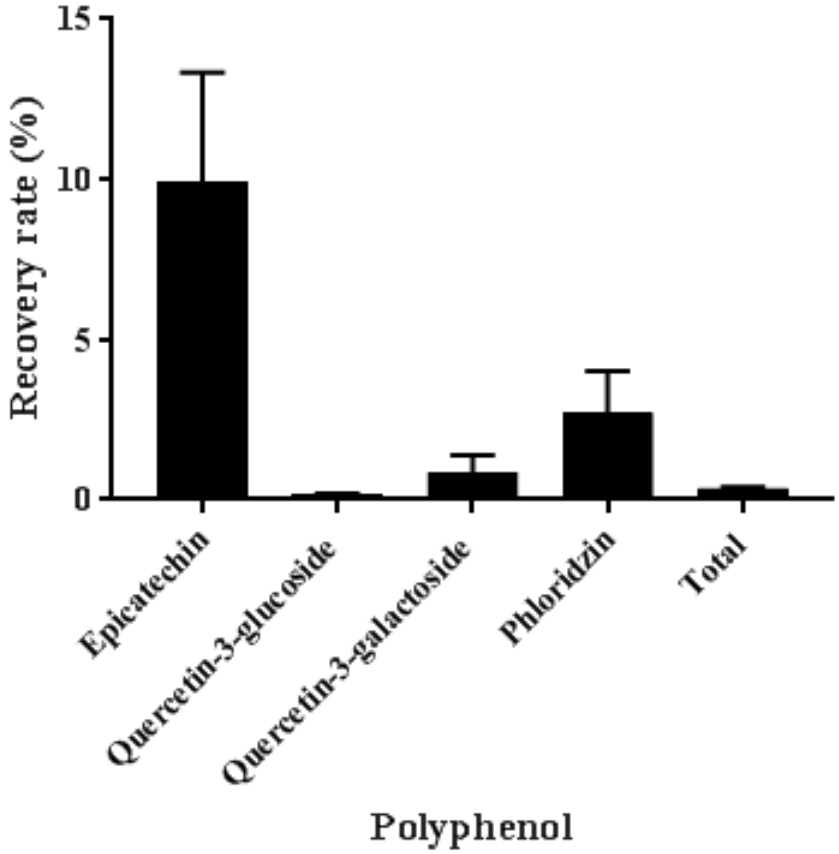

Figure 3.3 Polyphenol recovery rates (%) of rumen-digested AP determined by UPLC/MS. (n=3)

Table 3.2 Limits of detection and quantification, ions and retention times.
*nd – not detected (below lower detection limit), RT – retention time, LOD – limit of detection, LOQ – limit of quantification.

			Non-Digested		Digested	
Analyte	*m/z*	*RT*	*LOD*	*LOQ*	*LOD*	*LOQ*
		(min)		*(μg/mL)*		
Quercetin	317	2.55	0.0098	0.0294	0.0098	0.0294
Quercetin-3-glucoside	463	2.82	0.0098	0.0294	0.0098	0.0294
Quercetin-3-galactoside	463	2.84	0.0098	0.0294	0.0098	0.0294
Phloridizin	435	3.10	0.0024	0.0072	0.0024	0.0072
Caffeic acid	179	2.15	0.0098	0.0294	0.0098	0.0294
Coumaric acid	163	2.63	0.0024	0.0072	0.0012	0.0037
Chlorogenic acid	353	2.34	0.0024	0.0072	0.0024	0.0072
Epicatechin	289	2.43	0.0024	0.0072	0.0024	0.0072

Ontario AP is a good source of polyphenolic compounds (1562 ± 355.2mg/kg DW). AP contains an abundance of quercetin glycosides, of which, their metabolite quercetin, has been shown to possess powerful antioxidant and anti-cancer properties. In its present, unprotected state, AP could be minimally useful as dairy cattle supplement in an attempt to deliver PP into the cow's milk. 24hr *in vitro* rumen incubation extensively degraded original PP present in AP, likely due to microbial activity. Shorter incubation periods should be explored to better understand AP PP stability in the rumen environment. Additionally, evidence from other studies shows that the PP metabolites created likely still retain beneficial properties described for their parent compounds. Therefore, it would be necessary to measure activity of rumen digested AP PP by examining their free radical scavenging activity and/or their *in vitro* effects on models of

human disease, such as cancer. The next steps would involve analyzing the *in vivo* rumen digestion, absorption and resulting plasma metabolites of PP AP in lactating dairy cows before steps are taken to analyze PP transfer to milk.

CHAPTER FOUR

THE EFFECT OF APPLE POMACE POLYPHENOLS ON PROLIFERATION OF HUMAN COLON CANCER (HT-29) CELLS

4.1 Abstract

The effect of quercetin (Q), the aglycone of the most abundant polyphenol (quercetin-3-glucoside) in Ontario apple pomace, on the proliferation and apoptosis of human colon cancer cells (HT-29) was evaluated. Actively proliferating HT-29 cells were grown to 80% confluency in 25cm^2 culture flasks and then seeded at 2x10^{4}cells/well in a 96 well flat-bottom microplate. They were incubated for 24hr at 37°C and 5% CO_2 to allow for attachment and growth, and were then serum-starved for 24hr. Q dihydrate was dissolved in 0.25% DMSO (v/v) in serum-free medium at 400, 200, 100, 50, 25, 12.5, 6.3, and 3.1uM and its IC_{50} was determined. Irinotecan (CPT-11) was used as a positive control, and its IC_{50} was determined by dissolving it in 0.25% DMSO at concentrations of 200, 100, 50, 25, 12.5, 6.3, and 3.1uM. 0.25% DMSO in serum-free medium was used as the negative control, and wells with no cells were used as blanks. Treatments and controls were applied to the cells and incubated for 72hr at 37°C and 5% CO_2. Sulforhodamine B (SRB) assay was performed to evaluate effect on cell proliferation. The average IC_{50} of CPT-11 and Q, determined from 3 individual experiments were 6.45±2.05 and 2.85±0.15μM, respectively. At its highest concentration of 400μM in cell culture, Q inhibited 82±0.54% of cell growth, while CPT-11 inhibited 98±0.25% at half of that concentration (200μM).

4.2 Introduction

Colorectal cancer (CRC) is the second type of cancer with the highest mortality, with approximately 1.4 million cases diagnosed and 693 000 deaths worldwide in 2012 (Torre et al. 2015). Although hereditary and sporadic types of CRC exist, dietary factors are responsible for 70-90% of cases (Shannon et al. 1996; Glade 1999; Ahmed 2004). A commonly accepted preventative factor of CRC is the consumption of a diet rich in fruits and vegetables. This is largely attributed to the high content of micronutrients such as polyphenols (PP) in edible plant matter, which have been shown to possess powerful anti-cancer properties *in vitro* and *in vivo* (Manson 2003). Apple pomace (AP), the pulp and skin residue from the apple juice and cider industry, is rich in PP, therefore representing a potential source of bioactives for the production of value-added products for human health.

There are several common genetic mutations in CRC that affect multiple signaling cascades, such as mitogen-activated protein kinase (MAPK) pathways involved in communication between the cell surface and nucleus, transcription factors involved in mRNA production, cell cycle factors involved in the regulation of cell proliferation, and factors involved in regulation of apoptosis, which allow for carcinogenesis to take place (Manson 2003). Two missense mutations are found in the HT-29 cell line utilized in this study, one resulting in oncogenic BRAF tyrosine kinase and the other giving rise to a mutated p53 (mut-p53) tumor suppressor protein. BRAF results in increased signaling through ERK/MAPK pathway and therefore activates anti-apoptotic genes, while mut-

p53 alters DNA repair, cell cycle progression, and apoptosis (Gobert et al. 1999). Cancers bearing GOF mut-p53, such as HT-29 cells, are generally aggressive in nature and confer resistance to commonly used chemotherapeutics, such as 5-fluorouracil and oxiplatin (Weekes et al. 2009). For this reason, CPT-11 (irinotecan), normally a second line cancer drug, is used as a first line drug in the treatment of metastatic CRC. However, mut-p53 cancers can still be less responsive to CPT-11 than those bearing wt-p53 (Abal et al. 2004). It is therefore important to explore new anti-cancer agents, such as PP, which may be used prophylactically or in conjunction with current treatments.

A phenolic compound is any that possesses a hydroxyl substituted aromatic ring, and its polymer is referred to as a polyphenol (Craft et al. 2012). Of the several classes of PP present in nature, flavonoids are the largest, most ubiquitously represented, and for that reason, the best studied (Middleton et al. 2000). Daily flavonoid intake ranges widely from 23-1000mg/day (Kühnau 1976; Hertog et al. 1994) and tea, apples, and onions are their main sources in the human diet (Scalbert and Williamson 2000; Middleton et al. 2000). Quercetin, a major constituent of the flavonoid class, is highly abundant in apples, typically present in its glycoside form that is attached to a sugar moiety. Since flavonoid synthesis is UV stimulated, their concentration is highest in the skin and leaves of apples (Wolfe et al. 2003; Manach et al. 2004) Apple pomace is heavily produced in Canada (113 840-136 610 tons in 2009) and unnecessarily wasted due to unaffordable transportation costs associated with its high moisture content (70-75%). In [unpublished] results from our lab, as well as those from others, AP is a significant source of PP, particularly Q glycosides (~316-2480 mg/kg DW) (Lu and Foo

1997; Suárez et al. 2010; Rana et al. 2014). Therefore, AP presents an environmentally beneficial flavonoid source for the production of value added foods or nutraceuticals for human health.

Q consumed in the diet as its aglycone (without sugar moiety) survives gastric digestion to be passively absorbed across the small intestinal brush border. Its glycosides are largely freed of their sugar moiety by the brush border enzyme, lactase-phlorizin hydrolase (LPH), and absorbed as aglycones through passive diffusion. A smaller portion are transported across the brush border by sodium-dependent glucose transporter (SGLT1) intact, after which their sugar moiety is cleaved by cytosolic β-glucosidase (Wang et al. 2013). Due to the direct interaction of PP with the gastrointestinal tract (GIT) cells, many studies have looked at their affect on GIT cancers (Shannon et al. 1996; Glade 1999; Manson 2003; Van Der Woude et al. 2003; Ahmed 2004; Kim et al. 2005; Kim et al. 2010). Evidence of anti-cancer activity of Q exists in numerous *in vitro* (Van Der Woude et al. 2003; Kim et al. 2005; Lee et al. 2006; Kim et al. 2010) and *in vivo* studies with laboratory animals (Kim et al. 2014). In human CRC line harboring the BRAF mutation, Q displayed significant anti-proliferative and pro-apoptotic effects in a dose-dependent manner (Xavier et al. 2009). Other studies confirmed these effects in HT-29 cells (Van Der Woude et al. 2003; Kim et al. 2005) which provide evidence of Q's inhibitory effects on the PKB/Akt pathway and therefore removal of its downstream inhibition on pro-apoptotic (ex. caspases) and cell cycle inhibiting (ex. p21) factors.

In order to evaluate the use of AP PP in the production of value-added products

for human health, we examined the effect of Q aglycone compared to a commonly prescribed CRC drug, CPT-11, on the proliferation of HT-29 cells. The presence of a mut-p53 in this cell line makes them a suitable model of CRC that does not respond well to most chemotherapeutic agents.

4.3 Materials and Methods

4.3.1 Chemicals and reagents

All reagents used were of cell culture grade. Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin solution**,** dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), sulforhodamine B (SRB), acetic acid, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), and quercetin dihydrate, were obtained from Sigma (Oakville, Canada). Phosphate buffer saline 10x (PBS), L-glutamine, trypsin-EDTA, and trypan blue solution were obtained from Gibco (Bethesda, USA).

4.3.2 Cell line

Human epithelial colorectal adenocarcinoma cells (HT-29) were obtained from the American Type Culture Collection (Rockville, USA).

4.3.3 Cell culture

HT-29 cells were cultured in 25cm^2 flasks in DMEM supplemented with 10% (v/v) FBS, and 1% (v/v) 200nM L-glutamine and penicillin/streptomycin. Cells were

incubated in a humidified chamber kept at 37°C and 5% CO_2. Medium was replenished every 2-3 days. Cells were passed weekly during the exponential growth phase (~80% confluency) by trypsinization with trypsin-EDTA (25%). All cells used for experiments were >95% viable, as determined by Trypan blue dye exclusion.

4.3.4 Selecting a positive control drug

Cancers bearing mut-p53, such as HT-29 cells, are generally aggressive and resistant to commonly used chemotherapeutics (Weekes et al. 2009). CPT-11 is first line metastatic CRC drug derived from plant alkaloid camptothecin (CPT). CPT and its derivatives exhibit their anti-tumour activity by inhibiting DNA topoisomerase I (TOPO-I). This nuclear enzyme normally facilitates DNA replication and transcription by causing single-strand breaks in DNA which relieve torsional tension in the double helix ahead of the replication fork (Wang 1996). It is activated by carboxylesterase (present in intestinal mucosa, plasma and liver)-mediated hydrolysis to 7-ethyl-10-hydroxycamptothecin (SN-38), an analogue of CPT, which binds to and inhibits TOPO-I, thus impeding DNA re-ligation and resulting in DNA double-strand breaks, whose detection leads to apoptosis (Kjeldsen et al. 1992).

The mechanism of CPT-11 effect on cell cycle arrest and apoptosis in mut-p53 cells is still being elucidated. In cells with wt-p53, this tumour suppressor is capable of triggering a direct response to CPT-11, likely by activating the DNA-damage checkpoint in response to DNA double strand breaks (through TOPO-I inhibition). This involves the downstream activation of p21, a CDK inhibitor, eventually promoting apoptosis as a

result of sustained cell cycle arrest in the S phase (Abal et al. 2004). In mut-p53 cells, CPT-11 is less effective, but it does impose an arrest in cell-cycle progression during the G2/M phase, likely due to the inability of cells to complete DNA synthesis successfully. In the absence of a wt-p53, cell cycle machinery continues to progress and accumulates CDK1/cyclin B complexes (positive regulators of cell cycle progression into the M phase), which induce p21 (an inhibitor of CDK1), in a p53-independent manner, to suppress CDK1 activity and to block cells from progressing into G2/M phase (Abal et al. 2004). Due to its anti-proliferative effect on HT-29 cells, CPT-11 was chosen as a positive control for this experiment.

4.3.5 Determining cell seeding density

Seeding density depends on cell size, population doubling time, as well as treatment duration, making it experiment-specific. It was determined in triplicate for 96 well flat bottom microplates for a 24hr attachment period, 24hr serum starvation period, and a 72hr treatment period for the proliferation assay (SRB). A standard curve of cell number and absorption (Figure *4.1*) was first obtained by seeding 0, 200, 400, 800, 1600, 3200, 6400, 12800, 25600, 51200, and 102400 cells per well in triplicate and allowing for attachment, but not growth, overnight (12hr). The SRB assay was then performed as stated in section *4.3.7* (Skehan et al. 1990).

Actively proliferating cells (<80% confluent) were trypsinized and plated at $1x10^4$ and $2x10^4$ cells per well and allowed to attach and grow for 24hr. In a previous experiment, anything below $1x10^4$ cells/well did not survive serum starvation well, and

above $2x10^4$ cells per well was over-confluent at the end of the treatment period, resulting in cells that are no longer actively proliferating (Atcc 2014). Cells number was determined at two time points to ensure the number of cells exposed to the treatment after 24hr of serum starvation, and the cells of the untreated control at the end of the treatment period were both within the detectable and linear range of the standard curve. After growth and attachment, the medium was discarded and detached dead cells removed by rinsing with 300uL PBS solution per well. 100uL of new serum free medium (DMEM only) was added to each well to serum starve the cells for 24hr. To determine cell density at the beginning of the treatment period, the cells were quantified using SRB and the standard curve. Meanwhile, to determine the cell number at the end of the treatment period, the medium was removed, cells were rinsed with 300uL/well PBS, 100uL of new serum-free medium was added to each well, and incubated for 72hr. Cells were quantified using SRB and the standard curve.

4.3.6 Selecting a drug and polyphenol solvent and evaluating solvent toxicity

Dimethyl sulfoxide (DMSO) is a polar apriotic solvent that is miscible in a wide range of organic solvents. It is commonly used as a solvent in cell culture studies for water-insoluble compounds such as polyphenols and CPT (and its derivatives) (Kuntz et al. 1999; Wenzel et al. 2000; Van Der Woude et al. 2003; Kim et al. 2005; Kim et al. 2010). DMSO was the solvent of choice because both CPT-11 and quercetin hydrate were reported to have the highest solubilities in it compared to other solvents, such as ethanol (50mg/mL and 200mg/mL, respectively). Because DMSO has potential cytotoxic effects in cell culture (Wang et al. 2012), concentrations of <2% (v/v) are

usually used (Kuntz et al. 1999; Wenzel et al. 2000; Van Der Woude et al. 2003; Kim et al. 2010). Since this is likely to be influenced by cell type and experimental conditions, a cytotoxicity test was performed for HT-29 cells for each experiment. HT-29 cells were grown in $25cm^2$ culture flasks to 80% confluency and actively proliferating cells were detached with 25% trypsin-EDTA. They were seeded at 2×10^4 cells/well in a 96-well flat-bottom microplate and allowed to grow and attach for 24hr at 37°C and 5% CO_2. The medium was removed, cells washed with 300µL 1X PBS and the medium was replaced with 100µL serum-free medium for 24hr. DMSO was dissolved in serum free medium at 2.00, 1.00, 0.50, 0.25, and 0.13%. Negative controls contained DMEM, and blank wells contained DMEM with no cells. After the treatment period of 72hr, SRB was performed as per section *4.3.7*.

4.3.7 Determining cell proliferation using the sulforhodamine B Assay

The sulforhodamine B (SRB) assay is a widely used method for determination of cell density, based on the measurement of cell protein. It has been used to test the efficiency of natural compounds against herpes simplex virus type 1, as well as cancer cell sensitivity to radiation, among others (Plumb et al. 1989; Griffon et al. 1995; Pittayakhajonwut et al. 2005). SRB dye is an aminoxanthane compound with two sulfonic groups that binds to basic amino-acid residues under mildly acidic conditions and dissociates under basic conditions. The stoichiometric binding of the SRB dye results in absorbance values that are directly proportional to cell mass (Vichai and Kirtikara 2006). Results from the SRB assay performed with HT-29 cells exhibits a linear dynamic range over densities of 7500 – 180 000 cells per well (~1-200%

confluence) (Skehan et al. 1990). The SRB assay was chosen over the widely used tetrazolium (MTT) assay (Mosmann 1983) because it has been shown to have increased sensitivity (can measure ~2000 versus ~16 000 cells per well for MTT), better linearity with cell number and a higher reproducibility than MTT (Keepers et al. 1991). In contrast to SRB, MTT relies on metabolically active cells to reduce the salt to its formazan product (Mosmann 1983; Keepers et al. 1991). A disadvantage to MTT is that cell lines can differ in their ability to reduce the dye, and anti-proliferative agent treatment may affect their metabolism in different ways. With the use of SRB instead, which simply quantifies protein, these effects are avoided. SRB's protein quantification may present a potential for unintentional quantification of dead cell protein. However, Keepers et al. (1991) found that the number of cells quantified by MTT and SRB 24hr after lysis were not significantly different, meaning that cell debris was not stained by SRB.

HT-29 cells were grown in $25cm^2$ to 80% confluency and actively proliferating cells were then detached with 25% trypsin-EDTA. They were seeded at 2×10^4 cells/well in a 96-well flat-bottom microplate and allowed to grow and attach for 24hr at 37°C and 5% CO_2. The medium was removed, cells washed with 300µL 1X PBS and the medium was replaced with 100µL serum-free medium for 24hr. Irinotecan and quercetin were dissolved in 0.25% DMSO in serum-free medium (just DMEM) at concentrations of 200, 100, 50, 25, 12.5, 6.2 and 3.1uM and 400, 200, 100, 50, 25, 12.5, 6.3, 3.1uM respectively, and incubated for 72hr to determine IC_{50}. Negative controls contained 0.25% DMSO in DMEM, and blank wells contained growth medium only. After the treatment period of

72hr, the spent medium was aspirated and wells were rinsed with 300µL of 1X PBS per well. 200µL of DMEM was added to wells to reduce background, and the cells were fixed to the bottom of microwell plates by incubation with 50µL cold 50% trichloroacetic acid (TCA) at 4°C for 1hr. After rinsing the TCA from the wells with 300µL H_2O (5 times), plates were set to dry overnight. 50µL of 0.4% SRB dye, dissolved in 1% acetic acid (w/v) was then added to the wells and incubated for 30min, allowing for dye and protein binding to take place. The plates were then washed four times with 300µL of 1% acetic acid solution and allowed to completely dry for 2hr. The bound dye was then solubilized with 100µL of 10mM tris buffer, allowing for quantification of protein content in the cell mass by measurement of optical density (OD) at 570nm with a Synergy™ HT Multi Detection Microplate Reader (Bio-Tek Instruments, Winooski, Vermont, USA). Calculations were performed as per section *4.4.3*.

4.4 Calculations and Statistics

4.4.1 Seeding density

All absorbance values (A) were corrected for the blank reading. To obtain a standard curve of cell number to absorbance at 570nm specific to this cell line, the data was fitted to a log-log model in GraphPad Prism 7 (GraphPad Software, San Diego, California, United States) (R^2>0.99). Inter- and intra-day variation was expressed as %CV.

4.4.2 Solvent toxicity

After correction for the blank readings, viability of the control (0% DMSO) was normalized to 100% and values were expressed as % viability compared to control, calculated by equation *1*. Results were analyzed with one-way ANOVA and each treatment was compared to the control with Dunnet's multiple comparison test in GraphPad Prism v.7 (GraphPad Software, San Diego, California, United States) ($p<0.05$). Inter- and intra-day variation was expressed as %CV.

% cell viability = (A of sample / A of control) x 100% *Eq. 1*

4.4.3 Growth Inhibition and IC_{50}

All absorbance values were corrected for the blank reading and expressed as % inhibition of cell growth compared to control (no CPT-11 or Q), determined by Equation 2. Three individual experiments with 3 technical replicates for each treatment were performed, and a non-linear fit was determined in Solver by least squares method (Microsoft Corporation, Redmond, Washington, USA). The observed results were compared to control by repeated measures one-way ANOVA ($p<0.05$) and corrected for multiple comparisons with Dunnett's test in GraphPadPrism v. 7. (GraphPad Software, San Diego, California, USA).

% inhibition = [(A of control - A of treated) / A of control] x 100% *Eq. 2*

4.5 Results and Discussion

4.5.1 Seeding density

The seeding density of cells depends on cell characteristics, such as diameter and rate of cell growth, as well as experimental factors, such as size of well and treatment period. All of these factors influence the confluency of the cells, and the assay characteristics, such as sensitivity, influence the interpretation of these results. To determine cell seeding density, cells were quantified with interpolation of the standard curve and SRB assay for each experiment. In our experiments, the SRB assay was able to detect a difference of ≥3200 cells/well (Figure *4.1*). This is a slightly lower sensitivity than the 1000 cell/well difference that Skehan et al. (1996) found in HT-29 cells. Discrepancies like this could be a due to different rates of cell growth, as a result of differences in mediums used (RPMI-1640 versus DMEM) or in quality of FBS supplied. General laboratory differences in reagents, spectrophotometer ability or calibration of equipment, such as pipettes, could also be partially responsible. The inter- and intraday variation (represented as %CV) were less than 14 and 17%, respectively.

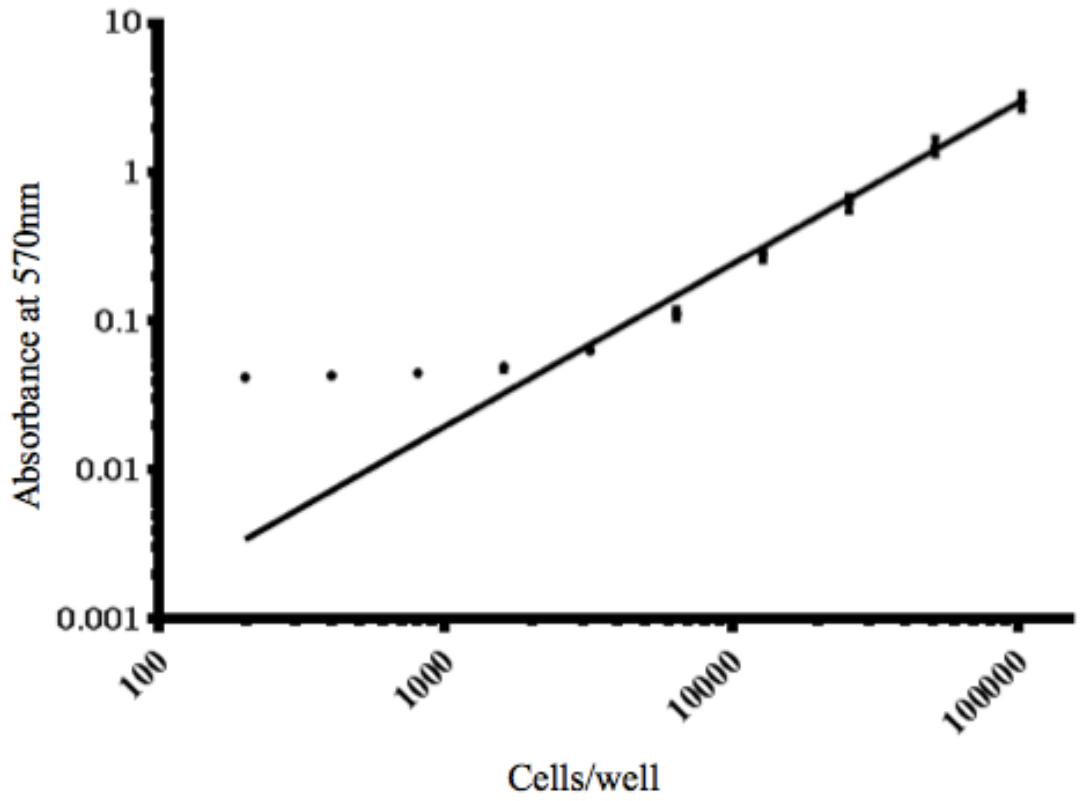

Figure 4.1 Standard curve of cell number plated per well versus absorbance, mean ± standard deviation, (n=3). $Y=10^{(-4.97 + 1.09*\log(X))}$, ($R^2 > 0.99$)

The average densities for all 3 experiments are shown in Table *4.1*. $2x10^4$ cells per well was the chosen seeding density, with 41 235 ± 2 977 cells per well at time of treatment application and 91 034 ± 7 479 cells per well in the control after the treatment period. The population doubling time in the serum free environment used in the experiment was therefore ~72hr, compared to ~23hr described for 10% fetal bovine serum containing medium for HT-29 cells (Atcc 2012). Although the doubling time increased, the cells were still actively growing.

Table 4.1 Mean cell number ± standard deviation (SD), interpolated from 3 individual experiments, (n=3).

Timepoint	Seeding density	Mean *(cells/well)*	SD
Treatment	10 000	15 529	658
application	20 000	41 235	2 977
Post-treatment	10 000	36 721	10 529
	20 000	91 034	7 479

4.5.2 Solvent toxicity

Although its biological effects have not been clearly defined, some of the known effects of DMSO include: producing cell fusion, inducing cell differentiation, increasing permeability across cell membranes, and functioning as a free radical scavenger (Capriotti and Capriotti 2012). It has additionally been used as treatment for leukemia, as it has been shown to cause cellular differentiation (and decrease cell proliferation) in human blood cancer cells (Lee et al. 2005; Santos et al. 2005; Wen et al. 2015). In our study, exposure to 0.25% DMSO for 72hr had no significant effect ($p>0.05$), while 2.00, 1.00, and 0.50% significantly decreased ($p<0.05$) and 0.13% significantly increased ($p<0.05$) the viability of HT-29 cells compared to the control (Table *4.2,* Figure *4.2*). The inter- and intraday variation (%CV) was less than 20% (Table *4.3*). 0.25% was therefore chosen as the acceptable level of DMSO for SRB experiments. The significant increase in cell proliferation with 0.13% (v/v) DMSO has been noted previously in human multiple myeloma cells (Wen et al. 2015). Wen et al*,* (2015) noted, with the use of the MTT assay, that exposure of Dox 40 MM cells to 0.1-0.4% DMSO (v/v) for 24hr, caused a significant increase in cell proliferation compared to control ($p<0.5$). This may be due to a growth hormesis effect, which states that agents that induce growth arrest at higher

concentrations cause a regulatory over correction by biosynthetic control mechanisms in response to sub-cytotoxic levels (Stebbing 1982). This biphasic modulation of cell growth by DMSO could also be due to the potential concentration-dependent antioxidative effects of free radical scavengers. Studies have shown that molecules classically deemed as antioxidants can actually have free radical scavenging or producing effects, depending on their concentration in the medium (Rice-Evans et al. 1996; Metodiewa et al. 1999; Van Der Woude et al. 2003).

Significant decrease in cell proliferation has been noted in studies with (Lee et al. 2005) or without (Teraoka et al. 1996; Roela et al. 2003; Wang et al. 2012) induction of cell differentiation. Similar to our study, Roela et al, (2003) found that 72hr exposure of CRC cells (LISP-1) to 2.5% DMSO (v/v) significantly inhibited cell proliferation (Roela et al. 2003). Teraoka et al (1996) confirmed these effects with a 72-120hr treatment of B lymphoblastoid cells with 1% DMSO. Both of these studies also noted a significant reversible cell cycle arrest in the G1 phase. Wang et al, (2012) reported a 50% decrease in growth compared to control after 48hr exposure of lung cancer cells to 5% DMSO (v/v). It is possible that the growth inhibitive properties in our study were a result of DMSO-induced arrest in the G1 phase of the cell cycle, possibly due to increased production of cell cycle inhibitors, such as p27. This CDK inhibitor, which causes cell cycle arrest in G1, was shown to be significantly increased in Chinese hamster cells in response to 96hr treatment with 1.5% DMSO (v/v) (Fiore and Degrassi 1999). In human leukemic cells, DMSO has been shown to induce phosphatase and tensin homologue (PTEN), an important anti-tumorigenic regulator of the PKB/Akt pathway, which induces

cell cycle arrest in G1 (Santos et al. 2005). Since PTEN expression is often absent in CRC (19-42% of cases), DMSO may possess important inhibitory actions on the PKB/Akt pathway (Manson 2003). There is a need for further elucidation of DMSO effects on cell cycle and proliferation machinery in colon cancer cells.

Table 4.2 Mean and standard deviation of % viability (relative to control) of HT-29 cells undergoing DMSO treatment (%v/v in medium). Different superscripts [(b-e)] signify significant difference compared to control (a) (0% DMSO, 100% viability)(p<0.05).

DMSO (%v/v)	Mean Viability (%)	Standard deviation (%)
0.13	109.3 [b]	6.4
0.25	102.1 [a]	6.5
0.50	86.85 [c]	3.2
1.00	60.12 [d]	6.4
2.00	15.38 [e]	2.3

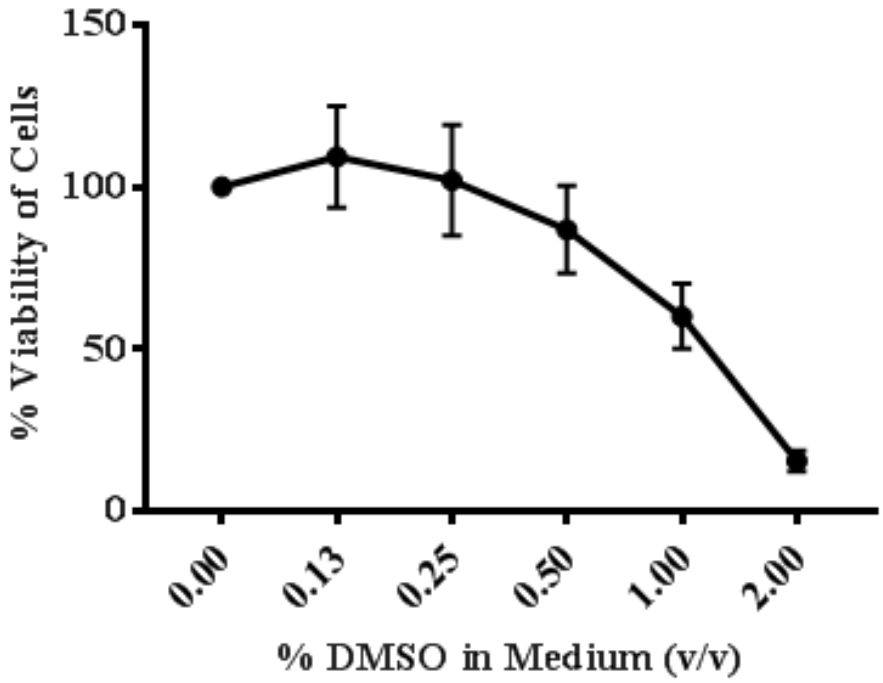

Figure 4.2 Cell viability (% relative to control) ± standard deviation of cells treated with DMSO (%v/v in DMEM), (n=3).

Table 4.3 Intra- and interday variation (%CV = coefficient of variation) for three separate experiments (n=3) testing for 72hr DMSO toxicity for HT-29 cells.

Treatment (%v/v DMSO)	Intraday variation (%CV)			Interday variation (%CV)
	Experiment 1	Experiment 2	Experiment 3	
2.00	19.6	15.6	16.0	15.2
1.00	14.1	11.3	17.3	10.6
0.50	19.8	15.8	11.3	3.67
0.25	19.5	16.0	13.5	6.36
0.13	16.2	15.8	10.0	5.85

4.5.3 CPT-11 and quercetin 72hr IC_{50} in HT-29 cells

All treatments significantly inhibited cell growth compared to control for both CPT-11 and Q (p<0.0001). The average IC_{50} for Q, determined from 3 individual experiments was 2.85±0.15μM, which is much lower than the values reported by Kuntz et al, (1999) (85.6±8.6μM) and Veeriah et al, (2006) (101.9μM) for the same cell line. Shorter treatment period (48hr compared to 72hr in our study) may have been responsible for the lower effectiveness of Q in Veeriah et al's study, as effects of Q on cell growth are generally time and dose-dependent (Kim et al. 2005). Additionally, use of serum in treatment medium (10% FBS) in both studies and use of 0.1% DMSO (v/v) by Veeriah et al (2006) may have increased cell growth and therefore made the cells more resistant to anti-proliferative agents. The use of high amounts of serum during treatment can provide inconsistent results due to its poorly defined, complex, and variable nature, as well as stimulatory effects on cell growth (Pirkmajer and Chibalin 2011). However, absence of serum in our treatment medium, compared to use of 0.1% (v/v) bovine serum albumin (BSA) by Kim et al, (2010, 2015), may have made our cells more susceptible to the anti-

proliferative effects of Q and CPT-11. This is due to the observation that serum starvation causes reversible G1 arrest in most cell cultures (Griffin 1976). Significant sequestration of cell growth is unlikely, however, as our cells continued to successfully grow in serum-free medium, although at a slower rate than in medium containing 10% FBS, as expected.

At its highest concentration of 400μM in cell culture, Q inhibited 82±0.54% of cell growth compared to control, while CPT-11 inhibited 98±0.25% at half of that concentration (200μM), proving to be a more effective CRC inhibitor. No other studies have looked at inhibition of cell growth at these levels for HT-29, but Kim et al (2005) showed similar inhibition at 100μM Q for 96hr in HT-29 cells. Determined by the MTT assay, cell growth was significantly inhibited by 81±3%, while the same dose inhibited cell growth by 75.0±0.8% after 72hr in our study (Kim et al. 2005). Exposure to the same dose of Q for 48hr significantly inhibited HT-29 cell growth by only 43.7%, as would be expected from a shorter treatment period (Kim et al. 2010). Van der Woude et al (2003) found significantly higher anti-proliferative potential of 100uM of Q in serum-free medium, as 24hr exposure significantly inhibited ~80% of cell growth compared to control (Van Der Woude et al. 2003). In this study, use of ascorbic acid to increase Q stability in medium may have altered its activity, and the use of 0.5% DMSO (v/v) could have contributed to the anti-proliferative effects observed. Additionally, determination of proliferation by a highly more sensitive assay (ELISA BrdU, Abcam), which can detect a difference of 40 cells/well, may have provided the authors with a different estimate of anti-proliferative potential of Q (Abcam 2016). Although differences in assay sensitivity

exist between MTT used by Kim et al, (2010, 2005) and SRB used in ours, our value for 72hr exposure is within the range determined for 48 and 96hr, as would be expected (Griffon et al. 1995).

The average 72hr IC_{50} determined for CPT-11 in HT-29 cells was 6.45±2.05μM. Conversely, Jang et al (2016) reported a 24hr IC_{50} of 62.5±0.18μM in HT-29 cells with the MTT assay (Jang et al. 2016). In the same study, exposure to 100, 50 and 25μM resulted in ~90, 60, and 50% inhibition of cell growth, compared to 96.8±0.37, 89.8±1.42, and 63.8±3.08% in our study, respectively. Naturally, a shorter time exposure in their study resulted in a lower efficacy of anti-proliferative properties, although not as much as would have been expected. After 72hr treatment with 1μM CPT-11, Abal et al (2004) found a ~65% decrease in cell growth in HT-29 cells (Abal et al. 2004). Surprisingly, our lowest treatment of 3.12μM for the same amount of time inhibited cell growth by only 42.8±7.2%. It may be possible that serum use (10% FBS) by Abal et al (2004) actually increased cell absorption of the drug, as has been suggested in studies of PP and protein interactions (Sahu et al. 2008).

The differences between our results and the other studies mentioned for Q and CPT-11 may stem from the use of different assays to quantify anti-proliferative potential. Use of the MTT assay requires normal cell mitochondrial function in order to metabolize the assay dye, and since PP are known to affect the ROS balance of mitochondria and therefore potentially their metabolic function, it may not be a well suited assay for studies of PP effects on cell proliferation (Masuda 2016). Additionally, mitochondrial enzymes

metabolize CPT-11 and HT-29 cell exposure to it causes a significant decrease in their mitochondrial membrane potential (Mathijssen et al. 2001; Grivicich et al. 2005). The SRB assay simply dyes protein of fixed cells, and may therefore be more appropriate to measure effects of PP and CPT-11 on cell viability (Skehan et al. 1990; Keepers et al. 1991). However, if CPT-11 and Q potentially affected mitochondrial metabolism of MTT dye, it would have been expected to result in an overestimation of the compounds' anti-proliferative activities in other studies compared to ours. Since the IC_{50} values obtained suggest otherwise, it is likely that inter-laboratory variation as well as much lower MTT sensitivity are partially responsible for the differences observed. Additionally, differences in serum inclusion in the treatment medium likely contributed to different cell growth rates, as well as possible effects on drug absorption across studies.

The response of cell growth inhibition in our study followed a 3-parameter Michealis-Menten curve (Equation 3). The equations determined for CPT-11 and Q are given in Equations 4 and 5, respectively, and the resulting curves, along with observed values are shown in Figure *4.3*.

$$y = (y_{max}\,[x]) / (IC_{50} + [x]) \qquad \textit{Eq. 3}$$

$$\%\ \text{inhibition} = (0.975\,[\text{CPT-11}]) / 6.26 + [\text{CPT-11}]) \times 100\% \qquad \textit{Eq. 4}$$

$$\%\ \text{inhibition} = (0.774\,[Q]) / 2.84 + [Q]) \times 100\% \qquad \textit{Eq. 5}$$

Drug dose-response curves are generally sigmoidal (4 or 5 parameters) in nature,

exhibiting a bottom (basal or no activity) and top (maximum activity) plateau (GraphPad 2015). Another reason for much lower IC_{50} values determined in our study is a different observed response-curve, which is likely attributable to complete lack of serum in treatment medium and different assay sensitivity compared to other studies. Lack of serum may have exacerbated the anti-proliferative potential of the agents at lower concentrations. This may be particularly true for Q, as it has been noted to cause arrest in the G1 phase in HT-29 cells, and may have therefore increased the possible serum starvation-induced cell cycle arrest at the same point. This could explain the lack of a 'bottom plateau' and sigmoidal response-curve in our study. However, none of the previously mentioned studies actually report on response curve shape used to determine the IC_{50} values, so no further conclusions can be drawn regarding their influence on the differences observed.

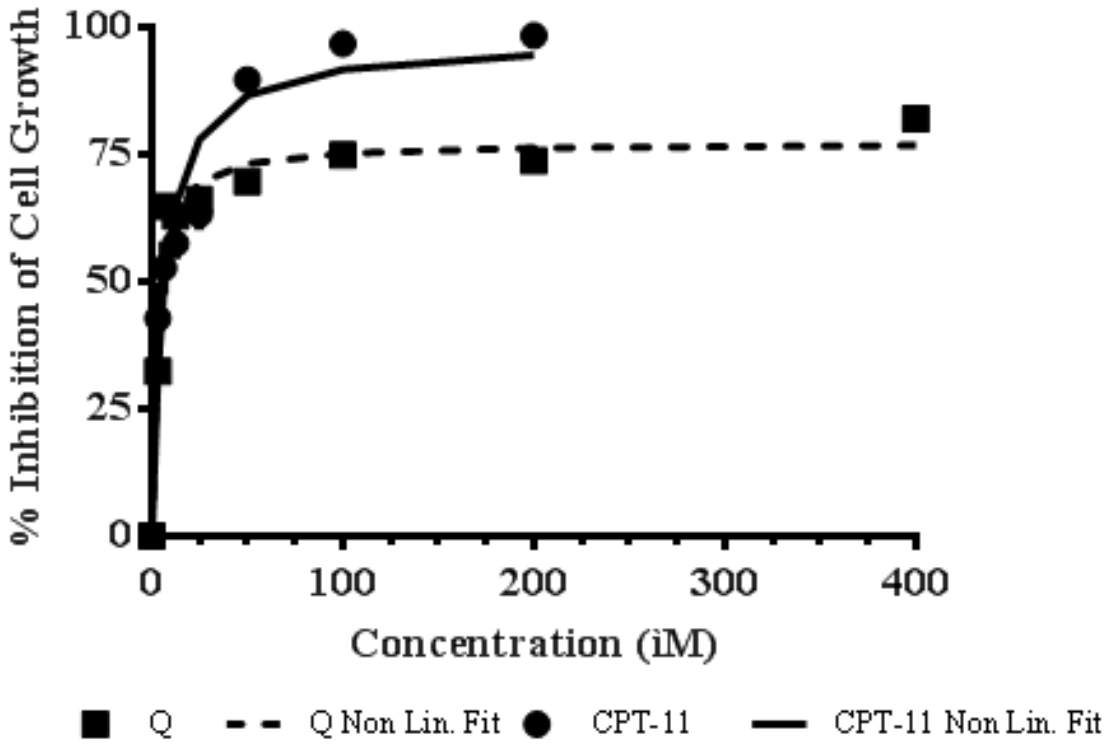

Figure 4.3 72hr inhibition of cell (HT-29) growth by CPT-11 and quercetin, mean SD, (n=27). Non-linear fit obtained by least squares method in Solver (Microsoft Corporation, 2011).

Quercetin is an effective anti-proliferative agent in mut-p53 cells. In this experiment, its effectiveness was higher than CPT-11 at lower concentrations (<25uM) in cell culture, above which CPT-11 proved to be more effective. Lower IC_{50} values determined by our study compared to others may be due to a combination of decreased sensitivity of the commonly used MTT assay by others, and differences in serum inclusion in treatment medium, which would have altered the cell cycle progression and therefore cell proliferation rates. Complete absence of serum in our treatment medium may have also exacerbated the growth-inhibiting effect at lower concentrations of the agents used, and therefore resulted in a different response curve than expected. There is a

need for better characterization of Q effects on activity of cell cycle regulators, as well as the influence of serum presence on its and CPT-11's anti-proliferative activity in cell culture.

CHAPTER FIVE

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The present research proved the potent anti-proliferative activity of apple pomace polyphenol, quercetin, against CRC cells *in vitro*. In order to better elucidate the effects of PP on oncogenic pathways in these cells, the need exists for development of standardized efficacy testing procedures of anti-cancer agents.

Extensive metabolism of AP PP during a 24hr *in vitro* rumen incubation was found in the present study. Therefore, with the long-term goal of developing a naturally-derived polyphenol-rich milk product in mind, a very likely chance exists that the final polyphenols secreted in the milk of a dairy cow will be conjugated metabolites with a fraction of the original compounds' activity, if any. Many steps of method development and validation will have to be taken along the way to production of the final product for human consumption.

Since evidence from other studies points to metabolism of polyphenols by rumen microbes and possible absorption in the rumen, future studies should evaluate the abundance of their metabolites *in vitro* and the extent of their absorption into the plasma of lactating dairy cows *in vivo*. Ideally, a time course of shorter incubation times should be explored, as evidence from other studies suggests some ruminal absorption of Q and its metabolites within 1hr of ingestion. Once major metabolites of AP in the ruminant plasma have been identified, their transfer to the mammary gland could be determined by

biopsy of glandular tissue, followed by solid phase extraction and HPLC/UPLC analysis. Significant methodology for the extraction of these metabolites would need to be developed, using *ex vivo* studies, such as those with human breast biopsy samples, as guidance (Maubach et al. 2003).

Transfer of AP PP to milk and the effect of AP inclusion on milk production parameters and composition would also need to be characterized through an *in vivo* feeding trial. The possibility exists for evaluation of transfer efficiency to milk coupled with anti-cancer effectiveness of metabolites secreted in milk by measuring the polyphenol-containing milk's anti-proliferative potential in CRC cells. Aseptic milk collection, possibly through a catheter, would have to be performed in order to prevent possible cell culture contamination. Additionally, pasteurization may have to be performed to sterilize the milk, but its effects on polyphenol structure, function, and stability would need to be evaluated. In addition to anti-proliferative assays, expression and activation of possible markers apoptotic markers, such as caspase-3, and regulators of MAPK pathways, could be quantified to further validate the health claims associated with PP containing milk products entering the market.

Depending on results of the above-mentioned studies, transfer efficiency of apple pomace polyphenols to milk in lactating dairy cows may need to be increased. This could be achieved through targeted nutrient delivery systems, such as encapsulating organogelators, which survive rumen digestion to deliver the intact apple pomace polyphenols into the small intestine for absorption. Research on rumen bypass potential

of these nutrient delivery systems containing plant phytochemicals is currently on-going in our laboratory.

Finally, sensory properties of PP-enriched milk would likely have to be improved, as PP possess a high protein-binding capacity, and will therefore result in undesirable precipitation of the milk protein-PP complexes. This effect could be masked by production of a cheese product, which would have to undergo sensory evaluation and validation of health-protective effects, also possibly through its effects on cancer cells.

CHAPTER SIX

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