Anthocyanins and Gingerols Exhibit Synergism in their Anticancer and Antioxidant Effects *in Vitro*

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

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ABSTRACT

Anthocyanins and gingerols are naturally occurring phenolic compounds abundant in berries and ginger, respectively. They have been reported to induce antioxidant effects, inhibit the growth of different cancer cell lines, and stimulate apoptosis by modulating different cellular signal pathways. However, low bioavailability limits the health benefits of anthocyanins. Meanwhile, gingerols potentially enhance bioavailability through regulating intestinal function and promoting absorption. This work is predicated on identifying a synergistic effect between these two compounds to increase their bioactive effects, the combined effect of which has not been reported. Evidence-based scientific evaluations are needed to explore their combined efficacy. This thesis aims to demonstrate and characterize the possible synergistic interaction between anthocyanins and gingerols (Ac-G). The Ac-G combinations were tested in cellular models *in vitro* for their anticancer and antioxidant effects at different effective doses and combination ratios.

In the first study, the growth inhibitory effects and selectivity of Ac-G combination and individual extracts were evaluated by measuring the cell viability of treated Caco-2, Hep G2, and HT-29 cancer cells compared to normal cells CCD33Co. Strong synergism was observed between anthocyanins and gingerols, at Ac-G w/w ratios of 16:1, 8:1, and 4:1, against the growth of Caco-2 and Hep G2 cells, at inhibition levels of ≥80%, as reflected by the low combination index (CI) values of 0.3−0.7, and dose reduction index (DRI) values up to 3-fold for anthocyanin and up to 103-fold for gingerols. Moreover, Ac-G combinations did not show toxicity on normal cells CCD-33Co. These results support the potential synergism of the anthocyanins and gingerols combination for an anticancer effect.

To explore the underlying mechanisms involved in the Ac-G synergistic interaction in inhibiting the growth of cancer cells, cell cycle and apoptotic pathways were explored. Significantly higher levels of cell cycle arrest at the S phase and more apoptotic cells were

observed for Caco-2 cells treated with Ac-G combinations (50+3.125)- (50+25) µg/mL, and for Hep G2 cells treated with Ac-G combinations (100+25) and (100+50) µg/mL. The synergism in the growth inhibitory effects of Ac-G combinations on Caco-2 cancer cells was mediated by p53-dependent and p53-independent pathways. Ac-G combinations induced apparent increases in the expression of p21 and reduced expression of the cyclin-cyclin dependent kinase (CDK) complex components, CDK-2, cdc25A, CHK1, and cyclin A. The apoptotic response was mediated by increased expression of Caspas3, Caspas8, Bid, and Bax. Thus, the substantial increase in the expression of the cellular CDK inhibitor p21 induced by the Ac-G combination represents remarkable anti-tumor efficacy and selectivity.

Ac-G combinations were subsequently tested for their immediate cellular antioxidant activity (CAA) and cytoprotective effects against oxidative stress on Caco-2 cells. Synergism was observed for all Ac-G combined dosages of (1+0.06) – (1+1) µg/mL in the CAA and the cytoprotective effects with synergistic effect indicator (SE) values of up to 1.6. These results might provide evidence for potential usage of the Ac-G combinations to support the endogenous antioxidant system protecting the normal physiological redox status. An evaluation of the cellular antioxidant defense mechanisms involved in the Ac-G synergistic interaction indicates that Ac-G combinations efficiently boosted the cellular antioxidant defense mechanisms by reducing cellular ROS generation, lipid peroxidation, and oxidant-induced activity of GPx enzyme and simulating cellular production of natural antioxidant compounds such as glutathione. The higher effects of Ac-G combinations on these exogenous and endogenous antioxidant biomarkers represent a high potential for synergistic antioxidant activities by combining anthocyanins and gingerols.

Overall, the present research demonstrates synergism between anthocyanins and gingerols in their anticancer and antioxidant activities in cell models. Furthermore, this study reported some

molecular mechanisms related to cell cycle arrest and apoptosis, and activation of antioxidant defense mechanisms involved in the Ac-G synergistic effects. Identifying effective combination ratios and doses and understanding these mechanisms could help better guide the use of combination strategies in novel supplements or food products. This gives justification for further *in vivo* studies. Positive results validated through animal models could enable Ac-G combination usage as natural health products and potential concurrent supplements for cancer patients.

PREFACE

This thesis is an original work done by Amna Emhemed Abdurrahim under the supervision of Dr. Lingyun Chen and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta.

This thesis consists of seven chapters. Chapter 1 provides a general introduction to the rationale of this research and its objectives. Chapter 2 includes a literature review on phytochemicals in general as natural health products, cancer initiation, progression, and prevention, an introduction to anthocyanins and gingerols and their bioactivities, and an introduction to synergism and methods to determine synergism.

Chapter 3 studied the synergism between anthocyanins and gingerols in inhibiting the growth of cancer cells *in vitro*. It has been published as Amna E. Abdurrahim, Feral Temelli, Vera Mazurak, Ramadan A. Benruwin, and Lingyun Chen. "Anthocyanin and gingerol extracts exhibit a synergistic effect to inhibit the proliferation of Caco-2, Hep G2, and HT-29 cells *in vitro*." in the journal of ACS Food Science & Technology 1, no. 9 (2021): 1642-1651. As the first author, I was responsible for the conceptualization, experimental design, performing the experiments and validation, data analysis, data interpretation and visualization, and writing the original draft, editing, and revising the manuscript. Dr. Temelli assisted in the methodology regarding the extract identification, and review and editing the manuscript. Dr. Mazurak assisted in the data interpretation and writing and editing the manuscript. Dr. Ben-Ruwin assisted in the methodology, data analysis and interpretation. Dr. Chen is the corresponding author, who was responsible for the conceptualization, experimental design, resources, supervision, reviewing, and revising the manuscript.

Chapter 4 was submitted for consideration for publication as Amna E. Abdurrahim, Vera Mazurak, and Lingyun Chen. “Mechanisms mediating the synergistic anticancer effects of combined anthocyanins and gingerols”. As the first author, I was responsible for the experimental design, performing the experiments, data analysis and interpretation, and modeling and writing the first draft of the manuscript. Dr. Mazurak provided insightful comments on the experimental design and the discussion of the results as well as in the revision of the manuscript. Dr. Chen is the corresponding author and was responsible for the experimental design, resources, supervision, revisions, and submission of the manuscript.

Chapter 5 and 6 were submitted to Food Research International for consideration for publication as Amna E. Abdurrahim, Vera Mazurak, and Lingyun Chen. “Gingerols synergy with anthocyanins to induce antioxidant activity *in vitro*”. As the first author, I was responsible for the experimental design, performing the experiments, data analysis and interpretation. and writing the first draft of the manuscript. Dr. Mazurak provided insightful comments on the results and discussion section as well as in the revision of the manuscript. Dr. Chen is the corresponding author and was responsible for the experimental design, resources, supervision, revisions, and submission of the manuscript.

Chapter 7 summarizes the key findings and points out the anticipated significance of this research. It also proposes recommendations for future studies.

This thesis is dedicated to

the memory of my dad, who taught me never to give up,

my beloved mom for her non-conditional love,

my kind-hearted siblings and husband,

and my lovely kids Hanan and Ibrahim

for your being in my life.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my God almighty for the everyday blessing and care in my life and during this scientific and life-enriching journey.

I am grateful to my supervisor Dr. Lingyun Chen, for providing me the opportunity to do my research studies in her lab and for her encouragement, insightful advice, and guidance throughout my Ph.D. studies. Thank you very much for encouraging me to keep up to bring out my best and love for what I do.

I want to extend my gratitude to Dr. Feral Temelli and Dr. Vera Mazurak for being my supervisory committee members and for providing guidance and invaluable suggestions to complete my research studies.

I feel blessed to express my sincere thanks to Dr. Michael Gaenzle for serving as the arm's length examiner and to Dr. Trust Beta for accepting to be the external examiner for my dissertation defense.

I would also like to thank my family for always believing in me and supporting me endlessly. Thanks for the constant motivation throughout my program.

I am thankful to my friends, colleagues, and professors, whom I met during my program, for their knowledge sharing, support, and fun moments that made this journey exciting.

I would like to thank the Libyan North American Scholarship Program, the Natural Sciences and Engineering Research Council of Canada (NSERC), and my family for their financial support to proceed throughout my study.

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LIST OF ABBREVIATIONS

AAPH	2,2′-azobis (2-amidinopropane) dihydrochloride
Ac	Anthocyanin
Ac-G	Anthocyanin: gingerol combination
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAA	Cellular antioxidant activity
Cdc25A	Cell division cycle 25 A phosphatase
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CI	Combination index
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CVD	Cardiovascular disease
Cy	Cyanidin
Cy-3-glc	Cyanidin-3-glucoside
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
Dp	Delphinidin
DRI	Dose reduction index
EC_{50}	The 50% effective concentrations in antioxidant activity
EGCG	Epigallocatechin gallate
EMEM	Eagle's minimum essential medium
ERK	Extracellular regulated kinase
ER	Endoplasmic reticulum
EVOO-PE	Extra-virgin olive oil phenolic extract

Fa	Affected fraction
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
Fu	Un-affected fraction
G	Gingerols
GRAS	Generally regarded as safe
GPx	Glutathione peroxidase
HDL	High-density lipoproteins
IC_{50}	The half-maximal (50%) inhibitory concentration
JNK	c-Jun N-terminal kinases
LDL	low-density lipoproteins
LTA4H	Leukotriene A4 hydrolase
LD_{50}	Lethal dose of 50%
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase kinase
mRNA	Messenger ribonucleic acid
MTT	3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide
Mv	Malvidin
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	Nuclear factor kappa B
NHP	Natural health products
Nrf2	Erythroid 2-related factor 2
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pg	Pelargonidin
Pg-3-rut	Pelargonidin-3-rutinoside
PI	Propidium Iodide
Pn	Peonidin
Pt	Petunidin
p53	Tumor suppressor protein, TP53

p21	Cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1
p27	Cyclin-dependent kinase inhibitor 1B
Q-3-glc	Quercetin 3-β-D-glucoside
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase-high performance liquid chromatography
SE	Synergistic effect
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant capacity
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
UVB	Ultraviolet light B

Chapter 1: Introduction and thesis objectives

1.1 The rationale of the research

Phytochemicals are natural bioactive ingredients. They are secondary metabolites in plants that protect them against pathogens and ultraviolet radiation (Scalbert et al., 2011). Plant foods such as vegetables, fruits, herbs, whole grains, and nuts provide nontoxic, inexpensive, and readily available sources of phytochemicals. Phenolic compounds are one of the most common groups of phytochemicals (Xiao & Bai, 2019). Epidemiological studies suggested a positive association between consuming a diet rich in fruits and vegetables and a lower incidence of chronic diseases (Mathew et al., 2004). In addition, phytochemicals have been shown to reduce the risks associated with chronic conditions, such as cardiovascular diseases and cancer (Gonzalez-Vallinas et al., 2013).

Synergistic interactions contribute to the health benefits of phytochemicals in fruits and vegetables (Alam et al., 2018). For example, research has demonstrated the efficacy of polyphenolic compounds and their potential to reduce many cancer aspects despite their low bioavailability. Further, the most recent animal and human studies suggested the synergistic effects within polyphenolic compounds and other phytochemicals to show considerably higher antioxidant, anticancer, and anti-inflammatory effects (Breda & Kok, 2018). Therefore, combining phytochemicals such as phenolic compounds has been suggested to strengthen their effects and provide better options to fight cancers such as colorectal cancer and other chronic conditions with lesser toxicity (Alam et al., 2018).

Over the past few decades, there has been a marked increase in chronic diseases due to sedentary lifestyles, fast food consumption, and stress. Conventional drug therapies exhibit various side effects, such as in the case of cancer therapies. This signifies the need to explore and discover

alternative preventive and therapeutic strategies. For prevention of chronic disease, lifestyle changes, including increasing dietary intake of nutrient rich foods, such as fruits and vegetables are recommended.

Nutraceuticals and dietary supplements have been recognized for their potential role in improving health quality and preventing disease. Canada, with more than 750 Canadian companies specialized in natural health products and functional foods in 2011, has emerged as a global supplier of nutraceuticals (Chopra et al., 2022). The Canadian nutraceuticals market is expected to grow by 5.62% between 2019 and 2024 due to the rising demand for nutraceuticals (Chopra et al., 2022). The highest amounts of fruits produced in Canada in 2019 after apples were blueberries, cranberries, and grapes, with a high content of polyphenolic compounds. The production of these fruits in Canada increased by 71, 79, and 80%, respectively, from 2009 to 2019 (Statistics Canada, 2022). This production increase has been driven by increased consumption of these fruits due to the increased public health consciousness and their reported health benefits, including the prevention of cancer and cardiovascular diseases. The health benefits of berries are attributed to the high phenolic content, mainly anthocyanins.

Anthocyanins are polyphenolic compounds responsible for the blue and red colors of plants. They are the glycosides of various anthocyanidins (Castaneda-Ovando et al., 2009). Studies have demonstrated bioactivities of anthocyanins, such as antioxidant and anticancer properties (Wang & Stoner, 2008). In light of their chemo-preventive efficacy against cancer, anthocyanins have shown potential for therapeutic applications (Lin et al., 2017; Thomasset et al., 2009). Fruits are a good source of anthocyanins; for instance, 100 g of berries can provide 200-1200 mg of anthocyanins (Bornsek et al., 2012). However, their low bioavailability has limited the health benefits of anthocyanins from food. The effective concentrations required to stimulate apoptosis

and inhibit malignant cell growth *in vitro* are about 10^3-folds higher than the detected concentrations in human plasma after consuming anthocyanins (Wang & Stoner, 2008). This low bioavailability of anthocyanins is attributed to their low permeability and their degradation into phenolic acids and phenyl aldehydes under physiological conditions (Mueller et al., 2017).

Gingerols are the main bioactive components in ginger (rhizome of *Zingiber officinale* Roscoe, Zingiberaceae) that has been used for thousands of years as food, spice, and traditional herbal medicine (Khan et al., 2016). Ginger is grown in tropical and subtropical regions, but due to its fresh and pungent taste, ginger has been used worldwide as a food seasoning (Masuda et al., 2004). Ginger has been reported in epidemiological and experimental studies to exhibit various bioactivities, such as antioxidant, anti-inflammatory, and anticancer activities. The main bioactive components that have been identified in ginger are 6-, 8-, and 10-gingerols and 6-shogaol, with 6-gingerol being the most abundant compound (Khan et al., 2016). The chemo-preventive effects of 6-gingerol present a promising alternative potential for therapeutic applications (Oyagbemi et al., 2010). Furthermore, ginger has been reported to enhance the absorption of several active components classified under nutrients, nutraceuticals, and different drug categories (Qazi et al., 2003). It exerts its effect either by regulating intestinal function by lowering food transit time in the gastrointestinal tract or promoting the absorption in the gut. Ginger stimulates bile acid production and secretion, and increases the activities of pancreatic lipase, amylase, and proteases in the gastrointestinal tract (Srinivasan, 2017). It also alternates brush border membrane fluidity and passive permeability property of the small intestine and increases the absorptive surface by increasing microvilli length and perimeter (Srinivasan, 2016).

Anthocyanins and gingerols are two groups of natural phytochemicals that show good antioxidant and anticancer activities demonstrated by *in vitro*, *in vivo*, and clinical studies (Pojer

et al., 2013; Srinivasan, 2017). Both anthocyanins and gingerols have been reported to induce antioxidant effects (Haniadka et al., 2013; Khan et al., 2016; Pojer et al., 2013; Toufektsian et al., 2008), inhibit the growth of different cancer cell lines, stimulate apoptosis and suppress inflammation (Lee et al., 2008; Lin et al., 2012; Mazewski et al., 2018; Radhakrishnan et al., 2014; Shin et al., 2009; Yi et al., 2005) through modulating different cellular signals pathways. Some pathways overlap, but some do not. The effects of anthocyanins and gingerols on some signaling pathways of colorectal cancer cell lines are summarized in **Figure 1.1**.

Collectively, this evidence suggests that there is potential for a synergistic effect between these two phenolic compounds. Toxicity is not observed at daily intake doses for either group. For example, 25 mg/kg per day of anthocyanins did not show any toxic effects in mice (Wallace & Giusti, 2015), and ginger is classified as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA) of USA (Khan et al., 2016). As far as is known, the combined effect of anthocyanins and gingerols has not been reported. Evidence-based scientific evaluations by appropriate models are needed to explore their combined efficacy. Therefore, it is worth investigating the potential synergism between anthocyanins and gingerols in terms of their antioxidant and anticancer activities. In addition, this may provide a strategy to counteract and address the issues associated with the poor bioavailability of anthocyanins and gingerols.

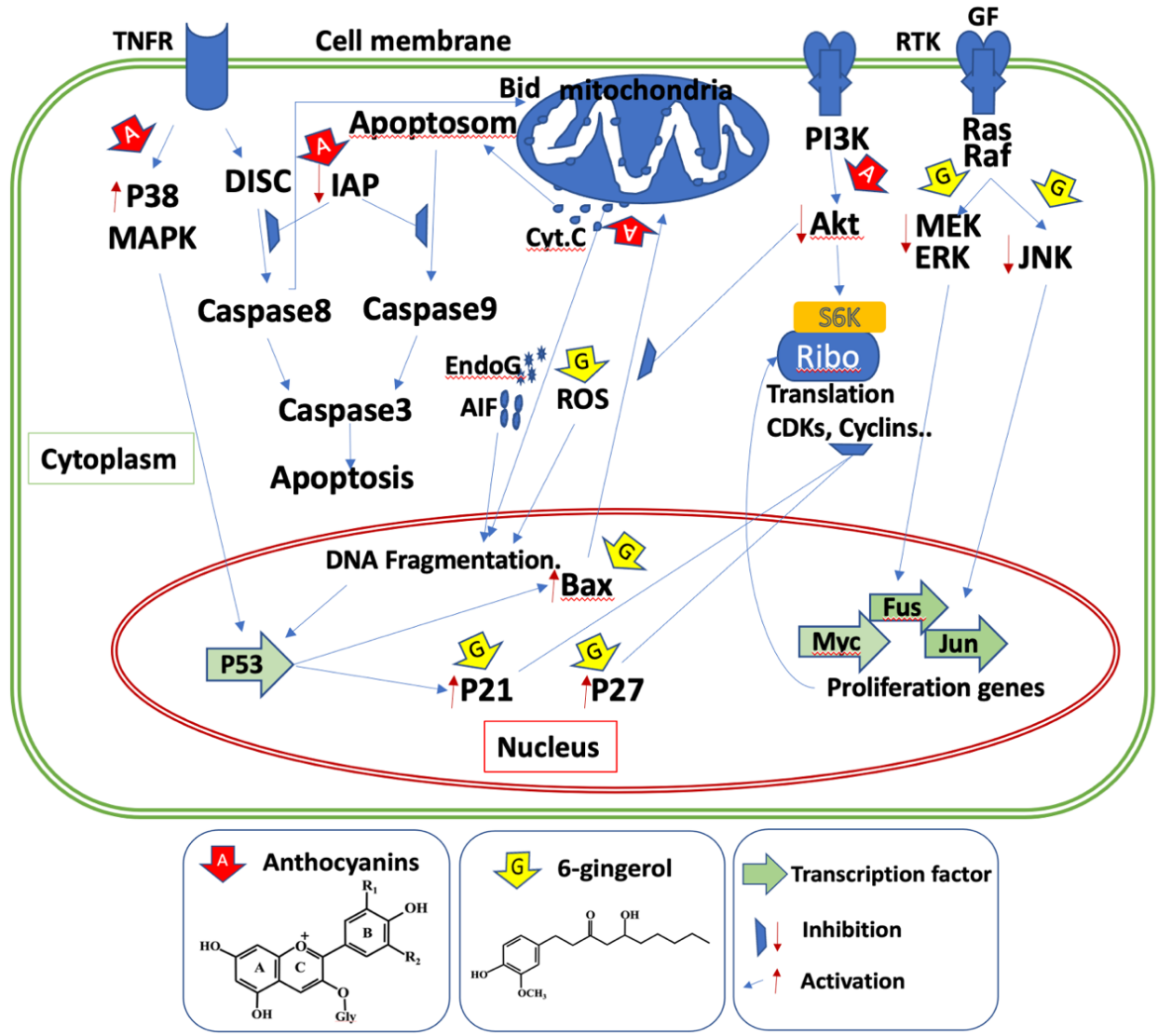


Figure 1.1. Effects of anthocyanins and gingerols on some signaling pathways of colorectal cancer cell lines from the literature (Lin et al., 2012; Mazewski et al., 2018; Radhakrishnan et al., 2014; Shin et al., 2009; Singh et al., 2016).

1.2 Hypothesis and objectives

The hypothesis is that combining anthocyanins and gingerols may lead to a synergistic effect against oxidative stress and cancer cell growth because the combined extracts may exert their effects through more cellular signaling pathways compared to each extract alone.

This work aimed to study the possible synergism between anthocyanins and gingerols to enhance their bioactivity against cancer cells and their antioxidant activity compared to individual anthocyanins or gingerols. The aim also includes providing insight into the molecular mechanisms and pathways involved if these synergistic interactions exist. To accomplish this goal, the specific objectives of this work were as follows:

1. To study the potential synergistic effect between anthocyanins and gingerols to inhibit the proliferation of cancer cells *in vitro*.
2. To study the potential synergism between anthocyanins and gingerols to induce cellular antioxidant activities *in vitro*.
3. To study the mechanisms involved in the synergistic effects of anthocyanins-gingerols combinations in inhibiting cell growth, inducing apoptotic response, and increasing their antioxidant activity.

1.3 Expected significance

One of the most popular topics of research these days is discovering additional antioxidant sources, mainly from natural products, and finding potential ways to enhance the bioavailability of such antioxidants. This study aimed to combine anthocyanins and gingerols from berries and ginger plants, respectively, to maximize their antitumor and antioxidant activities. If so demonstrated, this research would be the first to demonstrate synergism between anthocyanin and gingerol extracts for their anticarcinogenic and antioxidant activities. This work focused on *in vitro* cellular models as a first step in gaining the scientific basis for further animal and clinical studies. Furthermore, this research will help to understand the effective dosage and combination ratios of anthocyanins and gingerols to exert the synergistic effect. Therefore, these outcomes are beneficial in guiding applications development in the food and natural health products industries.

1.4 Chapter format

In this thesis research, the combined effects of anthocyanins and gingerols in inhibiting the growth of cancer cells and inducing the antioxidant response were investigated. First, human colorectal adenocarcinoma cells (Caco-2 and HT-29) and hepatocellular carcinoma cells (Hep G2) were chosen as models of colon and liver cancerous cells, respectively, and used to evaluate the synergistic anticancer activity of anthocyanins and gingerols. The normal colon fibroblast CCD33Co cell line was used to represent normal calls. Next, the mechanisms involved in the synergistic interaction between anthocyanins and gingerols were studied by analyzing the effects of anthocyanin-gingerol (Ac-G) combinations on the cell cycle development and apoptosis signaling pathways. Then, the synergism between anthocyanins and gingerols on the antioxidant activity was assessed. Finally, the mechanisms involved in the antioxidant response induced by Ac-G combinations against oxidative stress, including the enzymatic and non-enzymatic cellular defenses, were evaluated. The following chapters were designed to achieve the specific objectives of this study, with a brief description of each chapter.

Chapter 1 introduces the background, knowledge gap, the research rationale, and the hypothesis and objectives to test the hypothesis.

Chapter 2 provides a literature review covering the different aspects of this study, starting with a brief overview of the health benefits of phytochemicals and their use as natural health products. This was followed by an introduction to the cancer initiation and progression, emphasizing the oxidative stress as an initiator, cell proliferation, cell cycle stages, and apoptosis. Then, anthocyanins and gingerols were introduced, including their chemical structure, bioavailability, and bioactivities. Next, the antioxidant and anticancer activities of anthocyanins and gingerols and the underlying mechanisms were discussed. This chapter also introduced the concept of synergism,

evaluation methods, and examples of synergy between phytochemicals and some mechanisms involved.

Chapter 3 tests the synergistic effects of anthocyanins and gingerols to inhibit the proliferation of cancer cells *in vitro*. First, the effects of anthocyanins and gingerols alone and in combinations to inhibit the growth of cancer cells, Caco-2, Hep G2, and HT-29, compared to normal cells CCD33Co were evaluated using a biochemical assay. Then synergism indicators combination index (CI) and dose reduction index (DRI) were determined using the Chou-Talalay combination index method. Objective 1 is addressed in this chapter.

Chapter 4 further studies the anticancer effect of anthocyanins and gingerols and their combinations to explore the underlying mechanisms involved in synergistic interaction in such activities. First, using flow cytometry assays, different Ac-G combination doses were tested for their effect in inducing cell cycle arrest and apoptosis on Caco-2 and Hep G2 cells. Then molecular signals mediating the Ac-G synergism to modulate the cell cycle progression and induce apoptosis in Caco-2 cells were evaluated using gene expression assays. Objective 3 is partially addressed in this chapter.

Chapter 5 tests the synergism between anthocyanins and gingerols in their antioxidant effects *in vitro*. The cellular antioxidant activities (CAA) of combined and individual anthocyanins and gingerols were evaluated in Caco-2 cells using a fluorescent assay. In addition, the cytoprotective effects were evaluated as markers for the cellular antioxidant capacity against oxidative stress using biochemical assays. Then synergistic effect (SE) indicator was determined for both assays. Objective 2 is addressed in this chapter.

In Chapter 6, the binary antioxidant effects of Ac-G combinations on the regulation of antioxidant defense mechanisms were studied at the molecular level, such as ROS generation, lipid

peroxidation, cellular glutathione content, and the activities of some antioxidant enzymes. Objective 3 is partially addressed in this chapter.

Chapter 7 concludes the key findings and points out the anticipated significance of this work. It also proposes recommendations for future studies.

Chapter 2: Literature review

2.1 Phytochemicals as natural health products

2.1.1 Phytochemicals in foods

Phytochemicals are natural bioactive ingredients expressed in plants as secondary metabolites that play an important role in protecting their host against pathogens, ultraviolet radiation, and signal transduction. Although thousands of phytochemicals exist in nature, only some have been isolated and identified (Scalbert et al., 2011).

Phytochemicals are often referred to as phytonutrients. Consuming a diet rich in plant foods such as vegetables, fruits, whole grains, nuts, seeds, legumes, and tea provides a milieu of phytochemicals. Thus, they are non-toxic, inexpensive, and readily available. The most common groups of phytochemicals in food are polyphenols, carotenoids, flavonoids, coumarins, indoles, isoflavones, lignans, catechins, phenolic acids, and stilbenoids (Xiao & Bai, 2019). The recommendations from public health sectors are to increase the consumption of colorful fruits and vegetables, although there are no specific dietary recommendations in Canada, the United States, or the European Union (Wallace & Giusti, 2015).

Polyphenols are the most abundant antioxidants in the human diet. They are characterized by the presence of many phenol units in their structures, also known as polyhydroxyphenols, and usually exist in plants as glycosides. Polyphenols are classified according to their chemical structures into phenolic acids, flavonoids (isoflavones, flavones, flavanols, and anthocyanidins), polyphenolic amides, and other polyphenols (Alam et al., 2018). A list of about 100 richest dietary sources of polyphenols, providing more than 1 mg of total polyphenols per serving, was established. These richest sources include various spices of dried herbs, cocoa products, dark-colored berries, olive, some seeds (flaxseed), nuts (chestnut, hazelnut), and some vegetables

(Pérez-Jiménez et al., 2010). Research has demonstrated the efficacy of polyphenolic compounds and their potential to reduce many cancer aspects despite their low bioavailability. Moreover, the most recent animal and human studies suggest synergistic effects within these compounds and other phytochemicals to show considerably higher antioxidant, anti-cancer, and anti-inflammatory effects (Breda & Kok, 2018).

2.1.2 Health benefits of phytochemicals (antioxidant and anticancer properties)

Phytochemicals (phytonutrients) are non-nutritive compounds but show disease-preventive properties. They have been proven to exert antioxidant, anti-inflammatory, anticancer, immunomodulatory, antimicrobial, and antiviral properties, all of which are beneficial to human health. Their health benefits have been revealed by epidemiological studies, where a consistent relationship has been established between heavy consumption of fruits and vegetables and a reduced risk of developing several diseases, including different types of cancer. Subsequently, more studies identified specific dietary phytochemicals that exert anticancer properties (Fantini et al., 2015; Russo, 2007).

Dietary phytochemicals act as antioxidants through their function by reducing generated reactive oxygen species (ROS), regulating the detoxifying enzymes, and activating different pathways (**Figure 2.1**). Most dietary phytochemicals protect the cells from the effects of ROS being generated continuously inside the cell and reduce oxidative DNA damage. They also regulate several detoxifying enzymes (e.g., GST, nicotinamide adenine dinucleotide phosphate oxidase (NADPH), and heme oxygenase). Moreover, they can regulate different transcription factors, including nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB), and they show an ability to interfere with the interaction between the transcription factors and their regulators. Dietary phytochemicals trigger different pathways, including PKC, PI3K,

mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases (JNK-MAPK), beta-catenin, and transforming growth factor-β (TGF-β) serine-threonine kinase (Russo, 2007).

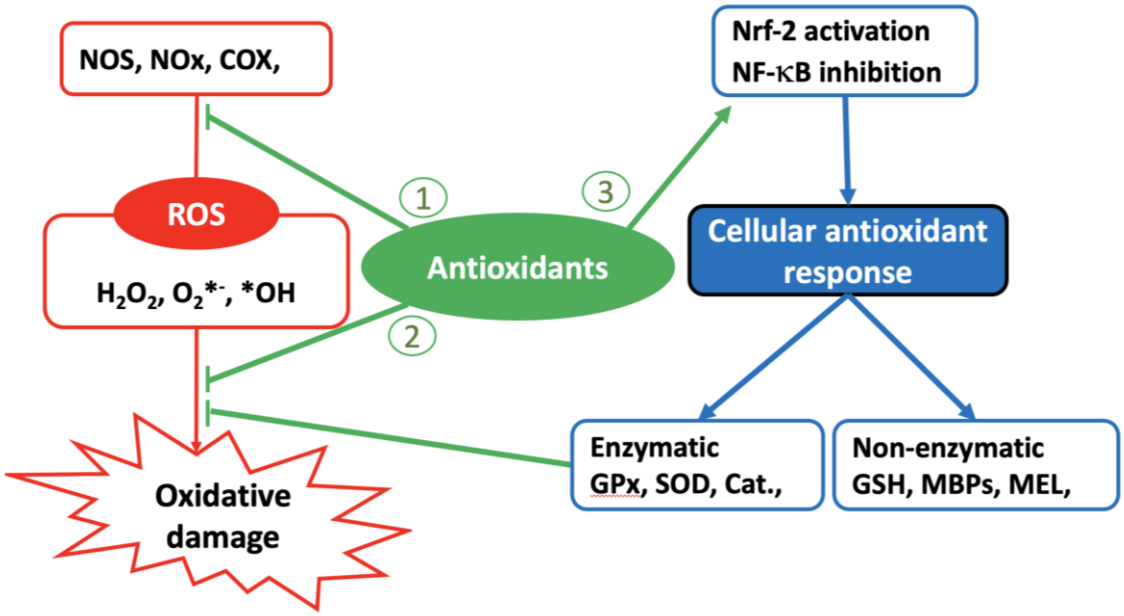


Figure 2.1. Different mechanisms of action of natural antioxidants, (Modified with permission from López-Alarcón & Denicola (2013)).

The relationship between dietary phytochemicals, reducing the risk of common chronic diseases, and improving health quality has been proven. For example, Battino et al. (2019) discussed and presented the evidence from *in vitro*, *in vivo*, and human studies about the role of some functional foods in the Mediterranean diet (olive oil, berries, and honey) rich in phytochemicals in the prevention of cancer and cardiovascular diseases. Dietary administration of 360 mg freeze-dried powder of black raspberries inhibited oral carcinogenesis in patients with biopsy-confirmed oral squamous cell carcinomas (OSCC) via inhibiting the pro-inflammatory and anti-apoptotic pathways (Knobloch et al., 2016). Anthocyanins were detected in all analyzed OSCC tissues. The extra-virgin olive oil phenolic extract (EVOO-PE), with a total phenol content

of 764 mg gallic acid equivalents/kg, inhibited the growth of breast cancer cells MCF-7, with an IC_{50} of 0.5 mg/mL, and induced intracellular ROS generation and apoptosis (Reboredo-Rodríguez et al., 2018). In another study, EVOO-PE inhibited cell proliferation on human urinary bladder cancer cells (T24 and 5637) with IC_{50} of 32 and 56 μg/mL, respectively, and showed a marked growth arrest at the G2/M phase (Coccia et al., 2016). Black raspberry extract supplementation reduced the high-fat diet-induced atherosclerotic formation in mice by improving blood lipid profile, reducing serum levels of triglycerides, total cholesterol, and lipid peroxides, reducing some inflammatory mediators, and inhibiting NF-κB (Kim et al., 2013).

Circadian rhythm is the biological activity rhythm in the body directed by the internal circadian clocks and affected by external signals. It is known that any disruption to the circadian rhythm leads to the development of chronic diseases, including obesity, metabolic syndrome, and cancer. Some phytochemicals have been proven to function as circadian rhythm modulators and demonstrated the ability to prevent and treat circadian rhythm disorder. These include quercetin, cinnamic acid, resveratrol, and caffeine (Xu & Lu, 2019).

Several chronic diseases, such as obesity, cardiovascular disease, diabetes, and fatty liver disease, are developed from metabolic syndrome. The most effective approach to managing this syndrome is by modifying diet and lifestyle. A relationship between phytochemicals and metabolic pathways, gut microbiota, obesity, and inflammation has been reported. For example, Alvarez-Suarez et al. (2014) reported that consumption of strawberries improved plasma lipids profile (triglycerides, total cholesterol, LDL, and HDL in blood, and platelet function in healthy subjects. A prospective longitudinal cohort study by Muraki et al. (2013) proved that a higher intake of the whole blueberry, grapes, and apple fruits was significantly associated with a reduced risk of type 2 diabetes. Zhao et al. (2019) reported the effects of microbial metabolites of dietary

phytochemicals in modulating glucose metabolism and confirmed the role of dietary bioactive phytochemicals in preventing and managing type 2 diabetes mellitus. Moreover, Xiao and Bai (2019) explained that phytochemicals with dietary fiber contribute to modulating gut microflora first by inhibiting the pathogens and promoting the growth of beneficial bacteria and then by influencing the beneficial bacteria to produce their metabolites, which could improve gut health, lipids profile, glycemic control, insulin resistance, and inflammation.

The immunomodulatory potential of phytochemicals in the tumor microenvironment has been reported in animal studies but remains to be determined in human clinical trials. Carotenoids, curcumin, resveratrol, epigallocatechin gallate (EGCG), and β-glucan, among phytochemicals, exhibited promising immune-modulating effects by enhancing natural killer and cytolytic T cells and inhibiting myeloid-derived suppressor cells (Pan et al., 2019).

Epidemiological studies suggested that higher consumption of fruits and vegetables, the major sources of antioxidant phytochemicals, could reduce cancer incidence (Willett & Trichopoulos, 1996). Furthermore, in a retrospective cohort study, the use of complementary alternative medicine (traditional food supplements, special diets, and vitamins) in patients with cancer was associated with a reduction in hospitalizations and antibiotic requirements; however, it was not associated with significant changes in hepatic and renal function (Chan et al., 2012). Furthermore, the use of phytochemicals in cancer chemoprevention has recently gained ample acknowledgment as alternatives to chemotherapeutic agents (Oyagbemi et al., 2010).

Phytochemicals have been divided into two main groups in terms of their anticarcinogenic activity: one group gives chemo-preventive effects by preventing carcinogens from attacking their cellular targets and then blocking cancer initiation through several mechanisms, including scavenging ROS, enhancing carcinogen detoxification, modifying carcinogen uptake and

metabolism, and enhancing DNA repair. The second group is cancer-suppressing agents, which inhibit cancer promotion and progression after the formation of pre-neoplastic cells by upregulating the cell cycle, signal transduction, and transcriptional processes and inducing apoptosis (Russo, 2007).

Among phytochemicals, polyphenolic compounds provide protection against cardiovascular diseases, neurodegenerative disorders, and various forms of cancer. Dietary sources of plant origin, including fruits, vegetables, herbs, spices, cereals, legumes, nuts, and olives, are rich in polyphenolic compounds. The anticancer potential of polyphenolic compounds has been well documented and their ability to modulate multiple biological mechanisms involved in cancer initiation and progression through direct interaction or modulation of gene expression has been well studied (Fantini et al., 2015; Khan et al., 2020). For example, curcumin, a polyphenolic compound in the *Curcuma* plant, has shown an anti-proliferative effect in many cancers. Curcumin induces anti-cancer activities through various pathways involved in mutagenesis, cell cycle regulation, apoptosis, and metastasis, such as inhibiting the transcription factor NF-κB and down streaming gene expression of c-myc, Bcl-2, Cyclin D1, COX-2, NOS, TNF-α, and interleukins (Alinezhad et al., 2017). Quercetin, a polyphenolic compound extensively observed in different foods, including nuts, teas, and vegetables, has been evidenced to prevent ovarian cancer through mechanisms including anti-proliferation, cell cycle arrest, anti-inflammation, and pro-oxidation (Vafadar et al., 2020). Quercetin induced apoptotic and necrotic cell death in prostate cancer cells LNCaP, DU-145, and PC-3, without affecting normal prostate epithelial cells, by modulating ROS, Akt, and NF-κB pathways and affecting the mitochondrial integrity (Ward et al., 2018).

Poor bioavailability is the main issue that prevents polyphenols from being used as an anticancer agent, mainly when single polyphenols are used. Therefore, combining polyphenols or

polyphenols and anticancer drugs are suggested approaches to counteract the issue of low bioavailability (Fantini et al., 2015). Moreover, as polyphenols have existed in the human diet for thousands of years without any side effects, it is thought that combining active polyphenols with targeted therapy may help in overcoming drug resistance and reducing side effects in many cancers (Alam et al., 2018).

2.1.3 Phytochemicals in functional food and natural health products (NHP)

The vast diversity of phytochemicals in the structure and sources make them renewable resources for discovering their novel biological activities and potential functional foods (Xiao & Bai, 2019). For example, phenolic compounds gained high importance due to their antioxidant activities and possible usage in processed foods as natural antioxidants. As a result, they attracted more attention to be used in many industrial applications as food preservatives and natural colorants. Furthermore, the food industry has increased interest in developing functional foods from plant polyphenols, and they are inspiring the need for more information about their bioavailability and efficacy (Ignat et al., 2011).

Establishing the scientific rationale for using phytochemicals as potential nutraceutical ingredients was the primary focus of many studies. Recent advances in identification, quantification, and standardizing extraction techniques of phytochemicals helped to incorporate phytochemicals in food products with higher stability and establish the commercial use of phytochemicals in the food industry (Sharma et al., 2019).

The concept of functional foods and nutraceuticals is not very new in food and nutrition research. However, using nutraceuticals to prevent and cure human diseases has recently gained importance. Nowadays, pharmaceuticals are gradually shifting towards nutraceuticals to explore a natural remedy for each health problem. In addition, the food industries are also turning towards

using bioactive components as food additives to improve food products' flavor, texture, and nutritional value (Banerjee & Ray, 2019). Health Canada identifies functional foods as ‘Similar in appearance to conventional food, consumed as part of the usual diet, with demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions.’ (Jones & Bourque, 2003). Natural health products (NHPs) are naturally occurring substances used to maintain or restore good health. They are often made from plants, in addition to microorganisms, animal, and marine sources (Harrison, 2019). In Canada, the term NHPs is used for regulated health products such as vitamin and mineral supplements, plant-based health products, traditional medicines, homeopathic medicines, probiotics and enzymes, amino acids and essential fatty acids, and personal care products containing natural ingredients. NHPs come in various forms, including tablets, capsules, solutions, drops, creams, and ointments (Harrison, 2019). Health Canada is regulating NHPs under the drug and health products regulations. Clear and easy to read and understand labels are required as an essential tool that assists consumers in making informed health choices when selecting and using NHPs (Health Canada, 2022).

The use of nutraceuticals and functional foods worldwide has been reported to continue to increase due to several factors, such as the historical and recent reports of significant biological and clinical benefits of using these products. There is also a growing awareness of the potential role that nutraceuticals and dietary supplements play in improving health quality and reducing health risks. Production of functional foods has been recognized as a priority for the global food industry due to the changes in population demographics, such as increased education and life expectancy and the improvement in healthcare that increased the diet and health consciousness within consumers. Health consciousness has been the most important stimulating factor for the growth of the global nutraceutical and functional food industry (Basu et al., 2007). The most

significant limitations for the growth in this sector result from the need to properly assess and label the health effects of nutraceutical and functional foods (Basu et al., 2007).

As a result of the increased consumption of nutraceuticals and functional foods worldwide, they have become a multi-billion-dollar industry and anticipated to expand rapidly over the next decade. According to a report by Grand View Research, the global nutraceuticals market was valued at US$ 454.55 billion in 2021 and is expected to grow at a compound annual growth rate (CAGR) of 9.0% from 2021 to 2030. With more than 750 Canadian companies specialized in natural health products and functional foods in 2011, Canada has emerged as a global supplier of nutraceuticals (Chopra et al., 2022). For example, several nutraceutical formulations from local Canadian berries such as cranberries, wild blueberries, and strawberries have been developed by registered producers such as Nutra Canada Inc., which was established in 2008. Functional foods and NHPs market size in Canada was estimated at US $18.29 billion in 2018 (Grand View Research, 2022), and it is expected to grow by 5.62% between 2019 and 2024 due to the rising demand for nutraceuticals (Chopra et al., 2022).

In Canada, berries and grapes are important fruits recognized for their high content of polyphenolic compounds. They are the most produced fruits in Canada in 2019 after apples, with quantities of blueberries (194,176 tons), cranberries (171,534 tons), and grapes (133,934 tons) shown in **Figure 2.2.A** (Statistics Canada, 2022). The production of blueberries, cranberries, and grapes in Canada increased by 71, 79, and 80%, respectively, from 2009 to 2019 (**Figure 2.2.B**) (Statistics Canada, 2022). The health benefits of berries are attributed to the high phenolic content, mainly anthocyanins.

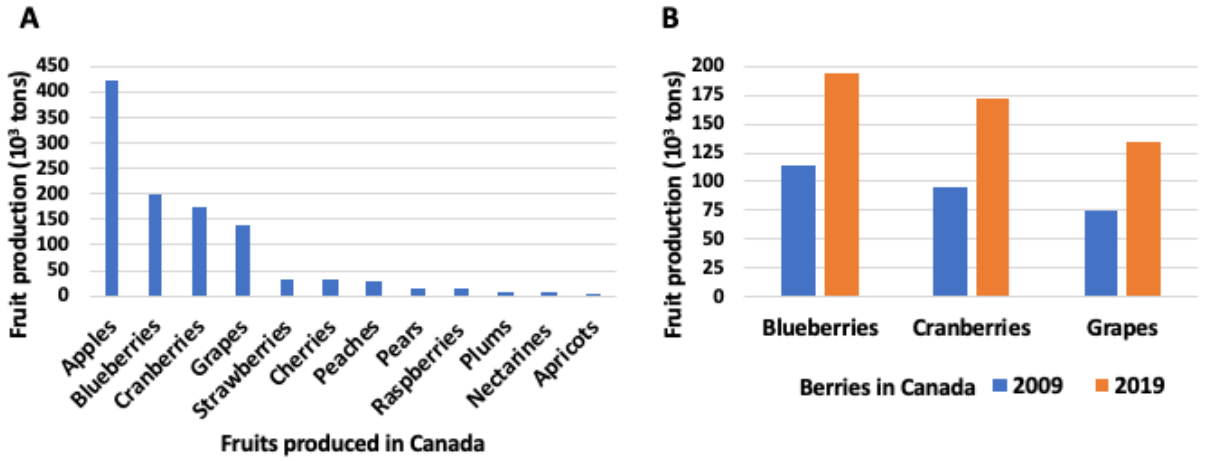


Figure 2.2. (A) Total fruit production in Canada in 2019, (B) Berry production in Canada in 2009 and 2019 (Statistics Canada, 2022).

2.2 Cancer initiation, progression, and prevention

2.2.1 Cancer initiation and progression

Cancer is defined as a large group of diseases that can affect any part of the body. They can be described as malignant tumors or neoplasms. The main feature of identifying cancer is the rapid growth of abnormal cells beyond their usual boundaries, which later can invade other body parts and organs. This process is referred to as metastasizing and is considered a major cause of death from cancer (WHO, 2021).

Carcinogenesis is a multistep process that requires accumulation of several mutations. As normal cells change progressively to neoplastic cells, they acquire new functional capabilities to allow them to survive and proliferate. Such functional capabilities form general hallmarks and explain the complexities of neoplastic diseases. These hallmarks include the independence of growth signal, insensitivity of growth suppressors, enabling limitless replication, resisting programmed cell death (apoptosis), inducing angiogenesis (formation of new blood vessels), and activating tissue invasion and metastasis, in addition to evading immune destruction and

reprogramming of energy metabolism (Hanahan & Weinberg, 2011). Genome instability is the underlying mechanism for the genetic diversity and the inflammation of neoplastic diseases, which facilitate and promote the acquisition of these hallmarks (Hanahan & Weinberg, 2011). Tumors develop through various stages of initiation and progression characterized by an increased metastatic potential of cells to dissociate from the tumor mass, spread to other organs, adhere, and colonize (Alam et al., 2018). **Figure 2.3** represents the progression phases of carcinogenesis.

Although many mechanisms are involved in tumor development and progression, dysregulation of cell proliferation and suppression of apoptosis are considered the leading platforms that promote neoplastic progression (Evan & Vousden, 2001). Moreover, oxidative stress and inflammation are believed to play an essential role in tumor development (Oyagbemi et al., 2010).

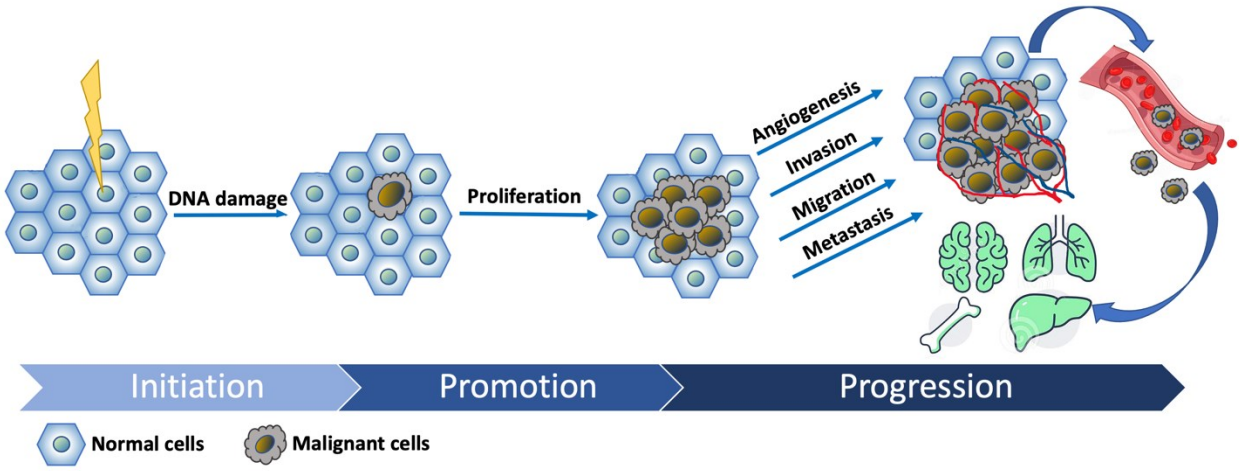


Figure 2.3. Progression phases of carcinogenesis (Modified with permission from Liu et al. (2015)).

2.2.2 Oxidative stress and diseases

Oxidative stress occurs in cells as a result of a physiological imbalance between the levels of free radicals (reactive species) and the body's endogenous antioxidants in favor of oxidants, where

the antioxidant defense mechanisms are overwhelmed by the increased production of free radicals. Then, the oxidative environment triggers the oxidation of cellular biomolecules such as DNA, protein, and lipids, leading to different disease conditions (Ighodaro & Akinloye, 2018; Rahman, 2007).

Free radicals are unstable, reactive chemical species that tend to interact with adjacent molecules, such as proteins, lipids, carbohydrates, and nucleic acids. Oxygen-free radicals, “reactive oxygen species” (ROS), are the main by-products formed in aerobic organisms' cells and the primary free radicals that damage biological systems. ROS can be generated endogenously through various mechanisms such as by-products of mitochondrial catalyzed electron transport reactions and in the inflammatory cell activation process. They can also be produced exogenously (Rahman, 2007).

It has been established that ROS have physiological roles in several cellular signaling systems, such as the cellular responses in the defense against infectious agents. Contrarily, increased production of ROS can be harmful and damage cellular structures. The body's endogenous antioxidant defense systems with input from co-factors and by ingesting exogenous antioxidants are capable of balancing the ROS and counteracting their oxidative damages (Rahman, 2007). However, if the generation of free radicals exceeds the protective effects of antioxidants and overwhelms the cellular defense mechanisms, then oxidative stress would be developed and could cause cell injuries (Rahman, 2007; Sies, 2018).

Physiologically oxidative stress was classified into two categories, oxidative eustress, and oxidative distress. Low-level oxidative stress could affect redox signaling and regulation and is called oxidative eustress; meanwhile, oxidative distress is characterized by a higher level of

oxidative stress that leads to disrupted redox signaling and oxidative damage to the biomolecules (Sies, 2018).

Oxidative stress is a precondition for the development of many chronic diseases, including cancer, cardiovascular disease (CVD), atherosclerosis, stroke, and neurodegenerative disorders. It can affect the whole body at cellular levels causing changes in the DNA, proteins, and lipids. For example, oxidation of low-density lipoproteins (LDL) initiates atherosclerosis leading to CVD, and DNA damage due to oxidation is the initial step in carcinogenesis. Moreover, oxidative stress could irritate the inflammation, which plays an essential role in the tumor development (Ighodaro & Akinloye, 2018; Oyagbemi et al., 2010).

2.2.3 Cell proliferation and cell cycle progression

Cell proliferation is a normal process that occurs regularly within all somatic cells through the cell growth and division cycle. A precise balance exists in normal cells to control and promote growth signals when proliferation is required, such as an injury or tissue turnover, and cell proliferation will be terminated when it is no longer needed (Evan & Vousden, 2001). Any errors that lead to dysregulation of this process could either drive cells to suicide through apoptosis or loss of control of cell division and keep proliferating, mostly leading to cancer progression. In tumor cells, the control of the cell proliferation process will be lost, cells may lose their ability to differentiate, and then cells would continually proliferate (Evan & Vousden, 2001; Hanahan & Weinberg, 2011).

The cell cycle process is a series of highly regulated events that occur in a cell leading to its replication and division, and it includes four phases (**Figure 2.4**). Through the cell cycle, a cell in G0 (resting) phase, after receiving specific stimuli, synthesizes mRNA and proteins required for DNA synthesis in the G1 (gap 1) phase and then replicates DNA in the S (synthesis) phase. In the

G2 (gap 2) phase, the cell replicates some of its organelles, and in the M (mitosis) phase, the cell divides its components into two identical cells. G2 and M phases are characterized by the double set of chromosomes and are recognized as the G2/M phase. After cell division, each daughter cell could begin a new cycle or stop dividing and enter a state of quiescence or senescence (G0). Cell cycle transitions between different phases are firmly controlled through checkpoints, which determine the optimal conditions to proceed to the next phase (Hanahan & Weinberg, 2011; Pillat et al., 2013).

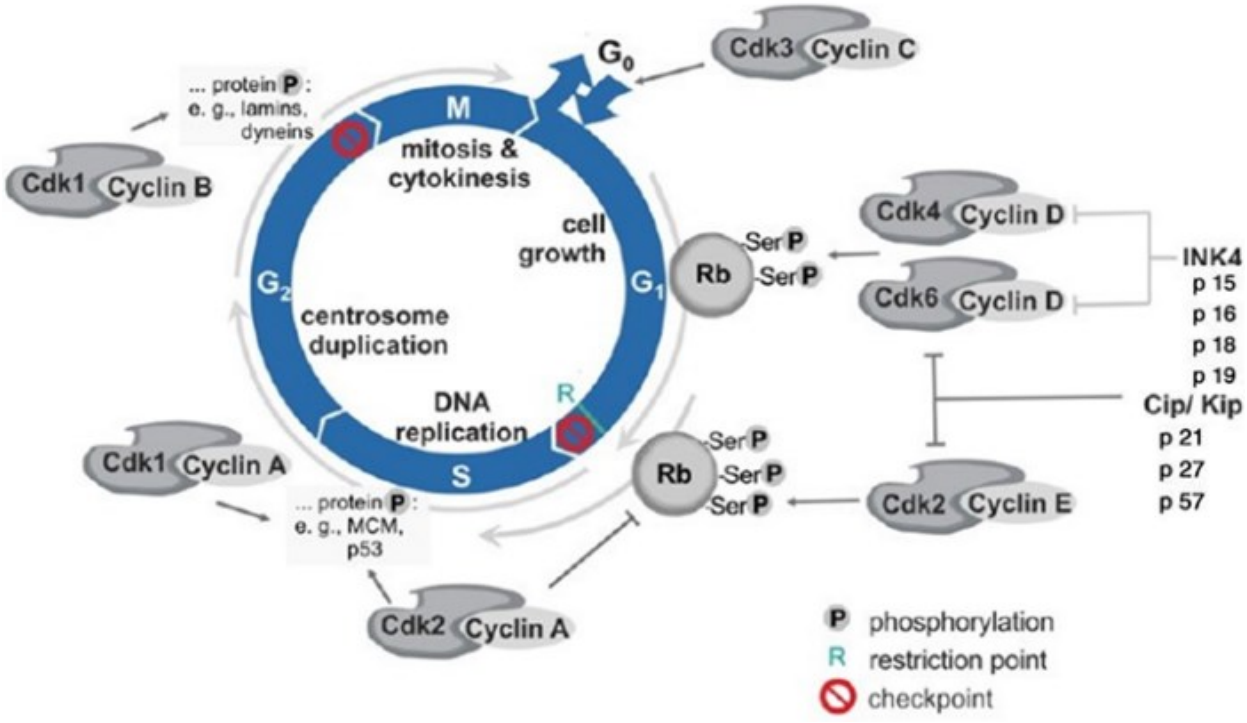


Figure 2.4. Overview of human cell cycle activation and transcriptional regulation through Cyclin- cyclin-dependent kinase (CDK) complexes (reprinted with permission from Graf et al. (2011)).

2.2.4 Programmed cell death (apoptosis)

Cell death could be regulated by specific molecular signals that can be modulated. It can also occur accidentally due to severe exposure to chemical or physical stresses (Galluzzi et al., 2016). The regulated (programmed) cell death (apoptosis) is a vital physiological process in the cells for normal development. It plays an essential role in eliminating damaged and tumor cells. Thus, apoptosis suppression is a highly acquired attribute in cancer cells (Evan & Vousden, 2001; Galluzzi et al., 2018).

In the apoptotic cell death, there are mainly two pathways intrinsic (mitochondrial) and extrinsic (FAS and FAS ligand) pathways (**Figure 2.5**). Cells receive either internal stimuli such as DNA damage, endoplasmic reticulum stress, or external stimuli such as ROS and growth factor withdrawal to activate some signal transduction cascades leading to their collapse. Multiple apoptotic cell death modes could be initiated and characterized by different molecular mechanisms, which exhibit considerable interconnectivity and overlapping (Galluzzi et al., 2018). Many cellular signals are involved in the apoptosis process. Bcl family of proteins, caspase signaling proteins, and p53 genes are key factors in regulating apoptosis.

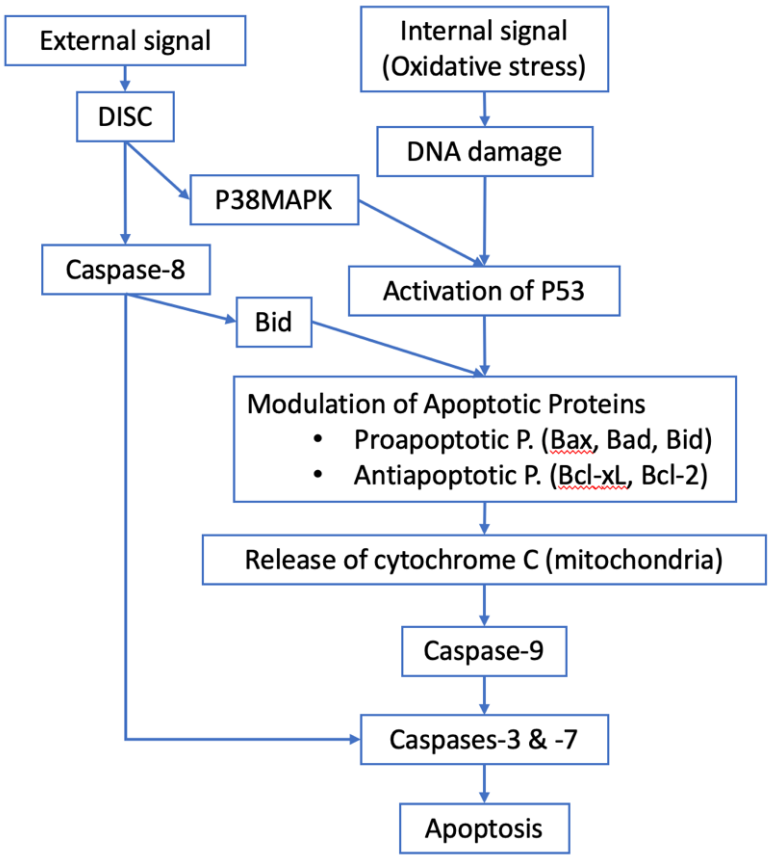


Figure 2.5. A flowchart of apoptosis extrinsic and intrinsic signaling pathways of caspase activation

2.2.5 Cancer preventive effects of phytochemicals

Cancer is the second leading cause of death globally, with an estimated 9.6 million deaths in 2018 (WHO, 2021). This makes cancer one of the most devastating diseases of this time. However, while it is clear that cancer is a gene expression disease, only a few cancers appear to be related to inherited gene mutations. Instead, research suggest that a combination of genetic and environmental (lifestyle and behavioral) factors are involved in the development of cancers (Singh et al., 2014). Diet is one of the major environmental factors affecting cancer development in addition to lifestyle habits (Johnson, 2007), which together cause geographical variations observed in the incidence of different cancers. For example, although the alimentary tract mainly, is

vulnerable to cancers, colorectal cancer is one of the most common causes of death from cancer in industrialized countries (Johnson, 2007). According to WHO, colorectal cancer and hepatic cancer are the second and fourth most common cause of cancer death worldwide, respectively. Statistics show that colorectal cancer is the third most common cancer (1.80 million cases) worldwide after lung (2.09 million cases) and breast (2.09 million cases) cancers (WHO, 2021). It is the second most common cancer in Canada, accounting for 13% of all cancers in 2016 (Canadian Cancer-Statistics Advisory Committee, 2016). Such increased incidence of colorectal cancer was connected to the high intake of energy-rich foods and low physical activity levels (Stewart & Wild, 2014). Research indicated that carcinogenesis typically develops over decades and is curable when diagnosed at early stages (WHO, 2021). Chemotherapy and radiation, in addition to surgical procedures, are considered the mainstream cancer therapies, although they are associated with significant side effects (Khan et al., 2020).

On the other side, a growing body of evidence shows that consumption of phytochemicals from fruits and vegetables reduces the risk of chronic diseases, such as cardiovascular diseases, type 2 diabetes, and cancer (reviewed by Breda and Kok (2018); Gonzalez-Vallinas et al. (2013) and Wang et al. (2014)). In support of that, two health claims were approved by Health Canada back in 2000 for 'fruits, vegetables and cancer', and in 2016 for 'vegetables and fruits and heart disease'. In those claims, Health Canada has concluded that there is sufficient and convincing scientific evidence supporting the protective effect of fruits and vegetables against many types of cancers, and their impact on reducing the risk of heart disease, respectively. For example, epidemiological studies suggest a positive association between consuming a diet rich in fruits and vegetables and a lower incidence of colon cancers (Mathew et al., 2004). Moreover, a prospective cohort study by Wirfält et al. (2009) reported that a vegetable and fruit pattern and a fat-reduced

foods pattern were associated with reduced colorectal cancer incidence in men and women. Subsequently, specific dietary phytochemicals such as resveratrol, quercetin, anthocyanins and gingerols have been identified with anticancer properties, exhibiting promising effects on cancer prevention and treatment through different molecular targets (Breda & Kok, 2018; Gonzalez-Vallinas et al., 2013). A major focus of the beneficial effects of dietary phytochemicals is on the antioxidant effect and the defensive functions against excessive oxidative stress and damages induced by the reactive species (Middleton et al., 2000), which are involved in the initiation process of chronic diseases, including cancer. For example, quercetin in onion, apple, and broccoli possesses strong antioxidant activities by scavenging free radicals and activating detoxifying enzymes (Jan et al., 2010). Combined mechanisms of actions are also reported for phytochemicals (Breda & Kok, 2018). For example, ellagic acid, the main polyphenol in pomegranate, has been reported to have antiproliferative and apoptotic effects in addition to functioning as an antioxidant while inducing cell cycle arrest to impact atherosclerosis and cancer in experimental models (Larrosa et al., 2006; Yu et al., 2005).

Combining phytochemicals was suggested to strengthen their bioactivities, thus providing better options to fight cancer. In addition, combining phytochemicals with drugs might help overcome drug resistance (Alam et al., 2018). For example, epigallocatechin gallate and sulforaphane combinational treatment reduced cell viability of both cisplatin-sensitive (A2780) and cisplatin-resistant (A2780/CP20) ovarian cancer cell lines alone or in combination with cisplatin (Chen et al., 2013). Moreover, several mixtures of phytochemical-containing plant extracts have been reported to provide synergy considering their antioxidant activities *in vitro* biological models (Wang & Zhu, 2017).

2.3 Introduction to anthocyanins (Ac)

2.3.1 Historical changes in the concept of anthocyanins

Although early observation records of anthocyanins and unconscious use of formic acid for their extraction were reported by Wray (1670), the paper by Willstätter and Everest (1913) on the isolation of pigments of cornflowers has played a dramatic role in the transformation of the knowledge state about blue and red coloring substances of flowers and blossoms. Willstätter and Everest (1913) have clarified that although anthocyanins are non-nitrogenous, they form salts with strong acids. More research was conducted and led to more knowledge about the molecular structure of the anthocyanins, and many of them have been purely synthesized in laboratories (Robinson, 1935). Then after, due to methodology development in chromatography, considerable addition in the knowledge accrued on the distribution and identification of anthocyanins in plants, and these findings were compiled in a taxonomic order by Harborne (1967).

Color additives, including naturally occurring and synthetic organic dyes, have been used a long time ago to enhance the aesthetic value of foods, beverages, and cosmetics. The interest in natural colorants has increased significantly due to safety concerns regarding synthetic dyes (Huck & Wilkes, 1996). Thus, there was a particular interest in the food industry for natural alternatives. The attractive colors and high anthocyanin content in many plant sources make them a potentially desirable source of natural colorant. However, the low stability of anthocyanins has limited their use, whereas anthocyanins exhibit low stability in the tissues of their host and the manufacturing processes (Markakis, 1982). For example, Nagai (1917) found that anthocyanin was easily decomposed to a colorless compound when mixed with plant juice containing oxidizing enzymes or with the aqueous solution of potassium permanganate and hydrogen peroxide. Nebesky et al. (1949) identified oxygen and temperature as the most specific accelerating agents

in the degradation of anthocyanins in blueberry, cherry, currant, grape, strawberry, and raspberry juices. Such findings drove the research for more exploration among different sources to find alternatives with desirable stability and low cost (Shenoy, 1993).

In addition to the color attributes of anthocyanins, they have been reported to exert beneficial, healthy physiological effects. Wang et al. (1956) reported the antioxidant activity of sweet-potato anthocyanin *in vitro* and *in vivo*, in addition, to the growth inhibition of cancer S180 in mic. Kamei et al. (1995) reported that anthocyanins extracted from petals of several edible and nonedible flowers showed a greater growth inhibitory effect on tumor cells *in vitro* than other flavonoids. Anthocyanins were more effective in suppressing tumor cell growth than commercially synthesized aglycones of flavonoids.

In the study of Tsuda et al. (1996), three anthocyanin pigments and their aglycons showed strong antioxidant activity in a liposomal system and reduced the formation of malondialdehyde by UVB irradiation. Moreover, researchers indicated that the pigment structure influence the extent of antioxidative activity in the scavenging effect of hydroxyl radicals (·OH) and Superoxide anion radicals (O2−). Then, Cao et al. (1997) reported that more hydroxyl substitutions in the backbone structures of flavonoids, including anthocyanins, induced stronger antioxidant and prooxidant activities. Satué-Gracia et al. (1997) reported that several anthocyanins inhibited the oxidation of human low-density lipoproteins (LDL) and lecithin-liposome system, and antioxidant mechanisms such as hydrogen donation, metal chelation, and protein binding were suggested. Then, a study by Narayan et al. (1999) demonstrated the potency of anthocyanin to inhibit both enzymatic and non-enzymatic lipid peroxidation, thus preventing auto-oxidation of lipids in the food as well as lipid peroxidation in biological systems. Wang et al. (1999) reported that tart cherries anthocyanins and their aglycon exhibited *in vitro* antioxidant activities superior to vitamin

E and comparable to the antioxidant activities of tert-butylhydroquinone and butylated hydroxytoluene at 2 mM concentrations; in addition to their anti-inflammatory activities. The potential usage of phenolic compounds, including anthocyanins, in processed foods as a natural antioxidant, gave them high importance (Karakaya, 2004). Thereafter, food industries have increased interest in developing functional foods from plant polyphenols which inspired the need for more information about their bioavailability and efficacy (Milbury et al., 2002).

Pharmacokinetic study of dietary anthocyanins in humans was ignored in the past because of the assumption that the most common naturally occurring anthocyanins in plants, such as 3-O-glycoside and 3, 5-di-O-glycoside are non-absorbable in humans (Milbury et al., 2002). But later, developments in analytical methodology showed that anthocyanins were absorbed in their unchanged glycated forms, and they were bioavailable to humans (Cao & Prior, 1999). Moreover, Milbury et al. (2002) and Mazza et al. (2002) confirmed that anthocyanins could be absorbed in humans in their original glycosylated forms. These findings make anthocyanins different when compared to other polyphenols. Later, glucuronide, methyl, and sulfo-conjugate metabolites were detected and documented (Felgines et al., 2003; Kay et al., 2005; Wu et al., 2002), and researchers attributed the presence of unconjugated anthocyanins in circulation to the large dose interventions of anthocyanins, leading to the saturation of metabolic pathways. They also attributed the exclusive identification of anthocyanins as unmetabolized parent compounds to the instability of metabolized anthocyanins and insufficient extraction and detection systems (Kay, 2006). Animal studies demonstrated that absorption of anthocyanins happens in the stomach (Talavera et al., 2003) and intestine (Talavera et al., 2004). Fleschhut et al. (2006) indicated the important role of gut microflora in the biotransformation of anthocyanins. Glycosylated and acylated anthocyanins were rapidly degraded to the corresponding phenolic acids derived from the B-ring of the

anthocyanin skeleton after anaerobic incubation with a human fecal suspension (Fleschhut et al., 2006). Tsuda et al. (1999) reported a significant increase in plasma concentrations of protocatechuic acid in plasma to an 8-fold higher concentration than the parent anthocyanin following intake of cyanidin glycosides. Moreover, the accumulation of anthocyanins in tissues like the liver, eye, and brain tissues was reported by Kalt et al. (2008). Thus, these data proved that anthocyanins could be bioavailable for human health and wellness.

2.3.2 Chemical structure and properties of anthocyanins

Anthocyanins are secondary plant metabolites belonging to the flavonoid group of the polyphenols, responsible for plants' blue, red and purple colors (Fleschhut et al., 2006). They are glycosides of anthocyanidins. The anthocyanidins (aglycons), the basic structures of the anthocyanins (**Figure 2.6**), consist of a heterocyclic ring (C) that contains oxygen, bonded with an aromatic ring (A), and connected from the other side by a carbon-carbon bond to a third aromatic ring (B) (Castaneda-Ovando et al., 2009). The sugar moiety of anthocyanins is usually conjugated to the skeleton of anthocyanidin via the C3 hydroxyl group in ring C. The conjugated bonds in the anthocyanin structures are responsible for fruits and vegetables' bright red and blue colors (Wang & Stoner, 2008).

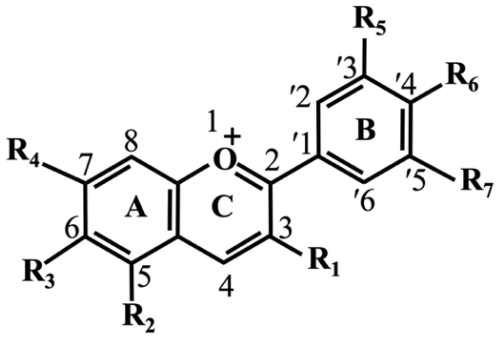
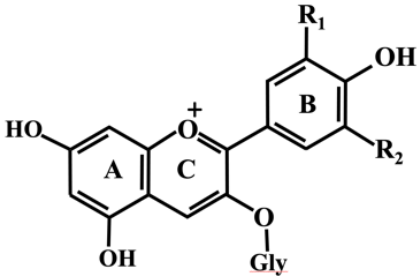


Figure 2.6. The general structure of anthocyanidins.

A huge variety of anthocyanins are found in nature. Up to 500 different anthocyanins have been reported. They differ in the number of hydroxyl groups, the nature and the number of bonded sugars, the aromatic carboxyl groups bonded to the sugar, and the positions of all these bonds. This enormous variety of anthocyanins makes them a complex but interesting group. The most abundant anthocyanins in fruits are the glycosides of cyanidin (Cy), delphinidin (Dp), malvidin (Mv), peonidin (Pn), and petunidin (Pt) (Castaneda-Ovando et al., 2009). Names and abbreviations of the most common anthocyanins are listed in **Table 2.1**.

Among these six common anthocyanin groups, the derivatives of the three non-methylated anthocyanins (Cy, Dp, and Pg) are the most common, with about 69% of the anthocyanins occurring in fruits. With that, Cy derivatives have the highest occurrence in fruits and vegetables with about 50% occurrence, and the most common cyanidin is the cyanidin-3-glucoside (Cy-3-glc) (Castaneda-Ovando et al., 2009).

Table 2.1. The general structure of anthocyanins and structural identification of the most common anthocyanins (Thomasset et al., 2009).

Name (anthocyanidins)	Abbreviations	Substitution pattern (anthocyanins)		
		 Gly = Glucose, galactose, or arabinose	R_1	R_2
Cyanidin	Cy		OH	H
Delphinidin	Dp		OH	OH
Malvidin	Mv		OCH_3	OCH_3
Pelargonidin	Pg		H	H
Peonidin	Pn		OCH_3	H
Petunidin	Pt		OCH_3	OH

2.3.3 Sources

Anthocyanins are widely available in fruits and vegetables in human diets. They exist in high concentrations in many of them, such as berries, cherries, red grapes and currants, red wines, blood oranges, the black varieties of soybeans, rice, beans, and the red varieties of onions, potatoes, and cabbage (Fantini et al., 2015).

Berries are a good source of anthocyanins; 100 g of bilberries and blueberries can provide 1210 and 212 mg of anthocyanins, respectively (Može et al., 2011). The occurrence of anthocyanins differs between berry species and varieties of the same species. For example, strawberry and lingonberry have a simple anthocyanin profile with one major anthocyanin. Black currants have four major components; blueberry, crowberry, and bilberry have complex anthocyanin profiles (Rein, 2005). Bilberry contains fifteen different anthocyanins, monoglycosides of Cy, Dp, Mv, Pn, and Pt, with three different monoglycosyl substituents of 3-galactoside (3-gal), 3-glucoside (3-glc), and 3-arabinoside (3-ara) (Kähkönen et al., 2003).

2.3.4 Bioactivities of anthocyanins

The anthocyanin group of natural phytochemicals exhibited several potential health effects, such as reducing the risks of cardiovascular disease, diabetes, and cancer. These health benefits of anthocyanins are attributed to many bioactivities, including antioxidant, anti-inflammatory, and anti-carcinogenic properties (Kamiloglu et al., 2015).

2.3.4.1 Antioxidant activity of anthocyanins

Anthocyanins exert a broad range of biological activities, including anti-carcinogenesis and anti-mutagenesis, mainly attributed to their antioxidant activities. The positive oxygen atom (oxonium) in the C ring of the anthocyanin molecule makes it a potent hydrogen−donating antioxidant compared to the other flavonoids (Kong et al., 2003). Moreover, the antioxidant

activity of anthocyanins depends on the chemical structure of these molecules, such as the phenolic structure, the number of hydroxyl groups, the catechol moiety in the B ring, the oxonium ion in the C ring, the pattern of hydroxylation, methylation, acylation and glycosylation (Yang et al., 2011). For example, the powerful antioxidant effect of anthocyanins is attributed to the presence of hydroxyl groups in ring B. Substituents of the B ring contribute to the antioxidant efficiency of anthocyanins in the order of $-OH > -OCH_3 >> -H$ (Rossetto et al., 2007). Meanwhile, glycosylation of anthocyanin decreases the radical scavenging activity as compared with aglycone (Fukumoto & Mazza, 2000). Moreover, the radical scavenging potential of anthocyanins is influenced by the pH of the surrounding matrix or tissue (Borkowski et al., 2005).

Anthocyanins exert their antioxidant effects through different mechanisms. They have free radical-scavenging capacity due to their ability to donate hydrogen (electron), which can bind with different ROS such as superoxide ($O_2\bullet^-$), peroxide (ROO^-), and hydroxyl radicals ($\bullet OH$) (Borkowski et al., 2005; Fukumoto & Mazza, 2000). Anthocyanins also enhance the internal antioxidant defense systems through different pathways. They reduce the oxidative damage in DNA and increase the activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Pojer et al., 2013), which leads to an increase in the glutathione content (Toufektsian et al., 2008). A reduced oxidative DNA damage and a significant increase in glutathione were observed in individuals who consumed an anthocyanin-rich juice (Weisel et al., 2006). Anthocyanins also reduce the formation of endogenous ROS. Steffen et al. (2008) reported that polyphenols, including anthocyanins, inhibit the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which activates $NO\bullet$-preserving action and lowers $O_2\bullet^-$ generation or increases $O_2\bullet^-$ scavenging activity. Excessive activity of NADPH oxidase would stimulate different pro-inflammatory and cytotoxic processes, leading to endothelial dysfunction and an

increase in the $O_2\bullet^-$ levels, which are implicated in the development of cardiovascular diseases. Endothelial NADPH oxidases have been implicated in the proliferating and apoptotic processes involved in formatting capillary-like structures and angiogenesis (Cai, 2005), which are induced during cancer progression.

Anthocyanins showed comparable antioxidant activity to the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and the natural antioxidants α-tocopherol (Fukumoto & Mazza, 2000), vitamin C, and vitamin E (Bagchi et al., 1998). García-Alonso et al. (2004) reported that the antioxidant activities of 3-glucosides of delphinidin, petunidin, and malvidin were 2-2.5 times higher than ascorbic acid and 3-5 times higher than Trolox, where antioxidant activities were assessed using three different chemical methods, including ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), and oxygen radical absorbance capacity (ORAC) assays. The isolated compounds, Cy-3-glc, Pg, and pelargonidin 3-rutinoside (Pg-3-rut), from strawberries showed the highest antioxidant activities (4922-7156 μM Trolox/mg) compared to other phenolic compounds in this extract (Zhang et al., 2008).

The antioxidant activity of anthocyanins can be considered a strong point for the rationale of their health effects. Anthocyanins from bilberry and blueberry showed cellular antioxidant activity at low 50% effective concentrations (EC_{50}) of< 1 μg/L, nM range, on different cell lines, including Caco-2 and Hep G2 (Bornsek et al., 2012). Cy 3-glc acted as a powerful intracellular antioxidant agent and a cardioprotective agent at EC_{50} of 0.9 nM after being transported via bilitranslocase into endothelial cells (Ziberna et al., 2012). Vitamin E-depleted rats showed an exceptional plasma antioxidant capacity after being fed a diet supplemented with anthocyanin-rich extract compared to the control group. Lower hepatic endogenous hydroperoxide and 8-hydroxy-2-deoxyguanoside,

which are indicators of lipid peroxidation and DNA damage, respectively, were also observed in the anthocyanin-treated rats (Ramirez-Tortosa et al., 2001). Epidemiological studies suggest that consumption of anthocyanin may reduce oxidative damage, which is involved in the pathogenesis of atherosclerosis, cancer, cardiovascular disease, and diabetes (Wang & Stoner, 2008). A reduced oxidative DNA damage and a significant increase in the reduced glutathione were observed in individuals who consumed an anthocyanin-rich juice (Weisel et al., 2006). It was suggested that dietary anthocyanins in the gastrointestinal tract might act by quenching ROS in local cells, preventing damage to the epithelial barrier as well as inhibiting protein expression levels of cyclooxygenase-2 (COX-2) and inflammation (He et al., 2005).

2.3.4.2 Anticancer properties of anthocyanins

Anthocyanins have been shown to exhibit anticarcinogenic and anticancer properties against multiple cancer cell lines *in vitro* (Yi et al., 2005; Zhang et al., 2005) and different tumor types *in vivo* (Kong et al., 2003). Anthocyanins extracted from different sources, or their constituents were tested for their cancer growth inhibitory effects in different cell lines. For example, antiproliferation effects were observed when colorectal cancer cell lines HT-29 and HCT-116 were treated with anthocyanin-rich extracts from blueberries (Yi et al., 2005), strawberries (Zhang et al., 2008), purple and red corn (Mazewski et al., 2017), red grape, black lentil and sorghum (Mazewski et al., 2018). In addition, anthocyanins also induced growth inhibition and apoptosis of human leukemia U937 cells (Lee et al., 2009) and Hep G2 liver cancer cells (Zhan et al., 2016).

Anthocyanins showed selectivity in inhibiting the growth and inducing the programmed cell death of cancer cells, with relatively little or no effect on the growth of normal cells (Feng et al., 2007; Zhao et al., 2004). For example, anthocyanin extracts of grapes, bilberries, or chokeberries,

at doses of 25 to 75 μg/mL, inhibited the growth of human colon cancer cells HT29 but not the non-malignant colon derived NCM460 (Zhao et al., 2004).

Anthocyanins exhibited higher anticancer activity compared to other phenolic compounds. For example, the anthocyanins fraction extracted from red wine was significantly more effective than other flavonoid fractions in inhibiting cell growth of human intestinal carcinoma (HCT-15) and gastric adenocarcinoma (AGS) cell lines (Kamei et al., 1998). In addition, cyanidin-3-glucoside and pelargonidin isolated from strawberry extract reduced the cell viability of human oral, colon, and prostate cancer cells to 50% at 100 μg/mL doses compared to the anthocyanins crude extract at 250 μg/mL (Zhang et al., 2008).

Anthocyanins' chemical structure affects their growth inhibitory activity. Jing et al. (2008) observed that nonacylated mono-glycosylated anthocyanins have greater growth inhibitory effects on the human colorectal adenocarcinoma cell line (HT-29), while anthocyanins with pelargonidin aglycone and triglycosylation exhibited the lowest effect. Their study tested anthocyanin-rich extracts with different anthocyanin profiles from different sources. They observed various degrees of growth inhibition (purple corn > chokeberry and bilberry > purple carrot and grape > radish and elderberry) with a 50% growth inhibitory concentration of ~ 14-100 μg of cy-3-glu equ/mL.

The potential anticancer activity of anthocyanins *in vitro* is attributed to multiple mechanisms, including cell cycle arrest and inducing pro-oxidant activity, inflammation (COX-2 enzymes), and apoptosis (Pojer et al., 2013).

2.3.4.2.1 Anthocyanins induce cell cycle arrest

The growth inhibitory effect of anthocyanin extracts was associated with the cell cycle arrest at different cell cycle phases in different cell lines. A significant dose-dependent decrease in the G2 phase was observed on Hep G2 cells after treatment with 25, 50, and 100% blueberry extract;

this was accompanied by a significant increase in the S phase when treated with 25% blueberry extract (Zhan et al., 2016). In the study of Mazewski et al. (2017), anthocyanin extracted from black lentil, sorghum, and grape increased the HT-29 cell cycle arrest at the G1 phase compared to the untreated cells. Lazze et al. (2004) reported that Dp inhibited HeLa and Caco-2 cell growth and caused a significant reduction of cells in the G1 phase at a dosage of 200 mM, accompanied by an accumulation of cells in the G2/M phase.

Various signal transduction pathways are involved in the growth inhibitory effect of anthocyanin extracts. For example, Chen et al. (2005) found that Pn 3-glc and Cy 3-glc showed strong inhibitory effects on cell growth via down-regulating CDK-1, CDK-2, cyclin B1, cyclin E, and cyclin D1, in addition to the activation of caspase-3 on the breast cancer cells HS578T. In addition, anthocyanins' inhibitory effects on human colon cancer HCT-116 cells were associated with activation of P38 mitogen-activated protein kinases (p38-MAPK) and suppression of Akt (Shin et al., 2009).

2.3.4.2.2 Anthocyanins induce pro-oxidant effect

Contradictory to their antioxidant effects, anthocyanins could induce an accumulation of ROS in cancer cells. Cy-3-rut showed selective toxicity in the leukemia cell line HL-60 due to its pro-oxidant effects (Feng et al., 2007). Hou et al. (2003) observed the prooxidant effects of some anthocyanidins in human leukemia cells (HL-60), which led to inducing apoptosis. Out of the six tested anthocyanidins representing the most common anthocyanins, only delphinidin, petunidin, and cyanidin induced apoptosis and DNA fragmentation. They suggested that the ortho-dihydroxy phenyl structure of anthocyanidins may contribute to the prooxidant activity and induction of apoptosis (Hou et al., 2003). Moreover, both delphinidin and cyanidin, though neither pelargonidin nor malvidin, were cytotoxic only on the metastatic human colorectal cancer cell lines (LoVo and

LoVo/ADR) by inducing cellular ROS accumulation and inactivation of the glutathione antioxidant system (Cvorovic et al., 2010).

Antioxidant and pro-oxidant activities have been reported for anthocyanins. The conditions where they induce each effect are not fully understood. Several factors might affect this, such as dosage, environmental pH, and the presence of some molecules such as proteins or lipoproteins. In the literature, the effective dosage reported for anthocyanins to induce anticancer activities *in vitro* were 200 mM, reported by Lazze et al. (2004) and 100-250 μg/mL, reported by Zhang et al. (2008). Those anticancer activities are mostly associated with pro-oxidant effects. Those doses were higher than the effective dosages reported for their antioxidant effects, such as 1 μg/L, reported by Bornsek et al. (2012) and 0.9 nM, reported by Ziberna et al. (2012). There is a need to understand the effect of these factors and the mechanisms that would help control this to benefit different applications.

2.3.4.2.3 Anthocyanins induce apoptosis

Apoptosis was induced in different cell lines such as colorectal cancer cells HCT-116 and HT-29 (Mazewski et al., 2018) and human leukemia cells HL-60 (Chang et al., 2005; Feng et al., 2007) when they were treated with anthocyanin-rich extracts. *In vitro* experiments proved that anthocyanins exert an apoptotic effect through both intrinsic and extrinsic pathways. For example, Chang et al. (2005) reported that anthocyanin-rich extract of Hibiscus induced apoptosis in the human leukemia cell line HL-60 via the p38-FasL and the mitochondrial pathways through phosphorylation in p38 and c-Jun, increased expression of tBid, Fas, and FasL and cytochrome c release. In another study, Feng et al. (2007) found that the cyanidin-3-rutinoside-induced accumulation of the intracellular ROS in HL-60 cells lead to the activation of p38 MAPK and JNK, in addition to the activation of the mitochondrial pathway mediated by down-regulation of

bim. In the study of Hou et al. (2003), induction of apoptosis in HL-60 cells by anthocyanidins was attributed to the oxidative stress-induced JNK signaling pathway, which involves induced generation of intracellular hydrogen peroxide, DNA fragmentation, activation of JNK phosphorylation, and caspase-3 gene expression.

Anthocyanins have been reported to also up-regulate some cell cycle signals in addition to inducing ROS generation and apoptosis signals on cancer cells. For example, Leon-Gonzalez et al. (2018) reported the effect of anthocyanin-rich bilberry extract to induce apoptosis in acute lymphoblastic leukemia cells by enhancing the ROS formation that induced increased expression in p73, p21 (CDK inhibitory proteins), and cleavage of caspase-3, which is an apoptotic pathway. Moreover, in the study of Anwar et al. (2016), anthocyanin decreased Caco-2 cell proliferation through upregulating p21 expression and induced apoptosis by increasing the intracellular ROS and activating caspase-3 cleavage.

All these studies reflect different responses from different cell lines when treated with anthocyanins as a crude extract or individual constituents. This implies that anthocyanins could activate various mechanisms. However, evidence from human studies remains inadequate (Thomasset et al., 2009; Wang et al., 2007). Epidemiological studies about the anticancer effects of anthocyanins in humans did not provide convincing evidence (Wang & Stoner, 2008). Meanwhile, interventional trials did not provide sufficient results to suggest a positive correlation (Pojer et al., 2013). However, anthocyanins have been shown to inhibit the growth of colon cancer tumors when biopsies of normal and tumor tissues were taken from a pre-surgical model, 25 colon cancer patients, before and after consuming 20 g x 3/day of black raspberry powder for 2–4 weeks. In addition, a reduction in the proliferation rates and increased apoptosis were observed in colon tumors compared to the normal-appearing tissues (Wang et al., 2007). In animal studies, when

ApcMin mice, a model of intestinal carcinogenesis representing the human familial adenomatous polyposis, were treated with cy-3-glc or anthocyanin from bilberries, their adenomas were decreased by 45% and 30%, respectively (Cooke et al., 2006), and when they were treated with cherry extract anthocyanins (375 to 3000 mg/kg diet), 74% fewer cecal tumors than the control group was observed (Kong et al., 2003).

2.3.5 Anthocyanin stability

Anthocyanins are unstable. Several factors can affect their stability, such as their chemical structure and concentration, pH, storage temperature, light, oxygen, solvents, and the presence of enzymes, flavonoids, proteins, and metallic ions (Rein, 2005). Different chemical forms of anthocyanin have been reported depending on the pH of the solution (Castaneda-Ovando et al., 2009). Anthocyanins have a high susceptibility to degradation. They could degrade when the pH value is higher than 7, and the degradation depends on their substituent groups. Mostly, they are degraded into their corresponding phenolic acid and aldehyde compounds (Castaneda-Ovando et al., 2009). In neutral pH conditions, the presence of extra hydroxyl or methoxyl groups are the elements that influence the anthocyanidins' stability. Pg is the most stable in neutral media among the six most common anthocyanidins (Fleschhut et al., 2006). On the other hand, because of the sugar molecules, monoglycosides and diglycosides derivatives (anthocyanins) are more stable than aglycons (Fleschhut et al., 2006). The resonant structure of flavylium ions, the main particles in the pH1 solution, is proposed to cause the intensity of the color shown by these molecules (Castaneda-Ovando et al., 2009).

The chemical instability of anthocyanins is one of the major limitations of their application. Anthocyanins’ susceptibility to degradation partially explains their poor bioavailability. Moreover, the instability of anthocyanins leads to color loss. Therefore, increasing anthocyanin stability is a

major challenge for food applications and a key to improving their bioavailability. Thus, different approaches have been proposed to preserve anthocyanins and enhance their bioavailability.

Delivery systems of different formulations such as nanoparticulate systems, microencapsulation, and protein complexes were suggested to be utilized to potentially protect anthocyanins, increase their residence time in the gastrointestinal tract, and improve the bioavailability after ingestion. Such approaches for enhancing the bioavailability of anthocyanins were reviewed by Herrera-Balandrano et al. (2021). Each delivery system has its pros and cons. However, researchers pointed out the high potency of nanoparticle systems compared to other methods. For example, nanoparticles fabricated with food-grade materials, such as chitosan and β-lactoglobulin, were reported to improve anthocyanin stability during storage at various temperatures and pH conditions (Ge et al., 2018) and during *in vitro* digestion (Ge et al., 2019). In addition, Ovalbumin, dextran, and pectin nanogel improved anthocyanin stability through *in vitro* digestion model and enhanced the permeability of anthocyanins through a Caco-2 cell monolayer (Feng et al., 2019). Zhao et al. (2017) showed that nanoliposomes could protect anthocyanins in simulated gastric fluid and rapidly release them in the simulated intestinal fluid. However, converting such technological innovation into functional foods or natural health products that provide health benefits for consumers is still challenging and *in vivo* settings remain to be determined.

It is important to mention that anthocyanins are relatively stable in the stomach at low pH, and their absorption starts there, whereas the instability of anthocyanins has been observed at higher pH during intestinal digestion. Therefore, nanoencapsulation has the potential to improve anthocyanin stability and then increase their residence time in the small intestine for improved absorption. However, more evaluations are required through *in vivo* and clinical tests.

2.3.6 Bioavailability of anthocyanins

Low bioavailability is the main issue that might limit the health benefits of anthocyanins and their possible food applications. Kinetic studies confirmed the very low availability of consumed anthocyanins in plasma (0.02%) and in urine (0.03%) (Mueller et al., 2017). However, the most impressive feature is that anthocyanins are absorbed and eliminated rapidly but with poor efficiency (Manach et al., 2005). Such low bioavailability of anthocyanins is attributed to their low absorption due to their low permeability through the intestinal mucosa in addition to their rapid degradation into phenolic acids and phenyl aldehydes under physiological conditions (Mueller et al., 2017).

The colonic metabolism of anthocyanins is still unclear because of the complexity of food matrixes and their composition and the individual variations in metabolism and gut microflora (Faria et al., 2014). However, the gut microbiota has been reported to play an important role the biotransformation of anthocyanins and may produce a variety of compounds with different bio-availabilities and biological activities (Ávila et al., 2009). Phenolic acids are the major degradation products, and they should be counted for the bioavailability and bioactivities of anthocyanins (Fleschhut et al., 2006). At the same time, anthocyanins work as prebiotic agents, where they modulate the microbiota by affecting the relative viability of colonic bacterial groups and enhancing the growth of beneficial bacteria and the host-microbial interactions, which have been observed to contribute to human health at different levels (Faria et al., 2014).

Furthermore, anthocyanins in aqueous berry extracts exhibited high binding properties with human serum albumin, which works as a drug carrier protein in the plasma (Namiesnik et al., 2014). Therefore, research suggests that despite the low plasma concentration of anthocyanins after oral administration, they seem to have adequate capacity to modulate signal transduction and

gene expression *in vivo* to exert bioactivities in promoting health benefits (Fernandes et al., 2013). Moreover, Mueller et al. (2017) showed that anthocyanins mainly accumulate in organs such as the human gastrointestinal tract and liver. Although absorption of anthocyanins and their metabolites in the upper gastrointestinal tract and their degradation by gut microbiota have been studied, whether their metabolites present in the blood and organs are from degradation by gut microbiota or from endogenous enzyme-metabolism of anthocyanins was not distinguishable (Tian et al., 2019).

Experimental studies, *in vitro*, showed a significant bioactive effect of anthocyanins at doses exceeding the detected concentrations in human plasma. The detected concentrations in human plasma 10^{-8} to 10^{-7} M after consumption of anthocyanins are much lower than the effective concentrations of 10^{-6} to 10^{-4} M, which have been proven to stimulate apoptosis and inhibit malignant cell growth in *in vitro* experiments (Wang & Stoner, 2008). However, despite the known instability of anthocyanins, only a few studies attempted to estimate the total bioavailability of anthocyanins by measuring the different degradation products and potential metabolites formed *in vivo*, which exhibit dynamic kinetic profiles, by using ^{13}C-labelled anthocyanins (^{13}C-tracer). Intervention study by Czank et al. (2013) provided evidence that anthocyanins are more bioavailable and that their metabolites are present in the circulation for about 48 h after ingestion. They reported relative bioavailability of 12.38±1.38 %, which was excreted in urine and breath, with a total recovery of 43.9±25.9% of ^{13}C in urine, breath, and feces. The detected serum concentrations of metabolites reached 42-fold higher and occurred much later than the parent anthocyanin. Researchers also suggested that more metabolites are still undetected by the used technique and conditions. De Ferrars et al. (2014) also provided new insight into anthocyanins metabolism. They suggested that anthocyanin clearance in the body involves multiple processes,

including hepatic recycling, enterohepatic recirculation, and microbial metabolism associated with an extended period of intestinal absorption from the small and large intestine. Moreover, anthocyanins have been reported to accumulate in the tissues including liver, eye, and brain of pigs, which were fed diets supplemented with blueberries for 4 weeks (Kalt et al., 2008).

Individual food components and food matrix could affect the potential bio-accessibility of anthocyanins (Sengul et al., 2014). Several studies have reported that anthocyanin bio-accessibility depends on the structure of the anthocyanin and food components. Anthocyanidin structures such as backbone structure, glycosylation, hydroxylation, and type, number, and acylation of bounded sugar molecules affect anthocyanin stability, bio-accessibility, and bioavailability (Charron et al., 2009; Novotny et al., 2012). In food processing, in addition to food pH and temperature, the presence of other components, which were added or formed during technological processes could affect the anthocyanin content in the food and their bioavailability (Cavalcanti et al., 2011; Charron et al., 2009). Pineda-Vadillo et al. (2017) assessed the stability of anthocyanins included in dairy and egg matrices such as custard dessert, milkshake, pancake, and omelet after processing. Anthocyanins recovery after processing largely varied among matrices due to the interactions with other food components and treatments. Researchers indicated that incorporating anthocyanins into food matrices effectively protected them against intestinal degradation. In the study of Wiczkowski et al. (2016), the bioavailability of anthocyanin in volunteers who consumed fresh red cabbage was about 10% higher than in participants who consumed fermented red cabbage. Moreover, the intake of fresh cabbage induced higher plasma antioxidant capacity than fermented cabbage.

Co-ingested foods, including other antioxidants and macronutrients in consumed foods, have been reported to induce positive or negative effects on anthocyanins' bio-accessibility, consequently affecting their absorption and antioxidant capacity (Eker et al., 2019; Yang et al.,

2011). For example, Mullen et al. (2008) reported that eating strawberries with cream significantly delayed the absorption of anthocyanins with no significant changes in the anthocyanin quantities absorbed into plasma and excreted in the urine. Meanwhile, a delay was also observed in the excretion of the metabolites. A study by Nielsen et al. (2003) reported higher anthocyanin absorption from blackcurrant juice compared to an aqueous citric acid solution of purified anthocyanins in hyperlipidemic rabbits, suggesting that the inclusion of the anthocyanins in a food matrix increased their bioavailability. However, the interaction between anthocyanins and some plant cell wall polysaccharides like pectin was blamed for reducing the anthocyanin content in the juices compared to the fruits (Larsen et al., 2019). Meanwhile, Lin et al. (2016) reported that non-water-soluble pectin showed a relatively strong anthocyanin binding ability at low pH, which may enhance the bioavailability for colonic microflora fermentation and promote colonic health. Therefore, the plant species (anthocyanin source) and food matrix are the main factors determining anthocyanins' content, bio-accessibility, and bioavailability.

2.3.7 Dietary recommendations and toxicity of anthocyanins

There are no dietary recommendations from public health sectors for anthocyanins in Canada, the United States, or the European Union. However, recently, China has proposed 50 mg of anthocyanins/day (Wallace & Giusti, 2015). Moreover, toxicity was not observed at daily intake doses for anthocyanins, for example, 25 mg/kg per day of anthocyanins did not show any toxic effects in mice (Wallace & Giusti, 2015).

The bioactivities of anthocyanins have been clearly demonstrated in experimental studies, while epidemiological studies were controversial (Wang & Stoner, 2008). Although the low bioavailability of anthocyanins and their low stability could limit their health benefits, their

antioxidant activity at low concentration of 1 μg/L (Bornsek et al., 2012), and 0.9 nM (Ziberna et al., 2012) can be considered a strong point towards the rationale for the reported health effects.

2.4 Introduction to gingerols (G)

2.4.1 Sources

Gingerols (6-, 8-, 10-gingerols, and 6-shogaol) are the main bioactive components in ginger (rhizome of *Zingiber officinale* Roscoe, Zingiberaceae). The traditional use of ginger as a herbal medicine was attributed to the activity of gingerols, the homologous phenolic ketones, of which 6-gingerol is the major one (Khan et al., 2016).

Ginger has been grown in tropical and subtropical regions to be used for food, spice, and medicinal purposes (Khan et al., 2016). Due to its fresh and pungent taste, ginger has been used worldwide as a food seasoning (Masuda et al., 2004). In addition, the ginger rhizome has been used for thousands of years in traditional medicine to relieve symptoms associated with pregnancy, inflammation, rheumatic illness, and gastrointestinal irritations (Khan et al., 2016; Kim et al., 2007). Research indicated that ginger has medicinal and nutritional features in the prevention and treatment of many conditions and that ginger extracts have been listed for various diseases in pharmacopeias of different countries (Khan et al., 2016).

2.4.2 Chemical structure and properties of 6-gingerol

6-Gingerol, the major constituent in ginger, is a phenolic ketone compound with a structure of 1-[40-hydroxy-30-methyoxyphenyl]-5-hydroxy-3-decanone (**Figure 2.7**) (Kim et al., 2007). It was inferred that the active part of 6-gingerol was the aliphatic chain moiety containing a hydroxy group (Nakamura & Yamamoto, 1983). Masuda et al. (2004) revealed that the radical scavenging activity and the inhibitory effect on the oxidation of methyl linoleate under aeration and heating are influenced by the substituents on the alkyl chain. Meanwhile, the inhibitory effect on the 2,2′-

azobis (2-amidinopropane) dihydrochloride (AAPH)-induced liposome oxidation was attributed to the alkyl chain length. Masuda et al. (2004) also suggested their affinity to the substrates in addition to their antioxidant activity.

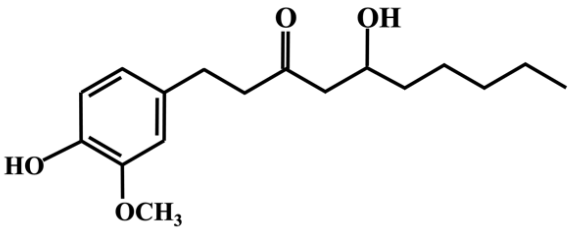


Figure 2.7. Chemical structure of 6-gingerol.

2.4.3 Bioactivities of gingerols

Ginger has been reported in observational, animal, and *in vitro* studies to exhibit antithrombotic, anti-inflammatory, anticancer, and antimicrobial activities (Khan et al., 2016). Furthermore, ginger showed beneficial properties in alleviating the toxicity of hepatotoxins. It was proven in preclinical studies to possess hepatoprotective effects against various foreign compounds, including alcohol, acetaminophen, heavy metals, fungicides, tetracycline, and organophosphorus compounds. Such protective actions are mediated through their free radical scavenging and antioxidant activities and modulating the levels of the detoxifying enzymes (Haniadka et al., 2013). Furthermore, ginger has been reported to enhance the absorption of several active components classified under nutrients, nutraceuticals, and different drug categories (Qazi et al., 2003). It exerts its effect either by regulating intestinal function or promoting the absorption in the gut through mechanisms such as stimulating the gastrointestinal tract and increasing the absorptive surface (Srinivasan, 2016). Moreover, toxicity was not observed at daily intake doses, and ginger is classified by USFDA as generally regarded as safe (GRAS) (Khan et al., 2016).

6-Gingerol, the most abundant compound in fresh ginger, exhibits a wide range of pharmacological effects, including antioxidant (Masuda et al., 2004), anti-inflammatory, anti-cancer activities (Kim et al., 2007; Yusof et al., 2009), antihepatotoxic (Haniadka et al., 2013), neuroprotective (Zeng et al., 2015), hypotensive, cardiotonic, antiplatelet, and antifungal effects (Khan et al., 2016). In addition, 6-gingerol showed a photoprotective effect against UVB-induced skin damage (Kim et al., 2007).

2.4.4 Antioxidant properties of gingerols

Ginger exhibited antioxidant activities. Ginger supplemented diet suppressed liver carcinogenesis in ethionine-induced rat hepatocarcinogenesis by scavenging the free radical formation and reducing lipid peroxidation by reducing the SOD activity and malondialdehyde (MDA) level and increasing the catalase activity (Yusof et al., 2009).

6-Gingerol prevented ultraviolet light B (UVB)-induced ROS production *in vitro* and *in vivo*. It showed a photoprotective effect against UVB-induced skin damage in hairless mice (HRS/J hr+/+) through suppressing intracellular levels of ROS and activation of NF-κB. Furthermore, 6-gingerol was observed to reduce UVB-induced intracellular ROS levels, in addition to activation of caspases-3, -8, and -9, cyclooxygenase-2 (COX-2), and Fas expression in human keratinocytes (HaCaT) *in vitro* (Kim et al., 2007). Moreover, ginger-supplemented diet suppressed liver carcinogenesis in ethionine-induced rat hepatocarcinogenesis by scavenging the free radical formation and reducing lipid peroxidation (Yusof et al., 2009).

2.4.5 Anticancer properties of gingerols

Gingerols have been identified as a group of natural phytochemicals with potential for cancer prevention (Srinivasan, 2017). Research has provided evidence about the anticancer properties of gingerols against different cancer cell lines *in vitro*. The potential cancer prevention activity of

gingerols are attributed to multiple mechanisms, including the antioxidant activity, inhibiting proliferation, inducing cell cycle arrest and apoptosis, and suppressing the inflammation (Srinivasan, 2017). For example, the antiproliferative efficacy of ginger crude extract and its most active phenolics, such as gingerols and 6-shogaol, on prostate adenocarcinoma cells PC-3 was reported (Brahmbhatt et al., 2013).

The 6-gingerol showed anti-proliferative activity in different human cancer cell lines, including colorectal (Lee et al., 2008), prostate, gastric, breast (Aggarwal et al., 2008), hepatoma (Yagihashi et al., 2008), and epidermoid carcinoma cells (Nigam et al., 2009). It was reported that 6-gingerol inhibited the proliferation of cervical cancer cells HeLa (Zhang et al., 2017), colon cancer cell lines HT-29 and SW837 (Yusof et al., 2015) at IC_{50} of ~ 28.3, 254, and 158 μg/mL, respectively, and inhibited the proliferation and invasion activities of hepatoma cells AH109A at concentrations of 25–200 μM (Yagihashi et al., 2008). 6-Gingerol also induced apoptosis in the colon cancer cell lines HCT-116 and HT-29 (Lee et al., 2008). Moreover, ginger has been reported to suppress liver carcinogenesis in ethionine-induced rat hepatocarcinogenesis (Yusof et al., 2009).

Gingerols showed selectivity in their cytotoxicity against cancer cells. Seven naturally isolated derivatives, including 4-, 6-, 8-, and 10-gingerols, along with several semi-synthesized 6-gingerol derivatives, were investigated and showed selective effects against colon cancer cells HCT-116 compared to normal cells TIG-1 and HF-19 (El-Naggar et al., 2017).

6-Gingerol has been reported to target various signal transduction pathways involved in carcinogenesis. For example, 6-gingerol inhibited the cell growth of human pancreatic cancer cell lines HPAC and BxPC-3 through cell cycle arrest at the G1 phase due to decreased expression of cyclin-A and CDK, p53-independent induction of p21, and reduced phosphorylation of retinoblastoma (Rb) proteins, and induced apoptosis in the BxPC-3 cells (mutant p53-expressing

cells) by increasing AKT phosphorylation (Park et al., 2006). In the study of Zhang et al. (2017), 6-gingerol inhibited the growth of human cervical adenocarcinoma HeLa cells and induced cell cycle arrest in G0/G1-phase by reducing the expression of cyclin-A, D1, and E1 and slightly decreasing CDK-1, p21 and p27. 6-Gingerol also exhibited anti-angiogenic activity by inhibiting both the vascular endothelial growth factor (VEGF)- and basic fibroblast growth factor (bFGF)-induced proliferation of human endothelial cells and arrested the cells in the G1 phase (Kim et al., 2005).

Interestingly, increased intracellular ROS was one of the observed mechanisms leading to cell cycle arrest and apoptotic response in the colorectal cancer cells LoVo when treated with 6-gingerol (Lin et al., 2012). Furthermore, 6-gingerol also decreased the mitochondrial membrane potential and subsequently induced apoptosis in human gastric adenocarcinoma cells AGS (Mansingh et al., 2018) and human epidermoid carcinoma cells A431 (Nigam et al., 2009) due to its effect in increasing the ROS generation.

Moreover, the nuclear factor-(kappa) B (NF-κB), which is a transcription factor that can be activated in an inflammatory response to different types of stress such as the pro-inflammatory cytokines tumor necrosis factor (TNF), viruses, (gamma)-radiation, and lipopolysaccharide, is considered an important link between inflammation and cancer. NF-κB, interleukin-8 (IL-8), and VEGF have been reported as molecular targets for antitumor activity of 6- gingerol (Oyagbemi et al., 2010). Gingerol extract exhibited anti-inflammatory activities in LPS-induced macrophages (RAW 264.7) by targeting the NF-κB signaling pathway (Liang et al., 2018). Photoprotective effects of 6-gingerol against UVB-induced skin damage have been attributed to the activation of NF-κB and suppression of intracellular levels of ROS (Kim et al., 2007). Furthermore, 6-gingerol and 6-gingerol derivatives inhibited the leukotriene A4 hydrolase (LTA4H). This pro-

inflammatory enzyme plays an important role in chronic inflammation-associated carcinogenesis in HCT115 (Jeong et al., 2009) and HCT-116 cell lines (El-Naggar et al., 2017).

Although 6-gingerol showed antiproliferative activity against various colorectal cancer cells, including HCT-116, SW480, HT-29, LoVo, and Caco-2, the mechanisms of 6-gingerol effects vary between the cell lines and involve multiple signals (Lee et al., 2008). Increased cell cycle arrest in the G1 phase, with a decrease in the S and G2 phases, was observed in HCT116 cells through downregulation of cyclin-D1, while these phases were not affected in SW480. Apoptosis levels were also significantly increased in HCT-116, SW480, and LoVo cells through upregulation of NAG-1 (Lee et al., 2008). Lin et al. (2012) observed cell cycle arrest in the G2/M phase in LoVo cells when exposed to 6-gingerol due to increased levels of p21 and p27, increased phosphorylation of p53, and decreased levels of CDK-1, cyclin A, and cyclin B1. Differently, in Colo205 cells, 6-gingerol did not show anticancer activity; meanwhile, 6-shogaol inhibited their growth and induced apoptosis through modulation of mitochondrial functions induced by ROS through up-regulation of Bax, Fas, and FasL, and down-regulation of Bcl-2 and Bcl-XL (Pan et al., 2008).

2.4.6 Bioavailability of gingerols

Fast metabolism of 6-gingerol has been reported in rats after a single oral dose. It was rapidly absorbed, distributed, and eliminated from plasma and other tissues, with the highest concentrations in the gastrointestinal tract (Jiang et al., 2008; Wang et al., 2009). The glucuronidation process was identified in pharmacokinetic studies as the primary mechanism in 6-gingerol metabolism and activity (Mukkavilli et al., 2017; Wang et al., 2009), which was confirmed to occur in the intestine when administrated orally (Wang et al., 2009).

2.5 Introduction to synergism

2.5.1 The principle of synergism and evaluation methods

The concept of drug combination has been previously used in treating various diseases, including acquired immune deficiency syndrome (AIDS) and cancer (Fu et al., 2016). When a combination of two or more agents exhibits higher bioactivity or therapeutic effects compared to the additive effect of individual agents at the same concentration, this effect could be described as synergism (Breda & Kok, 2018). Thus, simply, synergism is when agents A and B exhibit the same type of effect, and when combined (A+B), a higher effect than the additive effect of each agent alone would be obtained. Other situations could be observed when combined effects of two agents are additive or antagonistic, where the combination shows similar or lower effects, respectively, than the sum of individual agents. Even though synergism can be simply expressed as a percent or fold enhancement, synergism is different from enhancement; for example, when agent B has no effect by itself, but it could increase the effect of agent A, this is called an enhancement (Chou, 2008). The nature of combined drug action can be determined from dose-response curves and combination indices.

2.5.1.1 Dose-response curves

Dose-response (dose-effect) curves can be obtained by plotting the concentration of drugs against the effect. They provide a visual presentation as a qualitative measure of the combined drug actions.

2.5.1.2 Chou-Talalay combination index method

Chou and Talalay's method of combination index (CI) (effect-oriented) has been used widely to assess the potency of synergistic effects in a quantitative manner (Brahmbhatt et al., 2013; Majumdar et al., 2009; Parasramka & Gupta, 2012; Yang & Liu, 2009). The combination index

can be determined as effective doses at a specific growth inhibition level, affected fractions (Fa) X. It can be calculated using Eq. (2-1):

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} \qquad \text{Eq. (2-1)}$$

where $(Dx)_1$ and $(Dx)_2$ are the individual doses of agent 1 and agent 2, respectively, for X% affected fraction (Fa), and $(D)_1$ and $(D)_2$ are the doses in combination that exhibit the same inhibition level (Fu et al., 2016). These values for each agent can be obtained from the median-effect equation given in Eq. (2-2):

$$\frac{Fa}{Fu} = \left(\frac{D}{Dm}\right)^m \qquad \text{Eq. (2-2)}$$

where Fa is the affected fraction by dose D (reflecting the growth inhibition in the experiment), Fu is the unaffected fraction, Dm is the median effect dose (IC_{50}), and m is the slope of the median effect plot of y=log fa/fu vs. x=log (D) (Fu et al., 2016).

Chou-Talalay combination index (CI) method (Chou, 2008) considers the median-effect equation of the mass-action law and the combination index (CI) theorem. Obtaining the median-effect equation from experimental data allows for running a computerized simulation of the potency of synergism at other points using a software such as the *CompuSyn* software (Chou & Martin, 2005b). This method has been used for the qualitative and quantitative determination of synergism for *in vitro* (Brahmbhatt et al., 2013) and animal studies (Fu et al., 2016). CI values of less than, equal, or greater than one indicates that the interaction between the two agents is synergistic, additive, or antagonistic, respectively. Moreover, CI values less than 0.7 indicate clear, strong synergy, while over 0.85 suggest a moderate synergistic interaction (Chou, 2008; Fu et al., 2016). According to Fu et al. (2016), synergism at higher affected fractions is strongly related to the therapeutic efficacy, where a higher Fa reflects high growth inhibitory effects on the cancer cells.

2.5.1.2.1 Isobologram

Isobologram (dose-oriented) is another way for synergism determination. However, it only presents synergism visually and does not provide a quantitative determination of synergism (Fu et al., 2016). Isobologram equation could be obtained when the multiple drug effect equation equals one. When the combinations are in a constant ratio, the mixture behaves like a third drug; the isobologram could be presented as a classic isobologram with $(D)_1$ and $(D)_2$ doses on the x- and y-axis, respectively. For non-consistent combination ratios, a normalized isobologram could be constructed with the ratios $(D)_1/(Dx)_1$ and $(D)_2/(Dx)_2$ on the x- and y-axis, respectively. Isobologram could be constructed by the software at any effect level (Chou, 2008).

2.5.1.2.2 The dose-reduction index (DRI) equation and plot

Dose-reduction index (DRI) in a synergistic combination represents the degree of possible dose reduction for each agent in combination at a specific effective level compared to the concentration of each agent exhibiting the same effect level. This dose reduction index helps identify the dosage reduction level for the same effect and, consequently, the potential toxicity reduction of high doses (Fu et al., 2016). Because higher affected fraction values reflect a higher effect (e.g., growth inhibition), the increase in the DRI with the affected fraction value reflects a strong synergistic action (Heiduschka et al., 2014). DRI values of each agent at a specific combination dosage or different inhibition rate Fa (x) could be calculated by Eq. (2-3):

$$DRI1 = \frac{(\mathrm{Dx})1}{(\mathrm{D})1} \qquad \text{and} \qquad DRI2 = \frac{(\mathrm{Dx})2}{(\mathrm{D})2} \qquad \text{Eq. (2-3)}$$

2.5.1.3 The synergistic effect indicator (SE)

The synergistic effect (SE) indicator has been used to evaluate synergism in antioxidant combinations. SE considers the statistical significance between the experimental and expected effects of a combination to identify if the interaction is synergistic or antagonistic. The SE is

calculated as the ratio of the obtained effect of the combined agents in the experiment (experimental) (EE combination) and the expected effect (theoretical) (TE combination) as in Eq. (2-4) (Fuhrman et al., 2000; Luís et al., 2018):

$$SE = \text{EE combination} \,/\, \text{TE combination} \qquad \text{Eq. (2-4)}$$

The effect is considered synergistic if the SE is higher than 1 (SE >1), antagonistic when SE is lower than 1 (SE <1), and additive when SE is approximately 1. The TE combination is calculated based on the sum of the experimental effects (EE) of the individual agents, using Eq. (2-5) (Fuhrman et al., 2000; Luís et al., 2018):

$$\text{TE combination} = \text{EEa} + \text{EEb} - (\text{EEa} \times \text{EEb}/100) \qquad \text{Eq. (2-5)}$$

EEa and EEb represent the experimental effect values of individual agents a and b (Fuhrman et al., 2000).

2.5.2 Synergism between phytochemicals and mechanisms involved

The concept of synergism between different phytochemicals in fruits and vegetables was proposed more than a decade ago (Liu, 2003). Since then, a considerable amount of research has been conducted with a major focus on the synergism of resveratrol and curcumin together or with other phytochemicals such as quercetin, genistein, black and green tea polyphenols, and apigenin (Breda & Kok, 2018). However, most research has been conducted using experimental model systems, and understanding of synergism for other phytochemicals is still very limited. For example, quercetin and ellagic acid, combined with resveratrol, showed synergism against leukemia cells through alterations in cell cycle progression and induction of apoptosis (Mertens-Talcott & Percival, 2005). Synergism was observed when apple extract was combined with quercetin 3-β-D-glucoside (Q-3-G) in inhibiting the proliferation of breast cancer cells MCF-7 with a CI value of 0.42 at Fa of 95% (Yang & Liu, 2009). When garcinol was combined with

curcumin in ratios of 1:10-1:2.5 against pancreatic cancer cells (PaCa), they exhibited synergism with CI values of 0.7-0.9 at Fa of 50%. But, when the same combination ratios were reversed (curcumin: garcinol), higher levels of synergism were observed (CI of 0.2-0.6) (Parasramka & Gupta, 2012).

Individual phytochemicals have been studied extensively and their ability to exert an effect via modulating different signaling pathways in cancer cells has been shown both *in vitro* and *in vivo* (Fantini et al., 2015). The rationale for combining more than one agent is to attack cancer cells through multiple targets and to impact different pathways that may provide cumulative or additive effects and reduce toxicity while individual agents target one or a few molecular mechanisms (Breda & Kok, 2018). Evidence regarding the benefits of combined polyphenols against different types of cancer is increasing in the literature (Breda & Kok, 2018; Fantini et al., 2015). For example, curcumin and resveratrol combination showed better chemo-preventive response than individually in a mouse model of lung cancer by maintaining adequate zinc and triggering cell cycle arrest by reducing the induced enzyme activity of the inflammation marker COX-2, even to normal levels, and increasing the levels of cell cycle inhibitor p21 more than individual treatments (Malhotra et al., 2011).

Curcumin and resveratrol in combination showed a synergistic effect in inhibiting the growth of colon cancer cells *in vitro* and *in vivo* due to their effect in inhibiting proliferation, stimulating apoptosis, and reducing NF-κB activity (Majumdar et al., 2009). This study reported CI values of 0.43-0.9 at Fa of 29-81% in colorectal cancer cells HCT-116. Furthermore, *in vitro* results of this study suggest that curcumin and resveratrol together cause a greater inhibition in activation of the epidermal growth factor receptor EGFR family as well as IGF-1R, than that observed with either

agent alone; they also suggest that the growth inhibitory properties of individual and combined forms are independent of p53 status.

Moreover, evidence is available for the synergism between plant-based compounds, including resveratrol, EGCG, quercetin, genistein, and pro-anthocyanidin, with different chemotherapeutic drugs against cancer (Fantini et al., 2015). Such combinations have been proven to enhance efficacy and reduce toxicity (Heiduschka et al., 2014), and overcome resistance (Chen et al., 2013) more than the drug or compound alone. For example, resveratrol synergizes with etoposide, a chemotherapeutic drug, to inhibit the growth of head and neck squamous cell carcinoma (HNSCC) cells by inducing apoptosis *in vitro* (Heiduschka et al., 2014). Likewise, curcumin enhanced the effect of chemotherapeutic agent 5-fluorouracil against colorectal cancer cells HCT-116 and HT-29 cell line by inhibiting NF-κB and decreasing the expression of the COX-2 protein (Du et al., 2006; Shakibaei et al., 2013).

2.5.3 Anthocyanins' synergy with other phytochemicals

Research focusing on the anthocyanin synergistic effect is very limited. However, the 50% growth inhibitory concentrations (IC_{50}) reported in the literature for anthocyanin mixtures were less than those for purified individual anthocyanins. For example, the IC_{50} of anthocyanin fractions extracted from blueberries (Yi et al., 2005) and chokeberry (Zhao et al., 2004) on Caco-2 and HT-29 cells ranged from 15 to 50 µg/mL. Meanwhile, IC_{50} of ~60-100 µg/mL were reported for Dp-3-glc, Cy-3-glc, Pt-3-glc, Pn-3-glc and Mv-3-glc on Caco-2 cells (Fernandes et al., 2013). Additive effects were observed when the anthocyanin fraction, extracted from chokeberry, was combined with other phenolics fraction in inhibiting the growth of human colorectal adenocarcinoma cells HT-29 (Jing et al., 2008).

2.5.4 Gingerols synergy with other phytochemicals

In the study of Yusof et al. (2015), the synergism between 6-gingerol and γ-tocotrienol against human colorectal cancer cells, including HT-29 and SW837, was observed with CI of 0.89 and 0.79 at Fa of 50%. The IC_{50s} of 6-gingerol on HT-29 and SW837 were reduced from 254 and 158 μg/mL, to 105 and 70 μg/mL, respectively, when combined with γ-tocotrienol (Yusof et al., 2015). Interestingly, high synergistic interactions of binary combinations of ginger phytochemicals were demonstrated by Brahmbhatt et al. (2013) with CI values ranging from 0.03 to 0.88 at Fa of ≤50%. Moreover, a significant enhancement of antiproliferative activity on prostate cancer cells PC-3 was noticed when the ginger extract was combined with its constituents, particularly 6-gingerol (Brahmbhatt et al., 2013).

Gingerol synergized with doxorubicin (DOX) drug against liver cancer cells Hep G2 and Huh7 and decreased its IC_{50} by 10- and 4-fold, respectively; the combination protected the cells from DOX-induced vascular damage. In addition, the combined effect of 6-gingerol and doxorubicin significantly induced cell cycle arrest at the G2/M-phase, while doxorubicin alone induced cell accumulation at S-phase and G2/M-phase (Al-Abbasi et al., 2016).

Phytochemicals are natural bioactive, non-nutritive, non-toxic, and readily available ingredients (Xiao & Bai, 2019). They are secondary metabolites in plants protecting them from harsh environment and pathogenesis. Foods such as vegetables, fruits, whole grains, nuts, seeds, and tea are rich in phytochemicals (Scalbert et al., 2011). The health benefits of dietary phytochemicals in reducing the risk of common chronic diseases and improving health quality have been revealed. Many phytochemicals have been proven to exert antioxidant, anti-inflammatory, and anticancer properties (Battino et al., 2019; Fantini et al., 2015; Russo, 2007). Polyphenols are the most abundant phytochemicals in the human diet. Research has demonstrated

their efficacy and potential to reduce many cancer aspects despite their low bioavailability (Breda & Kok, 2018). Anthocyanins are polyphenolic compounds with demonstrated antioxidant and anticancer properties and showed a potential for health applications (Lin et al., 2017; Thomasset et al., 2009; Wang & Stoner, 2008). However, the low bioavailability of anthocyanins could limit the health benefits of anthocyanins (Mueller et al., 2017). Gingerols also have been reported to exhibit antioxidant, anti-inflammatory, and anticancer activities (Khan et al., 2016). Furthermore, the absorption enhancement property of ginger for several active components has been reported (Qazi et al., 2003). The antioxidant and anticancer effects of anthocyanins and gingerols have been proven to be induced through modulating different cellular signals pathways, which showed high level of interconnectivity and overlapping. This indicates a good chance of having a synergistic effect between these two types of phenolic compounds, which is worth investigating due to their effectiveness, selectivity, and non-toxicity at daily intake doses. This may also provide a strategy to counteract their poor bioavailability. Researchers attributed the health benefits of phytochemicals in fruits and vegetables to the synergistic interactions between them (Alam et al., 2018). Research studies demonstrated the synergistic effects within some phytochemicals to show considerably higher antioxidant and anticancer effects (Breda & Kok, 2018), but research is still limited and focusing on a number of compounds. Investigation of more potential combinations and understanding of such synergistic interactions is still required.

Chapter 3: Anthocyanins and gingerols exhibit a synergistic effect to inhibit the proliferation of Caco-2, Hep G2, and HT-29 cells *in vitro*[1]

3.1 Introduction

Cancer is a leading cause of death globally, with nearly 10 million deaths in 2020 (WHO, 2021). The main feature that identifies cancer is the rapid growth of abnormal cells beyond their usual boundaries, which later can invade other body parts and organs (WHO, 2021). Although many mechanisms are involved in tumor development and progression, dysregulation of cell proliferation and suppression of programmed cell death (apoptosis) are considered the leading platforms that promote neoplastic progression (Evan & Vousden, 2001). Conventional drug therapies, such as cytostatic drugs, including alkylating agents, alkaloids, antibiotics, and antimetabolites, exhibit side effects. These include immediate and late signs of toxicity, and all organs of the body can be affected, including essential organs, such as the heart and brain. The chronic effects of chemotherapy include carcinogenicity, infertility, and drug resistance (Schirrmacher, 2019). Thus, alternative preventive and therapeutic strategies are being sought.

Prevention of cancer is an important public health objective (Wang et al., 2014). Increasing scientific evidence has shown anticancer effects of phytochemicals (Gonzalez-Vallinas et al., 2013), which may present new strategies for cancer prevention (Several examples were discussed in chapter 2). Identifying natural compounds that target signaling pathways associated with the growth of malignant tumors without cytotoxic effects on normal tissues would provide alternative

[1] A version of this chapter has been published as Amna E Abdurrahim, Feral Temelli, Vera Mazurak, Ramadan A. Benruwin, and Lingyun Chen. "Anthocyanin and gingerol extracts exhibit a synergistic effect to inhibit the proliferation of Caco-2, Hep G2, and HT-29 cells *in vitro*." in ACS Food Science & Technology, 2021, 1(9) 1642-1651.

solutions to reduce the risk of cancer and reduce the toxicity of conventional anticancer therapies (Kamiloglu et al., 2015).

Individual agents target one or a few molecular mechanisms. Thus, the combination of more than one agent impacts different pathways and may provide cumulative or additive effects (Breda & Kok, 2018). Since the suggestion of synergism between different phytochemicals in fruits and vegetables, a considerable amount of research has been conducted focusing on the synergism of resveratrol with other phytochemicals such as quercetin, curcumin, genistein, apigenin, and black and green tea polyphenols (Breda & Kok, 2018). Most research has been conducted using experimental model systems, and the understanding of synergism for other phytochemicals is still very limited.

Anthocyanins are polyphenolic compounds identified with having antioxidant, anti-inflammatory, and anticarcinogenic activities and are implicated in various health effects such as the reduced risk of cardiovascular disease, diabetes, and cancer (Wang & Stoner, 2008). However, their low bioavailability limits the health benefits of anthocyanins (Mueller et al., 2017); whereas *in vitro* studies show a significant bioactive effect of anthocyanins at doses exceeding the concentrations detected in human plasma (Wang & Stoner, 2008). On the other hand, an animal study by Kalt et al. (2008) indicated that anthocyanins accumulate in tissues, including the brain. Therefore, research suggests that despite the low plasma concentration of anthocyanins after oral administration, they seem to have adequate capacity to modulate signal transduction and gene expression *in vivo* to exert bioactivities in promoting health benefits (Fernandes et al., 2013).

Gingerols are phenolic compounds have been identified to exhibit antithrombotic, anti-inflammatory, anticancer, and antimicrobial activities in clinical trials and animal studies (Khan et

al., 2016; Rahmani, 2014). The 6-, 8-, and 10-gingerols and 6-shogaol are the main bioactive components in ginger (rhizome of the *Zingiber officinale)*, with 6-gingerol being the most abundant compound (Khan et al., 2016).

Both anthocyanins (Mazewski et al., 2018; Shin et al., 2009; Yi et al., 2005) and gingerols (Lee et al., 2008; Lin et al., 2012; Radhakrishnan et al., 2014) show anticarcinogenic activities by inhibiting cell proliferation, inducing apoptosis, and suppressing inflammation through modulating different cellular signals pathways. Some pathways overlap, but some do not. For example, both anthocyanins and gingerols inhibited cell proliferation by inducing G1 cell cycle arrest and apoptosis in cancer cell lines such as HCT-116 (Lee et al., 2008; Mazewski et al., 2018). Anthocyanins have been reported to increase DNA fragmentation in HT-29 and Caco-2 (Yi et al., 2005), suppress Akt, and activate p38-MAPK pathways in HCT-116 cells (Shin et al., 2009). On the other hand, 6-gingerol lightly affected the phosphorylation of p38 MAPK and the activation of NF-κB, but it effectively inhibited ERK1/2/JNK/AP-1 pathway and activated caspases 8, 9, 3 and 7 in SW-480 cell (Radhakrishnan et al., 2014). In another study, it induced G2/M cell cycle arrest in LoVo cells through diminishing cyclin A, cyclin B1, and cyclin-dependent kinase-1 (CDK-1) and increasing levels of cell cycle inhibitory proteins p27Kip1 and p21Cip1 (Lin et al., 2012). All this implies that multiple pathways could be activated when combining anthocyanins and gingerols, potentially leading to a synergistic effect for inhibiting the growth of cancer cells. Toxicity was not observed at daily intake doses for either group (more details in chapter 2). However, anthocyanins and gingerols have never been investigated for their combined bioactivities (Breda & Kok, 2018).

It was hypothesized that combining anthocyanins and gingerols may lead to a synergistic effect against cancer growth, as the combined extracts may exert their inhibitory effects through more

cellular signaling pathways than each extract alone. Specifically, this research focused on colorectal and hepatic cancer cell models to study the potential synergism between anthocyanins and gingerols. The selection of cell models was based on the consideration that colorectal cancer is the third most commonly occurring cancer in 2020 and the second cause of death due to cancer globally; liver cancer is the third most common cause of cancer death (WHO, 2021). In addition, both anthocyanins and gingerols are mainly accumulated in organs such as the gastrointestinal tract and liver (Jiang et al., 2008; Mueller et al., 2017). The objectives of this study were to investigate the effects of anthocyanins and gingerols alone and in combination to dysregulate cancer cell proliferation and whether their combination results in synergistic effects against the growth of cancer cells compared to normal cells.

3.2 Materials and methods

3.2.1 Materials

Bilberry extract powder (76% anthocyanin, MW: 466 g/mol) from bilberry (*Vaccinium myrtillus*) was purchased from Hangzhou Ningsi Biotech (Hangzhou, China). Ginger root extract powder from ginger (*Zingiber officinale*) (20% gingerols) was obtained from Nate Biological Technology Co., Ltd. (Xi'an, China). Human colon colorectal adenocarcinoma cells: Caco--2 [Caco2] (ATCC® HTB37™) and HT-29 (ATCC® HTB38™), hepatocellular carcinoma cells: Hep G2 [HEPG2] (ATCC® HB8065™), normal colon fibroblast CCD33Co (ATCC® CRL1539 ™), Eagle's Minimum Essential Medium (EMEM), McCoy’s 5A Medium, 0.25% trypsin/0.53 mM EDTA in HBSS and Penicillin-Streptomycin solution (100X) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from GIBCO (Burlington, ON, Canada). MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium

bromide), dimethyl sulfoxide (DMSO), HPLC grade acetonitrile, methanol, and formic acid were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Gingerol standards were also purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Anthocyanin standards were purchased from Polyphenols Laboratories AS (Sandnes, Norway).

3.2.2 Extract analysis by high-performance liquid chromatography (HPLC)

The constituents of the bilberry and ginger root extracts were solubilized in water and methanol (1mg/1mL), filtered through 0.2 µm syringe filters and analyzed by reversed-phase-high performance liquid chromatography (RP-HPLC) using an Agilent 1200 Series HPLC System equipped with a ZORBAX Extended-C18 column (2.6×250 mm, 5 µm) and diode array detector (G1315D) (Agilent Technologies Inc., Mississauga, ON, Canada). The chromatographic analysis of anthocyanins in the bilberry extract was performed according to Yao et al. (2015), and signals at 535 nm were recorded. Identification of gingerols in the ginger root extract was performed according to Ok and Jeong (2012). Then, the UV spectrum at 230 nm was recorded. Thus, anthocyanins and gingerols were identified by their retention times and the UV spectra of standards. Quantitative analysis was performed for each extract solubilized in water, methanol, or cell culture medium (1mg/mL), after filtration of the insoluble portion filtered through 0.2 µm filters. The standard solutions were prepared in concentrations ranging from 50 to 1000 µg/mL for anthocyanins and gingerols.

For quantifying the soluble portions of the bilberry and ginger root extracts, 2 mg dry extract were dissolved in 2 mL solvent (distilled water or methanol) (each in four replications), then vortexed for 5 min at room temperature, and filtered through 0.2 µm filters. An aliquot (1 mL) of each filtrate was transferred to a pre-weighed 2 mL tube. Samples solubilized in water were lyophilized, while those solubilized in methanol were dried first under vacuum for 2 h and then

freeze-dried. Tubes were weighed, and the dry weights of samples were calculated. The bilberry and ginger root extracts were stored at -20ºC and their constituents were analysed at different time intervals during the research program. There was no significant loss in the active components.

3.2.3 Cell culture conditions and treatments

Caco-2 and HT-29 cells were cultured using DMEM and McCoy's 5A medium, with 20 and 10% FBS, respectively. HepG2 and CCD-33Co cells were cultured using EMEM with 10% FBS. The pH range of those culture media was 7.2-7.5. All culture and experimental media were supplemented with 1% penicillin-streptomycin solution to prevent contamination. Cells were cultured in CORNING 75 cm^2 cell culture flasks. They were maintained in a humidified atmosphere of 5% CO_2 at 37ºC during the incubation periods. The medium was changed 2 to 3 times per week until ~80% confluency. For subculture and obtaining cell suspension, cells were harvested by trypsinization using 0.25% trypsin/0.53 mM EDTA solution. For cell counting, 20 µL of cell suspension was mixed with 20 µL trypan blue solution (0.4% w/v), loaded into a counting slide, and read by a BIO-RAD cell counter (Bio-rad Laboratories Ltd., Mississauga, ON, Canada). Then, the cell suspension was diluted to the desired cell count for each experiment and distributed into the wells.

For treatment preparation, 1000 µg/1 mL stock solutions of anthocyanins or gingerols were prepared by dissolving the dry bilberry or ginger root extracts, respectively, in the corresponding medium for each cell line with 5% FBS, filtrating the solutions through 0.2 µm filters (Fisher brand™ Basix™ Syringe Filters 0.2 µm, PES, Sterile, 25 mm) for sterilization before being diluted and applied on the cells. Applied concentrations in the experiments were calculated depending on the amounts of the active ingredients, anthocyanins and gingerols, detected in the water-soluble portions of the original bilberry and ginger root extracts.

3.2.4 Cytotoxicity and antiproliferation assessment

3.2.4.1 Cell viability by MTT assay

Cytotoxicity and antiproliferative activity of the anthocyanins and gingerols and their combinations were assessed by evaluating the cell viability using the colorimetric MTT assay, as described previously (Brahmbhatt et al., 2013). Cells were seeded into 96-well plates (Greiner bio-one-flat bottom) and incubated for 20 h for attachment. Then, seeding medium was replaced by fresh medium containing anthocyanins, gingerols or their combination. Treatments were applied in five replicates. Five control wells (medium and cells, no treatment) and five blank wells (only medium) were assigned for each plate. The plates were incubated with treatments for 24 or 72 h depending on the experiment. In the MTT assay, the water-soluble tetrazolium dye MTT (colorless) was metabolized to the insoluble purple formazan within the mitochondria of live cells (Brahmbhatt et al., 2013; Fotakis & Timbrell, 2006). Then, formazan crystals were dissolved in DMSO. The absorbance at 570 nm was recorded by a *SpectraMax M3* microplate reader (Molecular Devices, San Jose, CA, USA). Thus, the intensity of the developed color reflects the number of live cells. The cell viability percentages were calculated using Eq. (3-1)

$$Cell\ viability\ \% = \frac{(\text{Absorption of the sample} - \text{Absorption of the blank})}{(\text{Absorption of the control} - \text{Absorption of the blank})} * 100 \qquad \text{Eq. (3-1)}$$

3.2.4.2 Cytotoxicity experiment

For the cytotoxicity experiment, cell survival after exposure to anthocyanins and gingerols treatments at different concentrations was assessed by measuring the cell viability by MTT assay as described above. Cells were seeded at the density of 4×10^4 cells/well, and the incubation time with the treatments was set at 24 h (Fotakis & Timbrell, 2006). Treatment doses of anthocyanins, and gingerols were 40-1000 µg/mL and 5-200 µg/mL, respectively. The 50% lethal doses (LD_{50}) of anthocyanins and gingerols on the tested cell lines, Caco-2, Hep G2, and HT-29, were defined

as the concentration required to kill 50% of the treated cells compared with untreated cells (control) using Curve Fitting and Dose-Response Analysis features in Origin 2018b software.

3.2.4.3 Proliferation experiment

Cell proliferation was evaluated to assess the growth inhibitory effects of the individual and combined extracts on the Caco-2, Hep G2, and HT-29 cell lines. The toxicity of the extracts was also evaluated on the CCD-33Co cells as a model of normal human colon cells. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Cells were incubated with the treatments for 72 h to give time for cells to grow and proliferate. Cell viability was assessed using the MTT assay. Firstly, cells were treated with individual extracts at concentrations of 6-300 µg/mL anthocyanins and 3-100 µg/mL gingerols where no or low cytotoxicity was exhibited. The antiproliferation activity of each extract was quantified as the concentration required for inhibiting cell growth by 50% (IC_{50}). The IC_{50} values of the individual anthocyanins and gingerols on the three cell lines, Caco-2, Hep G2, and HT-29, were obtained.

3.2.5 Combination studies and determination of synergism

A broad range of anthocyanins and gingerols w/w combination ratios was evaluated to increase the chance of identifying synergistic effects. Several concentrations of each extract were initially selected to represent the range around the IC_{50} values: 40, 80, 160, and 240 µg/mL anthocyanins, and 2.5, 5, 10, 20, and 30 µg/mL gingerols. Then, twenty (two-way) combinations of these concentrations in different anthocyanin-to-gingerol (Ac-G) w/w combination ratios, from 96:1 to 4:3, were applied to assess their antiproliferative effects compared to the same dose treatments of individual extracts and the control, non-treated cells (Appendix 1). Subsequently, three Ac-G w/w combination ratios of 4:1, 8:1, and 16:1, which exhibited higher synergism indicators (lower combination index values), were chosen. After that, they were further tested at a series of 5-8

dilutions each, with concentrations ranging from 6-300 µg/mL anthocyanins and 3-75 µg/mL gingerols, on the selected cell lines. This approach allowed to obtain the median-effect equation (Eq. (3-2)) and the computerized simulation of synergism/antagonism at other dosage levels (Chou, 2008; Fu et al., 2016). In each of these experiments, the effects of individual extracts and their combinations were carried out simultaneously under the same experimental conditions.

Data were analyzed according to the Chou-Talalay combination index (CI) method (Chou, 2008), which considers the median-effect equation and the combination index (CI) theorem. This method has been used for the qualitative and quantitative determination of synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) for *in vitro* (Brahmbhatt et al., 2013) and animal studies (Fu et al., 2016). The combination index was determined for the effective doses at inhibition levels, affected fractions (Fa), 20, 50, 75, 90, and 97% for each cancer cell line at each of the three combination ratios. CI was calculated using Eq. (3-2):

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} \qquad \text{Eq. (3-2)}$$

where $(Dx)_1$ and $(Dx)_2$ are the individual doses of agent 1 and agent 2, respectively, for X% growth inhibition (Fa) and $(D)_1$ and $(D)_2$ are the doses in combination that exhibit the same inhibition level (Fu et al., 2016). These values for each agent can be obtained from the median-effect equation given in Eq. (3-3):

$$\frac{Fa}{Fu} = \left(\frac{D}{Dm}\right)^m \qquad \text{Eq. (3-3)}$$

where Fa is the affected fraction by dose D, Fu is the unaffected fraction, Dm is the median effect dose (IC_{50}), and m is the slope of the median effect plot of y=log [Fa/Fu] vs. x=log (D) (Fu et al., 2016). (Fa)s were calculated from the cell viability results of the antiproliferation experiment for the individual extract and their combinations as given in Eq. (3-4):

Affected fraction (Fa) = 100 - Cell viability (Fu) Eq. (3-4)

The dose reduction index (DRI) values of each agent at inhibition rate (x) could be calculated by Eq. (3-5):

$$DRI1 = \frac{(\mathrm{Dx})1}{(\mathrm{D})1} \quad \text{and} \quad DRI2 = \frac{(\mathrm{Dx})2}{(\mathrm{D})2} \qquad \text{Eq. (3-5)}$$

3.2.6 Statistical analysis

Each experiment was performed at least in triplicates (n = 3-5). The data were calculated using Excel, Microsoft Office 365 software and presented as mean ± SD. Those results were subjected to the computerized assessment of the potency of synergism using the *CompuSyn* software (Chou & Martin, 2005b) to identify the type of interaction within the combination treatments. Origin 2018b software was used for defining the LD_{50} and presenting the data.

3.3 Results

3.3.1 Constituents of the anthocyanin and gingerol extracts

Based on the HPLC analysis, fifteen anthocyanins were identified in the bilberry extract, which accounts for approximately 80% (798 µg in 1 mg) of the bilberry extract powder dissolved in water or cell culture medium. They are mono-glycosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin (**Figure 3.1.A**), which are the most abundant anthocyanins in fruits (Castaneda-Ovando et al., 2009). The major components in this extract were Dp-3-gal, Dp-3-glc, and cy-3-glc. The total water-soluble fraction of the original bilberry extract was (0.8 ± 0.08 mg/1 mg) (**Table 3.1**). Thus, the water-soluble fraction of bilberry extract contained 97% anthocyanins by HPLC analysis.

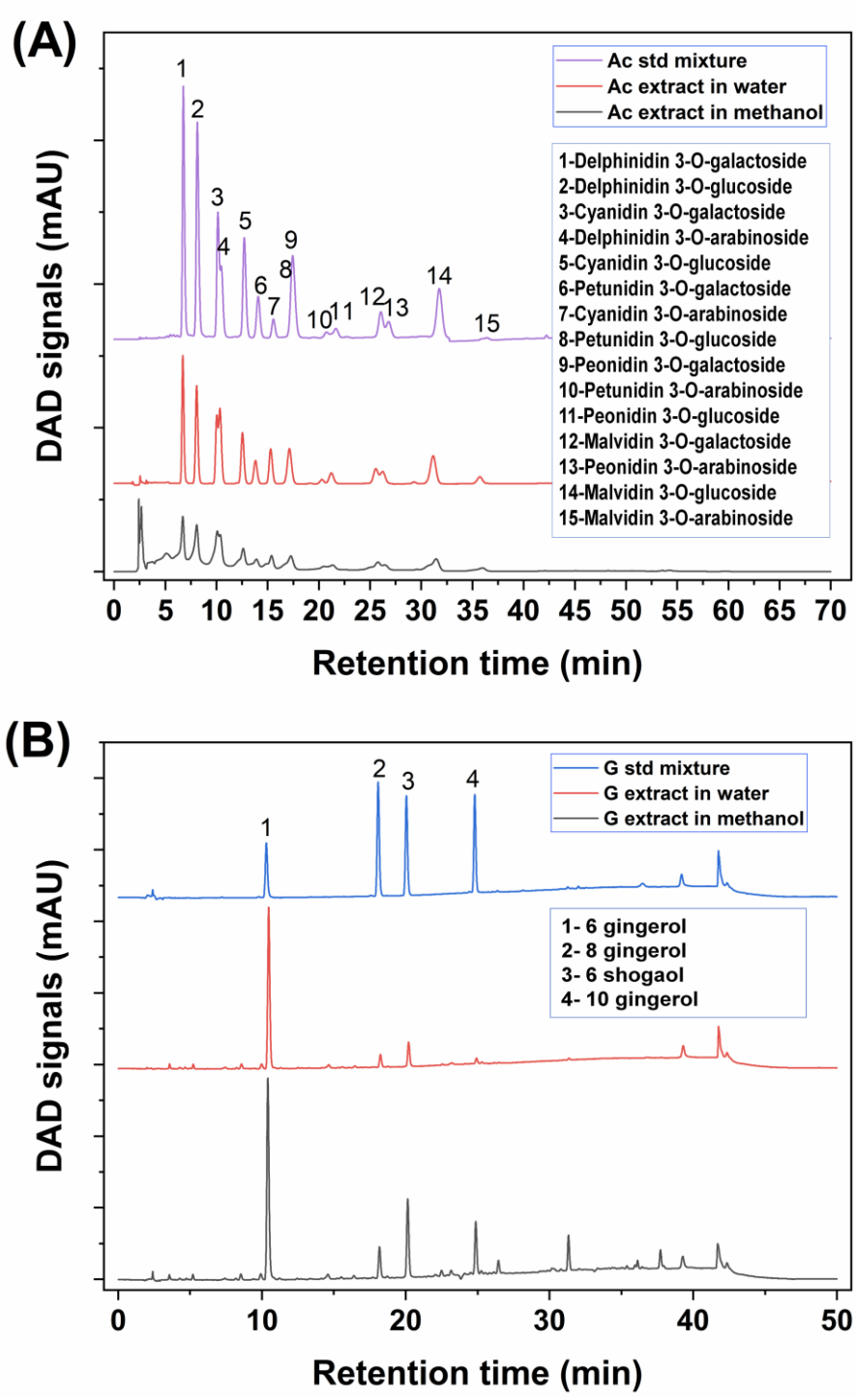


Figure 3.1. HPLC chromatograms of (A) Anthocyanin (Ac) rich bilberry extract with standards and (B) Gingerol (G) extract with standards.

Table 3.1. Soluble fractions of anthocyanin and ginger extracts and total amounts of anthocyanins and gingerols detected by HPLC in different solutions.

Sample and solvent	Soluble fraction (mean± SD) (mg in 1 mg extract)	HPLC (mean± SD) (μg in 1 mg extract)
Anthocyanins extract in water	0.8 ± 0.08	798 ± 27
Anthocyanins extract in methanol	0.8 ± 0.16	620 ± 88
Anthocyanins extract in medium		789± 91
Ginger extract in water	0.17 ± 0.06	106 ± 13
Ginger extract in methanol	0.83 ± 0.15	230 ± 8
Ginger extract in medium		93 ± 1

Gingerols detected in the water-soluble fraction of the ginger root dry extract were 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol (**Figure 3.1.B**). The total concentration of gingerols detected in this water-soluble fraction was 10.6% of the original ginger root extract powder, with 6-gingerol being the major component (90.9%). Gingerols detected in the water-soluble fraction of the ginger root extract account for about 63.8% of the water-soluble fraction (0.17 ± 0.06 mg/1 mg) (**Table 3.1**). Therefore, concentrations used in the treatments reported in the following sections are based on the anthocyanins and gingerols detected in the water-soluble fractions of the original extracts.

3.3.2 Cytotoxicity

Low cytotoxicity and high efficacy are the main factors in reflecting the potency of any treatment (Zhang et al., 2016). Therefore, the cytotoxicity of the anthocyanins and gingerols extracts on Caco-2, Hep G2, and HT-29 cells was tested. It was also evaluated at different concentrations with the purpose of selecting the appropriate dosage range, where no significant cytotoxicity was

observed. In general, anthocyanins and gingerols exhibited cytotoxic effects in a dose-dependent manner on all three cell lines (**Figure 3.2**). Hep G2 and HT-29 cell lines demonstrated higher tolerability (more cell viability) to anthocyanins compared to Caco-2 cells (**Figure 3.2.A**). LD_{50} of anthocyanins on the tested cell lines showed different effect levels in the order of Hep G2> HT-29> Caco-2 cells (**Table 3.2**). Meanwhile, lower cytotoxicity (higher LD_{50}) was observed for gingerols on Caco-2 cells than HepG2 and HT-29 cells (**Figure 3.2.B**). Cell viability of more than 80% was noticed for the bilberry extract at doses of ≤ 100 µg anthocyanins/mL and the ginger root extract at amounts of ≤ 60 µg gingerols/mL on the three tested cell lines. Depending on these results, concentrations of ≤ 300 µg/mL anthocyanins and ≤ 100 µg/mL gingerols, with no or low toxicity, were chosen to be tested individually and in combinations for their antiproliferative effects.

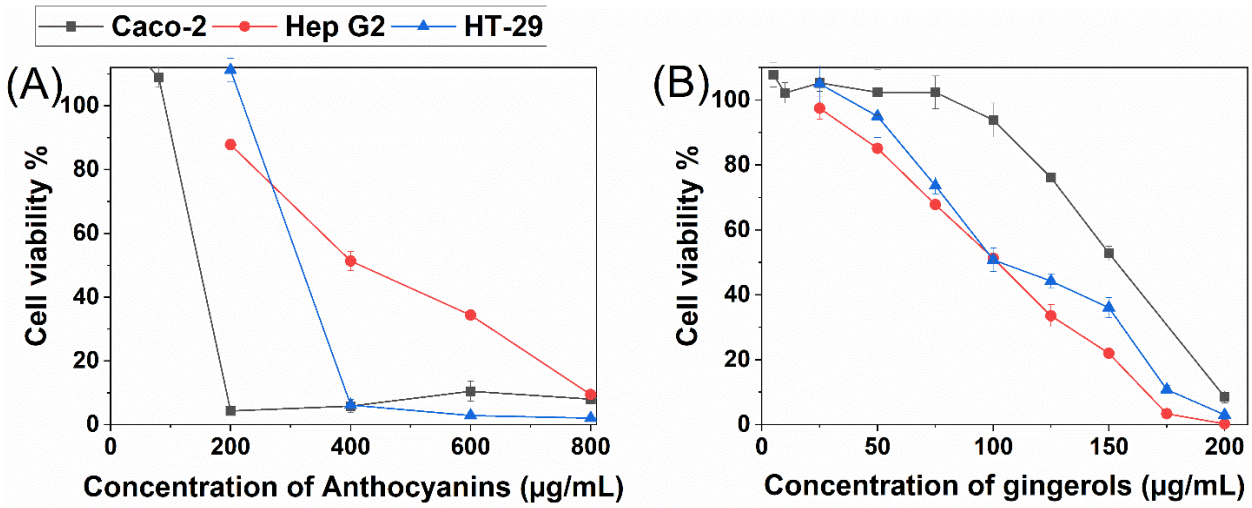


Figure 3.2. Cytotoxicity, as cell viability %, of anthocyanins (A) and gingerols (B) on Caco-2, HepG2, and HT-29 cell lines.

Table 3.2. The LD_{50} and the IC_{50} of anthocyanins (Ac) and gingerols (G) on Caco-2, Hep G2, and HT-29 cell lines.

Cell line	Caco-2		Hep G2		HT-29	
Extract	Ac	G	Ac	G	Ac	G
LD_{50} (µg/mL)±SE*	143±39.1	149.4±3.7	422±24	98.7±3.7	299.5±6.5	107.7±4.6
IC_{50} (µg/mL)	18.4	9.4	95.8	43.9	52	22.8

*SE: standard error (n=5)

3.3.3 Inhibitory effects on cancer cell proliferation (Dose effect curves and IC50)

Anthocyanins and gingerols alone and in combination inhibited the growth of Caco-2, Hep G2, and HT-29 cell lines after 72 h incubation in a dose-dependent manner (**Figure 3.3.A**, **B**, and **C**). The IC_{50} values for anthocyanins on the three cancer cell lines range from 18.4 to 95.8 µg/mL and from 9.4 to 43.9 µg/mL for gingerols (**Table 3.2**). To confirm that these extracts were not cytotoxic to normal cells, they were tested on the normal colon fibroblast cells, CCD-33Co. High cell viability levels of ~100% were observed in CCD-33Co cells at doses of 300 and 75 µg/mL of anthocyanins and gingerols, respectively (**Figure 3.3**.**D**). On the other hand, these doses showed high inhibitory effects on cancer cells. At lower doses, 100 µg/mL anthocyanins and 25 µg/mL gingerols inhibited the growth of Caco-2, Hep G2 and HT-29 compared to CCD-33Co by 97, 54, 61 and 18% and by 79.4, 31, 72.5 and 5.4%, respectively.

Combination experiments showed increased inhibitory effects after treatment with both extracts at some areas of the dose-effect curves in a cell type-specific pattern on the three cancerous cell lines. All r-values obtained from the data analysis of Caco-2 and Hep G2 cell lines were > 0.90, which are acceptable for tissue culture studies (Chou & Martin, 2005a). Only r-values of combination ratio 4:1 in HT-29 cell line data was < 0.90. The r-values of each dose-effect curve,

in addition to the highest growth inhibitory levels obtained from the experimental points, are summarized in (Appendix 2).

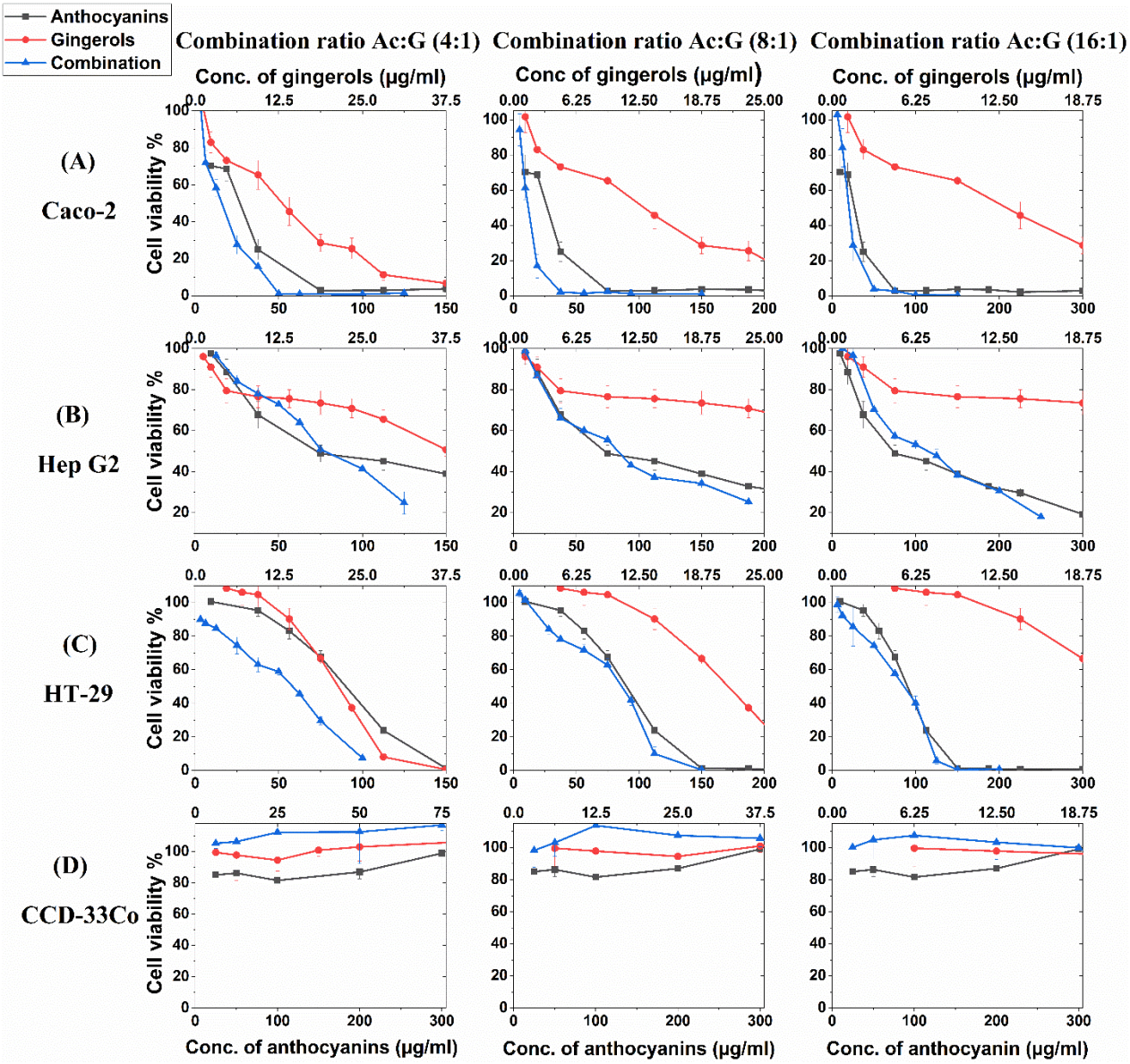


Figure 3.3. Dose effect curves of individual anthocyanins (Ac) and gingerols (G) and their combinations on the proliferation of (A) Caco-2, (B) Hep G2, (C) HT-29, and (D) CCD-33Co cell lines, at different Ac-G w/w combination ratios of 4:1, 8:1 and 16:1. The points represent the means ± SD (n=3-5).

For Caco-2 cells, an IC_{50} of 18.4 and 9.4 µg/mL was identified for anthocyanins and gingerols, respectively. Higher growth inhibition levels were observed for the three Ac-G w/w combination ratios of 4:1, 8:1, and 16:1 compared to the same concentrations of the individual anthocyanins doses ≤60 µg/mL (**Figure 3.3.A**), suggesting a synergistic effect of these compounds. Meanwhile, concentrations of ≥75 µg/mL anthocyanins exhibited high growth inhibition; thus, the growth inhibitory effects for the combinations might be higher than what was assessed.

Compared to the colorectal adenocarcinoma cell lines Caco-2 and HT-29, Hep G2 was less responsive to the anthocyanins and gingerols with IC_{50} of 95.8 and 43.9 µg/mL, respectively (**Table 3.2**). The dose-effect curves on Hep G2 cells (**Figure 3.3.B**) revealed a different response pattern towards the combinations compared to individual anthocyanins and gingerols. At inhibition levels of ≤50%, the three Ac-G w/w combination ratios of 4:1, 8:1, and 16:1, showed lower inhibitory effects than one or the two individual extracts, whereas, at higher inhibition levels of >50%, with a higher dosage, the three combinations inhibited the growth more than both individual extracts.

For HT-29 cells, IC_{50} values of 52 and 22.8 µg/mL were determined for anthocyanins and gingerols, respectively (**Table 3.2**). From the dose-effect curves on the HT-29 cell line (**Figure 3.3.C**), the growth inhibition was lower than what was observed in Caco-2 cells for both anthocyanins and gingerols. The growth inhibitory effects by the Ac-G combinations, at w/w ratios of 8:1 and 16:1, were similar or higher than that of anthocyanins alone at concentrations ≤150 µg/mL, while the mixture at the combination ratio of 4:1 exhibited higher growth inhibition compared to the individuals and the other two combinations. However, concentrations of the gingerols in this combination were also higher. Therefore, further data analysis was conducted to understand the combination indices.

3.3.4 Combination index (CI) and dose reduction index (DRI)

The CI indicator was calculated to reveal the type of interaction between anthocyanins and gingerols in the combinations to inhibit the growth of cancer cells. CI values of less than, equal to, or greater than one indicates that the interaction between the two extracts is synergistic, additive, or antagonistic, respectively. CI values of less than 0.7 indicate clear, strong synergy, while over 0.85 suggest a moderate synergistic interaction (Chou, 2008). Moreover, DRI in a synergistic combination represents the degree of possible dose reduction for each agent in the mixture at a specific effect level compared to the concentration of each agent exhibiting the same effect. Synergistic effects were observed (**Figure 3.4**) for the three Ac-G w/w combination ratios 4:1, 8:1, and 16:1, at high Fa of ≥ 80%, and ≥ 70% for Caco-2 and Hep G2 cell lines, respectively. The synergistic effect increased over the increase of the affected fraction ranging from slight to strong synergism.

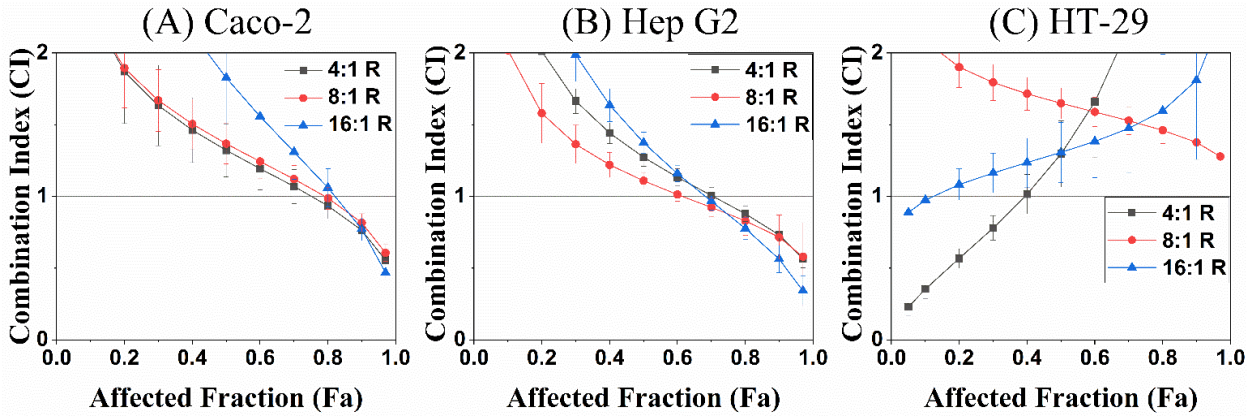


Figure 3.4. The Combination Index (CI) plot of the combined inhibitory effects of anthocyanins (Ac) and gingerols (G) at Ac-G w/w combination ratios of 4:1, 8:1, and 16:1 on (A) Caco-2, (B) Hep G2, and (C) HT-29 cell lines. Simulated curves of actual combination data (point)s ± SDA: Sequential Deletion Analysis.

Results from the Caco-2 cell line indicate strong synergism (CI: 0.7- 0.4) (**Table 3.3**) at high Fa of 90% and higher for all the three tested combination ratios with DRI values of 1.4-2.8 for anthocyanins and DRI values of 3.6-16.6 for gingerols. Similar trends of the CI curves (**Figure 3.4.A**) were observed for the three combinations. CI values (**Table 3.3**) revealed moderate antagonism to strong synergism between anthocyanins and gingerols when Fa levels increased from 50 to 97%, respectively.

Combination index curves of the combinations of anthocyanins and gingerols on Hep G2 cells (**Figure 3.4.B**) showed similar trends to those on Caco-2 cells described above with synergism at a wider window of Fa starting at 70%. Strong synergism (CI: 0.7- 0.3) was observed (**Table 3.4**) at Fa of 80% and higher for all three combinations at Ac-G w/w ratios of 4:1, 8:1, and 16:1. DRI values of 1.1-3 and 2.2-103 were observed for anthocyanins and gingerols, respectively, at Fa of 50 to 97%.

HT-29 cells exhibited different trends of the CI curves for the three Ac-G w/w combination ratios of 4:1, 8:1, and 16:1. In **Figure 3.4.C**, synergism was observed only at the Ac-G w/w combination ratio of 4:1 at low Fa of <50%. A high synergism indicator, CI of 0.56, was obtained at Fa of 20% (**Table 3.5**), where DRI of 2.47 and 6.16 were observed for anthocyanins and gingerols, respectively. Additive effect and strong antagonism were noticed at the Fa of 40-50% and 90-97%, respectively.

Table 3.3. The Combination Index (CI) and Dose Reduction Index (DRI) of combined anthocyanins (Ac) and gingerols (G) at Ac-G w/w combination ratios of 4:1, 8:1, and 16:1 at different effect levels on Caco-2 cell line.

Affected Fraction (Fa) %	Treatment dose (μg/mL)				Combination Index (CI) ±SDA*	Dose Reduction Index (DRI)	
	Anthocyanins (Ac)	Gingerols (G)	Combination Ac-G			Anthocyanins (Ac)	Gingerols (G)
			Ratio	Dose (Ac+G)			
50	18.4	9.4	4:1	16.3+4.1	1.31±0.18	1.1	2.3
			8:1	20.2+2.5	1.36±0.14	0.9	3.7
			16:1	29.9+1.8	1.81±0.32	0.6	5.0
75	35.0	16.9	4:1	23.1+5.7	1.00±0.10	1.5	2.9
			8:1	29.3+3.6	1.05±0.08	1.2	4.6
			16:1	36.8+2.3	1.18±0.16	1.0	7.3
90	66.6	30.2	4:1	32.8+8.2	0.76±0.06	2.0	3.7
			8:1	42.6+5.3	0.81±0.06	1.6	5.7
			16:1	45.2+2.8	0.77±0.07	1.5	10.7
97	140.8	59.7	4:1	49.2+12.3	0.55±0.05	2.9	4.8
			8:1	65.8+8.2	0.60±0.06	2.13	7.2
			16:1	57.4+3.5	0.47±0.04	2.45	16.6

*SDA: Sequential Deletion Analysis

Table 3.4. The Combination Index (CI) and Dose Reduction Index (DRI) of combined anthocyanins (Ac) and gingerols (G) at Ac-G w/w combination ratios of 4:1, 8:1, and 16:1 at different effect levels on Hep G2 cell line.

Affected Fraction (Fa) %	Treatment dose (μg/mL)				Combination Index (CI) ±SDA*	Dose Reduction Index (DRI)	
	Anthocyanins (Ac)	Gingerols (G)	Combination Ac-G			Anthocyanins (Ac)	Gingerols (G)
			Ratio	Dose (Ac+G)			
50	95.8	43.9	4:1	78.8+19.7	1.27±0.06	1.2	2.2
			8:1	83.4+10.4	1.10±0.05	1.1	4.2
			16:1	115.8+7.2	1.37±0.07	0.8	6.1
75	212.7	159.0	4:1	150.4+37.6	0.94±0.05	1.4	4.2
			8:1	160.0+20.0	0.87±0.08	1.3	8
			16:1	171.2+10.6	0.87±0.06	1.2	14.9
90	472.5	576.3	4:1	287.2+71.8	0.73±0.05	1.6	8.0
			8:1	306.8+38.3	0.72±0.15	1.5	15.0
			16:1	252.9+15.8	0.56±0.09	1.9	36.5
97	1196.3	2580.2	4:1	609.5+152.3	0.56±0.06	2	16.9
			8:1	654.7+81.8	0.59±0.23	1.8	31.5
			16:1	398.4+24.9	0.34±0.10	3.0	103.6

*SDA: Sequential Deletion Analysis

Table 3.5. The Combination Index (CI) and Dose Reduction Index (DRI) of combined anthocyanins (Ac) and gingerols (G) at Ac-G w/w combination ratios of 4:1, 8:1, and 16:1 at different effect levels on HT-29 cell line.

Affected Fraction (Fa) %	Treatment dose (μg/mL)				Combination Index (CI) ±SDA*	Dose Reduction Index (DRI)	
	Anthocyanins (Ac)	Gingerols (G)	Combination Ac-G			Anthocyanins (Ac)	Gingerols (G)
			Ratio	Dose (Ac+G)			
20	31.9	19.9	4:1	12.9+3.2	0.56±0.06	2.5	6.2
			8:1	50.5+6.3	1.89±0.14	0.6	3.2
			16:1	31.5+1.9	1.08±0.10	1.0	10.1
50	52.0	22.8	4:1	42.9+10.7	1.29±0.23	1.2	2.1
			8:1	66.7+8.3	1.65±0.10	0.8	2.7
			16:1	59.4+3.7	1.30±0.20	0.9	6.1
75	76.6	25.5	4:1	111.3+27.8	2.54±0.87	0.7	0.9
			8:1	83.2+10.4	1.49±0.09	0.9	2.5
			16:1	98.6+6.1	1.53±0.34	0.8	4.1
90	112.8	28.5	4:1	288.6+72.1	5.09±2.86	0.4	0.4
			8:1	103.9+12.9	1.37±0.09	1.1	2.2
			16:1	163.7+10.2	1.81±0.55	0.7	2.8
97	177.1	32.4	4:1	874.9+218.7	8.25±6.1	0.2	0.1
			8:1	134.4+16.8	1.31±0.09	1.3	1.9
			16:1	295.2+18.4	2.04±0.74	0.6	1.8

*SDA: Sequential Deletion Analysis

Even though the three Ac-G w/w combination ratios of 4:1, 8:1, and 16:1, showed similar trends of synergism on both cell lines Caco-2 and Hep G2, DRI values seem to be affected by the portions involved in the combination at each combination ratio. Interestingly, favorable DRIs for anthocyanins were observed for the Ac-G combination ratio of 4:1 at Fa of 50% and higher; meanwhile, the combined ratio of 16:1, which contains a higher anthocyanins portion, showed favorable DRIs only at Fa of ≥90%. These results suggest that a higher amount of gingerols in the combination increased the potency of DRI for anthocyanins within a wider window of Fa. On the other hand, higher DRI values of gingerols were observed for the Ac-G combination ratio of 16:1 than those observed for the combination ratios with a higher gingerol portion.

3.4 Discussion

This study was designed to evaluate the combined effects of anthocyanins and gingerols to inhibit the proliferative activity of Caco-2, Hep G2, and HT-29 cancer cells compared to the effects of each extract. This study reveals strong synergism between anthocyanins and gingerols for the three tested combination ratios in inhibiting the growth of Caco-2 and Hep G2 cells at high effect levels. Only those fractions of the original bilberry and ginger root extracts solubilized in the medium were tested, and no other chemical solvent was used. The purity of anthocyanins and gingerols in the water-soluble fractions of the extracts was determined. The water-soluble fraction of the bilberry extract showed a high purity of anthocyanins at ~97%, and thus the effects of bilberry extract are attributed to the anthocyanins. Meanwhile, the purity level of gingerols in the water-soluble fraction of the ginger root extract was not high, and gingerols comprised about 64% of the water-soluble fraction. Thus, other components, which make up about 36% of the water-soluble fraction, may also be contributing to the observed effects.

In this study, the growth of cancer cells was inhibited more by combining anthocyanins and gingerols than the individual extracts at the same dosage levels on the three tested cancerous cell lines. Synergism at higher affected fractions reveals high potency in inhibiting the growth of cancer cells, where a higher Fa reflects more potential for therapeutic efficacy (Fu et al., 2016). Thus, the strong synergism observed between anthocyanins and gingerols suggests a high potency of the Ac-G combination to inhibit cancer cell growth. Moreover, DRI values are increasing with the increments of the Fa values, reflecting a strong synergism (Heiduschka et al., 2014). For Caco-2 cells, the same Fa of 97% was achieved for anthocyanins at 2.86-fold reduced dosage when used in combination with gingerols at the Ac-G ratio of 4:1, where a DRI value of 4.84 was observed for gingerols.

The low CI values of Ac-G combinations observed on Caco-2 and Hep G2 cells were similar to those reported by Yang and Liu (2009) for the combination of apple extract and Q-3-glc to inhibit the proliferation of breast cancer cells (MCF-7) at Fa of 95%. Similar CI values but different trends of synergism were reported by Majumdar et al. (2009), with curcumin and resveratrol combination on colorectal cancer cells HCT-116, with CI of 0.43-0.9 for the Fa of 29-81%, respectively. Interestingly, high synergistic interactions of binary combinations of ginger phytochemicals to inhibit the proliferation of prostate cancer cells (PC-3) were demonstrated by Brahmbhatt et al. (2013), but Yusof et al. (2015) showed that 6-gingerol exhibited moderate synergism with γ-tocotrienol on human colorectal cancer cells including HT-29 and SW837.

DRI helps to reduce the dose for the same effect and consequently reduce the potent toxicity of high doses (Fu et al., 2016). To illustrate this concept, Fa of 97% for Hep G2 cells requires either 1196.3 µg/mL of anthocyanins or 2580 µg/mL gingerols; however, these concentrations were shown to be cytotoxic. Meanwhile, the same effect can be obtained with 398.4 µg/mL

anthocyanins combined with 24.9 µg/mL gingerols (Ac-G w/w ratio 16:1), which exhibited low cytotoxicity. DRIs of 3- and 103-fold were observed in this combination for anthocyanins and gingerols, respectively. At lower doses, 90% Fa requires that Hep G2 cells are treated with 472.5 µg/mL anthocyanins or 576.3 µg/mL gingerols, which are higher than the LD_{50} in the cytotoxicity study. A similar effect was obtained when anthocyanins and gingerols were combined at the ratio of 16:1 and doses of 252.9 and 15.8 µg/mL for anthocyanins and gingerols, respectively; however, concentrations here are much lower than the LD_{50} and considered nontoxic. The same (Fa)s of 97% and 90% were also obtained when combining anthocyanins and gingerols at the w/w combination ratios of 8:1 and 4:1, with desired but lower DRI values. Thus, the combinations at the Ac-G ratios of 4:1 and 8:1 exhibited synergism with considerable DRIs for both extracts, but the combination ratio of 16:1 showed higher DRIs, which brings the combined doses down to the nontoxic levels. This dose reduction is important for different applications. The different results observed by varying the Ac-G w/w ratio can be attributed to the effects exerted by each part of the combination at each specific combination ratio and dose. Such effects depend on the various molecular signal pathways that might be activated by different components at different concentrations, which is worthy of investigation in the future. In general, DRI values for gingerols were higher than those for anthocyanins. Since both anthocyanins and gingerols showed low bioavailability (Mueller et al., 2017; Mukkavilli et al., 2017), even a slight increase in the DRI of anthocyanins and/or gingerols extracts is considered beneficial because low available levels of both anthocyanins and gingerols could give higher bioactive effects when combined.

Selectivity of anthocyanins and gingerols and their combinations against cancer cells was evident in this study, since no cytotoxicity was observed on normal cells at concentrations that exhibited high inhibition on the three cancer cell lines. Cytotoxicity against cancer cells is

attributed to the higher metabolic rates, making them more susceptible to the cytotoxic effect of the phenolic compounds (Imani et al., 2021). Such selectivity has been evidenced for anthocyanins in previous studies, such as that extracted from black lentil, sorghum (Mazewski et al., 2018), and purple and red corn (Mazewski et al., 2017). 6-Gingerol also exhibited selectivity in cytotoxicity to cancer cells in a previous study (Radhakrishnan et al., 2014).

Previously reported effects of anthocyanins and gingerols as individual extracts were reproducible. In the present study, IC_{50} of anthocyanins on Caco-2 and HT-29 cell lines agree with the findings of Yi et al. (2005) that IC_{50} of anthocyanin fractions extracted from blueberries ranged from 15 to 50 μg/mL on Caco-2 and HT-29. Furthermore, IC_{50} of 25 μg/mL was observed for anthocyanin extract from chokeberry on HT-29 cells by Zhao et al. (2004). The IC_{50} values for gingerols, observed in this study, on Caco-2, Hep G2, and HT-29 cell lines are comparable to the IC_{50} of ~ 28.3 μg/mL for 6-gingerol extract on HeLa cells, reported by Zhang et al. (2017). In general, the effective concentrations of the gingerols in this study are comparable to the reported effective concentrations of 25–200 μM of 6-gingerol to inhibit the proliferation and invasion of AH109A hepatoma cells reported by Yagihashi et al. (2008). In this study, Caco-2 cells were the most sensitive to both extracts with the lowest IC_{50} compared with the other two cell lines Hep G2 and HT-29.

There was a considerable difference between the IC_{50} of both extracts on the two colorectal cancer cells Caco-2 and HT-29. Moreover, different trends of the CI curves for the three combinations on HT-29 cells were observed. Such differences might be because Caco-2 cells express wild-type p53, a key signal for cell growth and apoptosis, compared to HT-29 cells, which have a p53 mutation (Karpf et al., 2001). Thus, the growth inhibitory effects of anthocyanins and gingerols and their combinations could be p53 independent. Moreover, HT-29 cells are

heterogeneous and partially differentiate into mucous secretory cells and columnar absorptive cells, while Caco-2 cells differentiate into enterocytes with high homology (Gagnon et al., 2013). Lee et al. (2008) also reported 6-gingerol to inhibit the growth of several human colorectal cancer cells at different levels. They attributed this to the differences in the doubling time of various cell lines, where cells with short doubling times grow faster and are less susceptible to growth inhibition.

3.5 Conclusion

This research is the first to demonstrate synergism between anthocyanins and gingerols to inhibit the growth of cancer cells in *in vitro* models. A strong synergism between anthocyanins and gingerols on Caco-2 and Hep G2 cells was observed with CI values of 0.47 and 0.34, respectively, at a high effect level of 97%. DRI calculation indicated that the same growth inhibitory effect of ≥ 80% was achieved for anthocyanins at a reduced dosage of up to 3-fold and gingerols at a reduced dosage of up to 103-fold when they were combined. This research provides the rationale for further study of such synergistic effects using animal models and may provide reasonable evidence for further clinical testing. Positive *in vivo* results will enable combinations to be used as natural health products to lower the risks of cancer. Nevertheless, further research is required to understand the synergism mechanisms.

Chapter 4: Mechanisms mediating the synergistic anticancer effects of combined anthocyanins and gingerols[2]

4.1 Introduction

Carcinogenesis is a multistep process associated with the accumulation of several mutations involving activation of oncogenic genes and inactivation of tumor suppressor genes. Neoplastic cells acquire new functional capabilities, which enable them to survive and proliferate (Hanahan & Weinberg, 2011), which were discussed with more details in chapter 2. Dysregulation of cell proliferation (cell cycle) and suppression of programmed cell death (apoptosis) are considered hallmarks of neoplastic progression and pose primary targets for therapeutic intervention in all cancers (Evan & Vousden, 2001). There are several mechanisms through which these two dysregulations can occur. These processes are controlled mainly by the transcription factor p53 (a tumor suppressor protein, TP53) that regulates the expression of several genes associated with the cell-cycle arrest and induction of apoptosis. It controls the regulation of the cell cycle by p53-dependent pathway. P53 is activated in response to oxidative stress or DNA damage to repair DNA or induce apoptosis. The activation of p53 partly involves its phosphorylation by the mitogen-activated protein kinases MAPK member (p38-MAPK) (Aguda et al., 2007).

During cell proliferation, transitions between different cell cycle phases (G0/G1, S, G2, and M) are firmly controlled through checkpoints at the S and M phase initiation and during the M phase (Hanahan & Weinberg, 2011; Pillat et al., 2013). However, if interrupted, cells can be arrested at a specific stage and undergo apoptosis. Replicative stress or DNA damage can interrupt

[2] A version of this chapter will be submitted for consideration for publication as Amna E Abdurrahim, Vera Mazurak and Lingyun Chen. "Mechanisms mediating the synergistic anticancer effects of combined anthocyanin and gingerol extracts."

the cell cycle and prevent the progression of cells to the next phase. For example, cyclin-dependent kinase-2 (CDK-2) plays a critical role in the G1/S, S, and G2 phases. It is a crucial kinase for initiating DNA replication in the S phase by binding to cyclin E and pushing cells from the S phase to the G2/M phase by binding to cyclin A. Subsequently, cell division cycle 25 A phosphatase (Cdc25A) activates the CDK by removing the inhibitory phosphates from tyrosine and threonine residues (Dai et al., 2013). DNA damage could activate the M phase initiation checkpoint mechanisms by targeting the cyclin-CDK complexes, thus interrupting cell cycle progression and providing extra time for damage removal (Pillat et al., 2013). Cell-cycle inhibitors such as $p21^{WAF-1}$ and $p27^{KIP1}$ inhibit CDK proteins and negatively regulate the cell cycle when overexpressed. The p21 is one of the first identified targets of the transcriptional factor p53, and it can be transactivated by p53. Recently, it has become apparent that p21 could be stimulated by other pathways that are p53-independent such as cellular stress. Although p21 represents a critical pro-survival molecule, it also inhibits apoptosis (Abbas & Dutta, 2009; Aguda et al., 2007).

There are two main apoptotic pathways, intrinsic (mitochondrial) and extrinsic (FAS and FAS ligand). Cells receive either internal stimuli such as DNA damage, endoplasmic reticulum (ER) stress, or external stimuli such as reactive oxygen species (ROS) and growth factor withdrawal to activate signal transduction cascades leading to their collapse (Galluzzi et al., 2018). At the same time, apoptosis can be suppressed by survival signals generated internally or derived from neighboring cells (Tang & Kehrer, 2007).

Bcl family proteins, caspase signaling proteins, and p53 transcription factor are key factors in regulating apoptosis. Caspase cascades include initiator (caspases 8 and 9) and effector (caspases 3 and 7) caspases. The Bcl family of proteins include proapoptotic proteins such as Bad and Bax, which increase the permeability of the mitochondrial membrane and induce the release of

cytochrome c, which is required to activate caspase-9 (Tang & Kehrer, 2007). Bcl family proteins also include antiapoptotic proteins (pro-survival molecules) Bcl-2 and Bcl-xL, which can be induced in response to apoptotic stimuli such as DNA damage, a mitochondrial toxin, and trophic factor deprivation (Galluzzi et al., 2018; Tang & Kehrer, 2007). The Bcl family member Bid is responsible for the crosstalk between the extrinsic and intrinsic apoptosis pathways, which occurs mainly through Bid cleavage by active caspase-8 and its translocation to the mitochondria. It directly binds to Bax and induces its oligomerization or binds to and inactivates Bcl-2 and Bcl-xL. Caspase-8 is a common target for death-inducing signaling complex (DISC) activation through the death receptors such as FS-7-associated surface antigen (Fas) and tumor necrosis factor (TNF) receptors (Tang & Kehrer, 2007).

Cell cycle progression and cellular apoptosis are initiated and characterized by mechanisms exhibiting interconnectivity and overlap (Galluzzi et al., 2018). For example, activation of extracellular regulated kinase (ERK) (a survival factor) generally protects cells from apoptosis by regulating molecules such as Bcl-xL. The RAF/MEK/ERK pathway, activated by extracellular signals (growth factor), regulates cell-cycle progression. Meanwhile, growth factor withdrawal leads to sustained activation of c-Jun N-terminal kinases (JNK) and p38-MAPK and the inhibition of ERKs (Tang & Kehrer, 2007).

The ability of polyphenolic compounds to modulate multiple biological mechanisms involved in cancer initiation and progression through direct interaction or modulation of gene expression has been well studied (Fantini et al., 2015; Khan et al., 2020). Individual agents target a few molecular mechanisms, while the combination of more than one agent impacts multiple pathways and may provide cumulative or additive effects (Breda & Kok, 2018).

Synergism between anthocyanins and gingerols to inhibit the growth of colorectal (Caco-2) and hepatic (Hep G2) cancer cell models have been reported in the prior work (Chapter 3), and a strong synergism was observed at high inhibitory effect levels. The mechanisms underlying these effects have not been explored. Anthocyanins (Mazewski et al., 2018; Shin et al., 2009; Yi et al., 2005) and gingerols (Lee et al., 2008; Lin et al., 2012; Radhakrishnan et al., 2014) have been reported to inhibit cell proliferation, induce apoptosis, and suppress inflammation by modulating different cellular signaling pathways. This study aimed to define those pathways that function synergistically when gingerols and anthocyanins are combined. The focus is on the cell cycle progression and apoptosis pathways. It was hypothesized that combining anthocyanins and gingerols increase the effects of activating some signals triggered by one or the two individual extracts. Also, they may activate some non-active signals to promote their synergistic effect in inhibiting the growth of cancer cells. Therefore, the goal of this study was to evaluate some of the mechanisms mediating the antiproliferative effects of Ac-G combined treatment and the potential involvement of apoptotic effects.

4.2 Materials and methods

4.2.1 Materials

Bilberry extract powder, ginger root extract powder, Caco-2 cells, Hep G2 cells, DMEM medium, EMEM medium, FBS, trypsin-EDTA, PBS and penicillin-streptomycin solutions are the same as mentioned in the first study (Chapter 3, Section 3.2.1). In addition, propidium iodide (PI) and RNase A were purchased from Sigma-Aldrich Canada Co., (Oakville, ON, Canada).

4.2.2 Cell culture conditions and treatments

Caco-2 and Hep G2 cells were cultured, sub-cultured and harvested using the protocols described in Chapter 3 (Section 3.2.3). Cell suspension was diluted to the desired cell count of

2×10^5 cells/mL and distributed into the wells or plates for experiments. Caco-2 and Hep G2 cells were seeded in 6-well plates for cell cycle and apoptosis evaluation. Caco-2 cells were seeded in BioLite 100 mm Tissue Culture Dishes for gene expression experiments. Cells were incubated for 24 h for attachment and then treated and incubated for 48 h with the desired concentrations of individual and combined treatments of anthocyanins and gingerols in a 5% FBS medium. Control wells or plates (medium and cells, no treatment) were includes for each experiment and all washing steps and media change were applied on them as well. At the end of the incubation period, cells were trypsinized, collected, and washed twice with precooled PBS. Cell pellets were used for cell cycle and apoptosis experiments and RNA extraction.

Stock solutions of 1000 µg/mL of anthocyanins or gingerols were prepared by dissolving the dry bilberry or ginger root extracts, respectively, in the medium as mentioned previously in Chapter 3 (Section 3.2.3), diluted to the desired concentration, and applied on the cells. The modulatory effects of anthocyanins and gingerols as individuals and in combinations on the progression of cell cycle phases and apoptosis were evaluated on Caco-2 and Hep G2 cancer cells after incubation with treatments for 48 h using flow cytometry. Concentrations of 50 µg/mL anthocyanins with 3.125, 6.25, 12.5, 25, and 50 µg/mL gingerols at Ac-G w/w combination ratios 16:1, 8:1, 4:1, 2:1 and 1:1 were selected for Caco-2 cells, which caused ~ 90-97% growth inhibition in the first study (Chapter 3). Hep G2 cells were treated with 100 µg/mL anthocyanins combined with 12.5, 25, and 50 µg/mL gingerols at the w/w ratio of 8:1, 4:1, and 2:1. Individual anthocyanin and gingerol treatments were applied for comparison. In addition, cells incubated with only the growth media were used as a control for each experiment.

4.2.3 Cell cycle experiment

Cell cycle measurements were performed using flow cytometry of fixed cells stained with propidium iodide (PI). At the end of the incubation period, cells were detached, washed, and set in 70% (v/v) ethanol at 4ºC overnight. Then, cells were washed twice with PBS for rehydration and resuspended in the PI solution containing PI (50 μg/mL) and RNase A (100 μg/mL) in PBS; they were incubated for 30 min in the dark (Shin et al., 2009). DNA content was determined using the acoustic focusing cytometer (Attune NxT, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Data analysis for cell cycle profiles was carried out using the Flowjo software. More than 10,000 cells were evaluated for cell cycle measurement for each sample with triplicates of each treatment.

4.2.4 Cell apoptosis experiment

Apoptotic analysis was performed using the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) and flow cytometry. Briefly, the pellets of treated and non-treated cells were resuspended and stained with Annexin V-FITC and propidium iodide dye as instructed in the kit. Then, the cells were detected on an acoustic focusing cytometer (Attune NxT, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) within 1 h. More than 10,000 events were evaluated for each sample. All assays were performed in triplicates. Data were analyzed using the FlowJo software.

4.2.5 Real-Time PCR Analysis

According to the manufacturer's instructions, total RNA was extracted, separated, and purified using PureLink RNA Mini Kit (Invitrogen, Burlington, ON, Canada). On-column DNase treatment was conducted. RNA quantity and quality were assessed using NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Inc. Wilmington, DE, USA). Impure mRNA samples were

excluded from the analysis (absorbance at 260nm/280 nm ratio < 2). Complementary DNA (cDNA) was synthesized using 1 µg of total RNA and high-capacity cDNA transcription kit from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA). The same sample was used for the negative control but without reverse transcriptase. Quantitative PCR was performed using the Real-Time PCR system (StepOnePlus, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Expressions of target genes p53, p21 (CDKN1A), Caspase-3, Bcl-2 and Bax , Cyclin-A , CHEK1 (CHK1), cyclin-dependent kinase-2 (CDK-2), Cdc25 A, Caspase-8, Caspase-9, Bad, Bid, Bcl-xl, p27, ERK (P38MAPK), and JNK were determined by real-time PCR using TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays TP53 (Hs01034249_m1), CDKN1A (Hs00355782_m1), Caspase-3 (Hs00234387_m1), Bcl-2 (Hs00608023_m1), Bax (Hs00180269_m1), Cyclin-A (SCAPER) (Hs01065655_m1), CHEK1 (CHK1) (Hs00967506_m1), CDK-2 (Hs01548894_m1), Cdc25 A (Hs00948005_m1), Caspase-8 (Hs01018151_m1), Caspase-9 (Hs00962278_m1), Bad (Hs00188930_m1), BID (Hs00609632_m1), Bcl-xl (Hs00236329_m1), P27KIP1 (Hs00153277_m1), ERK (P38MAPK) (Hs01046830_m1), JNK (Hs01548508_m1), and TaqMan Endogenous control, Human GAPDH (Hs99999905_m1). Quantitative PCR reagents were purchased from Applied Biosystems (Life Technologies Inc. Burlington, ON, Canada). For cDNA sampling, 3 biological replicates and 2 technical replicates were applied for each treatment, which allowed sample comparison in one plate for assessing one target gene every run. Change in the mRNA gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Therefore, results are presented as the fold change of the target gene expression in treated samples relative to control samples, normalized to the reference gene (housekeeping gene, GAPDH).

4.2.6 Statistical Analysis

Each experiment was performed at least in triplicates (n = 3-5) and presented in the results as mean ± SD. The statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Significant differences were defined at the $p < 0.01$. The computer software Origin 2020 was used to analyze and present the data.

4.3 Results and Discussion

4.3.1 Effects of anthocyanin and gingerol combinations on the cell cycle

The modulatory effects of anthocyanins, gingerols, and their combined extracts on Caco-2 cell cycle progression are presented in **Figure 4.1**. **Figure 4.1.A** shows the portions of cells in each cell cycle phase: G0/G1, S, and G2/M phases. An accumulation of cells in the S phase was observed for different treatments compared to the control. This increase in the S phase arrested cells was accompanied by a decrease in the G0/1 phase and a slight decrease in the G2/M phase.

The accumulation in the S phase was observed at all the Ac-G combined treatments. In addition, accumulation in the individual anthocyanin (50 µg/mL) and gingerol (50 and 25 µg/mL) treatments were significantly ($p < 0.01$) higher than in the control cells (23.8%) (**Figure 4.1.B**). Moreover, the accumulation of cells in the S phase (33.1-33.8%) observed when cells were treated with 50 µg/mL anthocyanins combined with 6.25, 12.5, 25, and 50 µg/mL gingerols were significantly ($p < 0.01$) higher than the anthocyanins alone (30%) and the corresponding gingerol treatments (23.7-27.3%) (**Figure 4.1.B**).

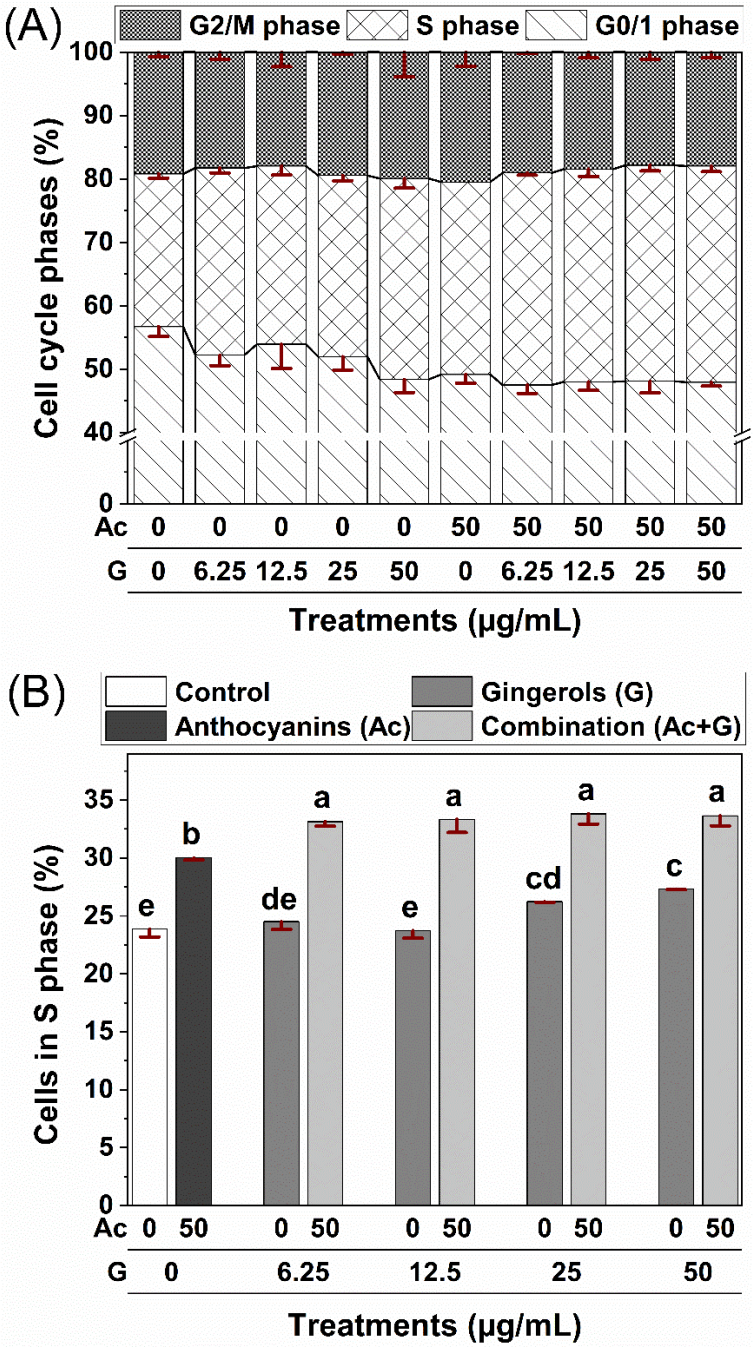


Figure 4.1. Cell cycle measurements of Caco-2 cells after incubation for 48 h with different treatments of anthocyanins (Ac) and gingerols (G) and their combinations, (A) percentages of cell cycle phases G0/1, S, and G2/M, (B) S phase percentages of cells treated with different Ac-G combinations compared to the individual treatments. Significant differences at $p < 0.01$ are designated by the letters a-e.

The increase of cells arrested in the S phase was also observed in Hep G2 cells under combined and individual treatments, accompanied by decreased cell percentages in both G1 and G2 phases (**Figure 4.2.A**). This increase in cell accumulation in the S phase was dose-dependent (**Figure 4.2.B**). A significant ($p < 0.01$) increase in the cells arrested at the S phase, from 19.2% to 28.5% and 23.7%, was observed on the cells treated with 100 µg/mL anthocyanins alone and in combination with 50 and 25 µg/mL gingerols, respectively (**Figure 4.2.B**). The increases in the cell accumulation in the S phase at all combined treatments, in addition to the individual doses of 100 µg/mL anthocyanins and 50 and 25 µg/mL gingerols, were significant ($p < 0.01$) compared to the control (**Figure 4.2.B**).

Anthocyanins and gingerols individually and in combination induced Caco-2 and Hep G2 cell cycle arrest in the S phase. Cell cycle arrest at the S phase means cells entered the S phase to duplicate DNA but could not progress further through the G2/M phase. Replicative stress or DNA damage might cause this. It has been reported that DNA damage activates mechanisms of the M phase initiation checkpoint by targeting the cyclin-CDK complexes, thus interrupting cell cycle progression to enable the removal of damage (Pillat et al., 2013).

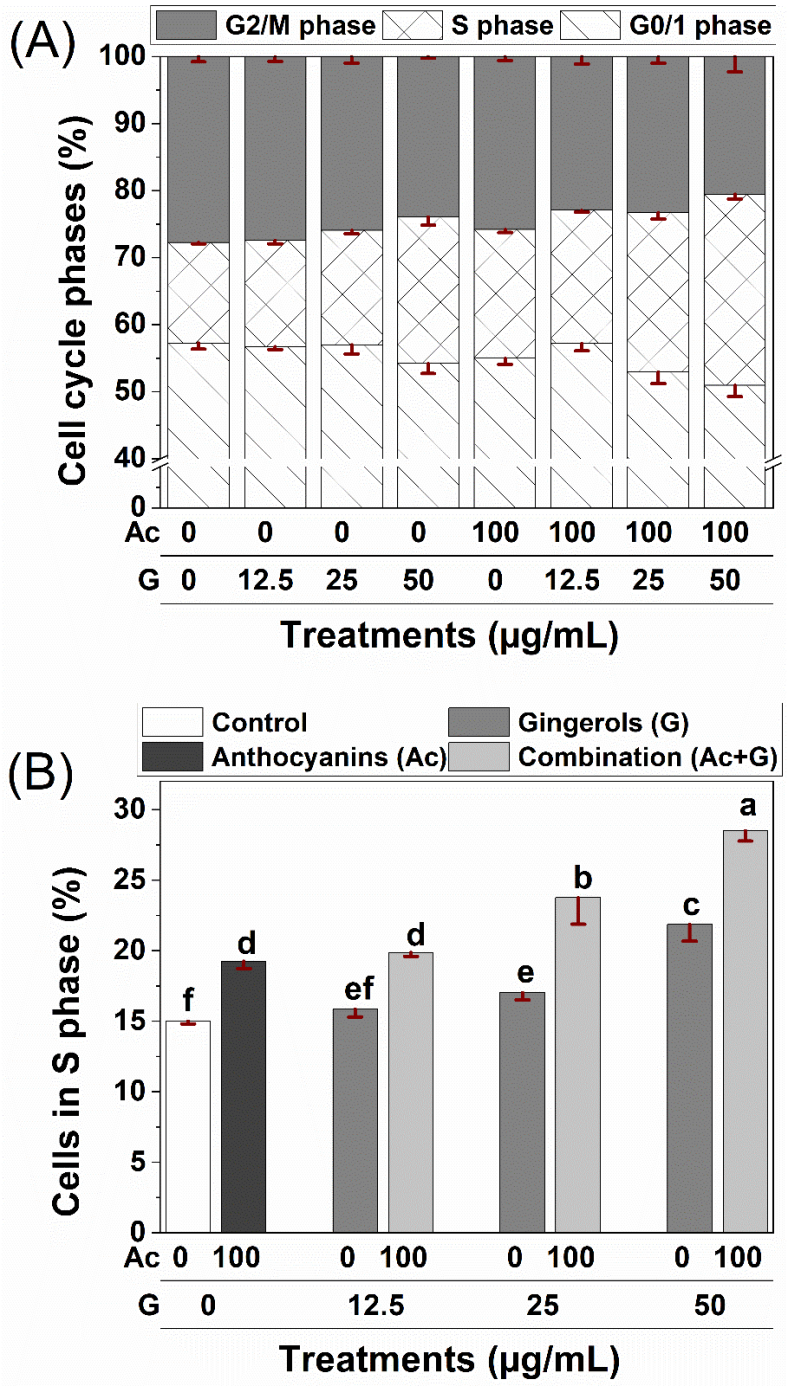


Figure 4.2. Cell cycle measurements of Hep G2 cells after incubation for 48 h with different treatments of anthocyanins (Ac) and gingerols (G) and their combinations, (A) percentages of cell cycle phases G0/1, S, and G2/M, (B) S phase percentages of cells treated with different Ac-G combinations compared to the individual treatments. Significant differences at $p < 0.01$ are designated by the letters a-f.

Published literature has identified both up and downregulation of cell cycle phases for the individual effects of anthocyanins or gingerols extracted from different sources or their constituents. For instance, for the colorectal adenocarcinoma cell lines, Mazewski et al. (2017) reported that anthocyanins extracted from black lentil, sorghum, and grape induced the arrest in the G1 phase of HT-29 cells compared to untreated cells. Lazze et al. (2004) reported that delphinidin inhibited Caco-2 cell growth and caused a significant reduction of cells in the G1 phase at a dosage of 200 mM, accompanied by an accumulation of cells in the G2/M phase. Lee et al. (2008) studied the effects of 6-gingerol and reported an increase in the G1 phase, and a decrease in the G2 phase in HCT116 and LoVo cells, while phase progression was not affected in SW480. Observations of cell cycle arrest at the S phase in Hep G2 cells treated with anthocyanins in this study were similar to that reported by Zhan et al. (2016), who observed that when Hep G2 cells were treated with 25% blueberry extract; this was accompanied by a significant decrease in the G2 phase.

The significant increase in cell cycle arrest at the S phase observed in this study under most Ac-G combinations compared to the individual treatments drives the consideration that synergism between anthocyanins and gingerols might, even partially, be attributed to their effects in suppressing the progression of the cell cycle to enter the G2/M phase. Given these results, the key regulatory factors in the cell cycle and those related to the S phase in the cell cycle were studied in Caco-2 cells to understand the possible molecular mechanisms involved in the synergistic interactions observed between anthocyanins and gingerols.

4.3.2 Effects of anthocyanin and gingerol combinations on cell apoptosis

Apoptosis plays an essential role in cell survival, growth, development, and tumorigenesis (Aguda et al., 2007). To assess the effects of anthocyanin and gingerol combinations in inducing

apoptosis of cancer cells, Caco-2 and Hep G2 cells were pretreated with individual and combined extracts for 48 h and examined for apoptosis using the Annexin V-FITC/PI labeling. Cells labeled with Annexin V-FITC represent early apoptosis, while cells labeled with PI represent late apoptosis and necrosis (early and late apoptosis are added and presented as apoptotic cells). Live cells are negative to both dyes.

Figure 4.3.A shows the percentages of apoptotic and living Caco-2 cells under different treatments. Cells treated with gingerols at doses of 3.125, 6.25, 12.5, and 25 µg/mL exhibited a dose-dependent increase in the apoptotic cell percentages (21.2-32.8%) (**Figure 4.3.B**). Meanwhile, the increases in the apoptotic cells when those doses of gingerols were combined with 50 µg/mL anthocyanins at Ac-G w/w combination ratios of 16:1, 8:1, 4:1, and 2:1 were higher but not dose-dependent. Anthocyanins treatment at 50 µg/mL induced significant ($p < 0.01$) apoptotic effects (31.9%) on the Caco-2 cells compared to the control (18.3%). However, Ac-G combinations of (50+3.125), (50+6.25), and (50+25) µg/mL showed significant ($p < 0.01$) increases in the apoptotic cells (42.9, 40.6, and 46.6%) compared to the individual anthocyanins and the corresponding gingerol treatments alone (**Figure 4.3.B**). Those Ac-G combinations represent the w/w combination ratios of 16:1, 8:1, and 2:1. Thus, the Ac-G combinations (50+3.125) and (50+25) µg/mL were chosen for the gene expression assays.

The observed level of apoptosis (31.9%) in Caco-2 cells treated with 50 µg/mL anthocyanins align with the previously reported apoptosis levels of (42.5% and 38.3%) when HT-29 and HCT-116 cells were treated with an anthocyanin-rich extract from black lentil (0.9 mg dry extract/mL ~ 52 µg/mL anthocyanins) (Mazewski et al., 2018).

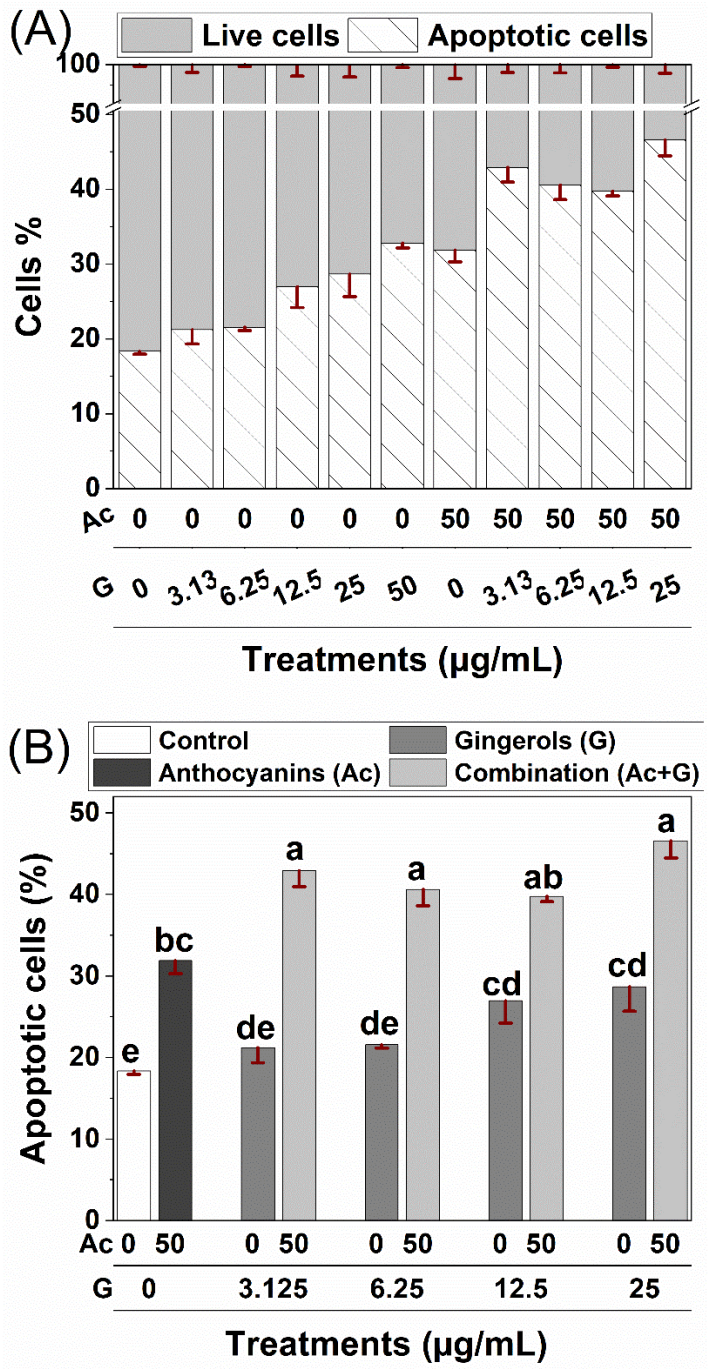


Figure 4.3. The apoptotic effects of anthocyanins (Ac) and gingerols (G) and their combinations on Caco-2 cells, (A) the percentages of apoptotic and live cells after incubation for 48 h with different treatments, (B) the percentages of apoptotic cells when cells were treated with different combinations compared to the individual treatments. Significant differences at $p < 0.01$ are designated by the letters a-e.

Figure 4.4 shows the apoptosis results of Hep G2 cells. Significant ($p < 0.01$) differences were observed only at individual treatments of 50 µg/mL gingerol and 100 µg/mL anthocyanins, with apoptotic levels of 8.7% and 9.1%, respectively, compared to 5.8% observed in the control (**Figure 4.4.B**). However, when a dose of 100 µg/mL anthocyanins was combined with 50 µg/mL gingerols, the apoptotic effect was significantly ($p < 0.01$) increased to 12.7%. The apoptotic effects induced by individual gingerols at doses of 6.25, 12.5, 25, and 50 µg/mL and Ac-G combined treatments at 100 µg/mL anthocyanins were increased in a dose-dependent manner (**Figure 4.4.B**).

It has been reported that tumor progression and suppression differ depending on the type of cells and tissue and its position and function (Evan & Vousden, 2001). In the first study (Chapter 3), the observed effective dosage of IC_{50} was higher on Hep G2 than on Caco-2. Therefore, in this study, different dosages were used for the two cell lines, and the observed increases in the apoptosis levels, compared to the controls, were similar when cells were treated with the anthocyanins (1.6-1.7%), and when cells were treated with 2:1 Ac-G combinations (2.2-2.5%).

The results of apoptosis levels (5.79 and 9.06%) in Hep G2 cells for the control and 100 µg/mL anthocyanins treatment are comparable to the apoptosis levels reported by Zhan et al. (2016) for Hep G2 cells treated with plasma from rats fed with no, low and moderate dosages of blueberry juice.

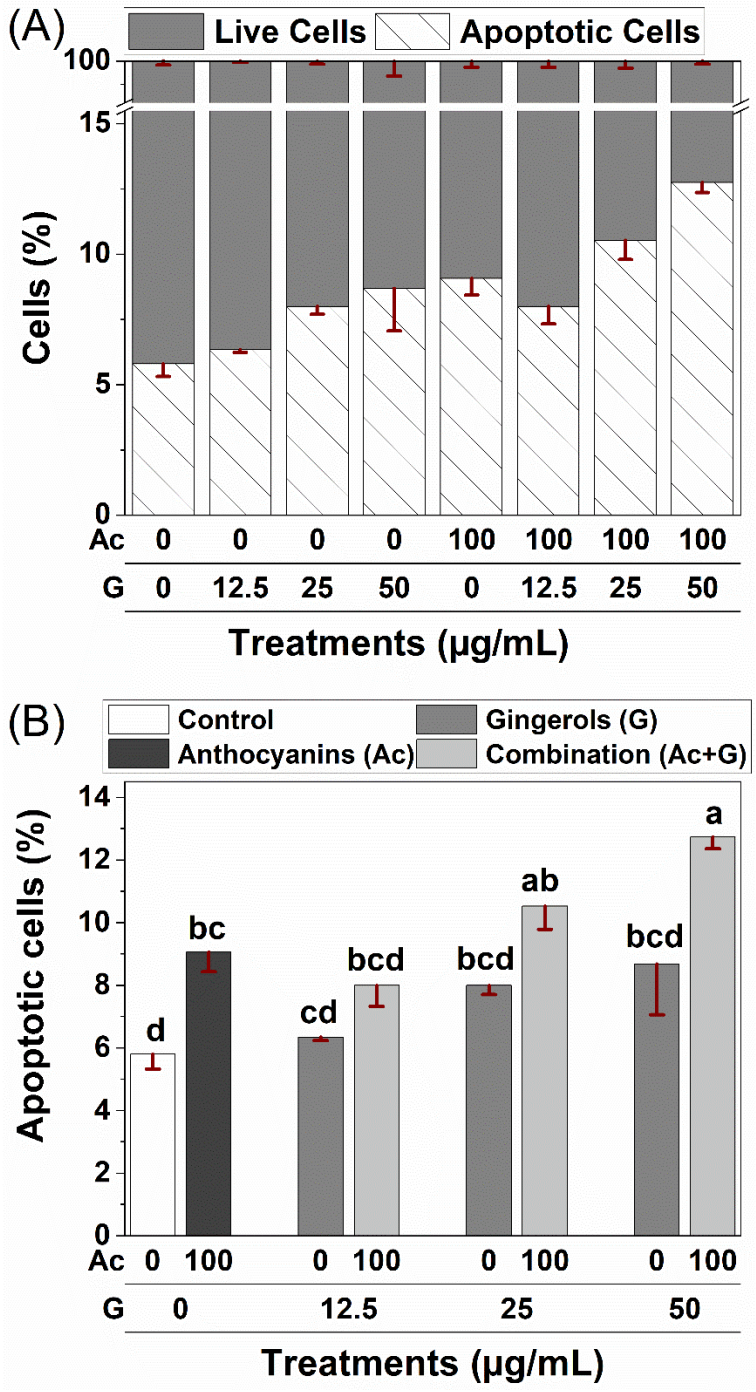


Figure 4.4. The apoptotic effects of anthocyanins (Ac) and gingerols (G) and their combinations on Hep G2 cells, (A) the percentages of apoptotic and live cells after incubation for 48 h with different treatments, (B) the percentages of apoptotic cells when cells were treated with different combinations compared to the individual treatments. Significant differences at $p < 0.01$ are designated by the letters a-d.

4.3.3 Effects of anthocyanin and gingerol combinations on the cellular signaling pathways

4.3.3.1 Effects of anthocyanin and gingerol combinations on the cell cycle-related genes

The effects of Ac-G combined treatments on cell cycle signaling of Caco-2 cells as determined by gene expression assays are shown in **Figure 4.5**, where a significant ($0 < 0.01$) increase in the expression of the p53 (tumor suppressor protein) gene was observed when cells were treated with Ac-G combinations and gingerols but not with the anthocyanins alone.

The expression of p21 in Caco-2 cells was increased significantly ($p < 0.01$) when cells were treated with anthocyanins and gingerols and their combination (**Figure 4.5**). A 6-fold increase in the expression of the p21 gene was observed for the Ac-G combination (50+25) µg/mL compared to the control, which is a substantial change compared to the individual treatments. Also, the (50+3.125) µg/mL Ac-G combination exhibited a significant ($p < 0.01$) higher folds of change than the control and the singular treatments. Despite the marked increase in p21 expression, slight differences were observed in the expression of p27 by different treatments.

In **Figure 4.5**, gingerols treatments induced an increase in the expression of p53 and p21, while anthocyanins induced an increase in p21 expression but not p53. Moreover, under Ac-G combined treatments, there was a significant increase in the expression of p21 and p53 compared to the control; however, the increased expression of p21 was even substantial compared to the individual treatments. Thus, the substantial increase of p21 expression observed under the Ac-G combined treatments of (50+25) and (50+3.125) µg/mL is suggested to be induced by p53-dependent and p53-independent pathways. This increased expression of p21 contributed to the synergistic interaction between anthocyanins and gingerols to inhibit Caco-2 cell growth.

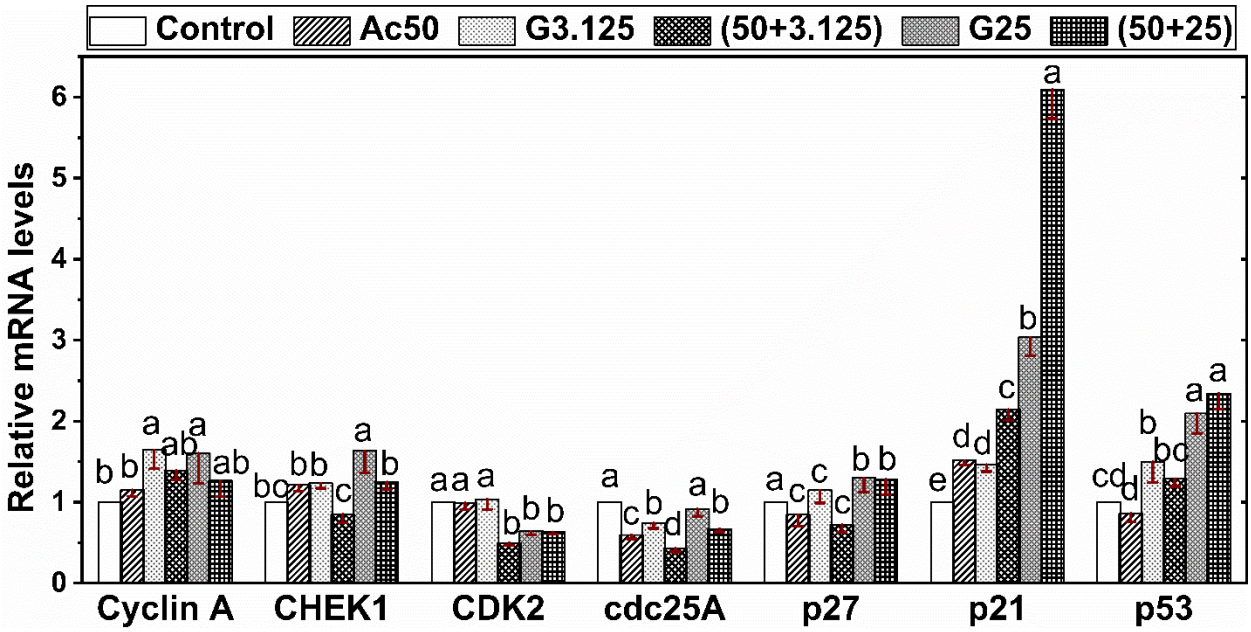


Figure 4.5. Cell cycle-related gene expression (relative mRNA levels) in treated Caco-2 cells after 48 h treatments with 50 µg/mL anthocyanins (A50), and 3.125 and 25 µg/mL gingerols (G3.125 and G25), and Ac-G combinations (50+3.125) (50+25). All the data are expressed as means ± SD (n=3). Significant differences at $p < 0.01$ are designated by the letters a-e.

Other signals are essential in the S phase and involved in the cell cycle progression. For example, the cyclin A−CDK-2−cdc25A complex is the key factor for the smooth entry of cells from the S phase to the G2/M phase, and CHK1 initiates the S phase checkpoint (Dai et al., 2013). In this study, a significant decrease in CDK-2 and cdc25A expression levels was observed in the cells treated with individual and combined extracts compared to the control, contributing to the cell cycle arrest at the S phase (**Figure 4.5**). Moreover, the reduced expression of CDK-2 and cdc25A in cells with Ac-G combined treatments, particularly Ac-G combination of (50+3.125) µg/mL, was significant ($p < 0.01$) compared to each extract treatment alone. Furthermore, anthocyanin and gingerol treatments slightly and significantly increased the expression levels of cyclin A and CHEK1, meanwhile, Ac-G combinations induced insignificant increase compared to

the control. Further, the observed decrease of CDK-2, cdc25A, CHEK1, and cyclin A levels in the cells treated with the combinations compared to the individual treatments also contributed to the higher effects of anthocyanins and gingerols combination to inhibit Caco-2 cell growth.

4.3.3.2 Effects of anthocyanin and gingerol combinations on the expression of apoptosis-related genes

Apoptosis can be initiated in response to the direct activation of the extrinsic (death receptor) or the intrinsic (mitochondrial) death pathways or indirectly by blocking essential survival signals (Tang & Kehrer, 2007). The influences of anthocyanin and gingerol treatments on the expression of apoptotic signaling in Caco-2 cells are shown in **Figure 4.6**.

Gene expression analysis of caspases (**Figure 4.6**) revealed a significant ($p < 0.01$) increase in the expression of the caspase-3 gene when cells were treated with an Ac-G combination of (50+25) µg/mL compared to the individual doses of both extracts and the control. Caspase-3 is an executioner caspase, which can be activated by either intrinsic or extrinsic apoptotic pathway (Tang & Kehrer, 2007). Although 50 µg/mL anthocyanins significantly reduced the expression of caspase-3, when it was combined with 25 µg/mL gingerols, which initially caused a significant increased expression of caspase-3, the caspase-3 expression was even significantly more increased. On the other hand, a significant ($p < 0.01$) decrease in caspase-9 expression was observed in Caco-2 cells treated with the individual 50 µg/mL anthocyanins and (3.125 and 25 µg/mL) gingerols compared to the control; Ac-G combination (50+3.125) showed even more reduction (**Figure 4.6**). Moreover, treating Caco-2 cells with individual gingerols and anthocyanins showed a significant and a slight increase, respectively, in the expression of caspase-8, while Ac-G combination of (50+3.125) µg/mL caused a significant ($p < 0.01$) reduction compared to the control and each extract alone, and significantly less than the control.

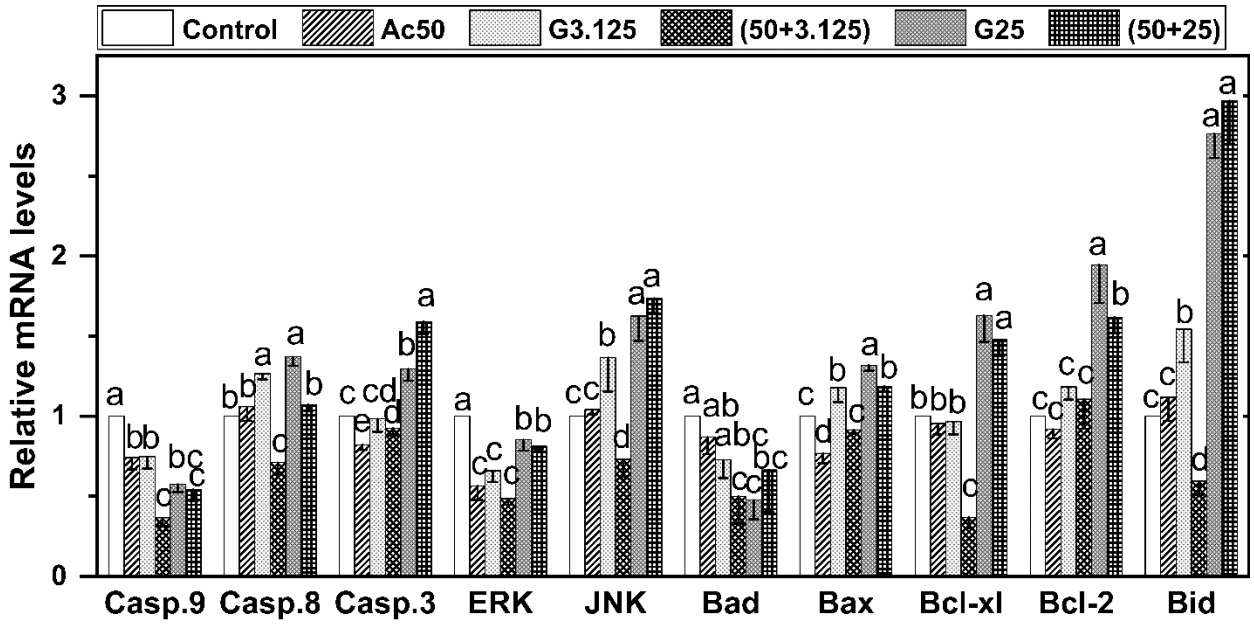


Figure 4.6. Cell apoptosis-related gene expression (relative mRNA levels) in treated Caco-2 cells after 48 h treatments with 50 µg/mL anthocyanins (A50), and 3.125 and 25 µg/mL gingerols (G3.125 and G25), and Ac-G combinations (50+3.125) (50+25). All the data are expressed as means ± SD (n=3). Significant differences at $p < 0.01$ are designated by the letters a-e.

The effects of anthocyanins, gingerols, and Ac-G combinations on the expression of some mitochondrial pathway-related genes are presented in **Figure 4.6**. Gingerol doses of (3.125 and 25 µg/mL) induced a significant ($p < 0.01$) increase in the Bax gene expression, but anthocyanins (50 µg/mL) significantly ($p < 0.01$) reduced the expression of this gene. When those doses of gingerol were combined with the anthocyanins, the expression of Bax was reduced considerably compared to the individual gingerols. On the other hand, there was a slight to a significant reduction in the expression of the Bad gene with combined and singular treatments. The decrease in the expression of the mitochondrial apoptotic signals, caspase-9, Bad, and to some extent Bax, suggested that the mitochondrial pathway was not involved in the apoptotic effects caused by Ac-G combinations; However, it might be involved in the apoptosis induced by the gingerol.

Results showed that anthocyanins slightly reduced the expression of the antiapoptotic Bcl genes Bcl-2 and Bcl-xL, while the 25 μg/mL dose of gingerols, individually and in combination with the anthocyanins, increased the expression of these genes, even though the effects of Ac-G combinations were in the middle. Only the Ac-G combination (50+3.125) μg/mL resulted in significant ($p < 0.01$) lower expression of Bcl-xL compared to the individual extracts and the control.

Interestingly, treatments of gingerols alone, at least one of the two doses, increased the expression of caspase-8, Bax, Bcl-xL, and Bcl-2; meanwhile, reduced expression of these signals was observed when gingerols was combined with anthocyanins, which induced non or reduced effects.

Anthocyanins and gingerols individual treatments caused a slight and a significant ($p < 0.01$) increase in the Bid signal transcription. Still, the Ac-G combination of (50+25) induced a slight increase compared to the 25 μg/mL gingerol treatment, while the Ac-G combination of (50+3.125) μg/mL caused a significant decrease compared to the non-treated cells. These results confer that Ac-G combined treatment of (50+25) μg/mL contributed to the apoptosis by increasing Bid, a Bcl member responsible for the crosstalk between the extrinsic and intrinsic apoptosis pathways (Tang & Kehrer, 2007).

There was a significant ($p < 0.01$) increase observed in JNK transcription in the cells treated with gingerol and Ac-G combined treatments, accompanied by a significant ($p < 0.01$) reduction in ERK (**Figure 4.6**), which might reflect the effect of external signal pathways.

The observed substantial increase in p21 expression of the combinations might contribute to the reduced expression of proapoptotic proteins (Bax and Bad) and the induced expression of antiapoptotic proteins (Bcl-2 and Bcl-xL). It has been reported that overexpression of p21 gives

resistance to apoptosis induction regardless of p53-dependency (Aguda et al., 2007). In the study of Mahyar-Roemer and Roemer (2001), overexpression of p21 protected human colon carcinoma cells against apoptosis induced by natural chemo-preventive and therapeutic agents. Moreover, it has been reported that the transcriptional factor p53 promotes the expression of the proapoptotic gene Bax and represses the transcription of antiapoptotic genes Bcl-xL and Bcl-2 (Aguda et al., 2007). Thus, the increased transcription of Bcl-xL and Bcl-2 observed in the results are suggested to be p53-independent.

The up-regulated transcription of caspase-3, caspase-8, and Bid signals under Ac-G combined treatment (50+25) μg/mL indicate the possible exogenous influence in inducing apoptosis. Moreover, down-regulation and up-regulation of the ERK and JNK transcription, respectively, suggest that other external signal pathways such as death receptors are involved in apoptosis induction. Oppositely, cells treated with the Ac-G combination of (50+3.12) μg/mL exhibited less expression than the control in all these signals.

In the literature, different effects on the cellular signaling of different cell lines were reported for anthocyanins and gingerols individually. For example, in human colon cancer cell lines, Anwar et al. (2016) reported a decreased Caco-2 cell proliferation through up-regulating p21 expression and induced apoptosis by increasing the intracellular ROS and activating caspase-3 cleavage. In another study by Shin et al. (2009), anthocyanins' inhibitory effects on HCT-116 cells were associated with activation of p38-MAPK and suppression of protein kinase B (PKB/Akt). Lin et al. (2012) reported that 6-gingerol induced G2/M cell cycle arrest in LoVo cells by diminishing cyclin A, cyclin B1, and CDK-1 and increasing cell cycle inhibitory proteins p27 and p21. In the study of Radhakrishnan et al. (2014), 6-gingerol lightly affected the phosphorylation of p38-

MAPK and the activation of NF-kappa B, but it effectively inhibited ERK1/2/JNK/AP-1 pathway and activated caspases 8, 9, 3 &7 in SW-480 cell.

4.3.4 Effects of anthocyanins and gingerols combinations on the intracellular ROS generation

The increased levels of cell cycle arrest at the S phase observed in Caco-2 and Hep G2 cells under Ac-G combined treatments compared to each extract alone drives the consideration that combining anthocyanins and gingerols induced a higher level of oxidative stress that may cause DNA damage. Thus, this study measured the effects of anthocyanins, gingerols, and Ac-G combined treatments on ROS generation in Caco-2 cells under normal conditions.

Ac-G combined treatments significantly reduced ROS levels compared to the control and the corresponding individual gingerol doses (**Figure 4.7**), but not to the anthocyanins. Further, only Ac-G combinations of (50+25) and (50+50) µg/mL showed a significant reduction in the ROS levels compared to the individual anthocyanins and gingerols treatments.

In the literature, anthocyanins and gingerols have been reported to induce apoptosis by modulating the expression of caspase cascades and increasing ROS generation. For example, Anwar et al. (2016) reported that anthocyanins induced Caco-2 cell apoptosis by increasing the intracellular ROS and activating caspase-3 cleavage. Likewise, Mansingh et al. (2018) reported the effects of 6-gingerol to cause an increase in the ROS generation leading to a decrease in mitochondrial membrane potential and subsequent induction of apoptosis on human gastric adenocarcinoma (AGS) cells. On the other hand, anthocyanins and gingerols have been reported to induce antioxidant activities as well (Bornsek et al., 2012; Khan et al., 2016; Schantz et al., 2010). The significant decrease in ROS levels observed under individual and combined treatments (**Figure 4.7**) reflects their antioxidant effects.

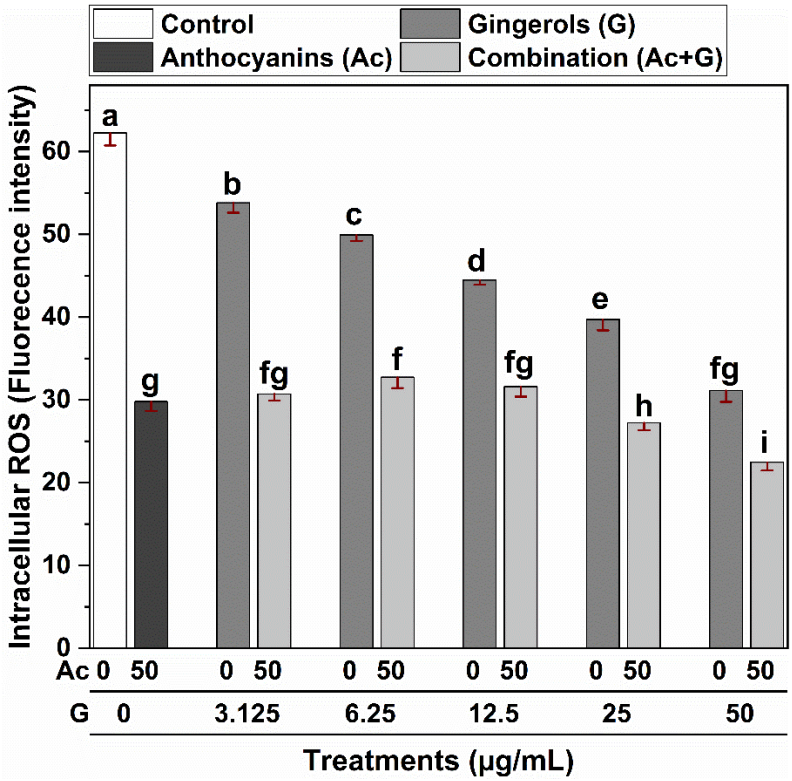


Figure 4.7. Intracellular ROS generation in Caco-2 cells treated with 50 µg/mL anthocyanins, and 3.125-50 µg/mL gingerols, and Ac-G combinations. All the data are expressed as means ± SD. Significant differences at $p < 0.01$ are designated by the letters a-i.

There is evidence that antioxidants can suppress the apoptotic response of diverse stimuli, and inversely, antiapoptotic Bcl family members can decrease ROS production in response to apoptotic stimuli (Aguda et al., 2007). Moreover, recent evidence highlights that ROS plays a dual role in mediating apoptosis as well as survival factor-induced cell proliferation and survival. ROS controls the redox balance required for proliferation signaling, including the MAPK family leading to the transcriptional upregulation of genes involved in cell proliferation (Tang & Kehrer, 2007).

4.3.5 Discussion of the synergistic effect

Several Ac-G w/w combinations (16:1, 8:1, 4:1, 2:1 and 1:1) of 50 µg/mL anthocyanins and 3.125- 50 µg/mL gingerols were tested for their effects on the cell cycle progression and apoptosis.

Significant ($p < 0.01$) increases to similar levels of cell cycle arrest at the S phase were observed in Caco-2 cells at all Ac-G combined treatments compared to the anthocyanins alone and the corresponding gingerol treatments (**Figure 4.1.B**). The increases in the apoptotic cells under Ac-G combined treatments were significant ($p < 0.01$) compared to the treatments of either anthocyanins or the corresponding gingerol alone at Ac-G combinations of (50+3.125), (50+6.25), and (50+25) μg/mL (**Figure 4.3.B**). This might contribute to the higher synergism observed in those Ac-G combinations, representing w/w combination ratios of 16:1, 8:1, and 2:1, in inhibiting cell growth. Therefore, Ac-G combinations of (50+3.125) and (50+25) μg/mL were tested for their effects in up and down regulating key cellular signals involved in the cell cycle arrest at S phase, and apoptosis.

In general, the effects of Ac-G combinations and individual treatments, observed in this study, on the cell cycle and apoptotic pathways are summarized in **Figure 4.8**. Ac-G combined treatments significantly up-regulated the mRNA expression levels of p21, p53, cyclin A, and JNK, and down-regulated CDK-2, cdc25A, and ERK expression levels. Gingerol treatments alone significantly upregulated p21, p53, JNK, cyclin A, and CHEK1; meanwhile, the significant effects of the anthocyanins treatment were on upregulating p21 and downregulating cdc25A and ERK. Results of apoptosis signals show that the Ac-G combination of (50+25) μg/mL induced a significant increase in the caspase-3 and Bid, Bax, Bcl-xL, and Bcl-2, while the Ac-G combination of (50+3.125) μg/mL induced a significant reduction in the expression of caspase-9, caspase-8, Bad, Bid, Bcl-xL, and caspase-3. Gingerols upregulated the mRNA expression levels of Bax, Bid, caspase-3, caspase-8, Bcl-2, and Bcl-xL, and down-regulated caspase-9 and Bad. At the same time, cells treated with anthocyanins showed significant reductions observed only in the expression of caspase-9, caspase-3, and Bax.

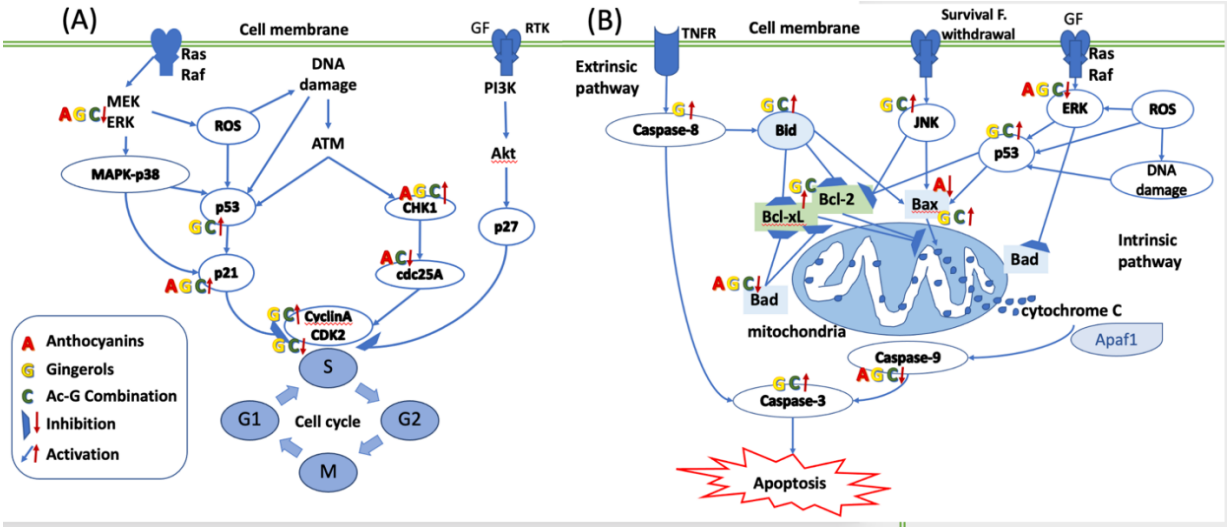


Figure 4.8. The proposed mechanism for the effects of Ac-G combinations on the cell cycle and apoptosis involves a series of pathways.

In this study, up-regulating p21 and down-regulating cyclin A-CDK-2-cdc25A complex prevented further progression to the G2/M phase and caused higher number of cells to be arrested in the S phase. The substantial increase in up-regulating p21 and down-regulating cyclin A-CDK-2-cdc25A complex induced by the (50+25) and (50+3.125) µg/mL Ac-G combined treatments compared to the corresponding individual treatments contribute to the synergistic interaction between anthocyanins and gingerol in inhibiting Caco-2 cell growth. Moreover, Ac-G combined treatment (50+25) µg/mL induced a significant increase in the expression of the caspase-3 gene compared to the control and individual treatments. In fact, anthocyanins treatment caused a reduction. Although gingerol treatment caused an increase compared to the control, the increase level was lower than the combination. The significant increase in the expression of the caspase-3 gene observed under (50+25) µg/mL Ac-G combined treatment contributes to the synergistic interaction in inducing apoptosis.

4.4 Conclusion

This work demonstrates that Ac-G combinations significantly increased the levels of cell cycle arrest at S phase and apoptosis, compared to each singular treatment, in Caco-2 cells at Ac-G w/w combination ratios of 2:1, 4:1, 8:1 and 16:1 at 50 μg/mL anthocyanins, and in Hep G2 cells at Ac-G w/w combination ratios of 2:1 and 4:1 at 100 μg/mL anthocyanins.

The synergism in the growth inhibitory effects of Ac-G combinations on Caco-2 cancer cells was mediated by p53-dependent and p53-independent pathways. These pathways were associated with an increased expression of the tumor suppressor protein p53, an apparent increase in the expression of the cell cycle inhibitory protein p21, and a reduced expression of some cyclin-CDK complex components CDK-2, cdc25A, CHEK1, and cyclin A. In addition, the external pathway mediated the apoptotic response with increased expression of caspase-3, caspase-8, Bid, and Bax.

The apparent increase in the expression of CDK inhibitor p21 induced by the Ac-G combination in this work represents an essential mechanism in inhibiting cancer cell growth and a potential natural alternative with remarkable anti-tumor efficacy and selectivity.

Chapter 5: Gingerols synergize with anthocyanins to induce antioxidant activity *in vitro*[3]

5.1 Introduction

Oxidative stress induced by free radicals has been implicated in the pathogenesis of multiple chronic health conditions such as atherosclerosis, cancer, neurodegenerative, and coronary heart disease (Ighodaro & Akinloye, 2018). Reactive oxygen species (ROS) are the main by-products formed in the cells of aerobic organisms and the primary free radicals that damage biological systems. They are generated endogenously as by-products of mitochondrial-catalyzed electron transport reactions and inflammatory cell activation processes. Free radicals can also be produced exogenously by environmental agents (Rahman, 2007). It has been established that ROS have physiological roles in many cellular signaling systems, such as the cellular responses in the defense against infectious agents and exerting control over redox regulated signaling. However, high production of ROS can be harmful by damaging and causing dysfunction of some cellular molecules and structures and inducing toxic mechanisms and disease processes (Kehrer & Klotz, 2015; Rahman, 2007). Antioxidant defense systems in the body are capable to tolerate ROS toxicity (Ighodaro & Akinloye, 2018). The imbalance between the antioxidant protective defenses and the free radicals causes oxidative stress and damages protein, lipid, and DNA structures.

Natural antioxidants have been used to mitigate oxidative stress as they could support antioxidant defenses that limit and prevent the toxic effects of ROS (Juan-Garcia et al., 2019). The antioxidant potential of phytochemicals from fruits, vegetables, and herbs is due to their ability to

[3] A version of this chapter in combination with chapter 6 will be submitted to Food Research International for consideration for publication as Amna E Abdurrahim, Vera Mazurak and Lingyun Chen."Gingerols synergy with anthocyanins to induce antioxidant activity *in vitro*"

scavenge ROS, protect the cells against oxidative stress-induced damage and modulate signal transduction of several signals involved in antioxidant responses to oxidative stress (Battino et al., 2019).

Anthocyanins are polyphenolic compounds and constitute the largest group of water-soluble pigments in fruits and vegetables. They have been reported to exert a wide range of biological functions, including anticarcinogenic and anti-inflammatory activities and the prevention of cardiovascular diseases (Khoo et al., 2017). The antioxidant function is the major mechanism related to their health benefits. Therefore, anthocyanins are used in functional foods and natural health products (Khoo et al., 2017). Naturally occurring anthocyanins show comparable antioxidant activity to the synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Fukumoto & Mazza, 2000), but are safely consumed at higher doses (Kahl & Kappus, 1993). Research suggests that, despite the low plasma concentration of anthocyanins after oral administration, they seem to have adequate capacity to modulate signal transduction and gene expression *in vivo* to exert bioactivities in promoting health benefits (Kalt et al., 2008; Mueller et al., 2017).

Ginger roots have been used for thousands of years as a food seasoning, as well as herbal medicine for health care and disease prevention. The traditional use of ginger as herbal medicine is attributed to the activity of gingerols, which are the main bioactive components in ginger (Khan et al., 2016). Ginger has been reported to exhibit antithrombotic, anti-inflammatory, anticancer, and antimicrobial activities in addition to its properties in alleviating the toxicity of hepatotoxins in experimental models (Khan et al., 2016). Such protective actions are mediated through free radical scavenging and modulating the levels of the detoxifying enzymes (Haniadka et al., 2013).

The concept of synergism between different phytochemicals in fruits and vegetables was proposed more than a decade ago (Liu, 2003). Synergism means higher effects when agents are combined than the sum of their individual effects, where more pathways could be activated (Breda & Kok, 2018). Several mechanisms of action were proposed for antioxidant synergy, such as scavenging ROS/RNS before they induce oxidative damages on cellular molecules, inhibition of oxidative enzyme activities, induction of defense enzymes expression, and modulating the expression of genes associated with redox processes (Wang & Zhu, 2017). Previous research has identified synergies of different mixtures of purified antioxidant components such as γ-tocopherol and ascorbic acid, polyphenols such as gallic acid, resveratrol, rutin, catechin, and epicatechin, and synthetic antioxidants such as BHT and BHA (Wang & Zhu, 2017).

Most of the antioxidant synergies have been observed in chemical systems, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays (Wang & Zhu, 2017; Wang et al., 2015). Despite the wide usage of these chemical antioxidant activity assays, it has been reported that the bioactivities observed in chemical systems might not appear in biological systems. Chemical antioxidant activity assays do not count for the antioxidant compounds' bioavailability, uptake, and metabolism, and some of them are performed at non-physiological pH and temperatures (Wolfe & Liu, 2007). Thus, living cells better mimic the synergy behaviors under physiological conditions than chemical models (Wang & Zhu, 2017).

Anthocyanins and gingerols exhibited high potential as antioxidants and they seem to have adequate capacity to modulate signal transduction and gene expression *in vivo* to exert bioactivities in promoting health benefits, despite their low bioavailability (Kalt et al., 2008; Mueller et al., 2017; Wang et al., 2009). Furthermore, achieving biological functions at a lower dosage of

anthocyanins and gingerols by combining them might enhance their health effects. Thus, the hypothesis is that combining anthocyanins and gingerols leads to a synergistic antioxidant effect against oxidative stress. Therefore, the objectives of this study were to investigate the effects of anthocyanins and gingerols alone and in combination to induce cellular antioxidant activity (CAA) and the cytoprotective effects. Furthermore, the combinatory antioxidant effects of anthocyanins and gingerols were explored for their potential synergistic interaction in Caco-2 cells.

5.2 Materials and methods

5.2.1 Materials

Bilberry extract powder, ginger root extract powder, Caco-2 cells, DMEM medium, FBS, MTT, DMSO and EDTA, PBS and penicillin-streptomycin solutions are same as mentioned in the first study (Chapter 3, Section 3.2.1). In addition, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and tert-butyl hydroperoxide (t-BHP) were purchased from Sigma-Aldrich Canada Co., (Oakville, ON, Canada). Dulbecco's Modified Eagle Medium (HEPES and no phenol red) DMEM/F-12, and Hanks' Balanced Salt Solution (HBSS) were purchased from GIBCO (Burlington, ON, Canada).

5.2.2 Cell culture conditions and treatments

Caco-2 cells were cultured, sub-cultured and harvested using the protocols described in Chapter 3 (Section 3.2.3). DMEM/F-12, HEPES, without FBS, and antibiotics solution were used for seeding the cells and preparing treatments in the experiments. Stock solutions of anthocyanins and gingerols (500 μg/mL) were prepared from bilberry and ginger root extracts in no FBS DMEM/F-12 medium as mentioned before Chapter 3 (Section 3.2.3), diluted to the desired concentration, and applied on the cells.

First, to increase the chance of identifying synergistic effects, a wide range of anthocyanins: gingerols combinations w/w (20:1-1:20, w/w) were tested in the CAA assay at doses of 0.1-100 μg/mL. Combined treatments did not show major enhancements compared to the individual treatments at high dosage, but synergism was observed at low doses. Therefore, based on preliminary trials, the dose of 1 μg/mL anthocyanins was chosen to be combined with gingerols at Ac-G w/w combination ratios of 16:1, 8:1, 4:1, 2:1, and 1:1 for further testing. Similar concentrations of individual treatments were applied as well.

5.2.3 Cellular antioxidant activity (CAA) assay

The cellular antioxidant activity (CAA) of anthocyanins and gingerols and their combination were assessed according to the protocol developed by Wolfe and Liu (2007). This assay is an *in vitro* cellular method that counts for different biological aspects such as the location of antioxidant compounds within cells, cellular uptake, and metabolism (Wolfe & Liu, 2007). Therefore, it is more biologically relevant than chemical antioxidant assays widely used in other studies. Briefly, Caco-2 cells were seeded into a Black 96-well culture plate at a density of 6×10^4 cells/well. After 24 h incubation at 37ºC, the media was removed, and cells were washed with PBS. Then, 100 μL of fresh FBS free medium containing the extract treatments and 25 μM DCFH-DA were added to each well, followed by incubation for 2 h at 37ºC. Control wells (medium with 25 μM DCFH-DA) were included, and all washing steps and media change were applied on them as well. The culture medium was removed, and cells were washed with HBSS. Then, 100 μL of AAPH (600 μM) in fresh medium was applied to the cells. Positive (cells treated with oxidant and DCFH-DA but without extract treatment) and negative (cells treated only with DCFH-DA) control wells were included. Cellular fluorescence of the sample, control, and blank wells was immediately monitored

each 5 min for 1 h at 37ºC using a SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA) at an emission and excitation wavelengths of 530 nm and 485 nm, respectively.

After subtraction of the blank fluorescence values, the integrated area under the fluorescence curve versus time was determined for each curve. Then the CAA of each treatment was calculated according to Eq. (5-1):

$$CAA\ unit\ \% = 100 - \int SA / \int CA * 100 \qquad \text{Eq. (5-1)}$$

$\int$ SA and $\int$ CA refer to the integrated area under the sample fluorescence and the control fluorescence curves, respectively, versus time.

5.2.4 Calculating the synergistic effect indicator (SE) of antioxidant combinations

The synergistic effects indicator (SE) was used to identify the type of interaction within the anthocyanin-gingerol (Ac-G) combinations. SE was calculated according to Fuhrman et al. (2000) and Luís et al. (2018). It was defined by comparing the CAA values of the combination obtained from the experiment (experimental) (EE combination) and the expected (theoretical) effect (TE combination) values, calculated as in Eq. (5-2):

$$SE = \text{EE combination} \,/\, \text{TE combination} \qquad \text{Eq. (5-2)}$$

where (SE > 1) indicates synergism, (SE < 1) indicates antagonistic effect, and (SE ≅ 1) is considered additive effect. The (TE combination) was calculated using the Eq. (5-3) as described by Fuhrman et al. (2000).

$$TE\ \text{combination} = \text{EE (Ac)} + \text{EE (G)} - (\text{EE(Ac)} \times \text{EE(G)}/100) \qquad \text{Eq. (5-3)}$$

EE (Ac) and EE (G) are the experimental CAA values of the anthocyanins (Ac) and gingerols (G) individual treatments, respectively.

5.2.5 Cytoprotective assay

MTT assay was used to evaluate the cell viability of Caco-2 cells exposed to oxidative stress after being treated with anthocyanins and gingerols and their combinations. MTT assay measures the water-soluble tetrazolium dye MTT (colorless) when metabolized to the insoluble purple formazan within the mitochondria of live cells (Fotakis & Timbrell, 2006). Caco-2 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and incubated for 20 h at 37ºC and 5% CO_2 humidified. Cells were pretreated with the extracts for 2 h (all control wells were treated with only medium), and then they were washed with PBS. Cells were then exposed to 350 μM t-BHP (100 μL/well) for 24 h at 37ºC. After incubation, the MTT assay was performed as described before (**Chapter 3, Section 3.2.4.1**), and the absorbance was measured at 570 nm. Control wells, with and without t-BHP treatment, were used as a positive and negative control, respectively. Results are expressed as a relative cell viability percentage compared to the negative control. Cytotoxicity of t-BHP on Caco-2 cells was tested first using the MTT assay.

5.2.6 Statistical analysis

Each experiment was performed at least in triplicates (n = 3-5). The data were calculated using Excel- Microsoft Office 365 software. Differences between groups were statistically analyzed using one-way ANOVA followed by the Tukey test for multiple comparison of the means. Significance was considered at $p< 0.05$, $p< 0.005$, and/or $p< 0.001$. Statistical analysis of data was carried out using Origin 2020 software. Data were presented as mean ± SD.

5.3 Results and Discussion

5.3.1 Cellular antioxidant activity (CAA)

The cellular antioxidant activities of the anthocyanins and gingerols and their combinations in reducing the 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced ROS generation in

Caco-2 cells were evaluated using the CAA assay. CAA assay is more biologically relevant than chemical antioxidant assays widely used in numerous studies. In this assay, the probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) is cleaved and oxidized in the cells to the fluorescent dichlorofluorescein (DCF) by the oxidizing agent AAPH (Wolfe & Liu, 2007). Thus, higher fluorescence reflects higher ROS levels.

The increased fluorescence observed in Caco-2 cells treated with the oxidant agent AAPH (positive control) compared to the negative control (untreated cells) is presented in **Figure 5.1**. Comparably, cells pretreated with anthocyanins or gingerols showed less fluorescence than the AAPH-positive control during the incubation time. Still, all combined treatments showed a greater reduction in fluorescence than the individual extracts. The Ac-G combinations of (1+0.125), (1+0.25), and (1+1) µg/mL prevented the AAPH-induced oxidation in Caco-2 cells, achieving levels closer to the negative control (**Figure 5.1.B**, **C**, and **E**).

Areas under fluorescence curves were integrated, and the CAA of each treatment was determined and presented in **Figure 5.2.A**. A slight, dose-dependent increase (12.3-20.5%) was observed in the CAA of Caco-2 cells treated with gingerols at 0.06-1 µg/mL. On the other hand, the CAA of anthocyanin-treated cells (10.8%) was significantly increased (25.9-31.4%) when anthocyanins treatment was combined with those concentrations of gingerols. The increases in the CAA of Ac-G combined treatments were not dose-dependent, and doses of (1+0.06) (1+0.125), (1+0.25) µg/mL (Ac-G w/w ratios of 16:1, 8:1, and 4:1) showed higher levels of CAA compared to their corresponding individual doses of gingerols compared to the other combinations.

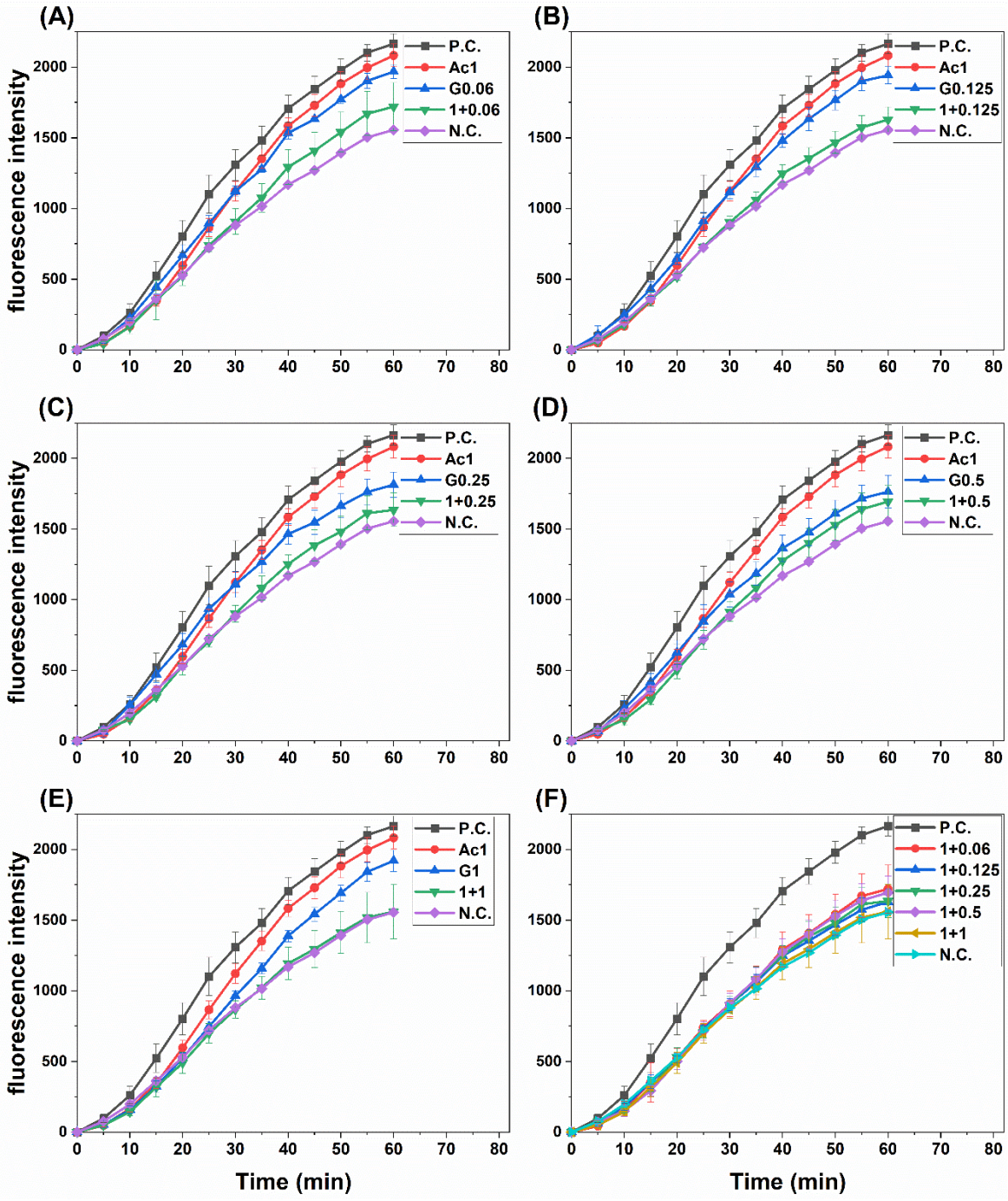


Figure 5.1. Peroxyl radical-induced oxidation of DCFH to DCF in Caco-2 cells and the inhibition of oxidation by pre-treatment with individual and combined anthocyanins and gingerols at different Ac-G combination doses (µg/mL), (A): 1+0.06, (B): 1+0.125, (C): 1+0.25, (D): 1+0.5 and (E): 1+1, over time. (F): presents combinations together. P.C.: positive (AAPH treated), and N.C.: negative (no extract nor AAPH treatment) controls. All values are means ± SD.

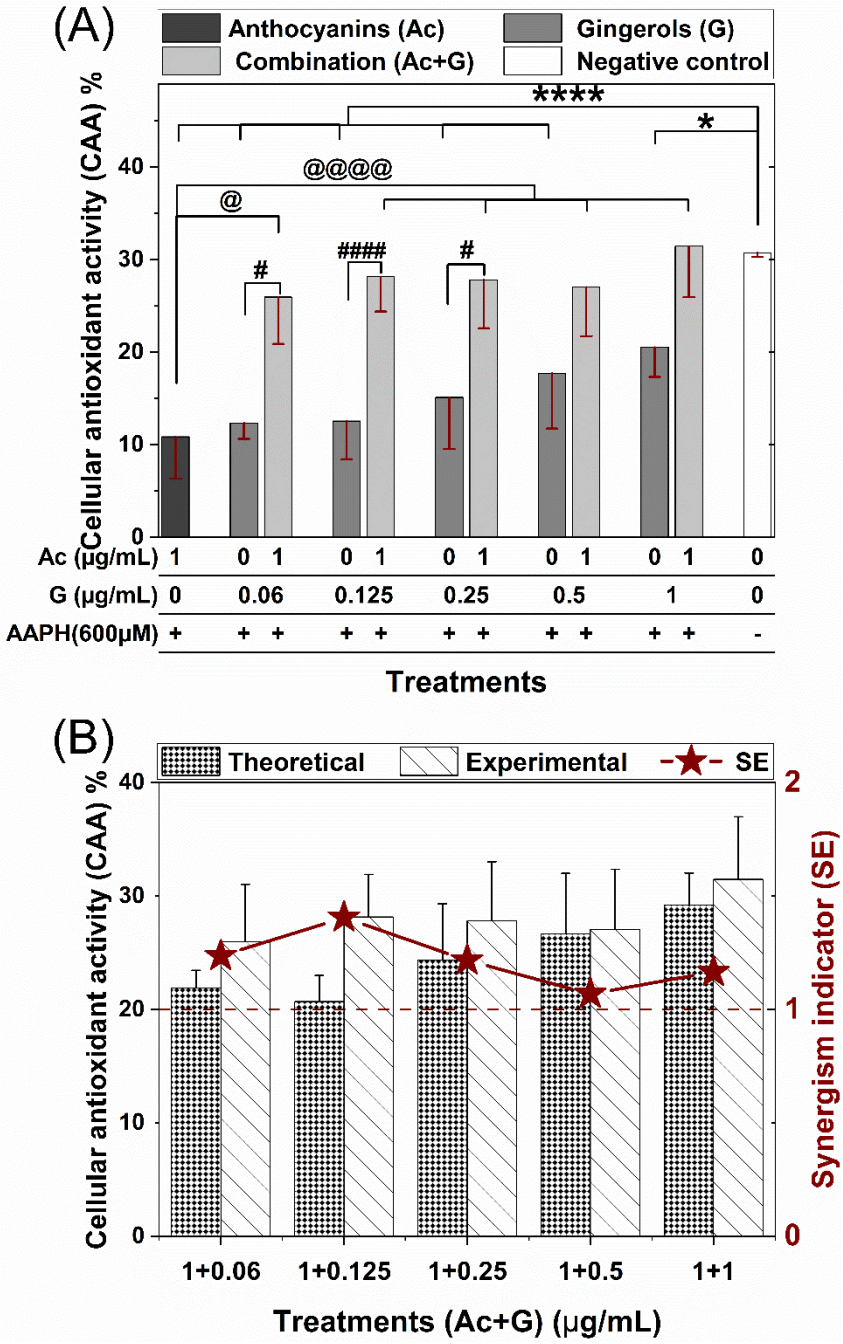


Figure 5.2. (A) The cellular antioxidant activity (CAA) of Caco-2 cells pretreated with individual and combined anthocyanins (Ac) and gingerols (G) compared to the control (no extract nor AAPH treatment). (* and ****), (# and ####), and (@ and @@@@) indicate significant differences compared to the control, the corresponding gingerols treatments, and the anthocyanins treatment, respectively, at $p < 0.05$ and $p < 0.001$, respectively. (B) The synergism indicator graph with a comparison of the experimental and the theoretical (calculated) cellular antioxidant activities of Ac-G combinations.

It should be mentioned that the reported CAA value (10.8%) in this research for the 1 µg/mL anthocyanins treatment was comparable to the CAA values of fruit extracts (blueberry, apple, and grape) evaluated by Wolfe and Liu (2007) in Hep G2 cells. However, these values were less than the CAA values (33.7-43.9%) in Caco-2 and Hep G2 cells for the bilberry and blueberry extracts reported by Bornsek et al. (2012). The reported CAA values were also comparable to the CAA values of crude vegetable extracts (beetroot, red pepper, broccoli, and carrot) on Hep G2 evaluated by Song et al. (2010). In this study, gingerols at doses of (0.06-1 µg/mL) exhibited CAA of 12.3-20.5%, which was higher than the CAA values reported by Sakulnarmrat et al. (2015) for the polyphenolic-rich fraction obtained from dry ginger powder on colon adenocarcinoma (HT-29) and stomach adenocarcinoma (AGS) cells. This diversity in the CAA might be caused by different constituents in each extract and different cell lines used in each study. It has been reported that the extent of the effect of antioxidants in tissue culture-based assays depends on the types of cells and the specific antioxidants in the mixture (Wang & Zhu, 2017).

The synergistic effect indicator (SE) for Ac-G mixtures was then calculated. SE is defined by comparing the CAA values obtained from the experiment and the expected effect values calculated out of the results of both individual treatments; thus, the SE > 1, SE < 1, and SE ≅ 1 indicate synergistic, antagonistic, and additive effects, respectively. In **Figure 5.2.B**, wider differences between experimental and theoretical values of the CAA were observed for the Ac-G combinations of (1+0.125), (1+0.06), and (1+0.25) µg/mL in favor of the experimental data. According to the synergism indicators in **Figure 5.2.B**, additive to synergistic effects (1.06-1.41) (SE >1) were observed for all tested combinations. Higher synergism indicator (SE: 1.41) was observed for the Ac-G combination of (1+0.125) µg/mL, followed by SE: 1.23 and 1.21 for the combinations of (1+0.06) and (1+0.25) µg/mL, reflecting the Ac-G w/w combination ratios of 8:1, 16:1 and 4:1,

respectively. Therefore, the synergism is more towards lower gingerol concentrations in the mixture.

5.3.2 Cytoprotective effects

The protective effects of anthocyanins and gingerols and their combinations against oxidative stress were further evaluated by measuring the cell viability of pretreated Caco-2 cells after exposure to oxidative stress using 350 μM t-BHP treatment for 24 h. The concentration of t-BHP was chosen based on the results of the cytotoxicity experiment on Caco-2 cells (Appendix 3).

In **Figure 5.3.A**, treating cells with t-BHP resulted in a 60% reduction in cell viability. Cells pretreated with anthocyanins at 1 μg/mL showed a significant ($p < 0.05$) increase in cell viability (55.1%) compared to the control (41.2%), while gingerols treatments (0.125-1 μg/mL) exhibited a slight dose-dependent increase in cell viability to 43.3-48.5%. Meanwhile, cells pretreated with combined anthocyanins and gingerols exhibited a significant ($p < 0.001$) dose-dependent increase in the cell viability (62.4 -73.6%) compared to the t-BHP control and the corresponding gingerols treatments. Compared to the anthocyanins treatment, only Ac-G combinations at the doses of (1+0.5) and (1+1) μg/mL showed significant increase at ($p < 0.01$) and ($p < 0.005$), respectively.

The synergistic effect indicator (SE) for Ac-G combinations was also calculated for the cytoprotective activity after 24 h exposure to t-BHP-induced oxidative stress. Synergistic effects were observed for all the tested combinations, with SE values in the range of 1.25-1.61 (SE > 1) (**Figure 5.3.B**). The Ac-G combinations with higher doses of gingerol (1+0.5) and (1+1) μg/mL (Ac-G w/w ratios of 2:1 and 1:1) exhibited higher levels of synergism (SE: 1.61 and 1.57), followed by Ac-G combinations (1+0.125), (1+0.25) and (1+0.06) μg/mL at SE values of 1.39, 1.27 and 1.25, respectively.

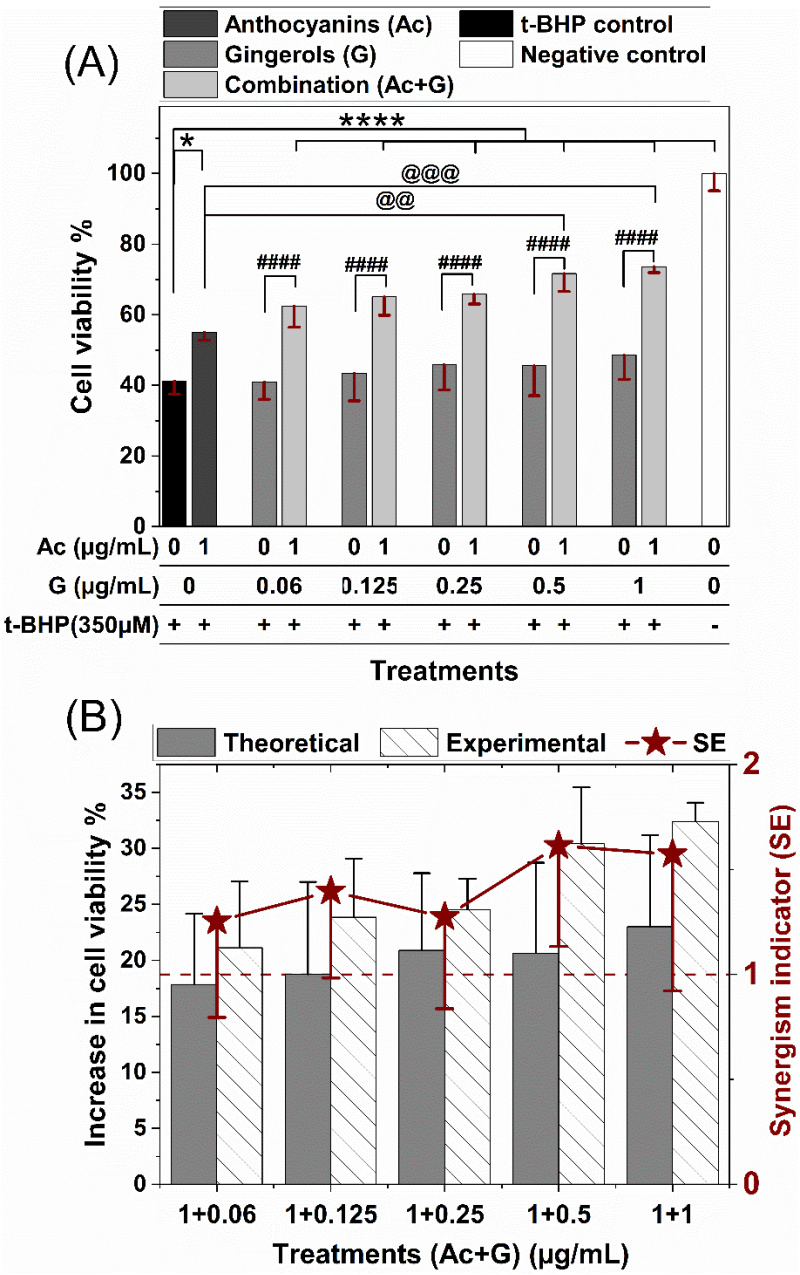


Figure 5.3. Protective effects of anthocyanins (Ac) and gingerols (G) and their combined treatments against 24 h t-BHP induced oxidative stress on Caco-2 cell viability. Values were expressed as mean ± SD. (A) Cell viability of extract pre-treated cells after t-BHP treatment. * and ****: Indicate significant differences compared to the t-BHP control, at $P < 0.05$ and $P < 0.001$, respectively. ####: Indicates significant differences compared to the corresponding gingerols treatments at $P < 0.001$. @@ and @@@: Indicate significant differences compared to the anthocyanins' treatment, at $P < 0.01$ and $P < 0.005$, respectively. (B) The synergism indicator (SE) graph with a comparison of the experimental and the theoretical (calculated) increase in the cell viability when cells were pretreated with Ac-G combinations.

These data indicate that anthocyanins and gingerols protected cell viability against excessive oxidative stress. Still, the protective effect was significantly increased when Ac-G combined treatments were applied to Caco-2 cells. Therefore, synergistic effects were observed for the Ac-G combination in both CAA and cytoprotective effect assays in this study using Caco-2 cells. Both data suggest a synergistic effect at certain combinations.

5.3.3 Discussion of the synergistic effect

Results showed that gingerol treatments significantly increased the CAA in a dose-dependent manner (**Figure 5.2.A**), while anthocyanins treatment significantly increased the cell viability of stressed cells (**Figure 5.3.A**). The combination of anthocyanins and gingerols provided cumulative and additive effects and evoked synergism.

Although the effects of Ac-G combinations with lower gingerol portions showed a higher effect in the short term by increasing the CAA levels (**Figure 5.2.A**) (reducing the induced ROS levels (**Figure 5.1.B, C, and E**)). Ac-G combinations with higher gingerol concentrations showed higher impact in preventing damaging effects of 24 h oxidative stress as observed in the cytoprotective data (**Figure 5.3.A**). Synergism in the CAA of Ac-G combined treatments was higher at doses of (1+0.125), (1+0.06), and (1+0.25) µg/mL, representing Ac-G w/w combination ratios of 8:1, 16:1, and 4:1 (**Figure 5.2.B**). However, higher levels of synergism in the cytoprotective effects were observed for the combined dosages of (1+0.5) and (1+1) µg/mL (Ac-G w/w ratios of 2:1 and 1:1) (**Figure 5.3.B**).

Anthocyanins synergy was previously reported when combining raspberries and adzuki beans extracts and raspberries and sumac extracts using chemical-based assays (Wang et al., 2011; Wang et al., 2015). In addition, positive interactions were reported between anthocyanins (Cy-3-glc, Md-3-glc, or pg-3-glc) and quercetin in FRAP assay, but not in the DPPH assay (Hidalgo et al., 2010).

As far as known, this study is the first to demonstrate the anthocyanins' synergy with gingerols to induce antioxidant activity in cell models. The low bioavailability of anthocyanins and gingerols limits their health benefits. Thus, achieving the same biological effects at lower dosage by combining these two types of phenolic compounds may provide a new strategy to overcome the common bioavailability issue. The possible synergistic mechanisms involved in the cellular responses to oxidative stress were assessed in the next study.

5.4 Conclusion

In conclusion, the Ac-G mixtures (1+0.06) - (1+1) μg/mL showed synergistic responses in CAA and cytoprotective activities in Caco-2 cells with SE values up to 1.61. The higher SE value of 1.41 was observed in the CAA at Ac-G combined dose of (1+0.125) μg/mL; while higher SE value of 1.61 was observed in the cytoprotective effects at Ac-G combined dose of (1+0.5) μg/mL. Therefore, the synergy derived from Ac-G formulations in their antioxidant and cytoprotecting effects is a promising health option for people, health professionals, and the food industry. Introducing those antioxidants together through dietary or supplementary sources could support the endogenous system to protect the cells from oxidation. It is most likely that diverse phenolic compounds in the mixture target multiple sites and act through various antioxidant mechanisms, which would be explored in the next study.

Chapter 6: Antioxidant defense mechanisms mediating the synergistic antioxidant effects of combined anthocyanins and gingerols[4]

6.1 Introduction

The antioxidant capability in the body is a complex defense network that includes powerful oxidative stress response systems. These consist of endogenous enzymatic and non-enzymatic systems, which act at different levels such as radical preventive, radical scavenging, and radical-induced damage repair (Ighodaro & Akinloye, 2018). For example, the cellular antioxidant enzymatic system includes several enzymes that convert the free radicals into non-radicals or less reactive species. Glutathione peroxidase (GPx) is a family of enzymes present at different cellular components that plays an essential role in detoxifying hydroperoxides through the glutathione (GSH) system. GPx catalyzes H_2O_2 and organic hydroperoxides into water and corresponding alcohol (Ng et al., 2007). Superoxide dismutase (SOD) converts superoxide radicals to H_2O_2 and oxygen (Je et al., 2015). Inducing the activities of antioxidant enzymes such as GPx and SOD is one of the critical mechanisms to defend against oxidative stress conditions in the cells. Moreover, excessive oxidative stress may inactivate the enzymes (Alía et al., 2005). GSH plays an essential role in maintaining intracellular redox status and detoxifying exogenous and endogenous compounds. Glutathione has two forms: reduced (GSH) and oxidized (GSSG) forms, and its protective action against reactive oxygen species (ROS) is facilitated by two antioxidant enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR) (Luberda, 2005). Glutathione is a tripeptide thiol typically produced and recycled in the body, but oxidative stress can change GSH,

[4] A version of this chapter in combination with chapter 5 will be submitted to Food Research International for consideration for publication as Amna E Abdurrahim, Vera Mazurak and Lingyun Chen.” Gingerols synergy with anthocyanins to induce antioxidant activity *in vitro*”

suppressing the antioxidant response. GSH can be depleted by some factors such as diet, medications, stress, infections, and pollution (Adeoye et al., 2018). The imbalance between the antioxidant protective defenses and the free radicals causes oxidative stress, damages protein, lipid, and DNA structures, and changes mitochondrial membrane potential.

Natural antioxidants could support the antioxidant defenses to prevent the toxic effects of free radicals (Juan-Garcia et al., 2019). Phytochemicals from fruits, vegetables, and herbs have the ability to scavenge ROS, protect cells against oxidative stress-induced damage and modulate signal transduction of several signals involved in antioxidant responses to oxidative stress (Battino et al., 2019). Anthocyanins and gingerols have been shown independently to induce antioxidant effects by inducing different antioxidant defence mechanisms. Anthocyanins have been reported to deactivate ROS, chelate metal ions (Khoo et al., 2017), and enhance the endogenous antioxidant defense systems. They reduce the DNA oxidative damage, increase the glutathione content, and the activities of the antioxidant enzymes SOD and GPx (Pojer et al., 2013; Toufektsian et al., 2008). Furthermore, anthocyanins reduce the formation of endogenous ROS by inhibiting the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase (Steffen et al., 2008). It was suggested that dietary anthocyanins in the gastrointestinal tract might quench ROS in local cells to prevent damage to the epithelial barrier and inhibit protein expression levels of COX-2 and inflammation (He et al., 2005). On the other hand, ginger has been reported to alleviate the toxicity of hepatotoxins in experimental models (Khan et al., 2016). The protective actions of ginger are mediated through free radical scavenging and modulating the levels of the detoxifying enzymes (Haniadka et al., 2013).

Synergism between anthocyanins and gingerols in their antioxidant activities has been demonstrated in the previous study (Chapter 5), and synergism was observed in the immediate

cellular antioxidant response against induced oxidative stress, and in their cellular protective effects. The concept of synergism between different phytochemicals suggests multiple pathways of cumulative or additive effects as underlying mechanisms (Breda & Kok, 2018). Several mechanisms of action were proposed for antioxidants synergy, such as scavenging ROS/RNS before they induce oxidative damage on cellular molecules, inhibition of oxidative enzyme activities, induction of defense enzymes expression, and modulating the expression of genes associated with redox processes (Wang & Zhu, 2017). Thus, it was hypothesized that combined anthocyanins and gingerols exert their antioxidant effects through more antioxidant defense mechanisms than each treatment alone, activating different non-active signals and inducing more effects on signals triggered by one or the two individual treatments to promote the synergistic effect. The objectives of this study were to investigate the molecular antioxidant defense mechanisms underlying the synergistic effects of combined anthocyanins and gingerols on Caco-2 cells, including ROS generation, lipid peroxidation, cellular glutathione content, and the activities of some antioxidant enzymes.

6.2 Materials and methods

6.2.1 Materials

Bilberry and ginger root extract powder, Caco-2 cell line, DMEM medium, FBS, and EDTA, PBS and Penicillin-Streptomycin solutions are the same as mentioned in the first study (Chapter 3, Section 3.2.1). The t-BHP, DMEM/F-12, and HBSS solutions are the same as mentioned in the third study (Chapter 5, Section 5.2.1). In addition, monobromobimane (mBBr) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

6.2.2 Cell culture conditions and treatments

Caco-2 cells were cultured, sub-cultured and harvested using the protocols described previously in Chapter 3 (Section 3.2.3). DMEM/F-12, HEPES, without FBS, and antibiotics solution was used for the treatments in the experiments. Stock solutions of the extracts and dilutions were prepared as mentioned in the third study (Chapter 5, Section 5.2.2).

6.2.3 Measuring intracellular ROS

ROS generation in the cells was evaluated using the Fluorometric Intracellular ROS Kit (Sigma-Aldrich MAK143). According to the manufacturer instruction, this assay kit detects intracellular ROS by localizing a fluorogenic sensors to the cytoplasm, reacting with ROS and resulting in a fluorometric product. Caco-2 cells were seeded in a transparent bottom black 96-well plate at a density of 4×10^4 cells/well and incubated for 20 h. After attachment, cells were incubated with the kit solution for 1 h. Then, concentrated solutions of anthocyanins, gingerols, or their combinations in the cell growth medium were added to the cells on top of the kit solution. Control wells treated with only medium were included. Then, the ROS production was monitored for 3 h at 37ºC using a fluorescence microplate reader SpectraMax M3 (Molecular Devices, San Jose, CA, USA). Fluorescence was assessed at excitation and emission wavelengths of 490 and 520 nm, respectively. The average fluorescence intensity after 2 h, which reflects the generated ROS, was calculated, and presented.

6.2.4 Determination of intracellular glutathione (GSH) levels

Intracellular GSH levels were measured under physiological and stressed conditions using the fluorescent dye Monobromobimane (mBBr) as described by Je et al. (2015) and Park et al. (2016). The mBBr reacts with glutathione nonenzymatically at low concentrations. It also reacts with thiol containing proteins. However, the staining conditions used for mBBr in this experiment gives

optimum GSH labelling with an acceptable level of background staining <30% (Hedley & Chow, 1994). Briefly, Caco-2 cells were seeded in a black 96-well plate at a 4 x 10^4 cells/well density and incubated for 24 h for attachment. First, cells were treated with individual and combined doses of anthocyanins and gingerols under normal conditions for 1 h. Control wells were treated with only medium. After that, all cells were washed (3x) with HBSS and labeled with 100 µL (40 µM) mBBr for 30 min at 37ºC in the dark. For measurements under a stressed condition, pretreated cells were washed and exposed to t-BHP at 350 µM for 3 h, then washed and labeled as described above. The t-BHP-treated and non-treated wells were included as positive and negative controls. Then, fluorescence due to mBBr-GSH interaction was measured at excitation and emission wavelengths of 360 nm and 465 nm, respectively, every 15 min for 2 h, using the microplate reader mentioned in Section 6.2.3. Results were obtained by subtracting the basal fluorescence readings of mBBr from the final readings. Therefore, the increase in fluorescence intensity indicates higher cellular content of GSH. The stock solution (5 mM) of mBBr was prepared in DMSO and stored at -20ºC in the dark. The mBBr stock solution was diluted immediately before labeling in the medium to prevent decomposition in aqueous solutions.

6.2.5 Determination of antioxidant enzymes

6.2.5.1 Cell treatment, cell lysis and determination of protein content

Caco-2 cells were seeded in 6 well plates at a 1x10^5 cell/mL density and grown to the confluence. For 2 h, cells were treated with extract treatments; then, they were washed with HBSS (3×) and incubated with (150 µM) t-BHP for 24 h. Positive and negative control wells were treated and not treated with t-BHP, respectively, and they were not treated with samples. Cells were washed twice with BPS and lysed on the ice using ice-cold 300 µL of the corresponding lysis solution (assay buffer/GPx assay, and 0.1 M Tris/HCl, pH 7.4 containing 0.5% Triton X-100, 5

mM β-ME, 0.1 mg/mL PMSF/SOD assay). Cell lysates were homogenized by pipetting and centrifuged (14000 × g, 10 min, 4ºC), after which the supernatant was stored at -80 °C and used for the antioxidant enzyme activity assays and protein content. Total protein was quantified using Pierce™ BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

6.2.5.2 Glutathione peroxidase (GPx) activity

The protective effects of anthocyanins and gingerols against oxidative stress on glutathione peroxidase activity were assessed. Oxidative stress was induced on the pretreated cells by t-BHP as described above. The GPx activity was measured using a Glutathione Peroxidase Activity Assay kit (Cat. #K762, BioVision, Inc., Milpitas, CA, USA). In the reaction, GPx reduces cumene hydroperoxide and oxidizes GSH to GSSG. The generated GSSG is reduced to GSH by glutathione reductase with NADPH consumption. SpectraMax M3 microplate reader (Section 6.2.3) was used to measure the decrease in NADPH at 340 nm, and the level is correlated to the GPx activity and presented as mU/mg protein.

6.2.5.3 Superoxide dismutase (SOD) activity

The total cytosolic and mitochondrial SOD activity in cell lysates was evaluated using a superoxide dismutase (SOD) activity assay kit (Cat. #K335, BioVision, Inc., Milpitas, CA, USA). In this assay, SOD activity was assessed based on superoxide anion generation by xanthine and xanthine oxidase reaction. The SOD assay kit utilizes a water-soluble tetrazolium salt solution that produces a water-soluble formazan dye upon superoxide anion reduction. SOD enzyme dismutases the superoxide anion into hydrogen peroxide and molecular oxygen and reduces the formation of the yellow-colored formazan dye, which can be detected spectrophotometrically at 450 nm. After adding the SOD enzyme to the sample wells, the plate was mixed thoroughly and incubated at 37ºC for 20 min. Then, the absorbance at 450 nm was recorded using the microplate reader

mentioned in Section 6.2.3. SOD activity (inhibition rate %) was calculated according to the manufacturer's instructions. Then, the activity was calculated as U/mg using the SOD standard curve and the protein content of the cell extract.

6.2.6 Lipid peroxidation inhibitory assay

Cellular lipid hydroperoxide content was determined, using the fluorescence probe diphenyl-1-pyrenylphosphine (DPPP), according to Bamdad et al. (2015). DPPP is a non-fluorescent molecule that reacts with hydroperoxide within the cell membranes to give the fluorescent diphenyl-1-pyrenylphosphine oxide (DPPP=O) (Okimoto et al., 2000). Briefly, Caco-2 cells were seeded into a black 96-well microplate at a density of 5×10^4 cells/well and incubated for 20 h at 37ºC for attachment. First, cells were incubated with 25 μM DPPP at 37ºC in the dark for 30 min. Then, they were washed with PBS (3×) and treated with different anthocyanins and gingerols for 1 h. Next, cells were washed and treated with t-BHP (150 μM) for 30 min. Here, t-BHP-positive, and negative controls were assigned and all media change and washing steps were applied. The fluorescence intensity of the DPPP oxide was measured using the microplate reader mentioned in Section 6.2.3. at excitation and emission wavelengths of 351 nm and 380 nm, respectively. Stock solution (13 mM) of DPPP was prepared by dissolving DPPP in DMSO and stored in small aliquates in the dark at -20ºC.

6.2.7 Statistical analysis

Data were calculated using Excel- Microsoft Office 365 software. Statistical analysis performed using one-way ANOVA followed by the Tukey test for multiple comparisons. Differences were identified at $p < 0.05$, $p < 0.01$, $p < 0.005$ and/or $p < 0.001$. Statistical analysis was carried out using Origin 2020 software. Data were presented as mean ± SD (n = 3-5).

6.3 Results and Discussion

6.3.1 Effects of Ac-G combined pre-treatments on the cellular antioxidant defense against oxidative stress

During oxidative stress, the endogenous antioxidative system (antioxidant enzymes and GSH) is overwhelmed by the high production of free radicals and ROS, leading to oxidative damage to cellular structures, including membrane lipids (Je et al., 2015). In this study, the individual anthocyanins and gingerols and their combined pre-treatments were evaluated for their capacity to induce antioxidant defense when exposed to oxidative stress by evaluating GSH content, GPx and SOD enzyme activities, and lipid peroxidation levels.

6.3.1.1 Reduced glutathione (GSH) content under stressed conditions

Cells exposed to oxidative stress induced by t-BHP for 3 h (t-BHP control) exhibited a significant ($p < 0.005$) reduction in the GSH levels when compared to the negative control (non-treated cells) (**Figure 6.1**). Combined treatments of 1 µg/mL anthocyanins with (0.125-1µg/mL) gingerols significantly ($p < 0.005$) prevented the reduction in GSH levels.

The Ac-G combinations showed significantly ($p < 0.005$) higher GSH levels compared to the individual anthocyanins only for anthocyanins combined with 1 µg/mL gingerols, but this was not significant ($p < 0.05$) compared to the gingerol treatment alone. Meanwhile, only Ac-G combined doses of (1+0.125) and (1+0.25) µg/mL showed significantly higher GSH levels than the corresponding gingerols alone at ($p < 0.005$) and ($p < 0.05$), respectively. In addition, more differences between individual and combined treatments were observed at the Ac-G combinations of (1+0.125) and (1+0.25) µg/mL, which might contribute to the higher synergism indicator values obtained in the CAA at these combinations.

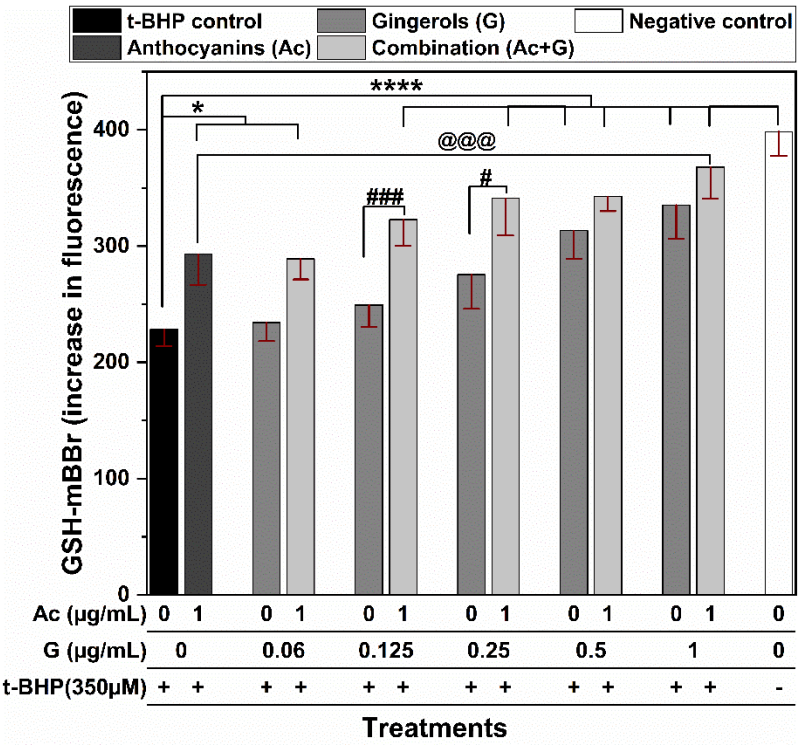


Figure 6.1. The effects of anthocyanins (Ac) and gingerols (G) treatments individually and in combination on the intracellular GSH content in Caco-2 cells after 3 h t-BHP-induced oxidative stress on the pretreated cells. * and **** indicate significant differences compared to the t-BHP control at $p < 0.05$ and $p < 0.001$, respectively. # and ### indicate significant differences compared to the corresponding treatments of gingerols at $p < 0.05$ and $p < 0.005$, respectively. @@@ indicates significant difference compared to the anthocyanins' treatment at $p < 0.005$.

6.3.1.2 Antioxidant enzymes

In **Figure 6.2.A**, exposing Caco-2 cells to 150 µM t-BHP for 24 h induced oxidative stress caused a 2-fold increase in the activity of the GPx enzyme (t-BHP positive control). Even though many phenolic compounds contribute to the antioxidant defense mechanisms in the cells, their beneficial effects are still dependent on the type and source of the specific stressor (Alía et al., 2005; Slamenova et al., 2013). The t-BHP is stable in aqueous solutions and induces constant cellular stress (Alía et al., 2005), which gives a long sustained oxidative effect on the cells. In addition, t-BHP is a hydroperoxide, and GPx catalyzes H_2O_2 and organic hydroperoxides (Ng et

al., 2007). Thus, the presence of t-BHP caused hydroperoxide oxidative stress and increased the activity of the GPX enzyme. The increased GPx activity under oxidative stress (**Figure 6.2.A**) was also associated with decreased GSH levels (**Figure 6.1**). It is known that GPx relies on GSH (specific co-factor) for the necessary reducing equivalents of H_2O_2 (Ng et al., 2007).

The induced increase in GPx activity was significantly prevented when cells were pretreated with Ac-G combinations of (1+0.5) and (1+1) µg/mL at ($p < 0.005$) and (1+0.25) µg/mL at ($p < 0.05$). The two Ac-G combinations (1+0.5) and (1+0.25) µg/mL showed significantly ($p < 0.05$) lower GPx activity compared to the corresponding gingerol treatment alone. Meanwhile, only the Ac-G combination of (1+0.5) µg/mL exhibited significantly ($p < 0.05$) lower activity levels than the anthocyanins treatment alone. The Ac-G combination (1+0.5) µg/mL exhibited even similar levels to the negative control (cells without treatment), which indicates the synergistic effect at such a combined dose. It might contribute to the higher synergism levels observed for this combination in the cytoprotective effect.

Treating Caco-2 cells with t-BHP caused a 4-fold decrease in the activity of the SOD enzyme compared to the negative control (**Figure 6.2.B**). Mitochondria are the primary producer of ROS and the main target. Accumulation of ROS in mitochondria induces the expression of mitochondrial SOD enzyme (Mn-SOD), which plays the leading role in protecting the mitochondria from oxidative stress (He et al., 2017). The substantial reduction in the SOD activity observed in the stressed cells can be attributed to the inactivation of the enzyme molecules in the cytoplasm induced by the oxidant. A lower reduction in the SOD enzyme activity was observed when cells were pretreated with anthocyanins and gingerols and their combinations, indicating their effectiveness in protecting the enzyme molecules from oxidation. The SOD activity was significantly higher for the cells pretreated with the Ac-G combined dosages of (1+0.25), (1+0.5)

and (1+1) µg/mL at ($p < 0.005$), and for the (1+0.125) µg/mL at ($p < 0.05$) when compared to the t-BHP control. Compared to the anthocyanins treatment, only Ac-G combined dosages of (1+0.25) and (1+1) µg/mL showed significance at ($p < 0.05$) and ($p < 0.005$), respectively. Moreover, wider differences were observed for the Ac-G combined treatments of (1+0.25) and (1+0.125) µg/mL compared to the corresponding gingerol treatments. The higher effects induced by these Ac-G combinations in preventing the SOD inactivation can be attributed to the synergism between anthocyanins and gingerols at these combination doses in their CAA.

In previous works, antioxidant enzymes were either decreased or increased when cells were treated with oxidants (Alía et al., 2006; Bamdad et al., 2015). In this research, a 2-fold increase in GPx activity was observed, accompanied by a dramatic decrease in the SOD activity in the t-BHP-stressed Caco-2 cells compared to the unstressed ones. Furthermore, pre-treatment of Caco-2 cells with the Ac-G combinations significantly changed the antioxidant enzymes GPx and SOD activities, bringing their levels comparable to those of negative controls (cells treated with only medium) at specific combination dosages. These results further support the synergism potential of anthocyanins and gingerols in their antioxidant effects.

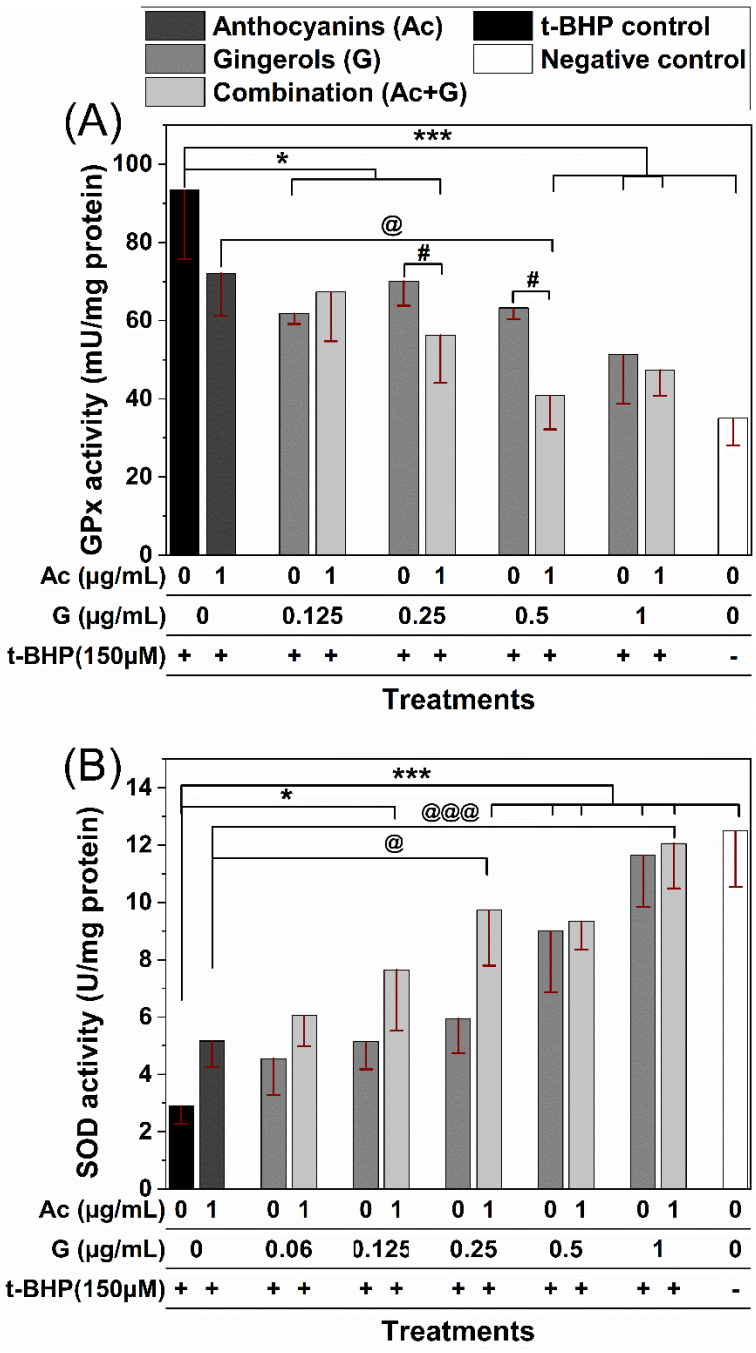


Figure 6.2. Effects of anthocyanins (Ac) and gingerols (G) pre-treatments, individually and in combination, on the antioxidant enzyme activity of GPx (A) and SOD (B) enzymes against 24 h t-BHP-induced oxidative stress in Caco-2 cells. (* and ***) and (@ and @@@) indicate significant differences compared to the t-BHP control and anthocyanins treatment, at ($p < 0.05$ and $p < 0.005$), respectively; # indicates significant differences compared to the corresponding gingerols treatments at $p < 0.05$.

6.3.1.3 Lipid peroxidation

The effect of the anthocyanins and gingerols combinations to prevent lipid peroxidation was evaluated when extract-treated Caco-2 cells were stressed by t-BHP. DPPP is a non-fluorescent molecule known to react with hydroperoxide within the cell membranes to produce diphenyl-1-pyrenylphosphine oxide (DPPP=O) that is fluorescent (Okimoto et al., 2000). In **Figure 6.3**, t-BHP-treated cells showed a significant ($p < 0.005$) increase in the fluorescence intensity compared to the non-treated cells (negative control). Comparably, almost all Ac-G combined pre-treatments significantly ($p < 0.05$) lowered the fluorescence intensity in cells under t-BHP oxidative stress. Further, the reduction in fluorescence intensity for the two Ac-G combinations of (1+0.5) and (1+1) µg/mL was more significant ($p < 0.005$). However, a significant difference ($p < 0.05$) was observed only for the Ac-G combination of (1+0.06) µg/mL compared to the corresponding individual gingerol treatments.

The inhibition of lipid peroxidation in the stressed Caco-2 cells by Ac-G combinations could also be related to the effects of these treatments in increasing the GSH levels and the preservation of the SOD enzyme. Moreover, the positive correlation between the inhibition of cellular lipid peroxidation (**Figure 6.3**) and the reduction in the GPx activities (**Figure 6.2.A**) of those cells could support the role of these treatments in scavenging the oxidants, where reduced ROS levels were observed under all those combined treatments.

All these results indicate higher effects of Ac-G combinations in supporting the endogenous system to reduce the impacts of the induced oxidative stress. To explore the effects of different treatments on the cells before being exposed to oxidative stress, ROS generation, and GSH content were evaluated in cells treated with combined and individual anthocyanins and gingerols.

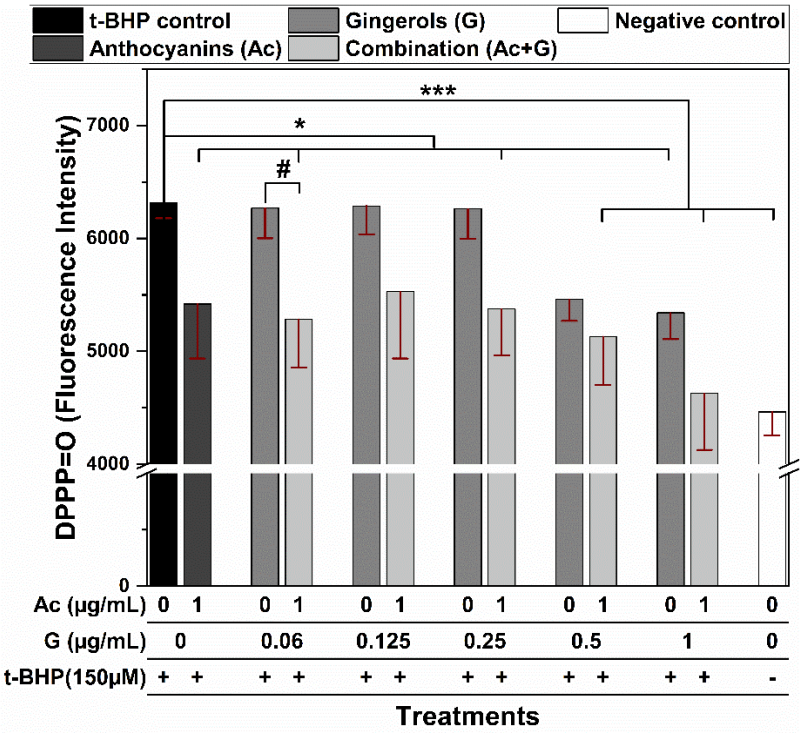


Figure 6.3. Fluorescence intensity of the DPPP-labelled Caco-2 cells pretreated with anthocyanins (Ac), gingerols (G), and the combinations (Ac+G) after oxidation induction by t-BHP. * and ***: indicate significant differences compared to the t-BHP control at $p < 0.05$ and $p < 0.005$, respectively; #: indicate significant differences compared to the corresponding gingerols treatments at $p < 0.05$.

6.3.2 Effects of Ac-G combined pre-treatments on the cellular ROS production and GSH level under normal conditions

6.3.2.1 ROS generation

Individual and combined treatments showed significant ($p < 0.001$) reduction compared to the non-treated cells as a control (**Figure 6.4.A**). Compared to the individual treatments, Ac-G combinations of (1+0.125) - (1+1) µg/mL (Ac-G w/w ratios of 8:1 - 1:1) significantly ($p < 0.001$) reduced ROS levels. However, Ac-G combinations of (1+0.125) and (1+0.25) µg/mL (Ac-G w/w ratios of 8:1 and 4:1) showed a higher level of reduction compared to each extract treatment alone and other combinations, which might contribute to the higher synergism levels observed in the CAA activity (**Chapter 5, Figure 5.2.B**) at these combinations. The kit used in this assay detects

intracellular ROS, especially superoxide and hydroxyl radicals. Thus, the results reflect the effects of the extracts on scavenging endogenous superoxide and hydroxyl radicals in the cells.

6.3.2.2 Reduced glutathione (GSH) content under normal conditions

The effects of anthocyanins, gingerols, and their combination on the intracellular GSH levels were measured under normal physiological conditions (no induced stress) (**Figure 6.4.B**). No significant changes were observed in the GSH levels of Caco-2 cells treated with individual anthocyanins and gingerols at doses of 1 µg/mL and 0.06-1 µg/mL, respectively. However, significant ($p < 0.05$) increases in the GSH levels compared to the control (untreated cells) were observed when cells were treated with the Ac-G combinations. Particularly, the increases in the GSH levels for the Ac-G combined treatments of (1+0.5) and (1+1) µg/mL showed more significance ($p < 0.001$) compared to the control. Moreover, they were significantly different compared to the corresponding gingerol treatments at ($p < 0.01$) and ($p < 0.001$), and to the anthocyanins at ($p < 0.05$) and ($p < 0.001$).

It was also observed that treating the cells with gingerols alone did not affect the levels of GSH (**Figure 6.4.B**) but reduced the ROS levels (**Figure 6.4.A**) in a dose-dependent manner. Meanwhile, when those doses of gingerols were combined with anthocyanins, the increase in GSH levels was significant and dose dependent. However, the reduction in the ROS levels was not dose-dependent, although significant. These results suggest that the synergistic effects applied by combined treatments are more towards lower doses in reducing the ROS generation may contribute to the higher synergism in the CAA at those treatments. Meanwhile, the synergistic effects applied by combined treatments are more towards higher doses in increasing the GSH levels, which might contribute to the higher synergism in the cytoprotective results for those treatments. These results suggest some of the mechanisms involved in the synergistic effects that helped the cells be better

prepared against oxidative stress when oxidant agents were applied in the CAA and the cytoprotective assays.

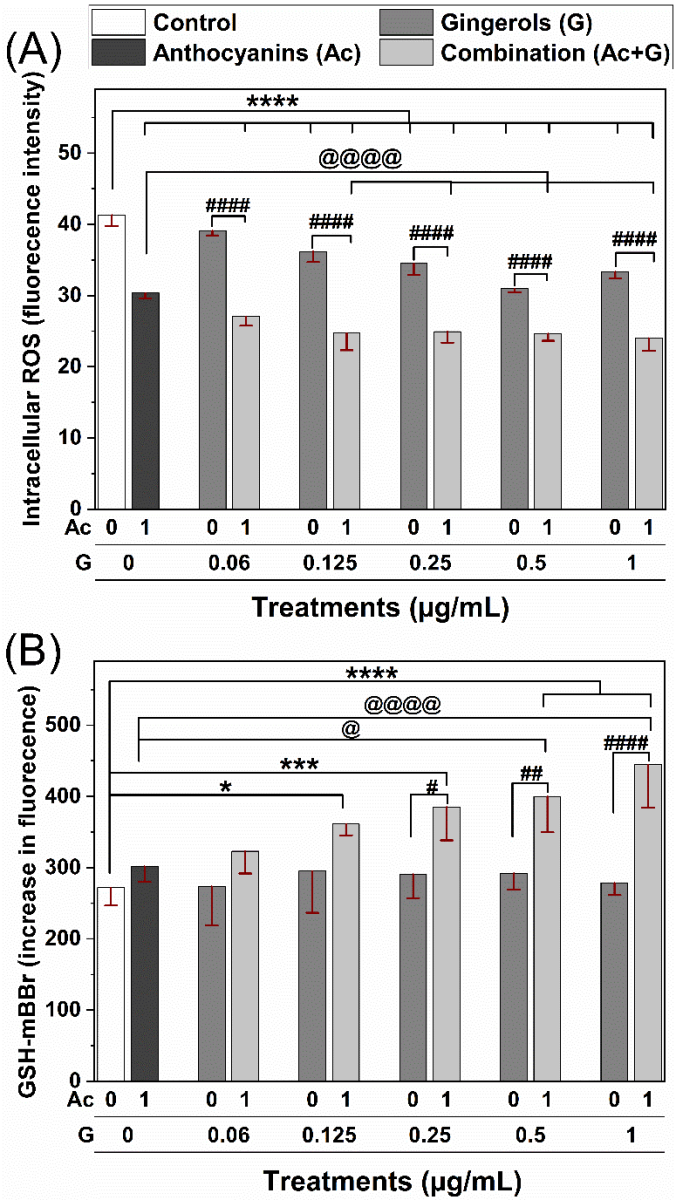


Figure 6.4. Effects of individual and combined anthocyanins (Ac) and gingerols (G) treatments on the intracellular (A) ROS generation, and (B) GSH content in Caco-2 cells. *, *** and **** indicate significant differences compared to the control at $p < 0.05$, $p < 0.005$ and $p < 0.001$, respectively. #, ##, and #### indicate significant differences compared to the corresponding treatment of gingerols at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. @ and @@@@ indicate significant differences compared to the anthocyanins treatment at $p < 0.05$ and $p < 0.001$, respectively.

6.3.3 Discussion of the synergistic effect

The Ac-G combination dosages of (1+0.125), (1+0.06), and (1+0.25) µg/mL showed higher ROS reduction levels than the individual gingerol treatments (**Figure 6.4.A**). Those combinations, representing Ac-G w/w combination ratios of 8:1, 16:1, and 4:1, also exhibited higher synergism levels in the CAA (**Chapter 5, Figure 5.2.B**). Furthermore, the two Ac-G combinations of (1+0.125) and (1+0.25) µg/mL (Ac-G w/w ratios of 8:1 and 4:1) were significantly ($p < 0.05$) more effective in protecting the SOD enzyme and preserving its activity (**Figure 6.2.B**), as well as in reducing the oxidative stress-induced depletion in cellular GSH levels (**Figure 6.1**) when compared to the corresponding gingerol treatments.

Treating Caco-2 cells with the Ac-G combination dosages of (1+0.5) and (1+1) µg/mL (Ac-G w/w ratios of 2:1 and 1:1) significantly increased the levels of cellular GSH compared to the control and corresponding individual treatments (**Figure 6.4.B**), and also showed significant ($p < 0.005$) prevention of the t-BHP-induced increase in GPx activity (**Figure 6.2.A**). Notably, the Ac-G combinations of (1+0.5) µg/mL (2:1 Ac-G w/w ratio) could bring the GPx activity to levels comparable to that of the negative control. These two combination dosages exhibited higher levels of synergism in the cytoprotective effects (**Chapter 5, Figure 5.3.B**).

The combination of more than one agent impacts multiple pathways and may provide cumulative or additive effects to evoke synergism. For example, anthocyanins treatment significantly reduced ROS generation (**Figure 6.4.A**) and prevented the induced GSH depletion (**Figure 6.1**) in the cell. Its protective effects were more apparent by increasing cell viability (**Chapter 5, Figure 5.3.A**) and decreasing cellular lipid peroxidation (**Figure 6.3**). Meanwhile, gingerols induced a significant dose-dependent reduction in ROS generation (**Figure 6.4.A**). Gingerol treatments only at higher doses, 0.5 and 1 µg/mL, caused a significant decrease in GSH

depletion (**Figure 6.1**), induced GPx activity (**Figure 6.2.A**), and lipid peroxidation (**Figure 6.3**). They also caused an increase in SOD activity (**Figure 6.2.B**). Thus, anthocyanins and gingerols combinations target more pathways, induced more effects than each one alone, explaining the synergistic effect observed in this study. In addition, the significant effects of the Ac-G combinations in reducing ROS levels (**Figure 6.4.A**) and increasing the GSH levels (**Figure 6.4.B**) in the non-stressed cells can help the cells be better prepared against oxidative stress, and contribute to the synergistic effects.

GSH is an essential molecule in maintaining the intracellular redox status and cellular GSH levels can be depleted when exposed to oxidative stress. Intervention with exogenous antioxidants is required to support the endogenous antioxidant system in protecting the cells from oxidation and restoring the normal redox state. In this study, treating cells with individual anthocyanins or gingerols slightly changed the GSH levels (**Figure 6.4.B**), but they made the cells more tolerant and reduced the depletion of the cellular GSH level in the presence of oxidative stress (**Figure 6.1**). Meanwhile, Ac-G combinations increased GSH levels in non-stressed cells (**Figure 6.4.B**) and much better maintained the GSH levels under induced oxidative stress (**Figure 6.1**). The GSH values were comparable to the GSH levels in the control cells (non-stressed cells). The significant increases in the GSH levels in the non-stressed cells (**Figure 6.4.B**) represent the Ac-G combination effects in increasing the GSH levels even before exposing them to oxidative stress. Such significant increases in the GSH level observed at these Ac-G combinations may contribute to the higher synergism in the cellular response to maintain the redox status and protect the cells against induced oxidative stress.

The low bioavailability of anthocyanins and gingerols limits their health benefits. Thus, achieving the same biological effects at a lower dosage by combining these two types of phenolic

compounds may provide a new strategy to overcome the common bioavailability issue. The Ac-G combination at the doses of (1+0.125), (1+0.25), and (1+0.5) µg/mL could be considered for new natural health products and functional food product development with enhanced antioxidant effects to prevent oxidative stress and associated chronic health conditions.

6.4 Conclusion

In conclusion, Ac-G combinations protected the cellular redox status against induced oxidative stress by increasing GSH content. They positively affected the cellular enzymatic antioxidant defense system by reducing the induced GPx enzyme activity and maintaining SOD enzyme activity. Moreover, Ac-G combinations significantly reduced the cellular ROS generation and increased GSH levels under physiological conditions, which helped the cells be better prepared against oxidative stress when exposed to oxidative stress. Thus, Ac-G combinations could support the cellular antioxidant defense systems at different levels.

In this study, biomarkers associated with the antioxidant activity and redox pro-survival pathways have been investigated in cell-based assays, which could help better guide the supplementary usage of such a combination; however, the metabolism of antioxidants synergy should be evaluated *in vivo*. Thus, additional research on animal models is required to study the synergistic effects between anthocyanins and gingerols in inducing the antioxidant capacity, which may provide reasonable evidence for further clinical testing.

Chapter 7: Conclusions and Recommendations

7.1 Summary and conclusions

Previous research has demonstrated the efficacy of polyphenolic compounds and their potential to reduce the risk factors of many cancer aspects despite their low bioavailability. Further, the most recent animal and human studies suggested the synergistic effects of phenolic compounds to induce their health benefits, including antioxidant, anticancer, and anti-inflammatory effects. Therefore, combining phytochemicals such as phenolic compounds has been suggested to strengthen their effects against chronic conditions, including cancer, and provide alternative options with less toxicity. Combined usage of phenolic compounds could also help overcome their low bioavailability.

Anthocyanins and gingerols are two groups of phytochemicals with several bioactivities inducing a wide range of health benefits. There is a potential synergistic effect between these two types of phenolic compounds, which might increase their bioactivities and provide a strategy to counteract and address the issues associated with the poor bioavailability of anthocyanins and gingerols. The combinatory effects between anthocyanins and gingerols has not been reported in previous literature. Therefore, this thesis research aimed to assess the combined effects of anthocyanins and gingerols in inhibiting cancer cell growth and inducing cellular antioxidant activity compared to individual anthocyanins or gingerols. Moreover, this thesis research targeted characterizing the combined effects of different Ac-G w/w combination ratios and providing insight into the molecular mechanisms involved in Ac-G synergism.

Synergism between anthocyanins and gingerols to inhibit the growth of cancer cells in *in vitro* models was demonstrated for the first time (Chapter 3). Synergism was observed for the three Ac-G w/w combination ratios 16:1, 8:1, and 4:1 at combined concentrations of 32-57 µg/mL

anthocyanins and 2-12 µg/mL gingerols with combination index (CI) values of 0.81-0.47 at an affected fraction of 90-97% for Caco-2 cells, and at combined concentrations of 150-654 µg/mL anthocyanins and 10-152 µg/mL gingerols with CI values of 0.94-0.34 at affected fractions of 75-97% for Hep G2 cells. A strong synergism was observed for the Ac-G w/w combination ratio of 16:1 with CI values of 0.47 and 0.34 on Caco-2 and Hep G2 cells, respectively, at a high effect level of 97%. In addition, dose reduction index (DRI) results indicated that the same growth inhibitory effect of ≥80% was achieved with up to 3-fold dosage reduction for anthocyanins, and up to 103-fold dosage reduction for gingerols when they were combined. Furthermore, Ac-G combinations showed high potential for inhibiting cancer cell growth with no toxicity to normal cells. Because low cytotoxicity and high efficacy are the main factors that reflect the potency of any treatment application (Zhang et al., 2016), Ac-G combinations exhibit a high potential for inhibiting cancer growth.

Synergism between anthocyanins and gingerols to induce the cellular antioxidant effects *in vitro* was also demonstrated in the cellular antioxidant activity (CAA) and cytoprotective effects with the SE values up to 1.61 (Chapter 5). A higher synergistic effect indicator (SE) value of 1.41 in the CAA was observed at the Ac-G combined dose of (1+0.125) µg/mL (8:1 Ac-G w/w ratio) (lower gingerol portion), while the highest SE of 1.61 was observed in the cytoprotective effect at the combined dose of (1+0.5) µg/mL (2:1 Ac-G w/w ratio) (higher gingerol portion). It is most likely that diverse phenolic compounds in the mixture target multiple sites and act through various antioxidant mechanisms. Synergism observed in the antioxidant effect was at lower effective dosages than those effective dosages that inhibited cancer cell growth. Therefore, having synergism between anthocyanins and gingerols, in such activities, at different combination ratios provides a wider base for developing new natural health products and food applications.

Furthermore, the underlying mechanisms involved in the synergistic effects of anthocyanins and gingerols to inhibit the growth of cancer cells and induce the cellular antioxidant activity were investigated (Chapters 4 and 6). Ac-G combinations significantly increased the cell cycle arrest at S phase mediated by an apparent increase in the expression of the cell cycle inhibitory protein p21, accompanied by an increase in p53 and a reduced expression of some cyclin-CDK complex components (Chapter 4). In addition, Ac-G combinations significantly increased the apoptosis levels. The apoptotic response was, even partially, mediated by the external pathway with increased expression of Caspase3, Caspase8, Bid, and Bax. Therefore, this synergistic effect was mediated by p53-dependent and p53-independent pathways. The apparent increase in the expression of CDK inhibitor p21 induced by the Ac-G combination in this study represents an essential mechanism in inhibiting cancer cell growth. Thus, Ac-G combinations represent a potential natural alternative with remarkable anti-tumor efficacy and selectivity.

Moreover, Ac-G combinations significantly reduced the cellular ROS generation and increased GSH levels under physiological conditions (Chapter 6). They also protected the cellular redox status against induced oxidative stress by increasing GSH content, reducing the induced GPx enzyme activity, and maintaining SOD enzyme activity. The effects of different combinations on various biomarkers differ. Thus, Ac-G combinations could support the cellular antioxidant defense systems at different levels. Understanding the mechanisms involved in the synergistic effects of anthocyanins and gingerols could help better guide the supplementary usage of such a combination and gives justification for further *in vivo* studies.

7.2 Significance of the present research

This research is an addition to the knowledge about the antioxidant and anticancer effects of the combined phytochemicals, anthocyanins and gingerols. This thesis research is the first to demonstrate the synergism between anthocyanins and gingerols for their anticancer and antioxidant activities. Findings of this research have generated new fundamental knowledge and provided new insights into the synergistic effects of combined anthocyanins and gingerols in inhibiting the growth of cancer cells and inducing antioxidant effects, as well as the mechanisms involved in the synergistic interactions in their activities.

Synergy derived from the Ac-G combinations represents a promising healthy option for people, health professionals, and the food industry. Combining those two extracts might provide a strategy to increase the bioactive effects of anthocyanins and gingerols at lower dosages. Thus, introducing those antioxidants together through dietary or supplementary sources could support the endogenous system to protect the cells from oxidation, restore the normal physiological redox state, and improve daily performance.

The findings of this research can be used to develop value-added products that will commercially benefit blueberry farmers. Canada is the world's second-largest producer of blueberry fruits (Protzman, 2021). Canada's blueberry production in 2019 reached 194 thousand tonnes (Statistics Canada, 2022). The characterization of combinatory effects of different Ac-G ratios in this work provides guidance for their utilization in food applications and supplementary health products. For example, the Ac-G combination could be considered as functional ingredients in foods such as yogurt and beverages. Adding ginger extract to anthocyanin-rich drinks, such as berry juices, would enhance their nutritional value and add desirable pungent flavor.

Moreover, this research provides a rationale for further studying the synergistic effect among anthocyanins and gingerols using animal models. They may provide reasonable evidence for further clinical testing. Positive *in vivo* results will enable combinations to be used as natural health products to reduce oxidative stress and lower cancer risk.

Further, the strong synergism levels observed for the Ac-G combination at high growth inhibitory effects and the high efficacy in inducing the programmed cell death of cancer cells signify the high potency of such combination to be used in inhibiting cancer progression potentially. Future studies could enable new natural health products or functional food applications.

This research helps to gain some preliminary understanding of the mechanism involved in the synergistic effect, such as the biomarkers associated with the cell survival, growth and apoptosis pathways, antioxidant activity and cellular redox status, and cell viability. For example, several CDK inhibitors (CDKIs) were developed as effective anti-tumor chemotherapies. Still, due to their low specificity and severe side effects, CDKIs have not been approved yet for cancer treatment, and further research is ongoing to overcome their issues (Zhang et al., 2021). In this study, the substantial increase in the expression of the cellular CDK inhibitor p21 induced by Ac-G combined treatment represents a potential natural alternative with remarkable anti-tumor efficacy and selectivity. However, more research is required to understand the mechanisms of such effects better.

Moreover, synergism between anthocyanins and gingerols from different plant sources is a good example for many other phytochemicals to be tested for potential synergism in their anticancer, antioxidant and other activities. This knowledge supports the suggestion that

combining phytochemicals from different food resources is useful as a dietary strategy to improve public health.

7.3 Recommendations and future directions

- Antioxidant effects of phenolic compounds play an essential role in preventing several health conditions such as carcinogenesis and neurodegeneration. Therefore, demonstrated synergism in the antioxidant activity of the Ac-G combination should be further investigated in the future for their effects to induce anti-carcinogenesis and anti-neurodegeneration.
- A vast and complex network of signals controls the survival and growth of cancer cells. This study demonstrated the synergistic effect of Ac-G combined treatments in inducing the cell cycle inhibitory signals that inhibited cancer cell growth. However, the investigated apoptotic signals refer more to the role of the combination as an external stimulus in the induced apoptosis response. Thus, death receptors and related signals are suggested to be investigated as a potential apoptotic pathway in future work.
- In this study, synergistic effects of anthocyanins and gingerols have been proven in cell culture models *in vitro.* More in-depth research is still required to understand the possible chemical interactions involved in the behavior of binary combinations and their combined effects, for example, evaluating the impact of the combined extracts on the stability and the permeability of each other. Like other phytochemicals (Hemalswarya & Doble, 2006), anthocyanins and gingerols might increase their effect by increasing the bioavailability or the stability of each other in the system, which needs to be validated in cell and animal models.
- The effects of ginger extracts on enhancing the absorption of several active components have been reported (Qazi et al., 2003). Therefore, their possible enhancement of the bioavailability of anthocyanins should be investigated.

- The findings of this research proved the synergism between anthocyanins and gingerols in inhibiting the growth of cancer cells and their antioxidant activities in cell culture modules. The significance of these findings can be explored in functional foods, natural health products, and dietary supplements to restore the normal physiological redox state in populations showing signs of redox imbalance. Such measures can be pursued following investigations using animal models and clinical trials to confirm the beneficial health effects of the anthocyanin-gingerol combinations and to validate the effective combination ratios and doses.
- Many other factors such as food matrix, digestion process, and gut microbiota could affect the bio-accessibility and thus the bioavailability of such combination. Therefore, the impacts of these factors on the health benefits of the Ac-G combinations need to be investigated in the future.
- Encapsulation attempts for Ac-G combined extracts are suggested to help prevent the effects of the digestion process and the gastrointestinal tract microflora on those phenolic compounds and prevent their degradation. Encapsulation could also help protect those phenolic compounds during food processing and storage.
- Although the relationship between colonic bacteria and anthocyanins is still not fully clear (Faria et al., 2014), recent studies have shown the importance of the interaction between the microbiota and anthocyanins during fermentation processes to improve the bioavailability of anthocyanins, which could be used for developing healthier food products using Ac-G combinations such as fermented dairy products with fruits.
- The demonstrated synergism of the Ac-G combination is a good example of the opportunity to further validate the synergism for other potential mixtures of phenolic compounds.

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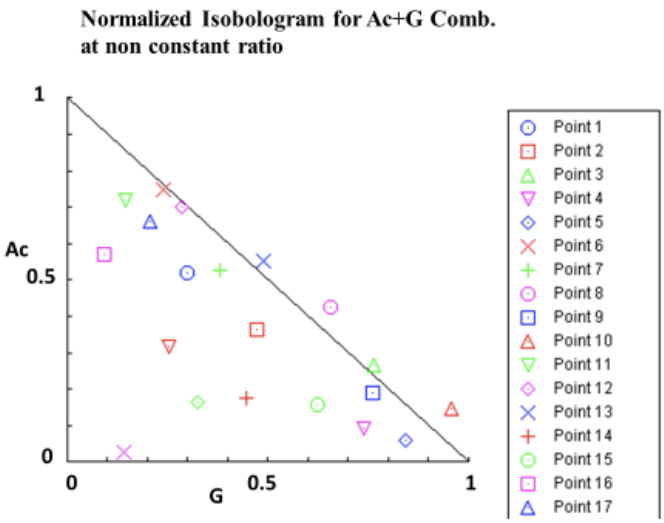
Appendices

Appendix 1

Combination indexes and normalized isobologram for anthocyanin-gingerol (Ac-G) combinations at the non-constant ratio on Caco-2 cells.

	Ac µg/ml	G µg/ml	Fa	CI
Point 1	40	2.5	0.35	0.82
Point 2	40	5	0.46	0.84
Point 3	40	10	0.56	1.03
Point 4	40	20	0.84	0.83
Point 5	40	30	0.90	0.91
Point 6	80	2.5	0.45	0.99
Point 7	80	5	0.56	0.91
Point 8	80	10	0.63	1.08
Point 9	80	20	0.83	0.95
Point 10	80	30	0.87	1.11
Point 11	160	2.5	0.68	0.86
Point 12	160	5	0.69	0.99
Point 13	160	10	0.75	1.04
Point 14	160	20	0.93	0.62
Point 15	160	30	0.94	0.78
Point 16	240	2.5	0.83	0.66
Point 17	240	5	0.80	0.87
Point 18	240	10	0.91	0.57
Point 19	240	20	0.96	0.49
Point 20	240	30	0.99	0.17

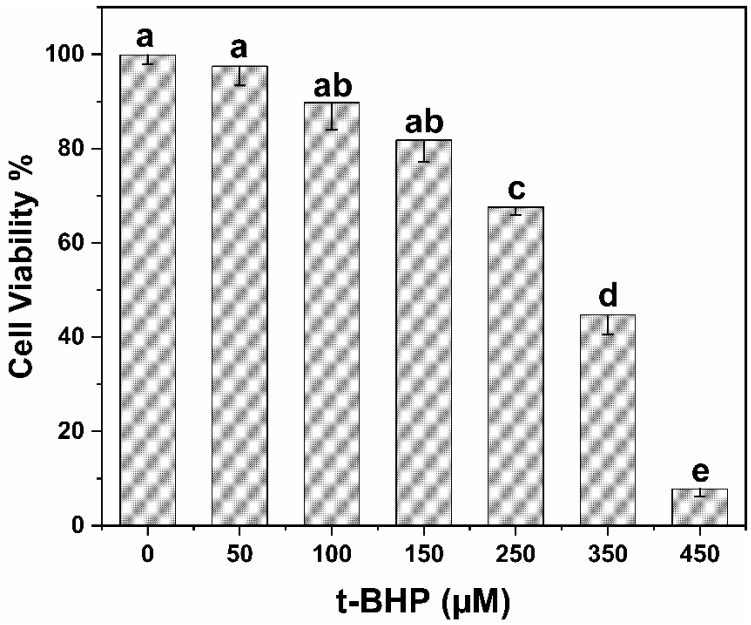


Appendix 2

The r-values of the dose-effect curves of different treatments on the three tested cell lines and the highest growth inhibitory effect levels obtained from the experimental points.

	The highest growth inhibitory effect %/ Cell line			r value/ Cell line		
The treatment	Caco-2	Hep G2	HT-29	Caco-2	Hep G2	HT-29
Anthocyanin Extract	96	89	99	0.94	0.98	0.94
Gingerol Extract	99	69	99	0.9	0.94	0.98
Combination R. 4:1	99	75	92	0.95	0.99	0.87
Combination R. 8:1	98	74	99	0.98	0.96	0.93
Combination R. 16:1	99	82	99	0.93	0.93	0.91

Appendix 3



Cell viability of Caco-2 cells exposed to different concentrations of t-BHP. Significant differences at $p < 0.05$ are designated by the letters a-e.