THE EFFECTS OF BLUEBERRY ANTHOCYANIDINS ON ANTIOXIDANT ENZYME

ACTIVITY IN HUMAN HEPG2 CELLS

by

ALEXIS ROSE GALAMBOS

(Under the Direction of Dr. Joan G. Fischer)

ABSTRACT

Anthocyanins are thought to have antioxidant effects in the body. The effects of two anthocyanidins, malvidin and delphinidin, on the activities of antioxidant enzymes, glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and other markers of oxidative stress were examined in HepG2 human hepatocellular carcinoma cells. In study one, cells were treated with anthocyanidins at concentrations of 0, 5 or 10 µmol/L for 24 h and 0 or 200 µmol/L tert-butyl hydroperoxide (t-BOOH) for 2 h. Study two used a combined dose of 10 µmol/L anthocyanidins and 0 or 200 µmol/L t-BOOH. Cell proliferation and GR activity were significantly increased by delphinidin; with 5 µmol/L having the greatest effect in oxidatively stressed cells. Malvidin also increased cell proliferation, but to a lesser extent than delphinidin. No effect of treatment was observed on GST and GPx activity. Finally, it appeared that combining these anthocyanidins had an antagonistic effect.

INDEX WORDS: Anthocyanin, Antioxidant, Oxidative Stress, Glutathione Reductase, Glutathione Peroxidase, Glutathione S-Transferase

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CHAPTER I

INTRODUCTION

Cancer is the second leading cause of death in the United States (Centers for Disease Control and Prevention, 2010) and it has been estimated that 30% of cancer deaths are related to dietary factors (American Cancer Society, 2010). Epidemiological studies have shown that diets high in fruits and vegetables are associated with a reduced risk of cancer at many sites (Cooke et al., 2005). The exact mechanisms by which fruits and vegetables help protect against cancer are not yet fully understood. There are many beneficial compounds in fruits and vegetables including vitamins, minerals, and phytochemicals that may be responsible for the health benefits. Phytochemicals are non-nutrient components found in plants. Anthocyanins are one class of phytochemicals and are the red, blue and purple pigments found in plants (Wang and Stoner, 2008). Average consumption of anthocyanins in the United States is estimated to be between 12.5 mg (Wu et al., 2006) to over 200 mg per day in people consuming many servings of fruit (Prior, 2003).

Oxidative stress is thought to be an important factor leading to the development of cancer. Reactive oxygen species (ROS) are formed from oxygen and are necessary in biological systems. It has been theorized, however that high concentrations of cellular ROS promote the development of many human diseases such as cancer, cardiovascular disease, diabetes and Alzheimer's disease (Halliwell and Gutteridge, 1999; Jacob and Burri, 1996). Mechanisms by which ROS are thought to lead to carcinogenesis include the mutation of DNA, the promotion of

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cell proliferation, invasiveness, angiogenesis and metastasis and the suppression of apoptosis (Halliwell, 2007).

Anthocyanins may be chemopreventive due, in part, to their direct antioxidant and anti-inflammatory activities, and/or their ability to increase the activities of the antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) and increase reduced glutathione concentration (Ramos, 2008; Alía et al., 2006). Anthocyanins have also been shown to inhibit cancer cell proliferation in many cell models. Studies have investigated the chemopreventive effects of anthocyanins using *in vitro* models, animal models or human epidemiological trials, and there have been conflicting results in human studies as to the benefits of anthocyanin intake (Ramos, 2008). *In vitro* cell models have shown increased antioxidant enzyme activity and decreased cell proliferation with anthocyanin treatment, but the concentrations used are generally high and may not be physiologically achievable in tissues. Many studies showing the beneficial effects of anthocyanin treatment have been conducted using intestinal cell culture models. Not much is known about the effect of anthocyanin treatment in liver cell culture models, though the liver is essential in anthocyanin metabolism (Talavéra et al., 2003).

This study investigated two common anthocyanidins, malvidin and delphinidin, in an *in vitro* HepG2 human hepatocellular carcinoma cell model. We tested the hypothesis that anthocyanidin treatment, at low concentrations, would increase the activity of the antioxidant enzymes GR, GPx and GST in the presence of the oxidative stressor tert-butyl hydroperoxide (t-BOOH). We further hypothesized that anthocyanidin treatment would counteract oxidative stress, thus salvaging cells and preventing lipid peroxidation, in the presence of t-BOOH. The

study examined the use of anthocyanidin concentrations that were lower than those previously used by most studies, and are closer to levels that are realistically attainable in the human diet.

Cells were treated with malvidin and delphinidin at concentrations of 0, 5 or 10 µmol/L for 24 hours followed by and 0 or 200 µmol/L of t-BOOH for 2 hours in study one. In study two, cells were treated with 5 µmol/L malvidin and 5 µmol/L delphinidin (10 µmol/L combined anthocyanidin) for 24 hours followed by and 0 or 200 µmol/L t-BOOH for 2 hours. Following treatment, GR, GPx and GST activities were assessed as well as cell proliferation and lipid peroxidation. It was found that cell proliferation and GR activity were significantly increased by delphinidin treatment; with a concentration of 5 µmol/L having the greatest effect on GR activity and cell proliferation in oxidatively stressed cells. Malvidin at 5 µmol/L also improved cell proliferation in stressed and unstressed cells, but did not have as pronounced an effect in stressed cells as seen with delphinidin treatment. No significant effect of anthocyanidin treatment was observed on GST and GPx activities or lipid peroxidation measured with TBARS. Finally, it appeared that the combination of these anthocyanidins had an antagonistic effect as increases in cell proliferation and GR activity were lost.

References

Alía, M., Ramos, S., Mateos, R., Granado-Serrano, A. B., Bravo, L., & Goya, L. (2006). Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Applied Pharmacology, 212*(2),110-118.

American Cancer Society. (2010). *Diet and physical activity: what's the cancer connection?* Retrived from http://www.cancer.org/Cancer/CancerCauses/DietandPhysicalActivity/diet-and-physical-activity

Centers for Disease Control and Prevention. (2010) *Deaths and mortality*. Retrieved from http://www.cdc.gov/nchs/fastats/deaths.htm

Cooke, D., Steward, W. P., Gescher, A. J., & Marczylo, T. (2005). Anthocyans from fruits and vegetables--does bright colour signal cancer chemopreventive activity? *European Journal of Cancer, 41*(13),1931-1940.

Halliwell, B. (2007). Oxidative stress and cancer: Have we moved forward? *The Biochemical Journal, 401*(1),1-11.

Halliwell, B. & Gutteridge, J. (1999). *Free Radicals in Biology and Medicine*. 3rd ed. New York: Oxford University Press.

Jacob, R. A., & Burri, B.J. (1996). Oxidative damage and defense. *The American Journal of Clinical Nutrition, 63*(6),985S-990S.

Prior, R. L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *The American Journal of Clinical Nutrition, 78*(3 Suppl,:570S-578S.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: dietary polyphenols and signaling pathways. *Molecular Nutrition and Food Research, 52*(5),507-526.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Lamaison, J. L., & Rémésy, C. (2003). Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *The Journal of Nutrition, 133*(12),4178-4182.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry, 54*(11),4069-4075.

CHAPTER II

LITERATURE REVIEW

Cancer

Despite technological advances, cancer is still the second leading cause of death in the

US (Centers for Disease Control and Prevention, 2010). Cancer is a group of diseases

characterized by uncontrolled cellular growth resulting from mutations in genes that tightly

regulate processes such as cell growth, division and death. The development of cancer can be

viewed as a multi-stage progression involving many genetic alterations (Hannahan and

Weinberg, 2000). It is estimated that about 5-10% of cancers result from direct inheritance of

genes associated with cancer (WCRF/AICR, 2007). The remaining cases of cancer are attributed

to alterations or damage amassed in DNA over time as a result of internal, and environmental

factors. Internal, or endogenous, factors attributed to carcinogenesis include inherited mutations,

genetic variation/mutation due to stand breaks, crossovers or deletions, oxidative stress and

chronic inflammatory disease. Environmental factors that may lead to carcinogenesis are often

related to lifestyle, including alcohol consumption, drug and tobacco use, physical activity and

food choices. Several other environmental carcinogens exist such as radiation, ultra violet light,

ozone, chlorinated hydrocarbons and heavy metals (WCRF/AICR, 2007).

In a healthy cell, there is a tightly regulated balance between cell proliferation,

differentiation and death; genes such as oncogenes and tumor suppressor genes regulate these

processes. Several mechanisms exist to ensure the preservation of these genes' sequences and the

cell's structure as cell division occurs (WCRF/AICR, 2007). No solitary gene has been found to

cause cancer. Rather, cancer develops when multiple genes are modified leading to advantageous growth and survival characteristics of one cell beyond that of adjacent cells. This process begins with initiation, or the exposure of a cell to the first genetic mutation. If this genetic mutation is left uncorrected, it can be passed on to its daughter cells thereby bestowing the potential for deviant growth. After initiation, promotion must also occur if cancer is to result. This involves the exposure of a cell to a promoting agent that causes further DNA damage, leading to additional mutation and potentially increased cell proliferation. These initiated and promoted cells are then able to produce growth factors, inflammatory mediators, and cytokines that substantiate their uncontrolled growth into malignant masses (WCRF/AICR, 2007).

Cancer cells have been shown to display some or all of the six hallmarks, or characteristics of cancer. These hallmarks are growth signal autonomy, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Growth signal autonomy enables cancer cells to divide without stimulation from growth factors. Instead, cancer cells produce their own signals that allow them to divide free of the growth constraints present in healthy cells (WCRF/AICR, 2007). Limitless replicative potential allows cancer cells to continue dividing long past the normal 60-70 times before death. Together, growth signal autonomy, evasion of growth inhibitory signals and unlimited replication all favor increased cancer cell proliferation. The ability of cancer cells to escape apoptosis also allows them to outlive their normal lifespan and further confer their pathogenic abilities to daughter cells. Due to their rapid and uncontrolled growth, cancer cells require increased oxygen and nutrients from the blood. In order to procure this increased supply, cancer cells release angiogenesis inducers that lead to the formation of new blood vessels. Finally, cancer cells are able to invade and spread to other

tissues. This is achieved by secreting enzymes (matrix metalloproteinases) that digest membranes, thereby allowing the cells to invade tissues.

Oxidative Stress

Oxidative stress is a result of a serious imbalance between reactive species (RS) production and the antioxidant defense system (Halliwell and Gutteridge, 1999). This imbalance can occur as a result of antioxidant deficiency, decreased antioxidant enzyme activity and/or a high cellular concentration of RS generated in response to disease, metabolism or the environment. Various types of RS are formed *in vivo* during the redox reactions of cellular respiration, and during immune responses. Reactive species can also be generated in response to radiation, ionization or by phase I enzymes during drug metabolism (Masella et al., 2005). Environmental exposures to oxidizing air pollutants such as ozone, tobacco smoke and fuel exhaust may also result in RS formation (Jacob and Burri, 1996). Reactive species consist of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive oxygen species are the byproducts of metabolic and immunological processes; examples include the oxygen radicals superoxide ($O_2 \bullet^-$), hydroxyl (OH•), hydrogen peroxide (H_2O_2), ozone (O_3) and singlet oxygen ($^1O_2$) (Weisman and Halliwell, 1996). Reactive nitrogen species refer to nitrogen radicals, including nitric oxide (NO•) and peroxynitrite.

ROS have beneficial roles *in vivo* through their involvement with energy production, phagocytosis, cell growth and intracellular signal regulation (Masella et al., 2005). However, ROS may also be harmful, as they can damage macromolecules such as lipids, proteins and DNA, causing membrane and DNA damage as well as enzyme inactivation. The hydroxyl radical is the most reactive of the ROS molecules and will bind and oxidize any DNA, lipids or proteins in its vicinity (Gaté et al., 1999). Through the Fenton reaction (Figure 1) and Haber-

Weiss reaction (Figure 1), hydroxyl radicals can be generated from the less reactive H_2O_2 and $O_2 \bullet^-$ as catalyzed by transition elements (iron) and other trace elements. While H_2O_2 is less reactive than $O_2 \bullet^-$ and $OH \bullet$, it is more highly diffusible and therefore able to cross the plasma membrane and induce cellular stress.

It has been shown that an increased concentration of cellular ROS promotes the growth of malignancies and the development of many human diseases such as cardiovascular disease, diabetes and Alzheimer's disease (Halliwell and Gutteridge, 1999; Jacob and Burri, 1996). Mechanisms by which RS are thought to lead to carcinogenesis include the promotion of cell proliferation, invasiveness, angiogenesis, metastasis and the suppression of apoptosis (Halliwell, 2007). Reactive species are able to cause structural changes to DNA through base pair mutations, rearrangements, deletions and insertions. These mutations can affect proto-oncogenes and tumor-supressor genes, therefore stimulating the promotion and progression of tumors (Weisman and Halliwell, 1996; Halliwell, 2007). ROS have also been shown to damage mitochondrial DNA (mtDNA) at levels higher than that seen in nuclear DNA. This increase can be explained by mtDNA's close proximity to the electron transport chain, and ROS species generated during metabolism. Further damage to mtDNA can occur from radicals generated during lipid peroxidation. It has also been postulated that increased mtDNA damage is due to inefficient repair or protection from DNA attack (Weisman and Halliwell, 1996).

In addition to causing direct oxidative damage, reactive species are also able to affect cytoplasmic and nuclear signal transduction pathways by activating transcription factors such as nuclear factor kappa B (NF-κB). Reactive species alter the activity of genes and proteins that respond to cellular stress and promote cellular processes such as proliferation, differentiation and apoptosis (Halliwell, 2007). Participation of RS in the initiation, promotion and progression of

cancer could thus involve their effects on cell cycle, gene expression, direct or indirect damage to DNA, apoptosis and other forms of cell death. It has been shown that some cancer cells produce large amounts of H_2O_2 (Issa et al., 2006). This overproduction may serve to yield higher levels of hydroxyl radicals in the presence of metal ions, causing DNA damage and mutations that may allow cancer cells to circumvent or inactivate regulatory checkpoints. This process could result in uncontrolled proliferation. H_2O_2 also serves as a cellular messenger that activates NF-κB (Issa et al., 2006). NF-κB activation in turn leads to activation of transcription genes involved in cell cycle progression and proliferation.

The consequences of ROS imbalance on tumor development have been illustrated in animal knockout studies. Elchuri et al. (2005) demonstrated hepatocarcinogenesis as a consequence of prolonged ROS imbalance in copper and zinc containing superoxide dismutase (CuZnSOD) knockout mice. Manganese SOD (MnSOD) is another important antioxidant enzyme responsible for scavenging $O_2\bullet^-$ produced in the mitochondria. Animal knockouts for MnSOD die shortly after birth, indicating the significance of mitochondrial ROS imbalance. Mice with 50% normal MnSOD survive past birth, but have increased incidences of lymphomas, adenocarcinomas and pituitary adenomas (Halliwell, 2007). Chu et al. (2004) showed that mice knockouts for the glutathione peroxidase (GPx) enzymes 1 and 2 developed intestinal cancers. In each of these studies animals experienced decreased antioxidant defense, increased reactive oxygen species (ROS) and increased incidence of cancer; indicating the importance of ROS concentration in carcinogenesis.

Antioxidant Defenses

In order to combat the constant generation of free radicals *in vivo*, aerobic organisms have developed antioxidant defenses in order to survive oxygen exposure (Halliwell and

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Gutteridge, 1999). These defenses can be induced by exposure to ROS, free radicals, and cellular signal molecules. Some antioxidant defenses, including antioxidant enzymes, catalytically remove free radicals and other reactive species. Other types of antioxidants scavenge ROS or minimize the availability of pro-oxidants, such as metal ions, by chelating them. Antioxidant enzymes include SOD, catalase (CAT), GPx, glutathione-S-transferase (GST) and glutathione reductase (GR). These antioxidant enzymes function together to inhibit the formation of, or metabolize, free radicals (Thomas, 2006). For example, GR is critical for recycling glutathione (GSH) and making this substrate available for GPx removal of H_2O_2. GPx is important in removing H_2O_2 produced by the dismutation of $O_2 \bullet^-$ by CuZnSOD. CAT, like GPx also converts CuZnSOD-formed H_2O_2 to water (Figure 2) (Paglia and Valentine, 1967). GR, GPx and GST are found primarily in the cytosol of cells. These enzymes function independently, but also in concert to remove RS from the cell.

Glutathione Reductase

Glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) and NADPH to GSH. This reaction is important for maintaining a high ratio of GSH to GSSG in normal cells:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$
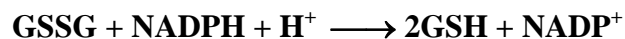
Glutathione has many roles in the cell. It acts as a cofactor for GPx, is involved in ascorbic acid metabolism, prevents protein sulfhydryl groups from oxidizing and cross-linking and chelates copper ions, thereby quenching their ability to release radicals into solution (Halliwell and Gutteridge, 1999). Glutathione itself is also a reducing agent. It is able to make molecules water-soluble; thereby allowing them to be excreted.

Glutathione Peroxidase

GPx is a family of antioxidant enzymes that consist of four protein subunits. Each of these subunits contains a selenium atom at its active site (Halliwell and Gutteridge, 1999). GPx exists primarily in the cytosol of cells; though it may also be found in the mitochondrial matrix and cell nucleus. GPx is widely distributed throughout animal tissues with the highest concentrations existing in the liver, kidney and adrenals. GPx removes H_2O_2 by coupling its reduction to H_2O with the oxidation of GSH (Paglia and Valentine, 1974):

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2H_2O$$
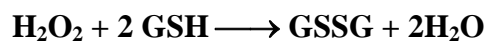
GPx can act on peroxides other than H_2O_2 and can thus catalyze the GSH-dependent reduction of fatty acid hydroperoxides and various synthetic hydroperoxides such as tert-butyl hydroperoxide (t-BOOH). Activity of GPx depends upon the availability of GSH. Activities of GPx will increase with exposure to high oxygen concentrations.

Glutathione-S-transferase

Glutathione is involved in the metabolism of foreign compounds, or xenobiotics, in animal tissues. Metabolism of xenobiotics is said to occur through two-step metabolism (Jung and Kwak, 2010). In the first step, phase I enzymes such as cytochrome P450, catalyze the introduction of functional groups into the hydrophobic regions of the xenobiotic, thus making the molecule more polar. This is known as biotransformation. In step two, phase II enzymes form conjugated metabolites using hydrophilic molecules such as GSH and glucuronic acid. This conjugation step makes the molecules water-soluble, thereby allowing them to be excreted. GST is considered to be a phase II enzyme as it catalyzes the conjugation of GSH to xenobiotics:

$$\text{RX} + \text{GSH} \longrightarrow \text{RSG} + \text{HX}$$
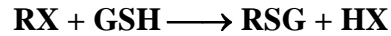
Like GPx, GST has been shown to remove peroxides from the body. Thus GST is considered to act as both an antioxidant enzyme and phase II enzyme. The GST-family of enzymes is expressed ubiquitously, with the greatest activity found in the testes, liver, intestine, kidneys and adrenal glands. GST enzymes are localized in both the cytoplasm and the endoplasmic reticulum. However, cytosolic GST activity has been shown to be 5 to 40 times greater than the microsomal activity (Klassen, Admur and Doull, 1986).

Antioxidant Response Element

Many phase II genes, including GST, are regulated through a *cis*-acting element located in their promoters known as the antioxidant response element (ARE) (Jung and Kwak, 2010). The ARE mediates transcriptional activation of genes in cells exposed to oxidative stress (Nguyen, Sherratt and Pickett, 2003). Proteins encoded by the ARE include enzymes associated with glutathione synthesis and drug metabolizing enzymes. There is a wide range of structurally diverse compounds known to activate the ARE. Examples include xenobiotics, flavonoids and other phenolic antioxidants and heavy metals. Proteins whose expression is mediated by the ARE have an endogenous role in regulating cellular redox status and protecting cells from oxidative damage. Enzymes such as GST, NADPH: quinone reductase and GPx, function to detoxify lipid and DNA base hydroperoxides. The ARE also induces enzymes involved in GSH biosynthesis and recycling such as γ-glutamate cysteine ligase (GCL) and GR, thus leading to increased cellular GSH levels that provide a buffer against oxidative insult.

Activation of the ARE is primarily controlled by nuclear factor E2-related factor 2 (Nrf2). Nrf2 is a basic leucine zipper transcription factor (bZIP) that is sequestered in the

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cytoplasm by the actin-binding protein Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap-1) (Hwang et al., 2011b). ROS react with the cysteine bonds on the Keap-1 protein leading to its dissociation from Nrf2. Free Nrf2 is then translocated from the cytosol to the nucleus, where it sequentially binds to the ARE. This binding results in a cytoprotective response, characterized by the upregulation of antioxidant enzyme expression. In this way, the ARE could be one of the ways that GR, GPx and GST activities are increased (Shih et al., 2007).

Several studies have suggested that Nrf2 nuclear translocation requires the activation of several signal transduction pathways. Such pathways include mitogen-active protein kinase (MAPK), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) (Hwang et al., 2011). Three MAPK cascades have been identified in mammalian cells including extracellular signal-regulated protein kinase 2 (ERK2), c-Jun N-terminal kinase 1 (JNK1) and p38 (Kong et al., 2001). Once activated, these MAPKs can phosphorylate transcription factors such as c-Jun and ATF-2 which ultimately lead to changes in gene expression.

Polyphenols

Diets rich in fruits and vegetables are associated with a reduced risk for coronary artery disease, stroke and cancer (Prior, 2003). This effect has been credited to the high presence of antioxidant vitamins such as vitamin E, C and A and more recently to the presence of polyphenolic compounds. Polyphenols are the most abundant class of dietary antioxidants and are found in a many plant foods. Over 8,000 polyphenols have been found in fruits, vegetables, seeds and liquids. These compounds are made up of multiple phenol rings with hydroxyl groups attached to the aromatic rings. These hydroxyl groups make it possible for polyphenols to donate electrons from their phenolic structure to RS (Issa et al., 2006). Polyphenols are partitioned into phenolic acids, flavonoids, stilbenes and lignans based on the number of phenol rings and

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functional groups (Manach et al., 2004). Flavanoids are further partitioned into flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. Numerous factors affect the content of polyphenols in plants such as ripeness, time of harvest, environmental factors, processing and storage.

Recently, polyphenols have been recognized as major potential players in cancer prevention (Guo et al., 2009). Polyphenols have been found to have multiple biological effects related to chemoprevention. Mechanisms may include antioxidant action, control of signal transduction pathways, decreases in inflammation, modulation of phase I and II enzymes and induction of apoptosis (Issa et al., 2006; Ramos, 2008).

For example, epigallocatechin 3-gallate (EGCG) (from green tea) and quercetin (from onions and green tea) were found to protect against oxidative damage to DNA induced by H_2O_2 (Issa et al., 2006). This suggests that polyphenols are able to act as antioxidants. Polyphenols may act as antioxidants by directly scavenging radicals, chelating metals involved in the Fenton reaction (figure 1) or modulating the activity of antioxidant enzymes such as GPx, GR and SOD (Ramos, 2008). Another chemopreventive mechanism of polyphenols is their ability to inhibit phase-I drug metabolizing enzymes, such as cytochrome P450, and increase the activity of phase II enzymes. Phase I enzymes participate in metabolizing xenobiotics such as drugs and carcinogens. Depending on the type of xenobiotic, phase I metabolism may cause the deactivation of the drug, but may result in the formation of another biologically active and damaging metabolite. In other words, chemical carcinogens may be activated through phase I metabolism (Issa et al., 2006). It may therefore be beneficial to inhibit phase I enzymes. EGCG, curcumin and capsaicin have all been found to inhibit phase I enzymes. Phase II enzymes are important for the deactivation and excretion of xenobiotics; therefore upregulation of these

enzymes may prevent carcinogenesis. Some polyphenols, such as curcumin and chlorogenic acid, have been shown to increase phase II enzyme activity (Issa et al., 2006; Ramos, 2008). Polyphenols have also been found to combat the abnormal cell growth of cancer cells by inducing cell cycle arrest. EGCG was found to induce cell cycle arrest in breast and cervical cancer cells (Ramos, 2008). Gallic acid was also able to attenuate cell cycle progression in leukemia cells.

Anthocyanins

Anthocyanins, a subclass of flavonoids, are the water-soluble red, purple and blue pigments found in many fruits and vegetables. Anthocyanins occur as glycosides or acylglycosides of their respective aglycone anthocyanidins (Wu et al., 2006). There are 17 anthocyanidins found in plants; 6 of which are commonly found in foods (Figure 3). Anthocyanins differ in the number and position of functional groups (methoxyl and hydroxyl) on their basic anthocyanidin structure, the type, number and position of sugar moieties and the extent of sugar acylation. Anthocyanin content in plants is proportional to color intensity; with the deep purple berries (blueberries, blackberries and black currants) having some of the highest concentrations (Table 2.1) (Prior, 2003). Cyanidin is the most common anthocyanidin (present in 90% of fruits) and is therefore one of the most researched (Youdim et al., 2000). It was estimated in a study by Wu et al. (2006) that cyanidin contributes about 47.7% of total daily anthocyanin intake in the United States with delphinidin, malvidin, petunidin, peonidin and pelargonidin contributing 20.7%, 15.4%, 9%, 6.9% and 3.3% respectively.

Anthocyanin intake in the Western diet has been estimated to range from 12.5 mg (Wu et al., 2006) to over 200 mg per day in people consuming many servings of fruit (Prior, 2003). It has been shown that anthocyanins have low bioavailability in human and animal models (<

0.1%) (Del Rio et al. 2010; Prior and Wu 2006); however a rodent study by Talavéra et al. (2005) was able to demonstrate that anthocyanins are absorbed in small amounts and delivered to tissues. In this study, rats were fed an anthocyanin rich diet for 15 days and then tissues from the stomach, jejunum, liver, kidney and brain were studied. It was found that the anthocyanins were absorbed across the stomach in intact forms. The mechanism of this absorption is still unknown. It was also found that the liver is a major site of enzymatic conversion as anthocyanins in the liver were found in a methylated form. Methylated forms of anthocyanins were also detected in the kidneys. Excretion of anthocyanins is also not well understood, however anthocyanin concentration in the urine was low when compared to amount consumed. From this study it was also found that anthocyanins are able to cross the blood-brain barrier.

Researchers are unsure of how anthocyanins are absorbed from the stomach. However in a recent study, anthocyanins were found as intact and methylated glycosides in the plasma and bile of rats (Talavéra et al., 2003). This study seemed to demonstrate that anthocyanin glycosides are quickly and efficiently absorbed from the stomach and are rapidly excreted into the bile in intact and metabolized forms. In another rat study, it was found that oral administration of anthocyanins from berries and berry extracts were absorbed into the blood stream in minutes (Miyazawa et al., 1999). It was determined that the time required to reach maximum anthocyanin concentration in the plasma ranged from half an hour to two hours. Studies yield conflicting results regarding anthocyanin absorption. In one study 58% of a dose of anthocyanins disappeared from the gastrointestinal tract as determined by recovery in the duodenum, ileum, cecum and colon of weanling pigs. However, the extent of anthocyanin disappearance varied depending on the anthocyanins, with 98% of cyanidin-3-glucoside disappearing, but only 22% of cyanidin-3-sambubioside. In the study by Talavéra et al. (2005), absorption of anthocyanins in

rodents depended on chemical structure with 10.7% of malvidin 3-glucoside being absorbed to 22.4% of cyanidin 3-glucoside being absorbed.

Several human studies have also been conducted to determine bioavailability and absorption of anthocyanins. Studies giving 150 mg to 2 g total of anthocyanins to volunteers in the form of berries, berry extracts or concentrates were reviewed by Manach et al. (2005). The concentration of anthocyanins measured in the plasma and urine after intake was very low, with most studies reporting relative urinary excretions of 0.004-0.01% of intake. Clearance of anthocyanins from the plasma also occurs quickly, as 6 hours after intake very little anthocyanin is detectable in the plasma. Two studies did report higher levels of anthocyanin excretion, up to 5%, after intake of red wine or strawberries. While the bioavailability of anthocyanins appears to be low, it has been postulated that availability may be underestimated. This could occur due to 1) important metabolites of anthocyanins are ignored and 2) methods used to measure anthocyanin metabolites are not optimized.

Anthocyanins: Proposed Mechanisms for Cancer Prevention

Aside from pigmenting plants, anthocyanins have been found to have multiple biological effects including antitumor properties. A review of epidemiological studies in humans by Cooke et al. (2005) suggested that foods high in anthocyanins possess cancer preventive properties. For example, in a cohort study of elderly individuals, those consuming large amounts of strawberries had an odds ratio of 0.3 for developing cancer at any site compared to subjects who refrained from high berry consumption (Colditz et al., 1985). Diets high in colored fruit and vegetable consumption have also been associated with a reduced risk of breast cancer and colorectal polyp recurrence. There are several proposed mechanisms by which anthocyanins are thought to elicit this chemopreventive effect. A review by Wang and Stoner (2008) suggests that anthocyanins

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are chemopreventive, in part, due to their ability to inhibit inflammation, cell proliferation, angiogenesis and induce apoptosis and differentiation in tumor cells. Anthocyanins are also thought be chemopreventive due to their antioxidant activity and ability to increase antioxidant and phase II enzyme activity.

Inflammation

There is a compelling body of epidemiological studies that show an association between inflammation and cancer. Key molecular players in the signaling of inflammation include NF-κB, tumor necrosis factor (TNF) and cyclooxygenase-2 (COX-2). These signaling molecules not only promote inflammation, but also promote cell proliferation, antiapoptotic activity, angiogenesis and metastasis (Ramos, 2008). It is thought that inhibition of COX-2, and blocking of the prostaglandin cascade may impact neoplastic growth/development by inhibiting proliferation, angiogenesis and metastasis. Wang et al. (1999) found that anthocyanins from tart cherries and the aglycone cyanidin could inhibit the activities of COX-1 and -2. Seeram et al. (2001) found that cyanidin was a potent inhibitor of cyclooxygenase activity *in vitro*. In addition, a study by Hwang et al. (2011a) found that an anthocyanin fraction from purple sweet potato led to decreased COX-2 and NF-κB expression in rats with dimethyl-nitrosamine induced liver injury. These studies suggest that anthocyanins are able to decrease the expression of signaling molecules involved in inflammation.

Angiogenesis

Angiogensis is the process of forming new blood vessels and is an important factor involved in tumor growth and metastasis (Wang and Stoner, 2008). One of the most potent angiogenesis-activating molecules belongs to the vascular endothelial growth factor (VEGF)

family. The anti-angiogenic properties of anthocyanins have been tested in endothelial and oral cancer cell lines as well as mouse epidermal cells. In endothelial and oral cancer cell models, anthocyanins decreased H_2O_2 and TNF-induced VEGF expression, and VEGF receptor expression. In the mouse epidermal cells, the anthocyanins led to down-regulation of VEGF expression through inhibition of the PI3K pathway. Anthocyanins may be able to inhibit or decrease angiogensis through a variety of signaling pathways.

Cell proliferation

Unregulated cell proliferation is essential for cancer evolution and progression. Therefore, many studies have investigated anthocyanins' anti-proliferative effects. Pure anthocyanins and anthocyanin-rich extracts have exhibited anti-proliferative activity towards multiple cancer cell types *in vitro*. A review by Wang and Stoner (2008) noted that cell proliferation was inhibited by the anthocyanins' ability to block various stages of the cell cycle via effects on cell cycle regulator proteins such as p53, p21 and cyclin d1. Loo (2003) suggested that anthocyanins may also inhibit cancer cell proliferation by scavenging H_2O_2 or inhibiting MAPK phosphorylation, thereby interrupting the flow of events leading to cell cycle progression and cancer cell proliferation by NF-κB and AP-1.

Many studies using anthocyanin extracts from pigmented fruits and vegetables have found that these extracts have significant antiproliferative activity (Table 2.2). Anthocyanin fraction concentration varied in these studies from 1 μg/mL to 2,000 μg/mL in cell culture models and from 100-500 mg/kg in rodent models. Most studies showed greater antiproliferative effects at the higher concentrations. One study by Yi et al. (2005) showed significant inhibition in Caco-2 colon cells using 1 μg/mL anthocyanins, with 50% inhibition occurring by

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concentrations of 15-50 μg/mL. In studies using models of oxidative stress induced by t-BOOH and H_2O_2, it was shown that anthocyanin extracts actually improved cell proliferation and prevented cell death (Heo and Lee, 2005; Hwang et al., 2011a). In addition, the Hwang et al. (2011a) study showed that purple sweet potato anthocyanin fraction alone at 10-800 μg/mL had no effect on cell proliferation in HepG2 cells. This was a unique finding as most studies showed significant decreases in cell proliferation with treatment levels from 1-250 μg/mL. In the study by Heo and Lee (2005) in PC-12 neuronal cells, treatment with 100-2,000 μg/mL strawberry anthocyanin extract in the presence of t-BOOH significantly, and dose-dependently, increased cell proliferation.

Studies looking at the effect of isolated anthocyanin and anthocyanidin treatment on cell proliferation have also been conducted (Table 2.2). Concentrations from 1-250 μmol/L have been investigated in many different cell lines. One study by Kim et al. (2008) showed that cyanidin and delphinidin were able to inhibit HT-29 colon cell growth at levels as low as 1 μmol/L. However, most studies show that anthocyanidins inhibit cell proliferation at concentrations of 25-100 μmol/L (Marko et al., 2004; Renis et al., 2008; Shih et al., 2005; Syed et al., 2008; Yun et al., 2009). In contrast to the study by Kim et al. (2008), several studies have demonstrated that anthocyanin treatment has no effect on, or increased cell proliferation (Hwang et al., 2011a; Lazzé et al., 2003, Lazzé et al., 2004, Renis et al., 2008; Seeram et al., 2003; Shih et al., 2007; Tokarev, 2010). Studies demonstrating a positive effect of anthocyanin treatment on cell proliferation were in cellular models of oxidative stress (Hwang et al., 2012; Tokarev, 2010). These studies showed that low levels of anthocyanins (0.1 mg and 1-10 μmol/L respectively), improved cell proliferation in oxidatively stressed cells. From these studies it seems that the

effect of anthocyanin treatment on cell proliferation depends on the cell line used, anthocyanin concentration used and whether or not an oxidative stressor is present.

Apoptosis

Apoptosis, or programmed cell death, also plays a key role in the development and growth regulation of normal cells. Apoptosis is often dysregulated in cancer cells, therefore targeting apoptotic pathways may aid in chemoprevention (Wang and Stoner, 2008). Anthocyanin-rich extracts and anthocyanidins have exhibited pro-apoptotic effects in many cell lines *in vitro* (Afaq et al., 2007; Heo and Lee, 2005; Seeram et al., 2006; Shih et al., 2005; Tokarev, 2010). Anthocyanins and anthocyanidins induce apoptosis through both the intrinsic mitochondrial pathway and the extrinsic FAS pathway (Wang and Stoner, 2008). Anthocyanins affected the intrinsic apoptotic pathway by increasing mitochondrial membrane potential, cytochrome *c* release and modulation of the caspase-dependent apoptotic proteins. Anthocyanins affected the extrinsic pathway by modulating expression of FAS and the FAS ligand, a transmembrane protein of the TNF family. These authors also noted that anthocyanin treatment may cause an accumulation of ROS in cancer cells, which in turn leads to apoptosis.

Antioxidant activity

The antioxidant effects of anthocyanins have been frequently demonstrated and are outlined in a review by Prior (2003). In a study by Youdim et al. (2000), blueberry anthocyanins were able to inhibit ROS development in red blood cells exposed to hydrogen peroxide both *in vitro* and *in vivo*. Tokarev (2010) also demonstrated the antioxidant effect of anthocyanins. Supplementation with delphinidin, even at low concentrations, significantly ($p < 0.05$) protected HT-29 cells from oxidative stress induced by H_2O_2, as measured by cell viability and DNA

fragmentation. Lazzè et al. (2003) demonstrated that 100 μmol/L cyanidin and delphinidin were able to protect liver cells from t-BOOH induced damage. In the Lazzè study, anthocyanidin treatment decreased single strand DNA breaks and reduced lipid peroxidation.

Various rodent models have also been used to demonstrate the antioxidant effects of anthocyanins. Hepatic lipid peroxidation was significantly decreased in mice consuming a diet consisting of 10% anthocyanin-rich cherry juice (Sarić et al., 2009). In rats with alcohol-induced liver damage, 500 mg/kg of anthocyanin-rich black rice extract significantly decreased hepatic and serum malondialdehyde levels. Dulebohn et al. (2008) demonstrated that rat diets supplemented with 1% blueberry flavonoids (containing primarily anthocyanins) showed significantly less DNA damage in liver tissues than controls. This suggested that blueberry flavonoids are able to prevent oxidative damage to DNA. The antioxidant effects of anthocyanins have also been demonstrated in human studies. Rowers consuming 150 mL of anthocyanin-rich chokeberry juice had significantly lower levels of lipid peroxidation versus the control (Pilaczynksa-Szczesniak et al., 2005). Taken together, the results of these studies suggest that anthocyanins have an antioxidant effect and are able to protect oxidatively stressed cells.

Prior (2003) noted that anthocyanins are able to act as direct antioxidants by donating electrons from their phenolic structure to ROS. This radical scavenging ability is due to the hydroxyl groups on the 3' carbon of the C-ring and the 3', 4' and 5' carbons of the B-ring. Rahman et al. (2006) demonstrated anthocyanin radical-scavenging activity by electron spin resonance using 5,5-dimethyl-1-pyrroline-N-oxide (*DMPO*). Superoxide radicals decreased in the presence of anthocyanins in a dose dependent manner due to the $O_2 \bullet^-$ scavenging by anthocyanins. The number and position of hydroxyl substituents in the aglycone B ring and

extent of O-methylation significantly affected activity with delphinidin having the highest superoxide radical scavenging ability and pelargonidin having the least.

Influence on antioxidant enzyme activity, GSH and phase II enzyme activity

Finally, anthocyanins are thought to be chemopreventive through their ability to increase the activity of antioxidant and phase II enzymes. Many studies have demonstrated that anthocyanin fractions and anthocyanins are able to increase antioxidant enzyme activity (Table 2.3). Animal studies examining antioxidant activity have typically used high concentrations of anthocyanin fractions; 125 mg/kg- 300 g/kg. Many of these studies found that anthocyanin fractions increased GPx and GR activities (Ajiboye et al., 2011; Han et al., 2006; Han et al., 2007; Hou et al., 2010). At these concentrations, anthocyanin fractions have also been shown to increase GSH levels. Despite the apparent benefit of anthocyanin fractions on antioxidant enzymes, some studies have failed to demonstrate increased activity. Palikova et al. (2010) treated rats with various cranberry anthocyanin-rich powders. Their study showed no effect on liver GPx or GST and significantly lowered GR and GSH versus the control.

The effect of anthocyanin fractions on antioxidants has also been assessed in humans. Spormann et al. (2008) found that 200 mL/day of anthocyanin-rich fruit juice significantly increased GSH levels in patients undergoing hemodialysis. Another study in male rowers observed that 150 mL of chokeberry juice/day increased GPx activity above the control as measured by blood draw 1 minute after exercise (Pilaczynksa-Szczesniak et al., 2005). These studies suggest that anthocyanins can increase antioxidant enzyme activity and GSH level *in vivo*. Not all studies, however, have demonstrated this effect. Duthie et al. (2006) did not see an

increase in GPx activity or GSH levels in healthy females consuming 750 mL of cranberry juice. The lack of findings may be attributed to the fact that these were non-stressed volunteers.

Antioxidant enzyme activity has also been investigated *in* vitro. *In vitro* studies have used a range of concentrations; 1-200 μmol/L. Many of these studies demonstrated increased GR and GPx activities. Of note is a study by Turner (2009), in which it was found that low concentrations of anthocyanidins are able to increase antioxidant enzyme activity. In this study it was observed that 5 μg/mL malvidin increased GR activity. Peonidin was able to increase GR activity as well but at a concentration of 10 μg/mL. This illustrates how structurally different anthocyanidins elicit different effects at the same concentration. In the Turner study it was also observed that a combined dose of malvidin and peonidin at 2.5 μg/mL and 5 μg/mL significantly increased the activity of glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) by 55%, 21% and 42% respectively above control in HT-29 cells. Supplementation of malvidin and peonidin alone had significant effects on antioxidant enzyme activity, but not as pronounced as when administered in combination, thereby suggesting a synergistic effect.

Shih et al. (2007) also demonstrated beneficial effects of anthocyanidin treatment on rat liver cells and non-cancerous breast cells. Supplementation with 50 μmol/L anthocyanidins improved antioxidant capacity by increasing the activity of GR, GPx and GST as well as increasing GSH. Not all anthocyanin concentrations confer a benefit to cells, however. Srivastava et al. (2007) treated HT-29 colon cells with 50-150 mg/mL blueberry anthocyanins. These high doses decreased GST and quinone reductase activity. This suggested that blueberry anthocyanins can actually act as prooxidants at high concentrations.

Anthocyanins also increase the activity of phase II enzymes. A study by Dulebohn et al. (2008) documented the effects of anthocyanin supplementation on phase II enzymes. Rats were supplemented with various blueberry fraction diets. In one treatment, rats were supplemented with 10% freeze-dried whole blueberries. While treatment resulted in increased liver glutathione S-transferase (GST) activity by 25% above that seen in the control diet, results were not significant. Reen et al. (2006) conducted a similar study in which rats were supplemented with 5 or 10% dried black raspberries. In this study they were able to detect a significant increase in GST activity. Shih et al. (2007) demonstrated that treatment of rat liver clone 9 cells with 50 µmol/L anthocyanidins significantly increased expression of GST and NADPH: quinone reductase in the presence of an oxidative stressor. The increase in these enzymes was found to occur through the activation of the ARE. This data suggested that anthocyanins act as chemopreventive phytochemicals and are able to stimulate the antioxidant system to resist oxidative injury. The researchers concluded that the promoting effect of the anthocyanins on phase II enzymes via ARE activation is a critical point in the modulation antioxidant defenses.

Rationale

Free radical damage to DNA resulting from oxidative stress has been linked to cancer (Weisman and Halliwell, 1996; Guo et al., 2009). Anthocyanins may be chemopreventive due to their antioxidant activity, anti-inflammatory activity, and ability to increase the activity of the antioxidant enzymes GPx, GR GST (Ramos, 2008; Alía et al. 2006). A limited number of studies have investigated the effects of anthocyanins on antioxidant enzyme activity, and results have been conflicting as to the benefits of anthocyanin intake (Ramos, 2008). Turner (2009) and Shih et al. (2007) found that low levels of anthocyanidin supplementation in HT-29 colon carcinoma cells and rat clone 9 hepatocyte cells, respectively, increased the activity of the

antioxidant enzymes GPx, GR and GST. Little is known about anthocyanin supplementation in the liver cell model. The liver plays a role in anthocyanin metabolism and research is needed to determine the effective concentrations of anthocyanins and the mechanisms responsible for their action.

Research question

Do anthocyanidins increase antioxidant enzyme activity and improve markers of cellular oxidative stress in HepG2 human hepatocellular carcinoma cells?

Hypothesis

Anthocyanidins will increase glutathione antioxidant enzyme activity and improve markers of cellular oxidative stress in HepG2 human hepatocellular carcinoma cells.

Specific aims

1. Assess the effect of anthocyanidins on the activities of GPx, GR and GST. *It is hypothesized that anthocyanidin supplementation will increase the activity of these antioxidant enzymes.*

2. Assess the effect of anthocyanidin supplementation on selected markers of cellular oxidative stress including thiobarbituric acid reactive substances (TBARS) assay and cell proliferation. *It is hypothesized that anthocyanidin supplementation will decrease the presence of TBARS and improve viability of cells subjected to oxidative stress.*

Fenton Reaction

$$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe_{3+}$$

Haber-Weiss Reaction

$$H_2O_2 + O_2\bullet^- \rightarrow OH\bullet + OH^- + O_2$$
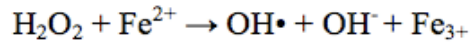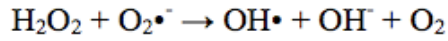
Figure 2.1: Fenton and Haber-Weiss Reactions
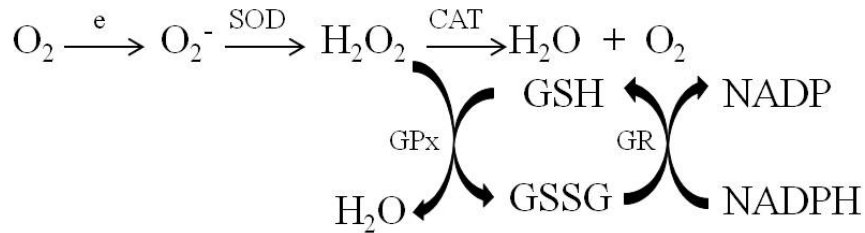
SOD = Superoxide dismutase; GPx = Glutathione peroxidase; GR = Glutathione Reductase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; e = Electron; O_2^- = Superoxide radical; H_2O_2 = Hydrogen peroxide; O_2 = Oxygen; H_2O = Water

Adapted from Sun, 1990

Figure 2.2: Interaction of antioxidant enzymes CuZnSOD, CAT, GR and GPx
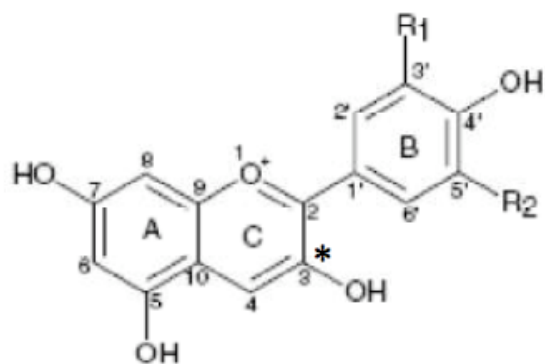
Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Pelargonidin	H	H

*Sugar moieties in glycosylated anthocyanins
are usually attached at this position

Figure 2.3: Structure of common anthocyanins

Table 2.1: Anthocyanin content in selected common foods[1,2]

Food	Anthocyanin/100g [2]
Cultivated blueberry	387 mg
Red cabbage	322 mg
Blackberry	245 mg
Cranberry	140 mg
Sweet cherry	122 mg
Concord grape	120 mg
Red radish	100 mg
Raspberry	92 mg
Eggplant	86 mg
Red onion	49 mg
Black beans	45 mg
Red grape	27 mg
Strawberry	21 mg
Red delicious apple	12 mg

[1]Data adapted from Wu et al., 2006 by Tokarev, 2010
[2]100 g of fresh weight or consumed form

Table 2.2. Studies on the effects of anthocyanin extracts and anthocyanins on cell proliferation

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Extracts							
Aquil et al., 2012	A549 human non-small-cell lung carcinoma	72 hr	None	Anthocyanin-rich extract of Indian Blackberry pulp	None	MTT Assay	Anthocyanin extract showed significant antiproliferative activity
Bishayee et al., 2010	Male Sprague-Dawley rats	22 wk	Oral	100 and 500 mg/kg body weight black currant skin extract (BCSE) (1.2% total anthocyanin)	Intraperitoneal injection of diethylnitrosa-mine (DENA) followed by promotion with phenobarbital	Immunohistoch-emial detection of proliferating cell nuclear antigen (PCNA)	BCSE at 500 mg/kg BW + DENA reduced PCNA
Boivin et al., 2007	Human cancer cell lines: AGS (stomach), MCF-7 and MDA-MB-231(mammary gland), PC-3 (prostate), Caco-2 (colon)	48 hr	None	50 µL/mL berry juice from raspberry, strawberry, seabuckthorn, cranberry, black, red and white currant, gooseberry, velvet leaf, low and highbush blueberry, serviceberry and blackberry	None	WST-1 Assay	Growth strongly inhibited by: raspberry, black/white currant, gooseberry, velvet leaf blueberry, buckthorn and cranberry; and moderately (28-56%) by low-bush blueberry and weakly by the remaining berries
Faria et al., 2010	MDA-MB-231 and MCF7 breast cancer cells	24 hr	None	50, 100 and 250 µg/mL blueberry anthocyanin extract (I) and anthocyanin-pyruvic acid adducts (II)	None	SRB Assay	250 µg/mL of both extracts significantly decreased proliferation in both cell lines, 50-250 µg/mL of extract II decreased proliferation in MDA-MB-231

Table 2.2 continued

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Heo and Lee, 2005	PC-12 neuronal cells	10 min	None	100, 300, 600 and 2,000 μg/mL strawberry anthocyanin extract	400 μM H_2O_2 for 2 hr	MTT Assay	Strawberry extract improved proliferation dose-dependently with 2,000 μg/mL increasing proliferation 127%
Hwang et al., 2011	HepG2 cells		None	10, 50 and 200 μg/mL Purple sweet potato anthocyanin fraction	300 μM t-BOOH for 24 hr	MTT Assay	AF alone at 10, 50, 200, 400 and 800 μg/mL did not affect cell proliferation. AF pretreatment at 50 and 200 μg/mL significantly prevented cell death
Jing et al., 2008	HT-29 colon cells	48 hr	None	14-100 μg/mL of anthocyanin-rich extract from purple corn, chokeberry, bilberry, purple carrot, grape, radish and elderberry	None	SRB Assay	Anthocyanins at 13.8 (purple corn), 31.2 (chokeberry), 32.2 (bilberry), 68.5 (purple carrot), 71.2 (grape), 107.7 (radish) and 130.3 μg/mL (elderberry) inhibited cell growth by 50%
Lala et al., 2006	Fischer 344 male rats	14 wk	Oral	3.85 g/kg monomeric anthocyanin from either chokeberry, bilberry or grape anthocyanin rich extract	20 mg/kg azoxymethane	Immunohistoch-emial detection of proliferating cell nuclear antigen (PCNA)	Bilberry and chokeberry anthocyanin-rich extract decreased colonic cell proliferation
Lo et al., 2007	7r5 rat embryonic aortic smooth muscle cell line	24 hr	None	0-6 mg/mL *Hibiscus* anthocyanins	None	MTT Cell Proliferation Assay	Dose dependent inhibition of cells

Table 2.2 continued

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Seeram et al., 2004	Human cancer cell lines: KB, CAL27 (oral), HT-29, HCT116, SW480, SW620 (colon), RWPE-1, RWPE-2, 22Rv1 (prostate)	48 hr	None	Total cranberry extract (TCE): 200 µg/mL and predominantly anthocyanin fraction: 1.2% TCE	none	CellTiter-Glo Luminescent cell viability Assay	Both TCE and anthocyanin inhibited in oral/prostate and colon cells
Yi et al., 2005	HT-29 and Caco-2 colon cells	48 hr	None	1 µg/mL-200 µg/mL blueberry anthocyanin fraction	None	MTT Cell Proliferation Assay	Significant inhibition in Caco-2 cells with 1 µg/mL, 50% inhibition in Caco-2/HT-29 by 15-50 µg/mL
Yi et al., 2006	HepG2 cells	48 hr	None	Anthocyanin fraction from 3 cultivars of blueberries and 4 cultivars of muscadine grapes	None	MTT Cell Proliferation Assay	50% Inhibition with 70–150 and 100–300 µg/mL in blueberries and grapes respectively

Anthocyanins

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Cvorovic et al., 2010	Caco-2, LoVo and LoVo/ADR colon cancer cells	68 hr	None	0.78-100 µM cyanidin (cyn) and delphinidin (del)	None	MTT Assay	Cyn and del were not cytotoxic in Caco-2 cells, but were in LoVo and LoVo/ADR cells
Hwang et al., 2012	ARPE-19 human retinal pigment epithelium cells	18 hr	None	0.1, 0.5 and 1 mg/mL anthocyanin oligomer	0.5 µM H_2O_2 for 24 hours	MTT Assay	Anthocyanins increased ARPE-19 cell proliferation and showed 60% viability against 0.5 µM H_2O_2
Hyun and Chung, 2004	U937 human monocytic leukemia cells	96 hr	None	0-200 µg/mL malvidin (mal) and cyn	None	MTT Assay	IC_{50} of 60 and 40 µg/mL for cyn and mal respectively
Kim et al., 2008	HT-29 colon cells	48 and 72 hr	None	1, 10 and 50 µM cyn, pelargonidin (pel) and del	None	[3H]thymidine	Cyn and del inhibited growth at 1µM over 48-72 hours, and pel at 50 µM over 72 h

Table 2.2 continued

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Lazzé et al., 2003	MH1C1 rat hepatoma cells and SMC rat smooth muscle cells	2 hr	None	100 μM del, del-3-glucoside, del-3-rutinoside, cyn, cyn-3-glucoside and cyn-3-rutinoside	200 μM t-BOOH for 1 hour	MTT Assay	In SMC cells, anthocyanins completely protective against t-BOOH induced cytotoxicity in MH1C1 line only cyn and del counteracted t-BOOH cytotoxicity
Lazze et al., 2004	Normal human fibroblast cells, Caco-2 colon cells and Hela uterine carcinoma cells	24 hr	None	50, 100, 150 and 200 μM del and cyn	None	MTT Assay	Cyn did not affect cell viability. Del decreased viability only in Hela cells at 150 and 200 μM
Marko et al., 2004	HT-29 colon cells	72 hr	None	0-300 μM mal, del, cyn, pel, peonidin (peon)	None	SRB Assay	Del and mal showed highest inhibitory effect with 25 μM decreasing proliferation to < 80% of the control
Renis et al., 2008	Caco-2 cells	48 hr	None	5, 10, 25, 50, 100 and 200 μM cyn and cyn-3-O-B-pyranoside	None	MTT Assay	5-10 μM did not exhibit damaging effects, 25-100 μM decreased cell proliferation
Seeram et al., 2003	MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), HCT-116 (colon)	48 hr	None	6.25, 12.5, 25, 50 and 100 μM mal, del, cyn, peon and pel	None	SRB Assay	No anthocyanidin led to significant inhibition on proliferation at 6.25-100 μM treatment level
Shih, Yeh and Yen, 2005	AGS gastric cells	48 hr	None	200 μM cyn, del, mal, pel, peon and glycosylated cyn, mal, pel and peon	None	MTT Assay	Mal, del and mal-3-glucoside lowered proliferation to 63, 67 and 69% of the control respectively

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Table 2.2 continued

Author	Model	Length	Route	Dose	Stress Induced	Assay Used	Results
Shih et al., 2007	Clone 9 normal rat hepatocyte cell line	24 hr	None	50 µM mal, del, cyn, peon, petunidin (pet), pel	None	MTT Assay	No anthocyanidin led to significant inhibition on proliferation at 50 µM treatment level
Syed et al., 2008	MDA-MB-231 and HCC1419 hepatocyte growth factor-Met expressing breast cells	48 hr	None	0, 60, 80, 100 and 120 µM del in MDA-MB-231 and 0, 100, 150, 200 and 250 µM del in HCC1419	None	MTT Cell Proliferation Assay	100 µM del in MDA-MB-231 and 150 µM in HCC1419 cells inhibited ($p < 0.05$) cell proliferation
Tokarev, 2010	HT-29 colon cells	4 hr	None	1, 5, 10, 25 µM del	100 µM H2O2 for 2 hours	MTT Assay	1-10 µM del significantly improved cell proliferation in H_2O_2 treated cells vs H_2O_2 alone
Yun et al., 2009	HCT116 colon cells	48 hr	None	30, 60, 120, 180 and 240 µM del	None	MTT Assay	Del treatment significantly inhibited cell proliferation from 60-240 µM
Zhang et al., 2005	AGS (stomach), HCT-116 (colon), MCF-7 (breast), NCI H460 (lung) and SF-268 (central nervous system (CNS)) human cancer cells	48 hr	None	12.5, 25, 50, 100 and 200 µg/mL del, cyn, mal, pel, pet and 4 glycosylated anthocyanins	None	MTT Assay	Anthocyanins at 200 µg/mL had no inhibitory effects. Cyn and del at 100-200 µg/mL inhibited MCF-7 only; pet at 200 µg/mL inhibited MCF-7 and AGS only; pel and mal inhibited all lines at 200 and 100 µg/mL respectively

Table 2.3. Studies on the effects of anthocyanin extracts and anthocyanins on enzyme activity and glutathione levels

Author	Model	Length	Route	Dose	Stress Induced	Enzymes/GSH	Results
Extracts							
Ajiboye et al., 2011	Male Albino Rats	14 d	Oral	200 mg/kg body weight (BW) Hibiscus anthocyanin extract (AE)	0.5 mL carbon tetrachloride (CCl_4)/kg BW	GPx GR GST	AE significantly induced GPx/GR activity and attenuated decreased activity from CCl_4 AE increased GST activity and attenuated decreased activity from CCl_4
Choi et al., 2009	Male ICR Mice	7 d	Oral	0, 200, 400 or 800 mg/kg BW/d anthocyanin fraction (AF) from purple-fleshed sweet potato	400 mg/kg BW Acetaminophen (APAP)	GST GSH	AF alone increased hepatic GST activity and GSH levels. AF pretreatment dose-dependently reduced APAP GSH depletion with 800 mg/kg returning GSH to control levels
Duthie et al., 2006	Healthy female volunteers aged 18-40	2 wk	Oral	750 mL cranberry juice /day (primarily cyanidin (cyn) and peonidin (peon) glycosides	None	GPx GSH	GPx was lower than the control, but not significant GSH did not change
Dulebohn et al., 2008	Male Sprague-Dawley Rats	3 wk	Oral	1% blueberry flavonoids diet	None	GST	24% increase in liver GST activity, though not found to be significant
Han et al., 2006	Male F344/DuCrj	4 wk	Oral	250 g/kg BW/d purple potato flakes from Kitamurasaki (KM) and Hokkai # 92 (H92)	None	GPx	GPx mRNA levels in H92 groups were significantly higher than control

Table 2.3 continued

Author	Model	Length	Route	Dose	Stress Induced	Enzymes/GSH	Results
Han et al., 2007	Male F344 Rats	4 wk	Oral	300 g/kg BW medium purple (87 mg anthocyanins/100g) or dark purple potato flakes (401 mg/100g)	High cholesterol diet (0.5% cholesterol)	GR GST GSH	Hepatic GR/GST activities and GSH levels in med/dark purple groups significantly greater than control
Hou, Qin and Ren, 2010	Male Wistar Rats	45 d	Oral	125, 250, 500 mg/kg BW black rice AE	3.7 g/kg BW/day ethanol	GPx GST GSH	Ethanol treatment increase GST and GPx, 500 mg/kg AE decreased activities (p < 0.01) 500 mg/kg AE restored GSH levels in ethanol treated rats
Hwang et al., 2011	HepG2 liver cells	24 hr	None	10, 50 and 200µg/mL purple sweet potato AF	300 µM t-BOOH for 24 hours	GST GSH	AF alone increased GST mRNA expression with 10-200 µg/mL AF AF significantly attenuated t-BOOH induced hepatic GSH depletion at 50-200 µg/mL
Hwang, Choi, et al., 2011	Male Sprague-Dawley Rats	4 wk	Oral	50, 100 or 200 mg/kg BW purple sweet potato AF	10 mg/kg BW dimethylnitrosamine (DMN) 3 x/week for 4 weeks	GST GSH	AF alone at 200 mg/kg increased GST and GSH 100-200 mg/kg AF + DMN significantly improved GST and GSH
Kim et al., 2011	Male Sprague-Dawley Rats	3 d	Oral	20, 50 or 80 mg/kg BW AE from *Rubus coreanus* twice/d	80 mg/kg BW Naproxen (nap) twice/d for 3 days	GPx	50 and 80 mg/kg significantly increased GPx activity compared to nap treated rats

Table 2.3 continued

Author	Model	Length	Route	Dose	Stress Induced	Enzymes/GSH	Results
Palikova et al., 2010	Male Wistar Rats	100 d	Oral	Cranberry powder containing 0.105, 0.440 and 0.610% anthocyanins	None	GPx GST GR GSH	No effect on liver GPx orGST GR and GSH were significantly lower than control in 0.105% anthocyanin powder
Pilaczynska-Szczesniak et al., 2005	Polish males on rowing team	4 wk	Oral	150 mL chokeberry juice/day (containing 23 mg/100 mL anthocyanins)	2,000 meter rowing exercise test	GPx	Group receiving chokeberry juice had lower GPx activity vs control after 1 minute of exercise
Šarić et al., 2009	Male CHA/Hr mice		Oral	Commercial food pellets with 10% or 50% cherry juice added (546 mg/L total anthocyanins)	Intraperitoneal (i.p.) injection of incomplete Freund's adjuvant	GPx	Liver GPx activity was increased in mice fed 10% and 50% sour cherry juice
Shih et al., 2010	Male SAMR1 and SAMP8 senescence-resistant and accelerated mice (respectively)	3 mon	Oral	50 mg/kg BW anthocyanin-rich blackcurrant extract (BE), 100 mg/kg BW anthocyanin-rich mulberry extract (ME) or 500 mg/kg BW ME	None	GR GPx GST	No difference in liver GR SAMP8 control had significantly lower liver GPx- salvaged with 50 mg/kg ME Liver GST significantly higher in 500 mg/kg ME vs controls
Spormann et al., 2008	Hemodialysis patients aged 21-79	4 wk	Oral	200 mL/day fruit juice from red grapes (40%) blackberries (20%) sour cherries (15%) black currant (15% and elderberries (10%)	None	GSH	Significant increase in GSH level and status during juice intake

Table 2.3 continued

Author	Model	Length	Route	Dose	Stress Induced	Enzymes/GSH	Results
Srivastava et al., 2007	HT-29 colon cells	6 hr	None	50, 100 and 150 µg/mL AF from blueberry cultivars	None	GST	Significant decrease in GST activity in treated cells versus control
Anthocyanins							
Cvorovic et al., 2010	Caco-2 (colon), LoVo (colon metastasis) and LoVo/ADR (doxorubicin-resistant metastatic colon)	24 hr	None	25, 50 and 100 µM delphinidin (del) or cyn	None	GR GSH	GR activity/GSH concentration not affected in Caco-2/ LoVo cells but significantly decreased in LoVo/ADR with 25-100 µM del/cyn.
Domitovic and Jakovac, 2010	Male Balb/C mice	2 wk	i.p.	10 mg/kg or 25 mg/kg del	2 mL/kg CCl4 i.p. 2/week for 7 weeks	GPx GSH	CCl4 reduced hepatic GPx activity and GSH, 25 mg/kg del significantly attenuated decreased GPx activity and GSH
Hwang et al., 2012	ARPE-19 human retinal pigment epithelium cells	24 hr	None	0.1, 0.5 and 1 mg/mL anthocyanin oligomer from fermented *Aspergilus niger* mold	0.5 µM H_2O_2 for 24 hours	GPx GST	1 mg/mL anthocyanins increased GPx activity significantly over H_2O_2 group 0.5 and 1.0 mg/mL significantly increased GST activity over H_2O_2
Kelsey et al., 2011	Cultured cerebellar granule neurons (CGNs) from 7 day old Sprague-Dawley rat pups	4 hr	None	100 and 200 µM callistephin (cal) and kuromanin (ku) anthocyanins	HA14-1 Bcl-2 inhibitor	GPx GSH	GPx activity dramatically increased with 100-200 µM cal/ku GSH levels in presence of HA14-1 were maintained with 200 µM cal/ ku

Table 2.3 continued

Author	Model	Length	Route	Dose	Stress Induced	Enzymes/GSH	Results
Lazzé et al., 2003	MH1C1 rat hepatoma cells and SMC rat smooth muscle cells	2 hr	None	100 µM del, del-3-glucosie, del-3-rutinoside, cyn, cyn-3-glucoside and cyn-3-rutinoside	200 µM t-BOOH for 1 hour	GSH	Anthocyanins did not show protective effect against t-BOOH induced GSH decrease
Shih et al., 2007	Clone 9 rat heptocyte cells	24 hr	None	50 µM cyn, del, malvidin (mal), peon, pelargonidin (pel), petunidin (pet), ku, oenin, peon-3-glucoside, and cal	None	GR GPx GST GSH	GR, GPx and GST activity and GSH/GSSG ratio increased with anthocyanin treatment (cyn, del, mal and ku showed most positive effects)
Turner, 2009	HT-29 colon cells	22 hr	None	5, 10 µg/mL mal or peon and 2.5, 5 or 10 µg/mL combined mal and peon	None	GR GPx GST	Mal at 5 µg/mL, peon at 10 µg/mL and combined at 2.5, 5 and 10 µg/mL significantly increased GR Combined 2.5 µg/mL significantly increased GPx Combined 2.5 and 5 µg/mL significantly increased GST
Vari et al., 2011	J774 A.1 macrophages	0-6 hr	None	25 µM protocatechuric acid (PCA) and anthocyanin metabolite	None	GR GPx	Increase in GPx mRNA expression 1-6 h after PCA treatment Increase in GR mRNA only at 2 hr after PCA
Zhu et al., 2012	HepG2 liver cells	24 hr	None	1, 10 and 100 µM Cyn-3-O-β-glucoside (C3G)	25 µM glucose	GPx GST GR GSH	No change in GPx/ GST/GR 10 µM C3G increased GSH 2 fold

References

Afaq, F., Zaman, N., Khan, N., Syed, D. N., Sarfaraz, S., Zaid, M. A., & Mukhtar, H. (2008). Inhibition of epidermal growth factor receptor signaling pathway by delphinidin, an anthocyanidin in pigmented fruits and vegetables. *International Journal of Cancer, 123*(7), 1508-1515.

Ajiboye, T. O., Salawu, N. A., Yakubu, M. T., Oladiji, A. T., Akanji, M. A., & Okogun, J. I. (2011). Antioxidant and drug detoxification potentials of Hibiscus sabdariffa anthocyanin extract. *Drug and Chemical Toxicology, 34*(2), 109-115.

Alía, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2005). Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology, 19*(2), 119-128.

Alía, M., Ramos, S., Mateos, R., Granado-Serrano, A. B., Bravo, L., & Goya, L. (2006). Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Applied Pharmacology, 212*(2), 110-118.

American Cancer Society. (2010). *Diet and physical activity: what's the cancer connection?* Retrieved from http://www.cancer.org/Cancer/CancerCauses/DietandPhysicalActivity/diet-and-physical-activity

Aqil, F., Gupta, A., Munagala, R., Jeyabalan, J., Kausar, H., Sharma, R. J., et al. (2012). Antioxidant and Antiproliferative Activities of Anthocyanin/Ellagitannin-Enriched Extracts From Syzygium cumini L. (Jamun, the Indian Blackberry). *Nutrition and Cancer, 64*(3), 428-438.

Boivin, D., Blanchette, M., Barrette, S., Moghrabi, A., & Béliveau, R. (2007). Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFkappaB by edible berry juice. *Anticancer Research, 27*(2), 937-948.

Centers for Disease Control and Prevention. (2010). *Deaths and mortality*. Retrieved from http://www.cdc.gov/nchs/fastats/deaths.htm

Choi, J. H., Choi, C. Y., Lee, K. J., Hwang, Y. P., Chung, Y. C., & Jeong, H. G. (2009). Hepatoprotective effects of an anthocyanin fraction from purple-fleshed sweet potato against acetaminophen-induced liver damage in mice. *Journal of Medicinal Food, 12*(2), 320-326.

Chu, F.-F., Esworthy, R. S., Chu, P. G., Longmate, J. A., Huycke, M. M., Wilczynski, S., & Doroshow, J. H. (2004). Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. *Cancer Research, 64*(3), 962-968.

Colditz, G. A., Branch, L. G., Lipnick, R. J., Willett, W. C., Rosner, B., Posner, B. M., & Hennekens, C. H. (1985). Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population. *The American Journal of Clinical Nutrition, 41*(1), 32-36.

Cooke, D., Steward, W. P., Gescher, A. J., & Marczylo, T. (2005). Anthocyans from fruits and vegetables--does bright colour signal cancer chemopreventive activity? *European Journal of Cancer, 41*(13), 1931-1940.

Cvorovic, J., Tramer, F., Granzotto, M., Candussio, L., Decorti, G., & Passamonti, S. (2010). Oxidative stress-based cytotoxicity of delphinidin and cyanidin in colon cancer cells. *Archives of Biochemistry and Biophysics, 501*(1), 151-157.

Del Rio, D., Borges, G., & Crozier, A. (2010). Berry flavonoids and phenolics: bioavailability and evidence of protective effects. *The British Journal of Nutrition, 104 Suppl 3*, S67-S90.

Dulebohn, R. V., Yi, W., Srivastava, A., Akoh, C. C., Krewer, G., & Fischer, J. G. (2008). Effects of blueberry (Vaccinium ashei) on DNA damage, lipid peroxidation, and phase II enzyme activities in rats. *Journal of Agricultural and Food Chemistry, 56*(24), 11700-11706.

Duthie, G. G., Duthie, S. J., & Kyle, J. A. (2000). Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutrition Research Reviews, 13*(1), 79-106.

Duthie, S. J., Gardner, P. T., Morrice, P. C., Wood, S. G., Pirie, L., Bestwick, C. C., et al. (2005). DNA stability and lipid peroxidation in vitamin E-deficient rats in vivo and colon cells in vitro--modulation by the dietary anthocyanin, cyanidin-3-glycoside. *European Journal of Nutrition, 44*(4), 195-203.

Duthie, S. J., Jenkinson, A. M., Crozier, A., Mullen, W., Pirie, L., Kyle, J., et al. (2006). The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *European Journal of Nutrition, 45*(2), 113-122.

Elchuri, S., Oberley, T. D., Qi, W., Eisenstein, R. S., Jackson Roberts, L., Van Remmen, H., et al. (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene, 24*(3), 367-380.

Faria, A., Pestana, D., Teixeira, D., de Freitas, V., Mateus, N., & Calhau, C. (2010). Blueberry anthocyanins and pyruvic acid adducts: anticancer properties in breast cancer cell lines. *Phytotherapy Research, 24*(12), 1862-1869.

Fritz, J., Kern, M., Pahlke, G., Vatter, S., & Marko, D. (2006). Biological activities of malvidin, a red wine anthocyanidin. *Molecular Nutrition and Food Research, 50*(4-5), 390-395.

Fritz, J., Roth, M., Holbach, P., Esselen, M., & Marko, D. (2008). Impact of delphinidin on the maintenance of DNA integrity in human colon carcinoma cells. *Journal of Agricultural and Food Chemistry, 56*(19), 8891-8896.

Gaté, L., Paul, J., Ba, G. N., Tew, K. D., & Tapiero, H. (1999). Oxidative stress induced in pathologies: the role of antioxidants. *Biomedicine and Pharmacotherapy, 53*(4), 169-180.

Granado-Serrano, A. B., Martín, M. A., Bravo, L., Goya, L., & Ramos, S. (2012). Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. *Chemico-Biological Interactions, 195*(2), 154-164.

Guo, W., Kong, E., & Meydani, M. (2009). Dietary polyphenols, inflammation, and cancer. *Nutrition and Cancer, 61*(6), 807-810.

Halliwell, B. (2007). Oxidative stress and cancer: Have we moved forward? *The Biochemical Journal, 401*(1):1-11.

Halliwell, B., & Gutteridge, J. (1999). *Free Radicals in Biology and Medicine*. 3rd ed. New York: Oxford University Press.

Han, K. H., Matsumoto, A., Shimada, K. I., Sekikawa, M., & Fukushima, M. (2007). Effects of anthocyanin-rich purple potato flakes on antioxidant status in F344 rats fed a cholesterol-rich diet. *The British Journal of Nutrition, 98*(5), 914-921.

Han, K. H., Sekikawa, M., Shimada, K. I., Hashimoto, M., Hashimoto, N., Noda, T., et al. (2006). Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats. *The British Journal of Nutrition, 96*(6), 1125-1133.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell 100*(1):57-70.

Heo, H. J., & Lee, C. Y. (2005). Strawberry and its anthocyanins reduce oxidative stress-induced apoptosis in PC12 cells. *Journal of Agricultural and Food Chemistry, 53*(6), 1984-1989.

Hou, D. X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3*(2), 149-159.

Hou, Z., Qin, P., & Ren, G. (2010). Effect of anthocyanin-rich extract from black rice (Oryza sativa L. Japonica) on chronically alcohol-induced liver damage in rats. *Journal of Agricultural and Food Chemistry, 58*(5), 3191-3196.

Hwang, J. W., Kim, E. K., Lee, S. J., Kim, Y. S., Moon, S. H., Jeon, B. T., et al. (2012). Antioxidant activity and protective effect of anthocyanin oligomers on h(2)o(2)-triggered g2/m arrest in retinal cells. *Journal of Agricultural and Food Chemistry, 60*(17), 4282-4288.

Hwang, Y. P., Choi, J. H., Choi, J. M., Chung, Y. C., & Jeong, H. G. (2011a). Protective mechanisms of anthocyanins from purple sweet potato against tert-butyl hydroperoxide-induced hepatotoxicity. *Food And Chemical Toxicology, 49*(9), 2081-2089.

Hwang, Y. P., Choi, J. H., Yun, H. J., Han, E. H., Kim, H. G., Kim, J. Y., et al. (2011b). Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression. *Food and Chemical Toxicology, 49*(1), 93-99.

Hyun, J. W., & Chung, H. S. (2004). Cyanidin and Malvidin from Oryza sativa cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G(2)/M phase and induction of apoptosis. *Journal of Agricultural and Food Chemistry, 52*(8), 2213-2217.

Issa A. Y., Volate, S.R., Wargovich, M. J. (2006). The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *Journal of Food Composition and Analysis, 19*:405-419.

Jacob, R. A., & Burri, B. J. (1996). Oxidative damage and defense. *The American Journal of Clinical Nutrition, 63*(6), 985S-990S.

Jing, P., Bomser, J. A., Schwartz, S. J., He, J., Magnuson, B. A., & Giusti, M. M. (2008). Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *Journal Of Agricultural And Food Chemistry, 56*(20), 9391-9398.

Jung, K. A., & Kwak, M. K. (2010). The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules,15*(10), 7266-7291.

Kelsey, N., Hulick, W., Winter, A., Ross, E., & Linseman, D. (2011). Neuroprotective effects of anthocyanins on apoptosis induced by mitochondrial oxidative stress. *Nutritional Neuroscience, 14*(6), 249-259.

Kim, J. M., Kim, J. S., Yoo, H., Choung, M. G., & Sung, M. K. (2008). Effects of black soybean [Glycine max (L.) Merr.] seed coats and its anthocyanidins on colonic inflammation and cell proliferation in vitro and in vivo. *Journal f Agricultural and Food Chemistry, 56*(18), 8427-8433.

Kim, S. J., Lee, H. J., Kim, B. S., Lee, D., Lee, S. J., Yoo, S. H., & Chang, H. I. (2011). Antiulcer activity of anthocyanins from Rubus coreanus via association with regulation of the activity of matrix metalloproteinase-2. *Journal of Agricultural and Food Chemistry, 59*(21), 11786-11793.

Klassen, C. D., Amdur, M. O. & Doull, J. (1986). *Toxicology: The basic science of poisons*. 2nd Ed. Macmillan, New York.

43

Kong, A. N., Owuor, E., Yu, R., Hebbar, V., Chen, C., Hu, R., & Mandlekar, S. (2001). Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metabolism Reviews, 33*(3-4), 255-271.

Lala, G., Malik, M., Zhao, C., He, J., Kwon, Y., Giusti, M. M., & Magnuson, B. A. (2006). Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats. *Nutrition and Cancer, 54*(1), 84-93.

Lazzé, M. C., Pizzala, R., Savio, M., Stivala, L. A., Prosperi, E., & Bianchi, L. (2003). Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells. *Mutation Research, 535*(1), 103-115.

Lazzè, M. C., Savio, M., Pizzala, R., Cazzalini, O., Perucca, P., Scovassi, A. I., et al. (2004). Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis, 25*(8), 1427-1433.

Lo, C. W., Huang, H. P., Lin, H. M., Chien, C. T., & Wang, C. J. (2007). Effect of Hibiscus anthocyanins-rich extract induces apoptosis of proliferating smooth muscle cell via activation of P38 MAPK and p53 pathway. *Molecular Nutrition and Food Research, 51*(12), 1452-1460.

Loo, G. (2003). Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (review). *The Journal of Nutritional Biochemistry, 14*(2):64-73.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*(5), 727-747.

Marko, D., Puppel, N., Tjaden, Z., Jakobs, S., & Pahlke, G. (2004). The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation. *Molecular Nutrition and Food Research, 48*(4), 318-325.

Martin, S., Favot, L., Matz, R., Lugnier, C., & Andriantsitohaina, R. (2003). Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochemical Pharmacology, 65*(4), 669-675.

Masella, R., Di Benedetto, R., Varì, R., Filesi, C., & Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *The Journal of Nutritional Biochemistry, 16*(10), 577-586.

Meyers, K. J., Watkins, C. B., Pritts, M. P., & Liu, R. H. (2003). Antioxidant and antiproliferative activities of strawberries. *Journal of Agricultural and Food Chemistry, 51*(23), 6887-6892.

Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., & Someya, K. (1999). Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. *Journal of Agricultural and Food Chemistry, 47*(3), 1083-1091.

Nguyen, T., Sherratt, P. J., & Pickett, C. B. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual Review of Pharmacology and Toxicology, 43*, 233-260.

Nichenametla, S. N., Taruscio, T. G., Barney, D. L., & Exon, J. H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition, 46*(2), 161-183.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine, 70*(1), 158-169.

Palikova, I., Vostalova, J., Zdarilova, A., Svobodova, A., Kosina, P., Vecera, R., et al. (2010). Long-term effects of three commercial cranberry products on the antioxidative status in rats: a pilot study. *Journal of Agricultural and Food Chemistry, 58*(3), 1672-1678.

Pergola, C., Rossi, A., Dugo, P., Cuzzocrea, S., & Sautebin, L. (2006). Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide: Biology and Chemistry, 15*(1), 30-39.

Pilaczynska-Szczesniak, L., Skarpanska-Steinborn, A., Deskur, E., Basta, P., & Horoszkiewicz-Hassan, M. (2005). The influence of chokeberry juice supplementation on the reduction of oxidative stress resulting from an incremental rowing ergometer exercise. *International Journal of Sport Nutrition and Exercise Metabolism, 15*(1), 48-58.

Prior, R. L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *The American Journal of Clinical Nutrition, 78*(3 Suppl), 570S-578S.

Prior, R. L., & Wu, X. (2006). Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Research, 40*(10), 1014-1028.

Rahman, M. M., Ichiyanagi, T., Komiyama, T., Hatano, Y., & Konishi, T. (2006). Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism. *Free Radical Research, 40*(9), 993-1002.

Ramirez-Tortosa, C., Andersen, Ø. M., Gardner, P. T., Morrice, P. C., Wood, S. G., Duthie, S. J., et al. (2001). Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biology and Medicine, 31*(9), 1033-1037.

Ramos-Escudero, F., Muñoz, A. M., Alvarado-Ortíz, C., Alvarado, Á., & Yáñez, J. A. (2012). Purple corn (Zea mays L.) phenolic compounds profile and its assessment as an agent

against oxidative stress in isolated mouse organs. *Journal Of Medicinal Food, 15*(2), 206-215.

Ramos, S., Rodríguez-Ramiro, I., Martín, M. A., Goya, L., & Bravo, L. (2011). Dietary flavanols exert different effects on antioxidant defenses and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. *Toxicology In Vitro, 25*(8), 1771-1781.

Renis, M., Calandra, L., Scifo, C., Tomasello, B., Cardile, V., Vanella, L., et al. (2008). Response of cell cycle/stress-related protein expression and DNA damage upon treatment of CaCo2 cells with anthocyanins. *The British Journal of Nutrition, 100*(1), 27-35.

Sarić, A., Sobocanec, S., Balog, T., Kusić, B., Sverko, V., Dragović-Uzelac, V., et al. (2009). Improved antioxidant and anti-inflammatory potential in mice consuming sour cherry juice (Prunus Cerasus cv. Maraska). *Plant Foods for Human Nutrition, 64*(4), 231-237.

Seeram, N. P., Adams, L. S., Hardy, M. L., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agricultural and Food Chemistry, 52*(9), 2512-2517.

Seeram, N. P., Zhang, Y., & Nair, M. G. (2003). Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. *Nutrition and Cancer, 46*(1), 101-106.

Shih, P. H., Chan, Y. C., Liao, J. W., Wang, M. F., & Yen, G.-C. (2010). Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (Morus atropurpurea L.) on senescence-accelerated mice and prevention of Alzheimer's disease. *The Journal of Nutritional Biochemistry, 21*(7), 598-605.

Shih, P. H., Yeh, C. T., & Yen, G. C. (2005). Effects of anthocyanidin on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. *Food And Chemical Toxicology, 43*(10), 1557-1566.

Shih, P. H., Yeh, C. T., & Yen, G. C. (2007). Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *Journal of Agricultural and Food Chemistry, 55*(23), 9427-9435.

Spormann, T. M., Albert, F. W., Rath, T., Dietrich, H., Will, F., Stockis, J.-P., et al. (2008). Anthocyanin/polyphenolic-rich fruit juice reduces oxidative cell damage in an intervention study with patients on hemodialysis. *Cancer Epidemiology, Biomarkers and Prevention, 17*(12), 3372-3380.

Srivastava, A., Akoh, C. C., Fischer, J., & Krewer, G. (2007). Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. *Journal of Agricultural and Food Chemistry, 55*(8), 3180-3185.

Sun, Y. (1990). Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radical Biology and Medicine, 8*(6), 583-599.

Syed, D. N., Afaq, F., Sarfaraz, S., Khan, N., Kedlaya, R., Setaluri, V., & Mukhtar, H. (2008). Delphinidin inhibits cell proliferation and invasion via modulation of Met receptor phosphorylation. *Toxicology and Applied Pharmacology, 231*(1), 52-60.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Gil-Izquierdo, A., Lamaison, J. L., & Rémésy, C. (2005). Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *Journal of Agricultural and Food Chemistry, 53*(10), 3902-3908.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Lamaison, J. L., & Rémésy, C. (2003). Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *The Journal of Nutrition, 133*(12), 4178-4182.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Manach, C., Lamaison, J. L., & Rémésy, C. (2004). Anthocyanins are efficiently absorbed from the small intestine in rats. *The Journal of Nutrition, 134*(9), 2275-2279.

Thomas, J. A. (2006). *Oxidant Defense in Oxidative and Nitrosative Stress. In: Modern Nutrition in Health and Disease.* 10 ed. Hagerstown: Lippincott Williams & Wilkins.

Tokarev, J. (2010). *Delphinidin alters hydrogen peroxide induced cytotoxicity and apoptosis in human colon adenocarcinoma cells*. MS Thesis, University of Georgia.

Turner, M. (2009). *Anthocyanins increase antioxidant activity in HT-29 adenocarcinoma cells*. MS Thesis, University of Georgia.

United States Department of Agriculture (USDA). (2003). *Database for the Flavonoid Content of Selected Foods*. Retrieved from http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html

Varì, R., D'Archivio, M., Filesi, C., Carotenuto, S., Scazzocchio, B., Santangelo, C., et al. (2011). Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages. *The Journal of Nutritional Biochemistry, 22*(5), 409-417.

Wang, C. J., Wang, J. M., Lin, W. L., Chu, C. Y., Chou, F. P., & Tseng, T. H. (2000). Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. *Food and Chemical Toxicology, 38*(5), 411-416.

Wang, H., Nair, M. G., Strasburg, G. M., Chang, Y. C., Booren, A. M., Gray, J. I., & DeWitt, D. L. (1999). Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *Journal of Natural Products, 62*(2), 294-296.

Wang, L. S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters, 269*(2), 281-290.

Wang, Q., Chen, Q., He, M., Mir, P., Su, J., & Yang, Q. (2011). Inhibitory effect of antioxidant extracts from various potatoes on the proliferation of human colon and liver cancer cells. *Nutrition and Cancer, 63*(7), 1044-1052.

Wätjen, W., Michels, G., Steffan, B., Niering, P., Chovolou, Y., Kampkötter, A., et al. (2005). Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. *The Journal of Nutrition, 135*(3), 525-531.

Weisman, H., & Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *The Biochemical Journal, 313 (Pt 1)*, 17-29.

Weng, C. J., Chen, M. J., Yeh, C. T., & Yen, G. C. (2011). Hepatoprotection of quercetin against oxidative stress by induction of metallothionein expression through activating MAPK and PI3K pathways and enhancing Nrf2 DNA-binding activity. *New Biotechnology, 28*(6), 767-777.

World Cancer Research Fund American Institute for Cancer Research (WCRF/AICR). 2007. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington, DC: AICR.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry, 54*(11), 4069-4075.

Yi, W., Fischer, J., Krewer, G., & Akoh, C. C. (2005). Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of Agricultural and Food Chemistry, 53*(18), 7320-7329.

Youdim, K. A., Shukitt-Hale, B., MacKinnon, S., Kalt, W., & Joseph, J. A. (2000). Polyphenolics enhance red blood cell resistance to oxidative stress: in vitro and in vivo. *Biochimica Et Biophysica Acta, 1523*(1), 117-122.

Yun, J. M., Afaq, F., Khan, N., & Mukhtar, H. (2009). Delphinidin, an anthocyanidin in pigmented fruits and vegetables, induces apoptosis and cell cycle arrest in human colon cancer HCT116 cells. *Molecular Carcinogenesis, 48*(3), 260-270.

Zhang, Y., Vareed, S. K., & Nair, M. G. (2005). Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences, 76*(13):1465-1472.

Zhu, W., Jia, Q., Wang, Y., Zhang, Y., & Xia, M. (2012). The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. *Free Radical Biology and Medicine, 52*(2), 314-327.

CHAPTER III

THE EFFECTS OF BLUEBERRY ANTHOCYANIDINS ON ANTIOXIDANT ENZYME

ACTIVITY IN HUMAN HEPG2 CELLS[1]

ABSTRACT

Anthocyanins are thought to have antioxidant effects in the body. The effects of two anthocyanidins, malvidin and delphinidin, on the activities of antioxidant enzymes, glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and other markers of oxidative stress were examined in HepG2 human hepatocellular carcinoma cells. In study one, cells were treated with anthocyanidins at concentrations of 0, 5 or 10 µmol/L for 24 h and 0 or 200 µmol/L tert-butyl hydroperoxide (t-BOOH) for 2 h. Study two used a combined dose of 10 µmol/L anthocyanidins and 0 or 200 µmol/L t-BOOH. Cell proliferation and GR activity were significantly increased by delphinidin; with 5 µmol/L having the greatest effect in oxidatively stressed cells. Malvidin also increased cell proliferation, but to a lesser extent than delphinidin. No effect of treatment was observed on GST and GPx activity. Finally, it appeared that combining these anthocyanidins had an antagonistic effect.

INTRODUCTION

Epidemiological studies have shown that diets high in fruits and vegetables are associated with reduced risk of cancer at many sites (Cooke et al., 2005). The exact mechanisms by which fruits and vegetables help protect against cancer are not yet fully understood. There are many beneficial compounds in fruits and vegetables including vitamins, minerals, and phytochemicals that may be responsible for the health benefits. Phytochemicals are non-nutrient components and include anthocyanins which are the red, blue and purple pigments found in plants (Wang and Stoner, 2008). Average consumption of anthocyanins in the United States is estimated to be between 12.5 mg (Wu et al., 2006) to over 200 mg per day in people consuming many servings of fruit (Prior, 2003).

Oxidative stress is thought to be an important factor leading to the development of cancer. Reactive oxygen species (ROS) are formed from oxygen and are necessary in biological systems. It has been theorized, however that increased concentration of cellular ROS promotes the development of many human diseases such as cancer, cardiovascular disease, diabetes and Alzheimer's disease (Halliwell and Gutteridge, 1999; Jacob and Burri, 1996). Mechanisms by which ROS are thought to lead to carcinogenesis include the mutation of DNA, the promotion of cell proliferation, invasiveness, angiogenesis and metastasis and the suppression of apoptosis (Halliwell, 2007).

Anthocyanins may be chemopreventive due, in part, to their direct antioxidant activity, anti-inflammatory activity, and/or ability to increase the activity of the antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) and increase reduced glutathione concentration (Ramos, 2008; Alía et al., 2006). Anthocyanins have also been shown to inhibit cancer cell proliferation in cell models. Studies have investigated the chemopreventive effects of anthocyanins using *in vitro* models, animal models or human epidemiological trials, and there have been conflicting results in human studies as to the benefits of anthocyanin intake (Ramos, 2008). Some *in vitro* studies have shown increased antioxidant enzyme activity and decreased cell proliferation with anthocyanin treatment, but concentrations used are generally high and may not be physiologically achievable in tissues. In addition, many of the studies showing the beneficial effects of anthocyanin treatment have been conducted in intestinal cells. Not much is known about the effect of anthocyanin treatment in the liver cell models, though the liver has been shown to play a role in anthocyanin metabolism (Talavéra et al., 2003).

This study investigated two common anthocyanidins, malvidin and delphinidin, in an *in vitro* HepG2 human hepatocellular carcinoma cell model. We tested the hypothesis that anthocyanidin treatment, at low concentrations, would increase the activity of the antioxidant enzymes GR, GPx and GST in the presence of the oxidative stressor tert-butyl hydroperoxide (t-BOOH). We further hypothesized that anthocyanidin treatment would counteract oxidative stress, thus salvaging cells and preventing lipid peroxidation, in the presence of t-BOOH. The study examined the use of anthocyanidin concentrations that were lower than those previously used by most studies on anthocyanins and are closer to levels that are realistically attainable in the human diet.

MATERIALS AND METHODS

Cells. The human hepatocellular carcinoma HepG2 cell line was purchased from ATCC (Manassas, VA). Cells were maintained in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) with 10% fetal bovine serum (FBS) in 75 cm^2 flasks. Cells were incubated at 37º C in 5% CO_2, 95% air with controlled humidity. Cells were passaged using a 1:4 ratio when they became confluent, and the media was changed every 2-3 days as needed. Studies were conducted using cell passages 12 to 37.

Anthocyanidin and t-BOOH Treatment. For experiment 1, a confluent monolayer of cells was incubated with two anthocyanidins, malvidin chloride or delphinidin chloride (Chromadex, Irvine, CA), at concentrations of 0, 5 or 10 µmol/L with 0.1% DMSO for 24 h. Each treatment was performed in triplicate. In experiment 2, a confluent monolayer of cells were incubated with either control media or a combination of 5 µmol/L malvidin and 5 µmol/L delphinidin (10 µmol/L combined) for 24 h. Each treatment was performed in four flasks. After the

anthocyanidin treatment period, the anthocyanidin-containing media was removed and cells were treated with 0 or 200 μmol/L *t*-BOOH in FBS-free media for 2 h (Alía et al. 2006). Cells were collected at the end of the 2 h for assessment of GR, GPx and GST activities. A concentration of 200 μmol/L t-BOOH was used to induce oxidative stress based on our preliminary studies using trypan blue that showed a significant decrease in cell viability compared to control (data not shown).

Cell Collection. Following both incubation periods, cells were trypsinized, collected, added to homogenizing buffer (pH 7.0, 0.05 M potassium phosphate, 0.001 M EDTA) and homogenized for 5 seconds on ice. The cell homogenate was then centrifuged in a J2-HS Beckman Centrifuge (Beckman Instruments Inc., Fullerton, CA) for 20 minutes at 4º C at 10,000 x g. The supernatant was then removed and stored at -80º C until analysis. The protein concentration of the supernatant was measured using the methods of Lowry et al. (1951).

Glutathione Reductase. GR activity was measured with a spectrophotometer according to the method of Xia et al. (1985) with oxidized glutathione (GSSG; 0.5 mmol/L) as the substrate. In the presence of GR, hydrogen is transferred from NADPH to GSSG. GR activity was determined by measuring the rate of oxidation of NADPH in the presence of GSSG. The reaction cuvette contained 0.8 ml NADPH (0.25 mmol/L), 0.1 ml GSSG (5 mmol/L), and 0.1 ml sample (50 mmol/L, pH 7.0). The reaction was started by the addition of GSSG. Samples were run in duplicate at absorbance 340 nm for 4 minutes, with readings taken every 60 seconds. One unit of GR activity is defined as the amount of GR to oxidize 1 umol NADPH/minute/mg protein. The molar extinction coefficient of NADPH at 340 nm is 6.22 mL/(umol x cm).

Glutathione S-Transferase. The activity of GST was assessed using the spectrophotometric methods of Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene (CDNB; 10 mmol/L) as the substrate. In the presence of GST, CDNB is conjugated to glutathione through the thiol group of the glutathione forming S-2,4-dinitrophenylgluathione. The reaction cuvette contained 0.8 mL GSH (6.24 mmol/L), 0.1 mL CDNB (10 mmol/L) and 0.1 mL sample (50 mmol/L, pH 7.0). The reaction was started by the addition of CDNB. Samples were run in duplicate at an absorbance of 340 nm for 3 minutes, with readings taken every 30 seconds. One unit of GST activity is defined as 1 nmole conjugate formed/minute/mg protein. The molar extinction coefficient for CDNB at 340 nm is 9.6 mL/nmol.

Glutathione Peroxidase. The activity of GPx was assessed using the spectrophotometric method of Paglia and Valentine (1967) with t-BOOH (t-BOOH; 0.3 mmol/L) as the substrate. GPx activity is measured indirectly through a coupled reaction with GR. Briefly, GSSG produced upon reduction of hydroperoxide by GPx to it its reduced state by GR and NADPH. Therefore, GPx activity can be measured by the rate of oxidation of NADPH in the presence of GSSG. The reaction cuvette included 0.8 ml of a mixture that contained potassium phosphate buffer, NADPH (2mmol/L), GSH (10 mmol/L), GR (10 IU/ml), and NaN_3 (10 mmol/L), 0.1 ml sample and 0.1 ml t-BOOH. The reaction was started by the addition of t-BOOH. Samples were run in duplicate for 4 minutes, with readings every 60 seconds. GPx activity was determined by change in absorbance at 340 nm. One unit of GPx activity is defined as one umol of NADPH oxidized/minute/mg protein. The molar extinction coefficient of NADPH at 340 nm is 6.22 mL/(umol x cm).

Lipid Peroxidation. The thiobarbituric acid reactive substances (TBARS) assay was used to assess lipid peroxidation (n=3). Cells were treated and collected as described above. TBARS

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was measured as described by Beuge and Aust (1978). Briefly, the homogenized cells were treated with thiobarbituric acid (TBA), trichloroacetic acid (TCA) and hydrochloric acid (HCl), then placed in boiling water for 15 minutes. Next the cells were centrifuged for 15 minutes at 4,000 x g (Beckman J2HS, JS-7.5.Palo Alto, CA). The supernatant was collected and absorbance was read at 535 nm using the spectrophotometer.

Cell Proliferation. The MTT Cell Proliferation Assay was purchased as a kit from ATCC and was used to assess cell proliferation following incubation with malvidin and delphinidin chloride and t-BOOH. In metabolically active cells, yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2-2,5-diphenyltetrazolium bromide) is reduced by dehydrogenase enzymes to produce NADH and NADPH. The result is purple formazan that can be measured by spectrophotometric means and is accepted as a reliable way to measure cell viability and proliferation. Cells were seeded in a 96-well plate (n=9 per treatment group) with a total volume of 0.1 mL/well and cell number of 1×10^5 cells/well. For experiment 1, malvidin or delphinidin chloride (Chromadex, Irvine, CA), were mixed with media and incubated with cells in concentrations of 0, 5 or 10 μmol/L for 24 h (n =9). In experiment 2, cells were incubated with either 0, a combination of 2.5 μmol/L malvidin and 2.5 μmol/L delphinidin chloride (5 μmol/L combined) or a combination of 5 μmol/L malvidin and 5 μmol/L delphinidin chloride (10 μmol/L combined) for 24 h (n=9). Anthocyanidin media mixtures and control media contained 0.1% DMSO. After the anthocyanidin treatment period, the anthocyanidin media was removed and cells were treated with 0 or 200 μM *t*-BOOH in media for 2 h (Alía et al. 2006).

The assay was performed as described in the MTT kit. Briefly, 10 μL MTT Reagent was added to each well and the plate was returned to the incubator for 2 hours. Next, 100 μL of detergent was added to all wells and the plate was gently swirled. The plate was then placed in a

dark room at room temperature for 4 hours. After this period, cell viability was measured at 570-655 nm with a microplate reader and was reported as percent compared to the control. Absorbance at this wavelength quantifies the number of metabolically active cells.

Statistical Analysis. Statistical analysis was conducted using Statistical Analysis Software (SAS Version 9.13, SAS Institute, Cary, NC). Treatment means, standard error of the mean (SEM) and analysis of variance were determined. Fisher's least significant difference test was used for post-hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Cell proliferation

The effects of pre-incubating HepG2 cells with 0, 5 and 10 μmol/L of malvidin and delphinidin on cells subjected to oxidative stress are shown in Figure 3.1 and Tables 3.1 and 3.2. It was observed that t-BOOH significantly reduced cell proliferation ($p < 0.0001$) by 39-48%, depending on anthocyanin treatment, confirming that t-BOOH causes oxidative stress in cells. Treatment with anthocyanidins prevented cell death in both unstressed and oxidatively stressed cells. A significant increase in cell proliferation for t-BOOH treated cells was observed in groups pre-incubated with 5 μmol/L malvidin and both 5 and 10 μmol/L delphinidin. In unstressed cells, addition of the anthocyanidins increased cell proliferation about 21%, while treatment of stressed cells increased cell proliferation about 42%. There was a weak trend towards an interaction between t-BOOH treatment, anthocyanidin concentration and anthocyanidin type ($p = 0.096$), reflecting a more pronounced effect of delphinidin on the cell proliferation of oxidatively stressed cells compared to malvidin. Delphinidin supplementation increased proliferation of stressed cells about 59%, compared to about 30% for malvidin. Pre-incubation with 10 μmol/L

malvidin did not have a significant effect on the proliferation of the stressed cells. This data suggests that low concentrations of these anthocyanidins may protect cells from oxidative stress and that delphinidin may have been more likely than malvidin to exert an antioxidant effect Interestingly, the beneficial effect of pre-incubation with anthocyanidins was lost when the anthocyanidins were combined at both 5 and 10 μmol/L (Figure 3.2; Tables 3.3 and 3.4).

Glutathione antioxidant enzyme activity

A significant increase in GR activity of 28-32% was seen in response to treatment with t-BOOH (Figures 3.3 and 3.4; Tables 3.4 and 3.5). Delphinidin treatment increased GR activity 14-18% in both oxidatively stressed and non-stressed cells, but post-hoc tests showed that this increase was only statistically significant with treatment at a concentration of 5 μmol/L. It was found that GR activity responded inconsistently to treatment with combined anthocyanidins (Figure 3.5; Table 3.7). The effect of 10 μmol/L combined anthocyanidins on GR activity was tested twice. While anthocyanidins increased GR activity with the first trial, this was not confirmed with the second trial. No significant effects of t-BOOH or anthocyanidin treatment on GPx activity were observed (Tables 3.5 and 3.6), although there was a slight trend for a positive effect of malvidin on GPx activity. In addition, there was a weak trend for an interaction between anthocyanidins and oxidative stress such that the combined anthocyanins increased GPx activity in unstressed cells, but not in stressed cells. Results were similar for GST activity, as neither t-BOOH nor anthocyanidin treatment caused a significant effect on the activity of this enzyme (Tables 3.5 and 3.6). Treatment with t-BOOH tended to increase GST activity, and combined anthocyanidins did not alter this change. However, the highest GST activity was found in stressed cells with combined anthocyanidins.

Lipid peroxidation

A significant effect of t-BOOH treatment was observed on lipid peroxidation in HepG2 cells (p = 0.021). With t-BOOH supplementation, it was found that TBARS formation increased; thereby confirming that t-BOOH causes oxidative stress in cells. However, pre-incubation with anthocyanidins had no significant effect on lipid peroxidation measured with TBARS, either alone, or in combination (data not shown). This may have been because TBARS concentrations were very low for all groups.

DISCUSSION

I examined the effect of anthocyanidin supplementation on (1) lipid peroxidation and survival of HepG2 cells subjected to oxidative stress, and (2) antioxidant enzyme activity in HepG2 cells. I hypothesized that anthocyanidin treatment would increase GR, GPx and GST activity while decreasing markers of oxidative stress. Anthocyanidins, at concentrations lower than typically used for in vitro studies (5 and 10 µmol/L), were applied to HepG2 cells exposed to t-BOOH. t-BOOH is an organic hydroperoxide which has been shown to induce cell death, DNA damage, lipid peroxidation and redox state alteration (Lazzé et al., 2003; Alía et al., 2005). Anthocyanidin supplementation did increase proliferation of cells subjected to oxidative stress, with a more pronounced effect seen with delphinidin. This finding is in agreement with other studies and may have been achieved through delphinidin's direct radical scavenging ability (Noda et al., 2002). Delphinidin has been found to be a potent radical scavenger, most likely due to the presence of three hydroxyl groups on the B ring (Rahman et al., 2006). This superior radical scavenging was demonstrated in a study by Shih et al. (2007), with 50 µmol/L of delphinidin suppressing H_2O_2 triggered apoptosis at significantly higher levels than malvidin. Delphinidin has also been shown to reduce cytotoxicity by inhibiting t-BOOH-induced formation

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of single strand DNA breaks (Nichenametla et al., 2006). These mechanisms may help to explain delphinidin's enhanced protection of cells over malvidin.

An unexpected result was an increase in the proliferation of unstressed cells with anthocyanidin supplementation. Such an increase in the proliferation of unstressed cells suggests that the anthocyanidins may be influencing cell cycle or decreasing apoptosis. Decreased apoptosis with anthocyanin treatment has been reported in other studies. Zhu et al. (2012) exposed HepG2 cells were exposed to high levels of glucose to simulate oxidative stress. With cyanidin-3-O-β-glucoside treatment at concentrations of 10-100 μmol/L, they were able to show decreased apoptosis as measured by Fas mRNA expression. Hwang et al. (2011a) also demonstrated decreased apoptosis with anthocyanin treatment in HepG2 cells. It was shown that supplementation of an anthocyanin fraction from purple sweet potato at 50 and 100 μg/mL led to a significant decrease in t-BOOH stimulated caspase-3 and -9 activities.

The increase in cell proliferation I observed in unstressed cells has not been reported often. A study by Tokarev (2010) showed improved cell proliferation with delphinidin treatment (1-10 μmol/L) in colon cells stressed with H_2O_2. Similarly, Hwang et al. (2011a) showed that retreatment of HepG2 cells with 50 and 200 μg/mL anthocyanins from purple sweet potato significantly improved cell proliferation in cells treated with 300 μmol/L t-BOOH. However, treatment with these same anthocyanin fractions (10, 50, 200, 400 and 800 μg/mL) did not affect HepG2 cell proliferation in unstressed cells. Seeram et al. (2003) also showed that anthocyanidin treatment with 6.25-100 μmol/L malvidin and delphinidin had no effect on cell proliferation in breast, central nervous system, lung or colon cell lines. Overall, most studies have found that anthocyanidin supplementation decreases proliferation of cells not subjected to oxidative stress.

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This discrepancy could be explained by differences in cell lines. In many studies examining the antiproliferative effects of anthocyanidins, colon cell lines were used. In addition, the majority of studies conducted used higher concentrations (25-300 μmol/L) of anthocyanidins than used in the current study. Finally, many studies have tested anthocyanin fractions and extracts from fruits and vegetables (Seeram et al., 2004; Boivin et al., 2007; Jing et al., 2008; Aquil et al., 2012; Faria et al., 2010) rather than isolated anthocyanidins. This decrease in proliferation could be due to a higher concentration of phytochemicals in the extracts, or a synergistic effect of the phytochemicals that is not seen with individual anthocyanidin treatment (Boivin et al., 2007).

It has been suggested by Issa et al. (2006) that the effects of anthocyanins may be synergistic, additive or even antagonistic depending on the cell type and concentration. In the current study, the combination of malvidin and delphinidin appeared to have an antagonistic effect. When these anthocyanidins were combined, their effects on cell proliferation were lost. This antagonistic effect may be, in part, due to the very different structures of the anthocyanidins. Delphinidin has three hydroxyl groups at the 3'-, 4'- and 5'-positions on the B ring, while malvidin has two methoxyl groups at the 3'- and 5'-positions (Wang and Stoner, 2008).

In the current study it was hypothesized that anthocyanidin supplementation would decrease lipid peroxidation assessed through TBARS. Results showed that t-BOOH significantly increased TBARS above the control, but anthocyanidins had no effect on lipid peroxidation. This is in contrast to the findings of Hwang et al. (2011a) and Kelsey et al. (2011). These studies showed that purple sweet potato anthocyanin fraction and anthocyanidin treatment, respectively, significantly and dose-dependently reduced oxidative stress-induced lipid peroxidation. The null

findings for TBARS may be due to the lower concentrations of anthocyanidins used in this study. It may take a greater concentration of compounds, such as used in the Hwang et al. (2011a) study, to impact this lipid peroxidation measure.

The second major hypothesis of this study was that anthocyanidin supplementation would increase antioxidant enzyme activity. This effect was only observed with GR, and delphinidin had the greatest effect. Since delphinidin had the most pronounced effect on proliferation of oxidatively stressed cells, this may indicate that the delphinidin-induced increase in cell proliferation of t-BOOH-stressed cells could be due, in part, to increased GR activity. GR activity increased by about 14-18% with delphinidin treatment. This increase in GR activity is similar to the 20% increase seen in the hepatic tissue of rats fed 400 mg/kg Brassica (Lampe et al., 2000). There was no significant effect of malvidin treatment on GR activity. These findings are in contrast to studies conducted by Turner (2009) and Shih et al. (2007) in which 13.5 μmol/L and 50 μmol/L malvidin, respectively, caused a significant increase in GR activity. These divergent results could be attributed to the cell lines used. Turner studied HT-29 colon cells while Shih et al. examined healthy clone 9 rat hepatocyte cells. The metabolism of anthocyanidins and enzymatic response of these cell lines may very well differ from that of human HepG2 liver cells. The higher concentration of anthocyanidins used by Shih et al. may also have contributed to the differing effects on GR activity in response to malvidin.

Inconsistent results of combined anthocyanidin treatment on GR activity were found. Cell collections for combined treatments were conducted at two different times, and in this case, a time effect of treatment was noted. For time one, it was found that anthocyanidin supplementation significantly increased GR activity, but this effect was not duplicated during the

second trial These results reflect the inconsistencies one can observe while conducting cell culture studies.

In contrast to my hypothesis, no significant effect of anthocyanidin treatment was found on either GPx or GST activity. These results are in agreement with Palikova et al. (2010) in which rats receiving an anthocyanin-rich cranberry powder did not have increased hepatic GPx or GST levels. In addition, Dulebohn et al. (2008) did not find a significant increase in hepatic GST activity in rats receiving a 1% blueberry flavonoid diet. The Dulebohn study did, however, demonstrate a 24% increase in GST activity with the flavonoid diet which was not observed in this study. In contrast, studies by Hou, Qin and Ren (2010), Ajiboye et al. (2011) and Hwang et al. (2012) all demonstrated increased GPx and GST activities with anthocyanidin supplementation. In these studies, anthocyanin treatment led to increased GPx and GST activity and attenuated decreased enzyme activity in the presence of a stressor. The contrasting results observed in these studies could be attributed to a number of possibilities including: use of *in vivo* versus *in vitro* models, different cell types, different lengths of treatment, different anthocyanins and different concentrations. These variations can make results difficult to compare. Different tissue will metabolize the anthocyanins differently, also potentially leading to different results. Metabolites found within liver tissue, for example, could vary from the metabolites that exist within the colon. These metabolites will most likely have differing effects on enzyme activity. This is of the utmost importance in *in vivo* studies where the environment is not as controlled and many physiological factors may play a greater role than seen *in vitro*.

In conclusion, cell proliferation and GR activity were significantly increased by the anthocyanidin delphinidin. The 5 μmol/L concentration showed a slightly greater impact on enzyme activity and cell proliferation in conditions of oxidative stress than the 10 umol/L

concentration. While malvidin supplementation at 5 µmol/L did improve cell proliferation in the stressed and un-stressed cells, it did not provide the added benefit that was seen with delphinidin treatment. No effect of anthocyanidin treatment at any concentration was observed on GST and GPx activity or lipid peroxidation. Finally, it appeared as though combination of the anthocyanidins led to an antagonistic effect as both the increases in cell proliferation and GR activity were lost when malvidin and delphinidin were combined.
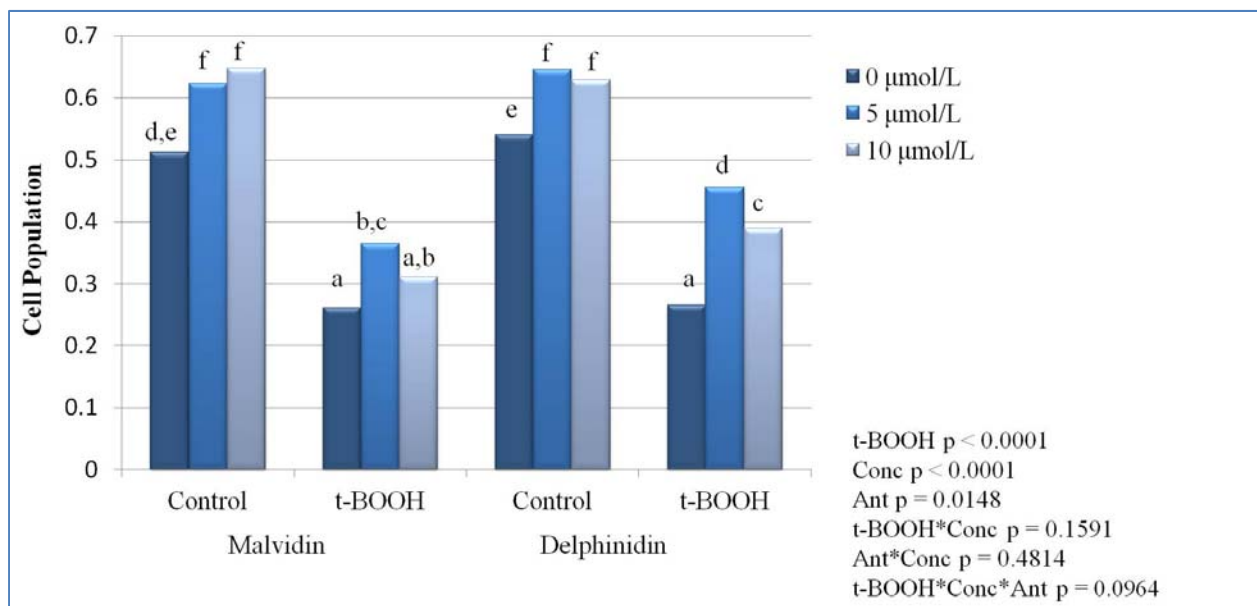
[1] Values are means ± SEM (n=9), means with different letters differ significantly ($p < 0.05$).
[2] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.

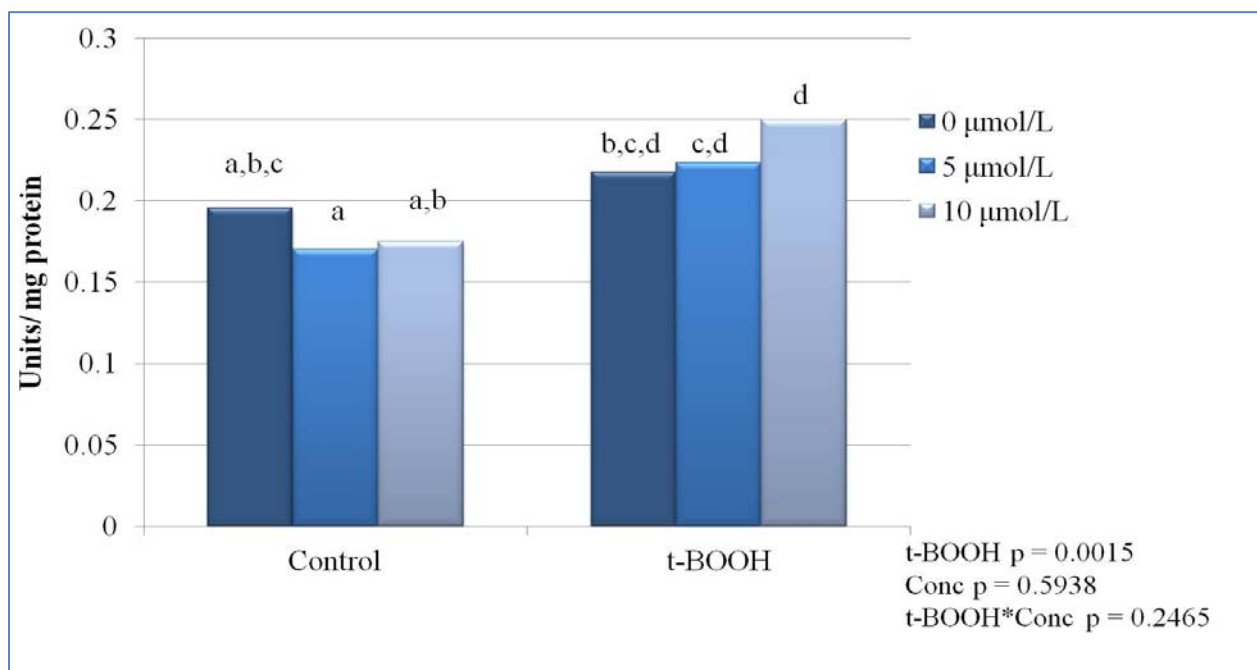**Figure 3.1** Cell proliferation of HepG2 cells treated with 0, 5 or 10 µmol/L malvidin or delphinidin chloride with or without t-BOOH.[1,2]
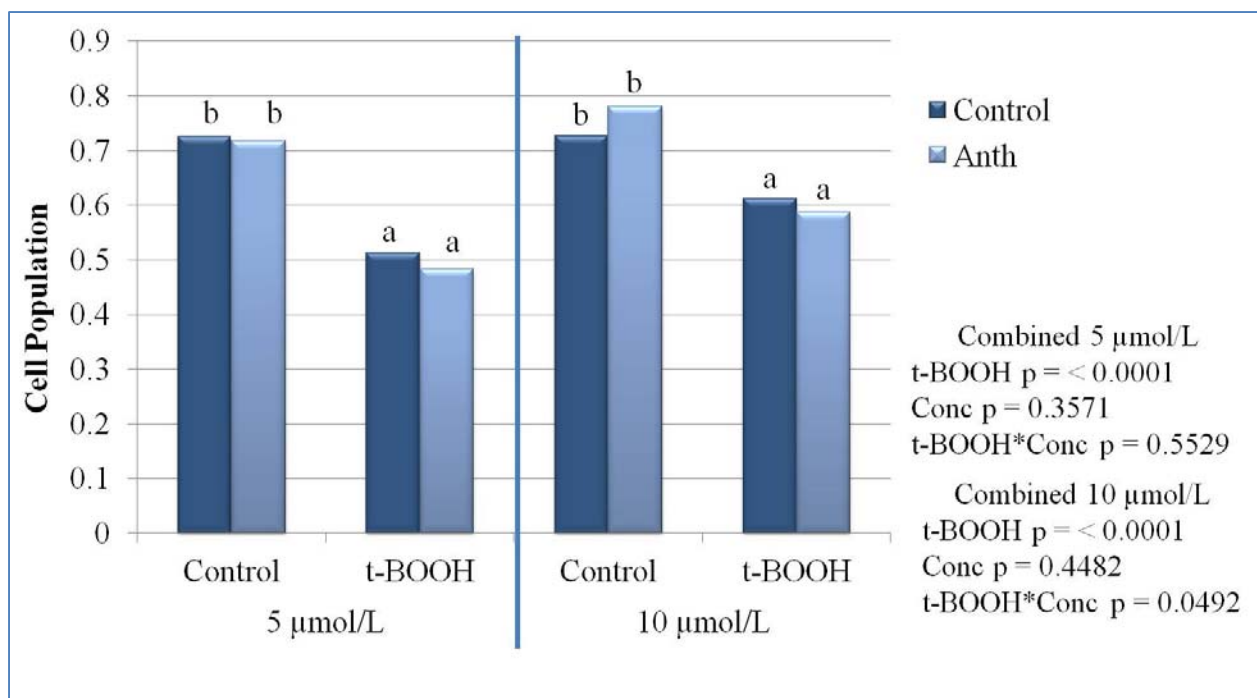
[1] Values are means ± SEM (n=9), means with different letters differ significantly ($p < 0.05$).
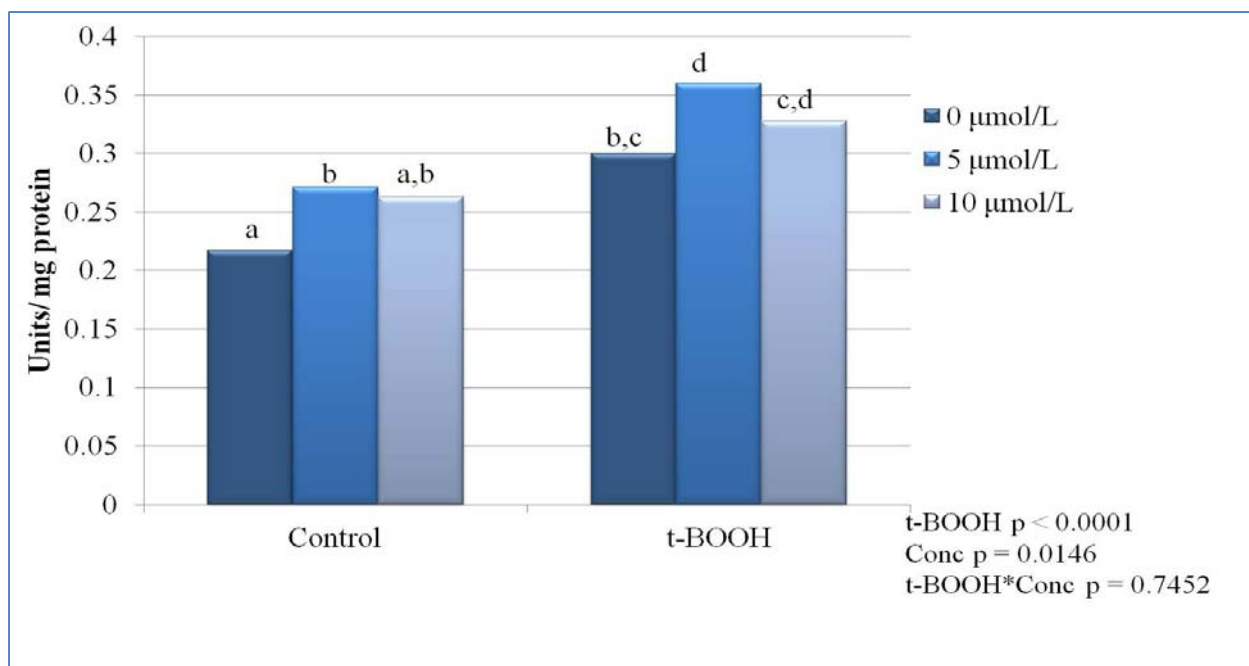[2] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.

Figure 3.2. Cell proliferation in HepG2 cells treated with 5 µmol/L combined or 10 µmol/L combined malvidin and delphinidin chloride with or without t-BOOH.[1,2]

[1] Values are means ± SEM (n=3), means with different letters differ significantly ($p < 0.05$).
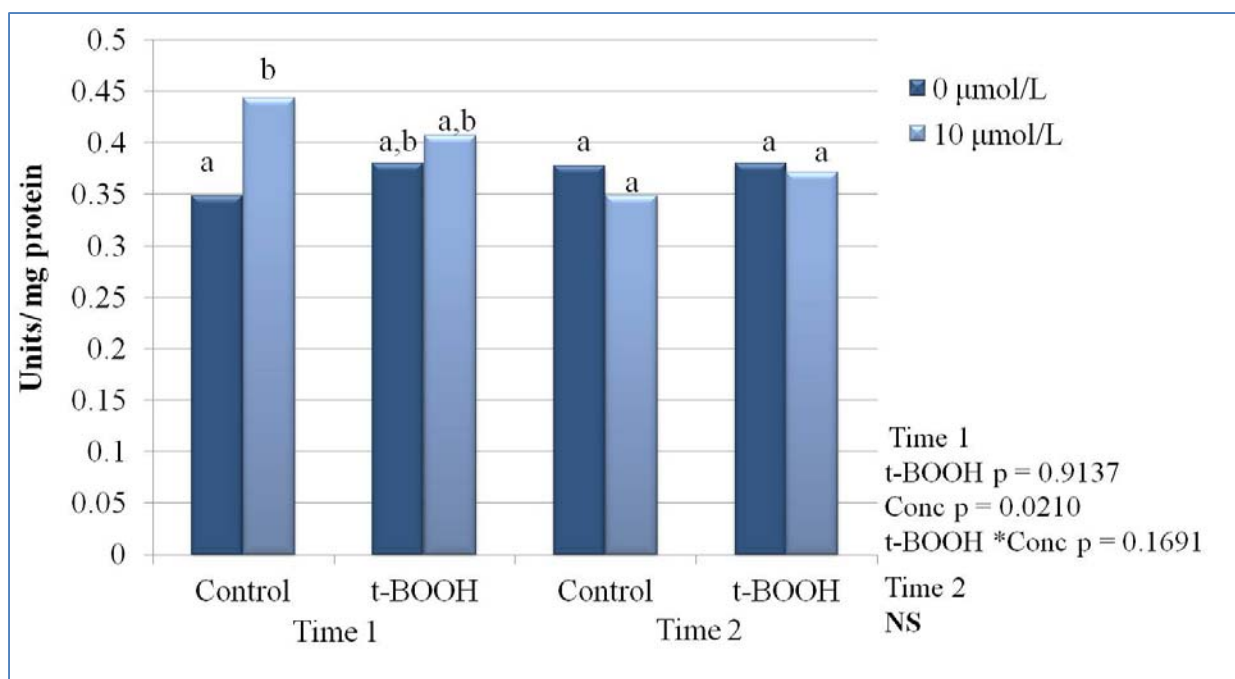[2] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.

Figure 3.3. Glutathione reductase activity (U/mg protein) in HepG2 cells treated with 0, 5 or 10 µmol/L malvidin chloride with or without t-BOOH.[1,2]

Figure 3.4. Glutathione reductase activity (U/mg protein) in HepG2 cells treated with 0, 5 or 10 µmol/L delphinidin chloride with or without t-BOOH.[1,2]

Figure 3.5. Glutathione reductase activity (U/mg protein) in HepG2 cells treated with 0 or 5 µmol/L malvidin + 5 µmol/L delphinidin chloride (10 µmol/L combined) with or without t-BOOH.[1,2,3]

TABLE 3.1

Cell proliferation in HepG2 cells treated with 0, 5 or 10 µmol/L malvidin chloride with or without t-BOOH.[1,2,3]

	Cell Proliferation[4]
Control	
0 µmol/L	0.512 ± 0.035^c
5 µmol/L	0.624 ± 0.013^d
10 µmol/L	0.648 ± 0.023^d
t-BOOH	
0 µmol/L	0.261 ± 0.021^a
5 µmol/L	0.365 ± 0.011^b
10 µmol/L	$0.311 \pm 0.023^{a,b}$
ANOVA (p-value)	
t-BOOH	< 0.0001
Conc	< 0.0001
t-BOOH*Conc	0.1143

[1] Values are mean ± SE (n=9).
[2] Means within a given column with different subscript letters differ significantly (p < 0.05).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.
[4] Values expressed as absorbance of purple formazan crystals in metabolically active cells, not cell number.

TABLE 3.2

Cell proliferation in HepG2 cells treated with 0, 5 or 10 µmol/L delphinidin chloride with or without t-BOOH.[1,2,3]

	Cell Proliferation[4]
Control	
0 µmol/L	0.540 ± 0.018^c
5 µmol/L	0.646 ± 0.024^d
10 µmol/L	0.629 ± 0.029^d
t-BOOH	
0 µmol/L	0.266 ± 0.024^a
5 µmol/L	0.455 ± 0.024^b
10 µmol/L	0.389 ± 0.029^b
ANOVA (p-value)	
t-BOOH	< 0.0001
Conc	< 0.0001
t-BOOH*Conc	0.2428

[1] Values are mean ± SE (n=9).
[2] Means within a given column with different subscript letters differ significantly ($p < 0.05$).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.
[4] Values expressed as absorbance of purple formazan crystals in metabolically active cells, not cell number.

TABLE 3.3

Cell proliferation in HepG2 cells treated with 0 or 2.5 µmol/L malvidin + 2.5 µmol/L delphinidin chloride (5 µmol/L combined) with or without t-BOOH.[1,2,3]

	Cell Proliferation[4]
Control	
0 µmol/L	0.724 ± 0.016^{b}
5 µmol/L	0.718 ± 0.015^{b}
t-BOOH	
0 µmol/L	0.513 ± 0.023^{a}
5 µmol/L	0.483 ± 0.021^{a}
ANOVA (p-value)	
t-BOOH	< 0.0001
Conc	0.3571
t-BOOH*Conc	0.5529

[1] Values are mean ± SE (n=9).
[2] Means within a given column with different subscript letters differ significantly ($p < 0.05$).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.
[4] Values expressed as absorbance of purple formazan crystals in metabolically active cells, not cell number.

TABLE 3.4

Cell proliferation in HepG2 cells treated with 0 or 5 µmol/L malvidin + 5 µmol/L delphinidin chloride (10 µmol/L combined) with or without t-BOOH.[1,2,3]

	Cell Proliferation[4]
Control	
0 µmol/L	0.727 ± 0.011^b
10 µmol/L	0.780 ± 0.032^b
t-BOOH	
0 µmol/L	0.612 ± 0.014^a
10 µmol/L	0.588 ± 0.011^a
ANOVA (p-value)	
t-BOOH	< 0.0001
Conc	0.4482
t-BOOH*Conc	0.0492

[1] Values are mean ± SE (n=9).
[2] Means within a given column with different subscript letters differ significantly ($p < 0.05$).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration.
[4] Values expressed as absorbance of purple formazan crystals in metabolically active cells, not cell number.

TABLE 3.5

Antioxidant enzyme activity in HepG2 cells treated with 0, 5 or 10 µmol/L malvidin chloride with or without t-BOOH.[1,2,3,]

	GR[4]	GPx[5]	GST[6]
		U/mg protein	
Control			
0 µmol/L	$0.195 \pm 0.007^{a,b,c}$	0.021 ± 0.006	0.1487 ± 0.0090
5 µmol/L	0.170 ± 0.004^{a}	0.025 ± 0.004	0.1039 ± 0.0226
10 µmol/L	$0.175 \pm 0.017^{a,b}$	0.026 ± 0.001	0.1045 ± 0.0045
t-BOOH			
0 µmol/L	$0.217 \pm 0.020^{b,c,d}$	0.022 ± 0.002	0.0995 ± 0.0273
5 µmol/L	$0.224 \pm .024^{c,d}$	0.031 ± 0.004	0.0871 ± 0.0197
10 µmol/L	0.250 ± 0.005^{d}	0.018 ± 0.006	0.1253 ± 0.0140
ANOVA (p-value)			
t-BOOH	0.0015	0.8667	0.3259
Conc	0.5938	0.2008	0.3048
t-BOOH*Conc	0.2465	0.4081	0.1926

[1] Values are mean ± SE (n=3).
[2] Means within a given column with different subscript letters differ significantly ($p < 0.05$).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione S-transferase
[4] 1 unit of GR activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[5] 1 unit of GPx activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[6] 1 unit of GST activity is equivalent to 1 nmole conjugate formed/minute/mg protein.

TABLE 3.6

Antioxidant enzyme activity in HepG2 cells treated with 0, 5 or 10 µmol/L delphinidin chloride with or without t-BOOH.[1,2,3]

	GR[4]	GPx[5]	GST[6]
	U/mg protein		
Control			
0 µmol/L	0.217 ± 0.010^a	0.024 ± 0.007	0.133 ± 0.0222
5 µmol/L	0.271 ± 0.013^b	0.0280 ± 0.002	0.122 ± 0.0151
10 µmol/L	$0.263 \pm 0.017^{a,b}$	0.026 ± 0.004	0.145 ± 0.0250
t-BOOH			
0 µmol/L	$0.300 \pm 0.002^{b,c}$	0.030 ± 0.003	0.121 ± 0.013
5 µmol/L	0.360 ± 0.032^d	0.022 ± 0.003	0.119 ± 0.015
10 µmol/L	$0.327 \pm 0.009^{c,d}$	0.026 ± 0.003	0.103 ± 0.024
ANOVA (p-value)			
t-BOOH	< 0.0001	0.9611	0.2561
Conc	0.0146	0.8808	0.9459
t-BOOH*Conc	0.7452	0.3882	0.5933

[1] Values are mean ± SE (n=3).
[2] Means within a given column with different subscript letters differ significantly (p < 0.05).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione S-transferase
[4] 1 unit of GR activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[5] 1 unit of GPx activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[6] 1 unit of GST activity is equivalent to 1 nmole conjugate formed/minute/mg protein.

TABLE 3.7

Antioxidant enzyme activity in HepG2 cells treated with 0, or 5 µmol/L malvidin + 5 µmol/L delphinidin chloride (10 µmol/L combined) with or without t-BOOH.[1,2,3]

	GR[4] Time 1[5]	GR Time 2	GPx[6]	GST[7]
	U/mg protein			
Control				
0 µmol/L	0.348 ± 0.035^a	0.377 ± 0.025	0.040 ± 0.003	0.034 ± 0.002
10 µmol/L	0.443 ± 0.021^b	0.341 ± 0.011	0.050 ± 0.004	0.036 ± 0.004
t-BOOH				
0 µmol/L	$0.380 \pm 0.020^{a,b}$	0.372 ± 0.017	0.047 ± 0.001	0.038 ± 0.005
10 µmol/L	$0.407 \pm 0.005^{a,b}$	0.364 ± 0.012	0.044 ± 0.006	0.048 ± 0.007
ANOVA (p-value)				
t-BOOH	0.9137	0.6237	0.8892	0.1242
Conc	0.0210	0.2176	0.4903	0.2041
t-BOOH*Conc	0.1691	0.4244	0.1028	0.4036

[1] Values are mean ± SE (n=8, except for GR where n=4).
[2] Means within a given column with different subscript letters differ significantly ($p < 0.05$).
[3] Abbreviations: t-BOOH, tert-butyl hydroperoxide; conc, concentration; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione S-transferase
[4] 1 unit of GR activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[5] Time effect of treatment was found for GR. Analysis was conducted separately for time 1 and 2.
[6] 1 unit of GPx activity is equivalent to 1 umol of NADPH oxidized/minute/mg protein.
[7] 1 unit of GST activity is equivalent to 1 nmole conjugate formed/minute/mg protein.

References

Ajiboye, T. O., Salawu, N. A., Yakubu, M. T., Oladiji, A. T., Akanji, M. A., & Okogun, J. I. (2011). Antioxidant and drug detoxification potentials of Hibiscus sabdariffa anthocyanin extract. *Drug and Chemical Toxicology, 34*(2), 109-115.

Alía, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2005). Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology, 19*(2), 119-128.

Alía, M., Ramos, S., Mateos, R., Granado-Serrano, A. B., Bravo, L., & Goya, L. (2006). Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Applied Pharmacology, 212*(2), 110-118.

American Cancer Society. (2010). *Diet and physical activity: what's the cancer connection?* Retrieved from http://www.cancer.org/Cancer/CancerCauses/DietandPhysicalActivity/diet-and-physical-activity

Aqil, F., Gupta, A., Munagala, R., Jeyabalan, J., Kausar, H., Sharma, R. J., et al. (2012). Antioxidant and Antiproliferative Activities of Anthocyanin/Ellagitannin-Enriched Extracts From Syzygium cumini L. (Jamun, the Indian Blackberry). *Nutrition and Cancer, 64*(3), 428-438.

Buege, J., & Aust, S. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*:302-310.

Boivin, D., Blanchette, M., Barrette, S., Moghrabi, A., & Béliveau, R. (2007). Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFkappaB by edible berry juice. *Anticancer Research, 27*(2), 937-948.

Centers for Disease Control and Prevention. (2010). *Deaths and mortality*. Retrieved from http://www.cdc.gov/nchs/fastats/deaths.htm

Cooke, D., Steward, W. P., Gescher, A. J., & Marczylo, T. (2005). Anthocyans from fruits and vegetables--does bright colour signal cancer chemopreventive activity? *European Journal of Cancer, 41*(13), 1931-1940.

Dulebohn, R. V., Yi, W., Srivastava, A., Akoh, C. C., Krewer, G., & Fischer, J. G. (2008). Effects of blueberry (Vaccinium ashei) on DNA damage, lipid peroxidation, and phase II enzyme activities in rats. *Journal of Agricultural and Food Chemistry, 56*(24), 11700-11706.

Faria, A., Pestana, D., Teixeira, D., de Freitas, V., Mateus, N., & Calhau, C. (2010). Blueberry anthocyanins and pyruvic acid adducts: anticancer properties in breast cancer cell lines. *Phytotherapy Research, 24*(12), 1862-1869.

Habig, W., Pabst, M., & Jakoby, W. (1974). Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry 249*:7130-7139.

Halliwell, B. (2007). Oxidative stress and cancer: Have we moved forward? *The Biochemical Journal, 401*(1):1-11.

Halliwell, B., & Gutteridge, J. (1999). *Free Radicals in Biology and Medicine*. 3rd ed. New York: Oxford University Press.

Hou, D. X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3*(2), 149-159.

Hou, Z., Qin, P., & Ren, G. (2010). Effect of anthocyanin-rich extract from black rice (Oryza sativa L. Japonica) on chronically alcohol-induced liver damage in rats. *Journal of Agricultural and Food Chemistry, 58*(5), 3191-3196.

Hwang, J. W., Kim, E. K., Lee, S. J., Kim, Y. S., Moon, S. H., Jeon, B. T., et al. (2012). Antioxidant activity and protective effect of anthocyanin oligomers on h(2)o(2)-triggered g2/m arrest in retinal cells. *Journal of Agricultural and Food Chemistry, 60*(17), 4282-4288.

Hwang, Y. P., Choi, J. H., Choi, J. M., Chung, Y. C., & Jeong, H. G. (2011a). Protective mechanisms of anthocyanins from purple sweet potato against tert-butyl hydroperoxide-induced hepatotoxicity. *Food And Chemical Toxicology, 49*(9), 2081-2089.

Issa A. Y., Volate, S.R., Wargovich, M. J. (2006). The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *Journal of Food Composition and Analysis, 19*:405-419.

Jacob, R. A., & Burri, B. J. (1996). Oxidative damage and defense. *The American Journal of Clinical Nutrition, 63*(6), 985S-990S.

Jing, P., Bomser, J. A., Schwartz, S. J., He, J., Magnuson, B. A., & Giusti, M. M. (2008). Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *Journal Of Agricultural And Food Chemistry, 56*(20), 9391-9398.

Kelsey, N., Hulick, W., Winter, A., Ross, E., & Linseman, D. (2011). Neuroprotective effects of anthocyanins on apoptosis induced by mitochondrial oxidative stress. *Nutritional Neuroscience, 14*(6), 249-259.

Lampe, J. S., Chen, C., Li, S., Prunty, J., Grate, M. T., Meehan, D.E., et al. Modultation of human glutathione S-transferases by botanically defined vegetable diets. *Cancer Epidemiology, Biomarkers and Prevention, 9*(8):787-793.

Lazzé, M. C., Pizzala, R., Savio, M., Stivala, L. A., Prosperi, E., & Bianchi, L. (2003). Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells. *Mutation Research, 535*(1), 103-115.

Lowry, O., Rosebrough , N., Farr, A., & Randall, R. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry 193*:265-275.

Nichenametla, S. N., Taruscio, T. G., Barney, D. L., & Exon, J. H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition, 46*(2), 161-183.

Noda, Y., Kaneyuki, T., Mori, A., & Packer, L. (2002). Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *Journal of Agricultural and Food Chemistry, 50*(1), 166-171.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine, 70*(1), 158-169.

Palikova, I., Vostalova, J., Zdarilova, A., Svobodova, A., Kosina, P., Vecera, R., et al. (2010). Long-term effects of three commercial cranberry products on the antioxidative status in rats: a pilot study. *Journal of Agricultural and Food Chemistry, 58*(3), 1672-1678.

Prior, R. L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *The American Journal of Clinical Nutrition, 78*(3 Suppl), 570S-578S.

Rahman, M. M., Ichiyanagi, T., Komiyama, T., Hatano, Y., & Konishi, T. (2006). Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism. *Free Radical Research, 40*(9), 993-1002.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: dietary polyphenols and signaling pathways. *Molecular Nutrition and Food Research, 52*(5):507-526.

Seeram, N. P., Adams, L. S., Hardy, M. L., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agricultural and Food Chemistry, 52*(9), 2512-2517.

Seeram, N. P., Zhang, Y., & Nair, M. G. (2003). Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. *Nutrition and Cancer, 46*(1), 101-106.

Shih, P. H., Yeh, C. T., & Yen, G. C. (2007). Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *Journal of Agricultural and Food Chemistry, 55*(23), 9427-9435.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Lamaison, J. L., & Rémésy, C. (2003). Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *The Journal of Nutrition, 133*(12), 4178-4182.

Tokarev, J. (2010). *Delphinidin alters hydrogen peroxide induced cytotoxicity and apoptosis in human colon adenocarcinoma cells*. MS Thesis, University of Georgia.

Turner, M. (2009). *Anthocyanins increase antioxidant activity in HT-29 adenocarcinoma cells*. MS Thesis, University of Georgia.

Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*(2):304-309 .

Wang, L. S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters, 269*(2), 281-290.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry, 54*(11), 4069-4075.

Xia, Y., Hill, K., & Burk, R. (1985). Effect of selenium deficiency on hydoperoxide-induced glutathione release from the isolated perfused rat heart. *Journal of Nutrition 115*:733-742.

Zhu, W., Jia, Q., Wang, Y., Zhang, Y., & Xia, M. (2012). The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. *Free Radical Biology and Medicine, 52*(2), 314-327.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Diets high in fruits and vegetables have been shown to decrease disease incidence; possibly due to phytochemicals found in plant foods that act as antioxidants. The anthocyanins found in blueberries have been extensively studied due to their high antioxidant capacity. Studies have examined their antioxidant and prooxidant effects in healthy and carcinoma cell models. It is theorized that one mechanism by which these anthocyanins act as antioxidants is by increasing the activity of antioxidant enzymes and decreasing oxidative stress *in vitro*. The effects of the anthocyanidins malvidin and delphinidin on the antioxidant enzymes GR, GPx and GST and cell proliferation have not been sufficiently examined.

OVERALL PURPOSE

The purpose of this study was to examine the effects of blueberry anthocyanidins on oxidative stress in HepG2 cells. The specific objectives of this research were to assess the effect of anthocyanidins on the activities of GPx, GR and GST and selected markers of cellular oxidative stress, including lipid peroxidation and cell proliferation in oxidatively stressed cells.

MAJOR FINDINGS

In this study, it was hypothesized that modest supplementation with the blueberry anthocyanidins malvidin and delphinidin would increase the activity of the antioxidant enzymes, GR, GPx and GST in HepG2 cells. We also hypothesized that anthocyanidin treatment would reduce oxidative stress.

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It had previously been demonstrated that individual anthocyanidins could alter antioxidant enzyme activity *in vitro* using colon cell lines. Little research, however, had been conducted on the effects of anthocyanidin treatment in a liver cell model. Our study found that significant effects of anthocyanidin treatment on cell proliferation, even at low concentrations, are plausible in liver cells. Delphinidin significantly improved cell proliferation in both stressed and non-stressed cells, with the greatest effect seen in preservation of cells subjected to oxidative stress. While malvidin supplementation at 5 μmol/L did improve cell proliferation in both stressed and non-stressed cells, the effect was not as pronounced.

It was also found that delphinidin, but not malvidin, significantly increased GR activity. These anthocyanidins did not enhance GST and GPx activity at these concentrations. Significance of effects varied depending on anthocyanidin concentration. This draws attention to the fact that the mechanism of action by which anthocyanidins increase enzyme activity is still largely speculative. Varying concentrations could affect the enzymes differently, with one concentration having an antioxidant effect, another concentration having a prooxidant effect, and a third dose having no effect. The same holds for anthocyanidin type. Here we saw that delphinidin was able to increase GR activity, while malvidin was not.

Finally, it appeared as though combination of these two anthocyanidins led to an antagonistic effect, as the impact of single anthocyanidins on cell proliferation and GR activity were lost when the anthocyanins were combined.

LIMITATIONS

This study was a cell culture study, therefore lacking in the homeostatic mechanisms seen in human tissue. It is thus hard to infer whether the reaction seen here would occur in the human body under tightly regulated conditions. Cell culture studies can be used to explore potential

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mechanisms by which anthocyanins may impact the body, but cannot be used to make dietary intake recommendations. Results cannot be applied to other cell types, or other anthocyanins.

Foods are composed of many different types and concentrations of anthocyanins. The anthocyanidins used in the current study were in the aglycone form, whereas whole foods consist of anthocyanins with attached sugar moieties. Such bonds affect bioavailability and reactivity, which was not examined in this study. Again, these results allow some idea of the mechanism through which anthocyanidins affect liver cells, but it is possible that the response in the body may be quiet different if the anthocyanins were consumed in whole foods and metabolized in the mammalian body. Also, if these compounds were consumed as a part of whole foods, many other phytochemicals are present, as well as vitamins, that could change the effects of the malvidin and delphinidin. The impact of these compounds could be additive, competitive, or synergistic. The findings of this study can, however, serve as a reference for future studies using anthocyanidins.

It is also important to note that this study utilized very low concentrations of anthocyanidins. If the study had been conducted using higher concentrations, it is likely that the results would be very different. Using low supplementation levels however is more likely to reflect the conditions present in the human body.

IMPLICATIONS

The results of this study showed mixed findings. The anthocyanins did impact the cell proliferation and in the case of delphinidin, GR activity. The other antioxidant enzymes did not appear to be affected by anthocyanidin treatment, though the results were inconsistent. For delphinidin, the lower concentration (5 umol/L) tended to show a greater impact on antioxidant enzyme activity and protection of oxidatively stressed cells than the higher concentration tested

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(10 umol/L). This has been reported by others and implies that it is possible that low, physiologically realistic concentrations of anthocyanidins may be able to cause a significant increase in activity of some enzymes. Delphinidin seemed to have a more beneficial effect than malvidin in this model, therefore fruits and vegetables with high delphinidin concentrations may have more favorable effects in the liver, but studies would have to be conducted *in vivo* to test this. The combination of these two anthocyanidins appeared to have an antagonistic effect on GR activity and protection of cells against oxidative stress. This is an important point to note since when eaten in food anthocyanins will be present as a mixture. It cannot be assumed foods with a wider mixture of anthocyanins will be more beneficial than those with fewer anthocyanidins, since interactions may be antagonistic rather than synergistic.

FUTURE RESEARCH

As with all research, the results from this study highlight other areas that should be investigated in future studies. This study showed that the two anthocyanidins examined did impact cell proliferation and that low concentrations of delphinidin increased GR activity. More research is needed on the direct and indirect antioxidant effect on the many anthocyanins consumed in foods and their metabolites.

Due to our findings of an antagonistic effect of combined malvidin and delphinidin treatment, it will be important for future studies to expand on these results and explore other anthocyanidins and anthocyanidin mixtures or complete blueberry fractions. Such research would be helpful to show an effect that would more closely resemble whole blueberry consumption in the human diet. Studies should be conducted at even lower levels of anthocyanidins to determine what effects, if any, these concentrations have on antioxidant enzyme activity and cell proliferation. Human and animal *in vivo* studies that examine these

same effects are greatly needed. It is important to see if these enzymes are equally affected by anthocyanins in the body and whether results can be replicated in a dietary model versus cell culture. Such research would allow findings to be applied to dietary recommendations.