

ANTHOCYANINS INCREASE ANTIOXIDANT ENZYME ACTIVITY IN HT-29  
ADENOCARCINOMA CELLS

by

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(Under the Direction of Joan G. Fischer)

ABSTRACT

Anthocyanins are thought to have antioxidant effects in the body. The effects of two anthocyanins, malvidin and peonidin, on activity of antioxidant enzymes, glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx), were examined in HT-29 human adenocarcinoma cells. Cells were treated with each anthocyanin or a combination of both at concentrations of 0, 5, and 10  $\mu\text{g/mL}$  in study one and 0, 2.5 and 5  $\mu\text{g/mL}$  in study two. While the data suggests that these anthocyanin concentrations may increase activity of each enzyme, effects were often anthocyanin and dose-dependent. A synergistic effect between malvidin and peonidin was observed. At 2.5  $\mu\text{g/mL}$ , the anthocyanins did not individually increase enzyme activity, however, a combined dose of 2.5  $\mu\text{g/mL}$  significantly increased activity, GR by 55%, GPx by 21%, and GST by 42%. This study demonstrated that malvidin and peonidin have the potential to increase antioxidant enzyme activity at 10  $\mu\text{g/mL}$  and below.

INDEX WORDS: Anthocyanin, Antioxidant, Glutathione peroxidase, Glutathione reductase, Glutathione-S-transferase



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## DEDICATION

To Dean Kindle  
He fought the good fight.  
His spirit will live on forever in the hearts of those who were privileged to know him.

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# CHAPTER I

## INTRODUCTION

Cancer is the second leading cause of death in the United States. Research shows that diets high in fruits and vegetables can decrease the risk of some cancers and other chronic diseases. While plant foods are high in fiber, vitamins, and minerals, it has been suggested that the phytochemicals found in plants contribute to their ability to decrease disease risk in humans. Phytochemicals are secondary plant metabolites and are abundant in plant foods. Some of the most abundant phytochemicals are flavonoids. Anthocyanins, a class of flavonoids, are the red, blue, and purple pigments found in plants. Foods high in anthocyanins include aubergine, blackberries, black currants, blueberries, black grapes, cherries, rhubarb, strawberries, red wine, plums, and red cabbage (Manach and others 2004). Average consumption of anthocyanins in the United States is estimated to be 12.5 mg/day/person (Wu and others 2006).

Oxidative stress is an improper balance between free radicals and antioxidants, which leads to oxidative damage. Free radicals are highly reactive molecules due to an unpaired electron. Anthocyanins act as antioxidants and counteract oxidative damage in plants (Wu and Prior, 2008). They have this same effect in the human body. When high levels of free radicals are present in the body, whether from normal metabolic processes and the immune system, or from environmental sources, antioxidants may not be able to offset all the potential oxidative damage. It is widely postulated that this damage to the cells in the body is linked to the progression of many chronic diseases, including cancer (Jacob and Burri, 1996). Damaged cells are normally repaired or destroyed by the body;

however, cancer cells bypass this normal process, resulting in uncontrolled cell proliferation and tumor growth (WCRF/AICR, 2007).

Phytochemicals found in fruits and vegetables may counteract these processes, thus preventing oxidative damage, protecting against disease progression, and interfering with the development of cancer (Cooke and others 2005). Anthocyanins have been found to be especially potent antioxidants. They function as antioxidants in a number of ways. Not only do they donate electrons to free radicals, but it is also thought that another mechanism of action involves increasing the activity of antioxidant enzymes (Youdim and others 2000). Antioxidant enzymes neutralize free radicals present in the tissues. These enzymes include superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and glutathione reductase (Thomas, 2006).

This study investigated the two common anthocyanidins, malvidin and peonidin, in an *in vitro* HT-29 human adenocarcinoma cell model. Anthocyanin bioavailability is low, thus the colon is exposed to high amounts of anthocyanins (Mazza and others 2002). We tested the hypothesis that the anthocyanins, malvidin and peonidin, would increase the activity of the antioxidant enzymes glutathione peroxidase, glutathione-S-transferase, and glutathione reductase, in human colon cancer cells. We further hypothesized that the single anthocyanins would individually increase the activity of the enzymes, and an anthocyanin mixture of equal parts of the two anthocyanins would have a synergistic effect. The study examined the use of anthocyanins at a physiological level in order to determine whether or not an effect was attainable at an anthocyanin dose that could be realistically achieved in a human diet.

This study demonstrated that malvidin and peonidin have the ability to increase antioxidant enzyme activity, although the effects are anthocyanidin- and concentration-dependent. Also, at the low concentration of 2.5  $\mu\text{g/mL}$ , the combined anthocyanidins synergistically increased GR, GPx and GST activities. Individual anthocyanidins, however, had little or no effect on enzyme activity.

## CHAPTER II

### LITERATURE REVIEW

#### **Background**

Cancer is the second leading cause of death in the United States. Diets high in fruits and vegetables have been shown to decrease the risk of some cancers, including cancers of the gastrointestinal tract and lung (WCRF/AICR, 2007). There are many compounds in fruits and vegetables that may be protective, including the phytochemicals (Kris-Etherton and others 2002). Phytochemicals have no nutritive value, but are thought to protect against cellular damage associated with the development of cancer.

Polyphenols are one class of phytochemicals that have been extensively studied for their suggested impact on disease prevention. Though widely studied, there are still many questions to be answered regarding the mechanisms by which polyphenols impact overall health and potentially reduce the risk of certain diseases. Anthocyanins have been identified as one group of polyphenols that may contribute to a reduction in disease risk.

#### **Cancer**

Cancer is defined as group of over 100 diseases that are characterized by uncontrolled cellular growth which results from changes in the genetic information of the cells (WCRF/AICR, 2007). When cells replicate, they are normally destroyed by the body if abnormalities are detected. This controlled cell death is called apoptosis (Johnstone and others 2002). The system is carefully regulated to prevent the growth of cancerous tumors, and normally works very effectively. However, the human body is

made up of a vast number of cells, which are replaced at a rapid pace. Errors are inevitable.

Most of the errors observed when cells divide can be attributed to genetic variation and mutation due to a deletion, strand breakage, or crossovers, but some of them are caused by the cellular environment. This environment is dictated by many lifestyle factors. High levels of cancer causing agents, carcinogens, can cause changes to the cell's DNA, which could potentially be copied when the cell is replicated, and if the error is not detected and removed, more mutated cells will be formed. Carcinogens are introduced into the body by a number of different factors including: ultraviolet light, cigarette smoke, ozone, chlorinated hydrocarbons, heavy metals, and x-rays (Jones and DeLong, 2000). These environmental factors along with other normal, metabolic chemicals produced in the body can cause damage due to their highly reactive nature, oxidizing cellular components at the site of exposure (Halliwell and Gutteridge, 1999).

Although there are many different types of cancer, the pathogenesis of the disease is the same throughout and the multi-stage process can be classified into initiation, promotion, and progression (WCRF/AICR, 2007). During initiation, the cell or tissue is exposed to carcinogen and DNA mutation results. If unrepaired, or cellular apoptosis does not occur this damage is passed on. Promotion occurs during replication of the initiated cell, and requires additional promotional factors. Once the replicated cells have become a tumor mass, unless interrupted, the progression stage occurs as they continue growing larger and ultimately metastasizing to distant organs. Cancer cells will display all or some of the six characteristics that have come to be known as the hallmarks of cancer (Hanahan and Weinberg, 2000). It is these hallmarks that make cancer such a

difficult disease to treat and control. These six hallmarks are growth signal autonomy, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Unlike normal cells, cancer cells do not need growth signals to grow. Growth signal autonomy refers to the fact that the cells can replicate without mitogenic growth signals. Cancer cells are also insensitive to antigrowth signals. Cancer cells also evade apoptosis, whereas normal cells, even without similar mutations, are only allowed to live for so long before they undergo cell death. Cancer cells have the ability to replicate indefinitely. Since cancer cells grow rapidly and without restraint, they require a large supply of nutrients. Angiogenesis refers to the development of new blood vessels and is needed by the tumor in order to supply enough nutrients. Lastly, unlike normal cells, cancer cells invade their surrounding tissue. When the cancer successfully invades the vascular or lymphatic systems, it can spread and metastasize to the rest of the body (Hanahan and Weinberg, 2000). This development is a common characteristic of most cancer deaths (WCRF/AICR, 2007).

### **Oxidative Stress**

It is theorized that free radicals can damage DNA, ultimately leading to cancer development (Jacob and Burri, 1996; Juranic and Zizac, 2005). Free radicals are highly reactive molecules with an unpaired electron. This makes them unstable and very potent oxidizers. They oxidize other molecules they come into contact with in the body by removing electrons. This then creates a new molecule that has an unpaired electron, causing the cycle to start over. Free radicals may be generated by environmental



carcinogens. However, many free radicals are formed naturally in the body as by-products of normal metabolic and immune system processes (Halliwell, 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are both free radicals found naturally in the body. ROS are byproducts of various metabolic and immunological processes and include superoxide ( $O_2^{\bullet -}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl ( $\bullet OH$ ), ozone ( $O_3$ ), and singlet oxygen (Jones and DeLong, 2000). Nitric oxide (NO) and peroxynitrite are both RNS. While all of these molecules are free radicals, they also contribute to normal metabolism and are therefore essential to proper cell function (Halliwell and Gutteridge, 1999).

Free radicals can damage carbohydrates, nucleic acids, proteins, and lipids. Damage to proteins and carbohydrates can lead to improper enzyme function (WCRF/AICR, 2007). Oxidation of proteins can cause them to fragment and alter protein binding. These protein changes are sometimes reversible. Damage to lipids can alter membrane functioning, disrupting the fluidity of the membranes (Thomas, 2006). As mentioned previously, damage to nucleic acids can cause mutations to the DNA (Halliwell and Gutteridge, 1999).

Oxidative stress is the term used to describe an improper balance between free radicals and antioxidants. Oxidative stress is defined as an imbalance between oxidizing agents and cellular antioxidant systems, which include antioxidant vitamins, cellular reduced glutathione and reduced coenzymes, as well as the enzymes that control glutathione dependent processes. It can occur if there are not enough antioxidants present, as in the case of a poor diet, or if there are simply too many free radicals

generated from metabolism and the environment (Halliwell and Gutteridge, 1999; Cooke and others, 2005).

Dietary antioxidants are defined as “a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species or both on normal physiological function in humans” (FNB, IOM, 2009). Antioxidants counteract the effects of free radicals by donating an electron to neutralize the free radical, thus rendering them harmless. Like free radicals, antioxidants are found both naturally in the body and in the environment. Food contains many antioxidants in the form of vitamins and phytochemicals. Many researchers believe that it is the antioxidants in fruits and vegetables that decrease chronic disease risk. Vitamins E and C are well known for their antioxidant properties. More recently, researchers have begun to study phytochemicals and their impact on cancer prevention and cancer treatment. Although their mechanisms of action are not yet fully understood, many of them have been shown to act as antioxidants in the body (Issa and others 2006; Moller and Loft, 2006). It is also thought that some may act as pro-oxidants in certain situations, particularly with cancer cells, possibly contributing to the apoptosis (Srivastava and others 2007). There are enzymatic and non-enzymatic antioxidants. Non-enzymatic antioxidants are typically “chain breaking” antioxidants, so named because they scavenge ROS and RNS in the body and break the chain of oxidation being caused by free radicals after it has already begun. Vitamin E and glutathione are “chain breaking” antioxidants and break the cycle of oxidation (Ou and others 2002).

## **Antioxidant Enzymes**

The body has an extensive system of antioxidant enzymes to safely remove free radicals formed by metabolism and the immune system. These enzymes protect the body's cells from ROS, RNS, and any other environmental free radicals by directly inhibiting the formation of, or metabolize, free radicals. Many of these antioxidant enzymes function together (Figure 1) (Thomas, 2006). Antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR). Superoxide anions, one type of free radical, are neutralized by superoxide dismutase into hydrogen peroxide and oxygen. Catalase converts hydrogen peroxide to water (Thomas, 2006).

GR, GST, and GPx are found primarily in the cytosol of cells. These enzymes function individually, but also together, to remove pro-oxidants from the cell. GPx, like catalase, converts hydrogen peroxide to water (Paglia and Valentine, 1967). It uses reduced glutathione to reduce hydrogen peroxide to water. This then produces oxidized glutathione. GR catalyzes the reduction of oxidized glutathione and NADPH back to reduced glutathione (Xia and others 1985; Carlberg and Mannervik, 1975). The reduced glutathione can be used to chelate metals or again as a substrate for GPx. GST is a conjugation enzyme that transfers glutathione to other molecules. Glutathione is itself a reducing agent. It makes molecules water-soluble, which allows them to be easily excreted. Making the molecules water-soluble is one way for the body to expel mutagens and carcinogens (Habig and others 1974). GST and GPx both remove peroxides from the body (Paglia and Valentine, 1967; Shih and others 2007). Thus GST is considered to be both a phase 2 enzyme and an antioxidant enzyme.

The antioxidant response element (ARE) reacts to chemical stress in the cell and induces GST and NADPH: quinone oxidoreductase, another antioxidant enzyme. It has been determined that ARE responds to increased ROS levels or decreased antioxidant levels, such as a decrease in glutathione. Activation of the ARE is primarily controlled by nuclear factor E2-related factor 2 (Nrf2). Nrf2 is normally bound to Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1). ROS react with cysteine bonds on the Keap1 protein and as a result the Nrf2 disassociates with Keap1. The Nrf2 is then free to move into the nucleus where it activates ARE, so ARE can in turn activate antioxidant enzymes. When the ARE is activated, it directs the formation of messenger-RNA to code for peptides and proteins to make antioxidant enzymes. Nrf2 is sensitive to levels of ROS and other pro-oxidants, but also antioxidants, including phenolic compounds, like anthocyanins. Therefore, the Nrf2/ARE pathway could be one more way anthocyanins increase the activities of GST, GR and GPx in the cells (Shih and others, 2007; Nguyen and others 2009).

## **Polyphenols**

Phytochemicals are non-nutritive molecules found in plant foods that convey a beneficial function in the body (Liu, 2004). There are many different classes of phytochemicals (Bravo, 1998). One class, the polyphenols, consists of secondary metabolites produced by plants during metabolism. Over 8000 polyphenols have been identified to date (Bravo, 1998). Polyphenols are found in a wide variety of foods such as berries, grapes, tea, coffee, chocolate, wine, olive oil, walnuts, peanuts, pomegranates, and many other fruits and vegetables. Within the polyphenols class, the structures of

different polyphenols vary widely and they are further broken down into different groups based on these structures. General polyphenolic classifications include phenolic acids, stilbenes, coumarins, tannins, and flavonoids (Liu, 2004). The flavonoids include a number of different subgroups, including anthocyanins, flavonols, flavones, flavanols, flavanones, and isoflavones. Flavonoids are widely studied since they are so commonly distributed in our foods (Manach and others 2004).

Studies have shown that flavonoids may have antioxidant and anti-inflammatory properties that may decrease disease risk. Flavonoids act as direct antioxidants by reducing free radicals. They directly quench free radicals, chelate transition metals, and stimulate the activity of antioxidant enzymes (Duthie and others 2000). They have also been shown to increase levels of glutathione. This in turn acts as an antioxidant directly by removing free radicals. Glutathione is also involved in many other enzymatic antioxidant pathways. Therefore, consumption of flavonoids can have a positive health impact by decreasing chronic disease risk. As with other complex disease processes, flavonoids impact many different levels of disease progression and therefore have numerous effects (Kris-Etherton and others 2004).

### **Anthocyanins**

This study will focus on one specific class of flavonoids, the anthocyanins. These red, blue and purple pigments are found in many foods, namely aubergine, blackberries, black currants, blueberries, black grapes, cherries, rhubarb, strawberries, red wine, plums, and red cabbage (Table 1) (Manach and others 2004). Although many foods contain anthocyanins, some in very high amounts, it is estimated that consumption of

anthocyanins in the western diet is approximately 12.5 mg/day (Wu and others 2006). Due to the high antioxidant capacity of anthocyanins, they are frequently studied for their antioxidant roles (Harris, 2006; Neto, 2007). Their presence in the diet in relatively high concentrations is another reason they are often studied (Cooke and others 2005). They have also garnered attention for anti-inflammatory and anti-tumor properties, and for the reduction in risk of cardiovascular diseases (Shih and others 2007). Their effects on control of cancer cell proliferation have been studied (Yi and others 2005). These studies have focused mainly on controlling cancer proliferation and progression, but few of them study the mechanisms by which these changes occur. In order to better understand the antioxidant activities of anthocyanins and their role in cancer treatment and prevention, it is important to know the mechanisms by which these chemicals cause these effects.

Anthocyanins vary in structure and are typically found glycosylated with one or more sugars (Figure 2). Anthocyanidins are aglycones. Structure is an important determining factor of the way in which ingested anthocyanins are utilized in the body. Some of the common anthocyanins are peonidin, malvidin, cyanidin, delphinidin, pelargonidin, and petunidin (Prior, 2003).

The bioavailability of anthocyanins has been examined by a number of research groups. In humans, Charron and others (2007), examined the concentration of anthocyanins being excreted in the urine of twelve, healthy volunteers. They were fed an anthocyanin free diet and then one of three treatments of red cabbage, 100, 200, or 300 g of cooked red cabbage. The results showed a linear response, with those eating higher levels of cabbage having greater excretion of anthocyanins, but the levels seen in the urine from the 200 or 300 g diet were not two and three times higher than the 100 g

treatment group (Charron and others 2007). It was also found that non-acylated anthocyanins were excreted at a significantly higher level in the urine. Another study by Kurilich and others (2005) also used twelve, healthy volunteers who were fed an anthocyanin free diet and then placed in treatment groups and fed 250 g raw purple carrots, 250 g cooked purple carrots, or 500 g of cooked purple carrots. This study measured blood levels of anthocyanins rather than urine concentrations. All three treatment groups showed very similar levels of anthocyanins in the blood (Kurilich and others 2005). And, although there are different forms of anthocyanins that may not have been measured using these tests, they do demonstrate that anthocyanins have a low bioavailability. Factors affecting bioavailability of anthocyanins include acylation and structure (Charron and others 2007; Yi and others 2006b). When anthocyanins are absorbed into the bloodstream, the process of absorption and excretion is fairly rapid. Both the liver and kidney modify anthocyanins by the processes of methylation and also mono-glucuronidation. This is done more by the liver than the kidneys (Talavera and others 2005). Excretion begins around 20 minutes after absorption, with bloodstream levels reaching their peak between 30 minutes and 2 hours. By entering the blood stream, anthocyanins are circulated throughout the entire body and levels can be measured in other tissues (Prior and Wu, 2006; Talavera and others 2005).

Although anthocyanins are absorbed to an extent, they are excreted to a large extent unmetabolized. The amount of anthocyanins seen in the urine compared to the amount ingested demonstrates the low levels of absorption (Cao and others 2000). The anthocyanins are passing through the body unabsorbed. The bioavailability of the various anthocyanins differs dependent upon the structure of the anthocyanin. The

specific anthocyanins are absorbed and excreted to differing degrees (McGhie and others 2003). This is true in both rat and human models. If the bioavailability of anthocyanins is relatively low, it follows that the colon would have a relatively high concentration of undigested anthocyanins present after ingestion of an anthocyanin rich food. Using the colon cancer cell model replicates these conditions, however the form that ingested anthocyanins are in when they reach the colon is still unclear.

Anthocyanins may have a protective role at various stages in the cancer process. They can act as antioxidants by directly reducing free radicals and preventing oxidative damage to DNA from carcinogens (Shih and others 2007). A number of studies have been conducted associating consumption of fruits and vegetables with decreased cancer risk and mortality (Doll, 1990; Dragsted, 1993). Antioxidant capacity and polyphenols were measured in a study looking at 100 foods to determine a correlation between the two. Fruits and vegetables showed a strong correlation between the two parameters (Wu and others 2006). Anthocyanins have been shown to act as antioxidants and chemopreventive agents during initiation, promotion, and progression of cancer (Hou, 2003). Studies have also found that they are stronger antioxidants than both vitamins E and C (Duthie, 2007). It has also been theorized that anthocyanins can increase apoptosis in mutated cells. Yi and others (2005, 2006a) demonstrated an increase in apoptosis of human liver and colon cancer cells in response to treatment with blueberry anthocyanins. The studies also showed inhibition of cell growth at high concentrations of blueberry extracts. Srivastava and others (2007) confirmed the enhancement of apoptosis by blueberry anthocyanin extracts. Other possible anticancer effects of anthocyanins include decreased cell proliferation, decreased cell growth, increased DNA damage, and



increased enzyme activity (Feng and others 2007; Cooke and others 2006). Preventative changes anywhere in the stages of cancer could lead to prevention of the development of cancer altogether.

Others have proposed that anthocyanins upregulate the phase 2 enzymes. It is well known that many other flavonoid compounds such as lycopene, quercetin and resveratrol can enhance GST activity (Breinholt and others 2003). Srivastava and others (2007) examined the effect of blueberry anthocyanins and tannins on the activation of GST and quinone reductase, another Phase II/antioxidant enzyme, in HT-29 colon cancer cells. They measured the effects that anthocyanin extracts had on the cells at concentrations of 50 to 150 mg/ml. These high doses were selected because they induced apoptosis in the colon cancer cells. However, the activity of the GST and quinone reductase decreased as a result of these high doses (Srivastava and others, 2007).

Dulebohn and others (2007) examined the effects of whole freeze dried blueberries and blueberry extracts on the enzyme activity in the colon and liver of rats. DNA and lipid damage were the main focus of the study. However, Dulebohn also examined the effect of these compounds on the activity of GST. Changes in GST activity seen in the colon were not significant and although liver GST activity was 26% higher in rats fed blueberries or blueberry extracts, this was not significant. Boateng and others (2007) treated rats with azoxymethane to induce colon cancer and then supplemented them with blueberries to 5% of their total diet or blueberry juice at 100mL/day. The treated rats in both groups had increased GST activity in the liver and decreased precancerous aberrant crypt foci. Reen and others (2006) conducted a similar study in which rats were supplemented with diets of 5% or 10% dried black raspberries.

They found a significant increase in GST activity. Patterson and others (2008) examined a concentration-dependent effect of malvidin and peonidin in human intestinal adenocarcinoma cells stressed with  $H_2O_2$  as measured by DNA damage and apoptosis. They hypothesized that low concentrations would scavenge  $H_2O_2$  and prevent DNA damage; and higher concentrations would induce oxidative stress and increase DNA damage. The results of the study showed some apoptosis, although at much lower concentrations than expected and not a significant increase at the very high concentrations. It has been hypothesized that increased activity in antioxidant enzymes actually protects the cells from apoptosis (Shih and others 2007). Shih and others (2007) examined the effects of 50  $\mu\text{mol/L}$  of 10 different anthocyanins on GST, GR, GPx, and apoptosis. They found increases in all enzymes from nearly all the anthocyanins used and decreased apoptosis. In this case, the enzymes were thought to be performing antioxidant roles in the cancer cells to shield them from harm, including apoptosis, or the cells could have been undergoing necrosis. If anthocyanins increase antioxidant enzyme activity in cancer cells, it will be important to determine whether or not this would be a protective effect for the cancer cells or prevent apoptosis, and therefore be a negative impact for the body. The current study will attempt to determine whether or not anthocyanins increase the activity of the antioxidant enzymes, GST, GPx, and GR in HT-29 colon cancer cells, thereby increasing the overall antioxidant effects of the anthocyanins.

## **Hypothesis**

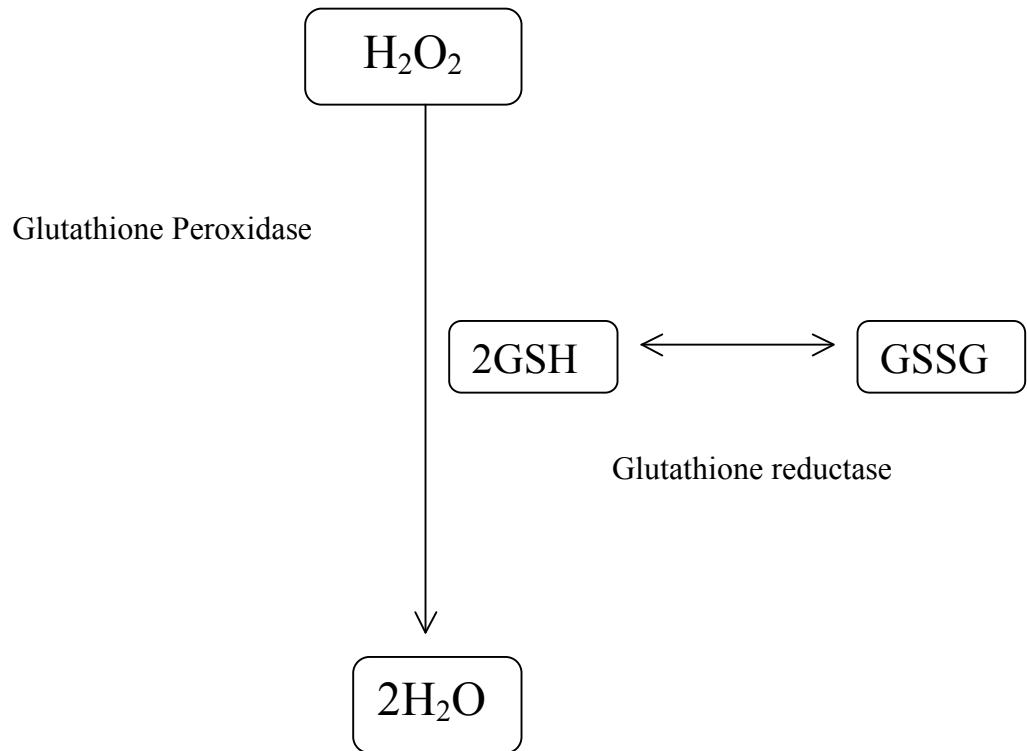
We tested the hypothesis that the blueberry anthocyanins, malvidin and peonidin, would increase the activity of the antioxidant enzymes, glutathione-S-transferase, glutathione peroxidase, and glutathione reductase, in human adenocarcinoma HT-29 cells. We further hypothesized that the single anthocyanins would individually increase the activity of the antioxidant enzymes, and that an equal mixture of the anthocyanins would have a synergistic effect on the antioxidant enzyme activity.

**Table 1.** Anthocyanin content of selected foods

Food	mg Anthocyanins /100g edible portion
Bilberry	380
Black Raspberry	324
Wild Blueberry	321
Cultivated Blueberry	163
Cranberry	93
Blackberry	90
Sweet Cherry	80
Red Grape	45
Red Raspberry	39
Strawberry	34

(USDA, 2003)

**Figure 1.** Antioxidant enzyme pathway



(Aitken and Roman, 2008)

**Figure 2.** Chemical structure of anthocyanidins

(USDA, 2003)

CHAPTER III  
DO BLUEBERRY ANTHOCYANINS INCREASE THE ACTIVITY OF  
ANTIOXIDANT ENZYMES?

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## **Abstract**

Anthocyanins, red, blue, and purple polyphenols found in high amounts in blueberries, are thought to have an antioxidant effect in the body. We examined the effects of the anthocyanins, malvidin and peonidin, on activity of the antioxidant enzymes, GST, GR, and GPx in HT-29 human adenocarcinoma cells. The cells were treated with malvidin and peonidin or a combination of malvidin and peonidin in concentrations of 0, 5, and 10  $\mu\text{g}/\text{mL}$  in study one and 0, 2.5 and 5  $\mu\text{g}/\text{mL}$  in study two. While data suggests that anthocyanins at these concentrations may increase activity of all three enzymes, the effects were often anthocyanin and dose-dependent. A consistent finding suggested a synergistic effect between malvidin and peonidin on enzyme activity. For all three enzymes, while malvidin and peonidin alone at 2.5  $\mu\text{g}/\text{mL}$  did not increase enzyme activity, the combination of the two anthocyanidins significantly increased enzyme activity above control. Malvidin and peonidin combined at a dose of 2.5  $\mu\text{g}/\text{mL}$  increased GR activity by 55%, GPx activity by 21%, and GST activity by 42%. This study demonstrated that malvidin and peonidin do have the potential to increase antioxidant enzyme activity at concentrations of 10  $\mu\text{g}/\text{mL}$  and below.



## **Introduction**

It is estimated that about 35% of cancer occurrences are directly related to diet (ACS, 2007). Diets high in fruits and vegetables have been shown to decrease the risk of many chronic diseases, including some types of cancers (WCRF/AICR, 2007). There are many theories as to which compounds in fruits and vegetables are protective. One possibility is the phytochemicals in fruits and vegetables. Phytochemicals have no nutritive value, but are thought to protect against cellular damage associated with the development of cancer. Many phytochemicals may act as antioxidants in the body, preventing damage from oxidative stress (Kris-Etherton and others 2002).

Oxidative stress refers to an improper balance between prooxidant free radicals and antioxidants. Free radicals are molecules that are unstable and are highly reactive due to an unpaired electron. It has been theorized that oxidative damage to DNA causes mutations ultimately leading to cancer (Jacob and Burri, 1996; Juranic and Zizac, 2005).

Although antioxidant activity varies, anthocyanins seem to be especially potent antioxidants, shown by one study to be 2-6 times more potent than some other phytochemicals (Prior and others 1998). They function as direct antioxidants by donating electrons to free radicals, thus rendering them harmless. It is also theorized that anthocyanins induce antioxidant enzymes (Shih and others 2007). Antioxidant enzymes are the natural defense system of the body against reactive oxygen and nitrogen species and include GPx, GST, and GR (Thomas, 2006). GPx and GR work in conjunction to reduce free radicals. GPx converts hydrogen peroxide to water, using reduced glutathione. Glutathione reductase then reduces the oxidized glutathione (Thomas, 2006). GST transfers the antioxidant glutathione to other molecules. Activity of these enzymes

may be upregulated by anthocyanins via the antioxidant response element (Shih and others 2007; Nguyen and others 2009).

There has been little research on the effects of anthocyanins on antioxidant activity. A previous study in our lab by Dulebohn and others (2008), examined the effect of blueberry supplementation on GST activity in Sprague-Dawley rats. Although a significant effect was not shown, there was a slight trend seen in GST activity. The blueberry, polyphenols, and 1% flavonoid groups had roughly 27% higher GST activity in liver tissue when compared to the control group. She suggested low anthocyanin bioavailability as one reason for the lack of significant results. Others (Boateng and others 2007; Reen and others 2006) found significant increases of GST activity in rats supplemented with high anthocyanin fruits. Srivastava and others (2007) examined the effect of blueberry anthocyanins and tannins on the activities of GST and quinone reductase in colon cancer cells. They used high concentrations that had previously been shown to increase apoptosis, but the activities of GST and quinone reductase decreased at these levels of supplementation.

This study investigated the use of the blueberry anthocyanins, malvidin and peonidin, in an in vitro colon cancer model. We tested the hypothesis that malvidin and peonidin would increase the activity of the antioxidant enzymes, GPx, GST, and GR, in HT-29 human adenocarcinoma cells. We further hypothesized that the single anthocyanins would individually increase the activity of the enzymes, and that an anthocyanin mixture of equal amounts of malvidin and peonidin would have a synergistic effect. The study examined the use of anthocyanins at a physiological level in order to

determine whether or not an effect was attainable at an anthocyanin dose that could be realistically achieved in a human diet.

## **Methods**

*Cells.* The colon adenocarcinoma HT-29 cell line was purchased from ATCC (Manassas, VA). Colon cancer cells were chosen because of the exposure of the colon to nonabsorbed anthocyanins. Cells were maintained in McCoy's 5A modified medium with L-glutamine (ATCC, Manassas, VA) with fetal bovine serum added at a final concentration of 10% in 75 cm<sup>3</sup> flasks. Cells were incubated at 37° C in 5% CO<sub>2</sub>, 95% air and controlled humidity. Cells were passaged when they became confluent, and the media was changed every 2-3 days, as needed.

*Anthocyanin treatment.* Two anthocyanins, malvidin chloride and peonidin chloride, or equal mixture of malvidin and peonidin chloride were mixed with media and applied to the cells in concentrations of 0, 5 (13.5 µmol/L) and 10 µg/ml (27 µmol/L) for study 1 and 0, 2.5 (6.75 µmol/L) and 5 µg/mL for study 2 (15). The anthocyanin media mixtures and control media contained 0.1% DMSO. The anthocyanins were purchased from Chromadex (Irvine, CA). Each treatment was performed in triplicate flasks. The treated cells were then incubated for 22 hours.

*Cell Collection.* Following the incubation period, cells were trypsinized, collected, added to homogenizing buffer (pH 7.4, 50 mmol/L potassium phosphate, 5 mmol/L EDTA) and homogenized for 5 seconds at 4° C. The cell lysate was then centrifuged in a J2-HS Beckman Centrifuge (Beckman Instruments Inc., Fullerton, CA) for 20 minutes at 4° C at 10,000g. The supernatant was ultracentrifuged in a Beckman

Optima LE-80K Ultracentrifuge (Beckman Instruments Inc., Fullerton, CA) at 4°C, 100,000 G for 1.16 hours. The supernatant following ultracentrifugation, which was the cytosol, was then removed and stored at -80°C until analysis. The protein concentrations of the cytosolic fractions were measured using methods by Lowry and others (1951).

*Glutathione Reductase.* The activity of GR was assessed using the method of Xia and others (1985), measuring the reduction of oxidized glutathione by NADPH. Briefly, 0.1 ml dilute cytosol was placed in a cuvette. 0.8 ml of 0.05 mol/L potassium phosphate buffer with 0.001 mol/L EDTA and 0.25 mmol/L NADPH was added to the cuvette and then 0.1 ml 5 mmol/L oxidized glutathione was added for a total volume of 1.0 ml in the cuvette. The substrate, oxidized glutathione, was added immediately preceding the spectrophotometric readings. Change in absorbance at 340 nm was read every minute for 4 minutes using a spectrophotometer (Beckman Instruments Inc, Fullerton CA). Change in absorbance was used to determine the concentration of NADPH that had been oxidized. One unit of enzyme activity was equal to 1 µmole of NADPH oxidized per min per mg protein (Xia and others 1985).

*Glutathione S-Transferase.* The activity of GST was assessed using spectrophotometric methods as described by Habig and others (1974) with 10 mmol/L 1-Chloro-2,4-dinitrobenzene as the substrate. This assay used 0.1 mol/L potassium phosphate buffer (pH 6.5), 6.24 mmol/L GSH, and 10 mmol/L 1-Chloro-2,4-dinitrobenzene. Briefly, 0.1 ml diluted cytosol was added to a cuvette. 0.8 ml of buffer with glutathione (final concentration 5 mmol/L) and 0.1 ml of 1-Chloro-2,4-dinitrobenzene in ethanol (final concentration 1 mmol/L) were added for a total volume of 1 ml. The velocity of formation of S-2,4-dinitrophenylglutathione at 25°C was

measured for 3 minutes at 340 nm. One unit of enzyme activity is equal to 1 nmol conjugate formed/minute/mg protein. The molar extinction coefficient for 1-Chloro-2,4-dinitrobenzene at 340 nm is 9.6/mM/cm.

*Glutathione Peroxidase.* The activity of GPx was assessed utilizing t-butyl hydrogen peroxide (TBH; 0.3 mmol/L) as the substrate using spectrophotometric methods. Briefly, 0.1 ml dilute cytosol was added to a cuvette. Then 0.8 ml of 50 mmol/L potassium phosphate buffer with 5 mmol/L EDTA, 1 mmol/L reduced glutathione, 0.2 mmol/L NADPH, 1 U/ml GSSGR, and 1mmol/L NaN<sub>3</sub> was added to the cuvette followed by 0.1 ml of 0.3 mmol/L t-butyl hydroperoxide (TBH) for a final volume of 1.0 ml in the cuvette. The substrate, TBH, was added immediately preceding the spectrophotometric readings. Change in absorbance at 340 nm was read every minute for 4 minutes. One unit of enzyme activity was defined as one umol of NADPH oxidized per minute per mg protein (Paglia and Valentine, 1967).

*Preliminary Tests.* Prior to treating the cells, assays were run to determine whether or not the cell line contained a measurable level of protein and enzyme activity. Once it was confirmed that the adenocarcinoma cell line displayed measurable enzyme activity levels, the cells were also tested homogenized and non-homogenized. It was found that homogenization nearly doubled enzyme activity and the process was therefore used on the treated cells.

*Statistics.* Statistical analysis was conducted using the Statistical Analysis Software (SAS Version 9.13, SAS Institute, Cary, NC). Treatment means, standard error of the mean, analysis of variance and least significant difference tests were conducted. The overall effects and interactions between anthocyanin type (using the combined

anthocyanins as one type) and concentration were determined using two way ANOVA. All data was tested for normality. When non-parametric tests were run, the analysis yielded a similar level of significance as ANOVA, so the data presented uses ANOVA. Fisher's least significance difference test was used to assess the difference between means for treatment groups. The statistical significance was  $p < 0.05$ .

## Results

The effects of the anthocyanins malvidin and peonidin on antioxidant enzyme activity at concentrations of 0, 5, and 10  $\mu\text{g/mL}$  are shown in Table 2 and Figures 3, 5, and 7. While there was a significant ( $p < 0.05$ ) interaction between anthocyanin type and dose, there was no effect of anthocyanin type or anthocyanin concentration alone on GR activity (Figure 3). Peonidin, at a concentration of 10  $\mu\text{g/mL}$ , increased GR activity 291% above control, while 5  $\mu\text{g/mL}$  malvidin increased GR activity 198% about control. The combined malvidin and peonidin treatment increased GR activity about 118% above control for both 5 and 10  $\mu\text{g/mL}$ . Similar to GR, there were no main effects of anthocyanin type or dose on GPx activity (Figure 5). However, there was a significant ( $p < 0.05$ ) interaction between anthocyanin type and dose on GPx activity. The combination of anthocyanins increased the GPx enzyme activity slightly above that of control, but only at 5  $\mu\text{g/mL}$ . GST activity (Figure 7) was not significantly affected by anthocyanins at 5 or 10  $\mu\text{g/mL}$  concentrations. There was a slight trend for anthocyanin concentration ( $p = 0.082$ ) in GST activity such that lower concentrations tended to show a larger increase in enzyme activity.

Table 3 and figures 4, 6, and 8 show the effects of malvidin and peonidin on

antioxidant enzyme activity at concentrations of 0, 2.5, and 5  $\mu\text{g}/\text{mL}$ . GR activity (Figure 4) was significantly ( $p<0.05$ ) affected by anthocyanin type with highest activity found with the combined anthocyanins. There was also a significant ( $p<0.05$ ) interaction between anthocyanin type and dose. While malvidin and peonidin alone did not increase activity above control, the mixture of both anthocyanidins at a concentration of 2.5  $\mu\text{g}/\text{mL}$  significantly increased GR activity about 55% above control. Like GR, using 2.5 and 5  $\mu\text{g}/\text{mL}$  concentrations, GPx activity (Figure 6) was significantly ( $p<0.05$ ) affected by anthocyanin type and there was a significant ( $p<0.05$ ) interaction between anthocyanin type and dose. Peonidin increased GPx activity slightly more than malvidin. The mixture of the two anthocyanins resulted in the highest GPx activity and a concentration of 2.5  $\mu\text{g}/\text{mL}$  significantly increased activity about 21% above control. GST activity (Figure 8) was only significantly ( $p<0.05$ ) affected by anthocyanin type. Once more, the combination of anthocyanins resulted in the largest increase in GST activity with a 2.5  $\mu\text{g}/\text{mL}$  concentration increasing enzyme activity about 42% above control.

We hypothesized that a combination of the two anthocyanins would have a synergistic effect. For all three enzymes, 2.5  $\mu\text{g}/\text{mL}$  of the combined malvidin and peonidin had the greatest effect. The 3x3 ANOVA analysis appeared to be synergistic, however, a 2x2 analysis using only cells treated with 0 and 2.5  $\mu\text{g}/\text{mL}$  for malvidin and peonidin was conducted. This examined all of the 2.5  $\mu\text{g}/\text{mL}$  treatment groups and the control, and then compared the single anthocyanins to the mixture and the control. Slinker (1998) recommends this analysis with the suggestion that synergism has occurred only when the interaction between malvidin and peonidin is significant. The analysis confirmed a significant synergistic malvidin x peonidin interaction. The individual

anthocyanins did not show a significant effect above control. The mixture of malvidin and peonidin was significant ( $p < 0.05$ ) for GR, GPx, and GST. This confirmed the ANOVA results and showed that there was a synergistic effect of the two anthocyanins.

## **Discussion**

The results of our study indicate that anthocyanins, malvidin and peonidin specifically, do have the potential to impact the activity of GST, GR, and GPx. Our results show that these effects are dependent on the specific anthocyanin and dose used. One significant finding was that anthocyanin concentration did not have a consistent effect on enzyme activity across anthocyanin types. This result was unexpected and shows how important anthocyanin structure is. In interpreting the results, it is important to remember that structure plays a very significant role in bioavailability and metabolism of anthocyanins (Prior and Wu, 2006). Our results confirm that different anthocyanins may have completely different biological effects. Thus it is important to study many different anthocyanins and anthocyanin mixtures before making conclusions about the effects of anthocyanins on biological processes.

The synergistic effect found between malvidin and peonidin suggests that although individual anthocyanins do have an effect, a combination of anthocyanins, or maybe even a whole berry extract, would have a greater or lesser effect. Individual anthocyanins have been shown to have an effect on antioxidant enzymes, but there may be unknown factors within the metabolism of the anthocyanins that cause them to have this synergistic effect. And while it is essential to study individual anthocyanins to determine mechanism of action, studying whole berries is also important because it can



be more directly correlated to blueberries and other high anthocyanin foods in the diet (Bravo, 1998).

The highest levels of anthocyanins tested in this study did not have an effect on enzyme activity. Instead, the lower concentrations of anthocyanins tended to have the greatest effects, increasing antioxidant enzyme activity. Since the lowest concentration of 2.5  $\mu\text{g}/\text{mL}$  is close to feasible physiological levels, this may be similar to results of others that have shown slight increases in GST activity in rats fed high anthocyanin foods (Boateng and others 2007; Dulebohn and others 2008; Reen and others 2006). Previous research (Srivastava and others, 2007) showed that high concentrations of anthocyanins (50-150  $\mu\text{g}/\text{mL}$ ) decreased GST activity. While some of our results did not vary greatly from the control, there were no significant decreases from the control, even at the higher levels. This could be due to the fact that 10  $\mu\text{g}/\text{mL}$  was the highest concentration used in this study and the much higher concentrations have a very different effect on the enzymes.

In contrast, Shih and others (2007) examined the effects of 50  $\mu\text{mol}/\text{L}$  of 10 different anthocyanins on GST, GR, GPx activities. They found increases in all enzymes with nearly all the anthocyanins used. It is possible that different concentrations have different effects on the ARE, a control point for expression of these enzymes, causing differing results. It is also important to note that the cell type used by Shih et al. was the clone 9 rat liver cell, which may metabolize anthocyanins differently. Lastly, the effects found in the Shih et al. study varied among the 10 different anthocyanins, showing again that effects are based on structural differences among anthocyanins.

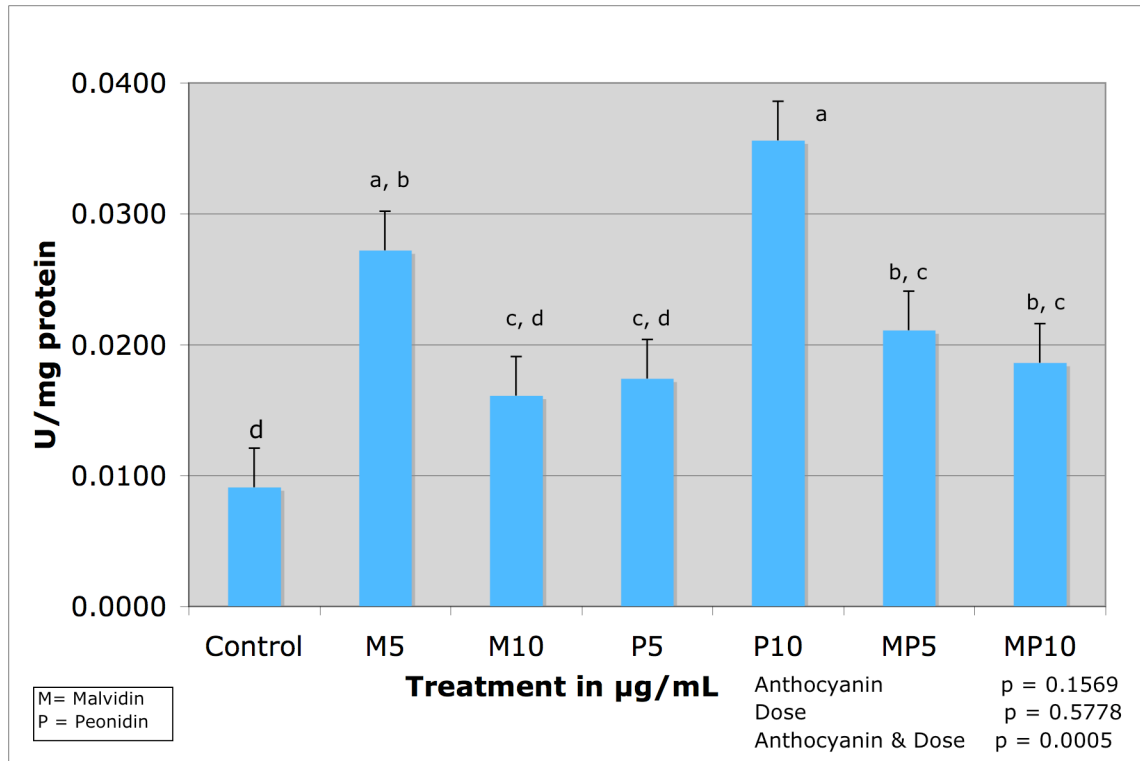
It is important to note that the results are not always consistent among studies for a number of possible reasons, including: use of *in vivo* versus *in vitro* models, different cell types, different lengths of treatment, different anthocyanins and different concentrations. These variations can make results difficult to compare. Our results show that anthocyanin type and dose interact significantly, and this should be considered when looking at results from different studies. Cell type is also of particular importance, whether *in vivo* or *in vitro*. Different tissue will metabolize the anthocyanins differently and potentially have different results. Metabolites found within liver tissue, for example, could quite possibly be very different than the metabolites that exist within the colon. These metabolites will most likely have differing effects on enzyme activity. This is of utmost important *in vivo* where the environment is not as controlled and other factors may play a greater role in the results that are obtained. The concentration of antioxidant enzymes will also vary with different tissue types.

One area of current interest is the study of the impact of flavonoid-induced antioxidant enzyme activity on cellular apoptosis. Apoptosis, a beneficial process, may be induced via oxidative stress (Shih and others 2007). There is interest in whether flavonoid induction of antioxidant enzymes is associated with a decrease in the apoptotic process. However, results of these studies, thus far, are conflicting and may be dependent on concentration and flavonoid structure (Leunga and others 2006; Srivastava and others 2007; Shih and others 2007). This is an area for further research.

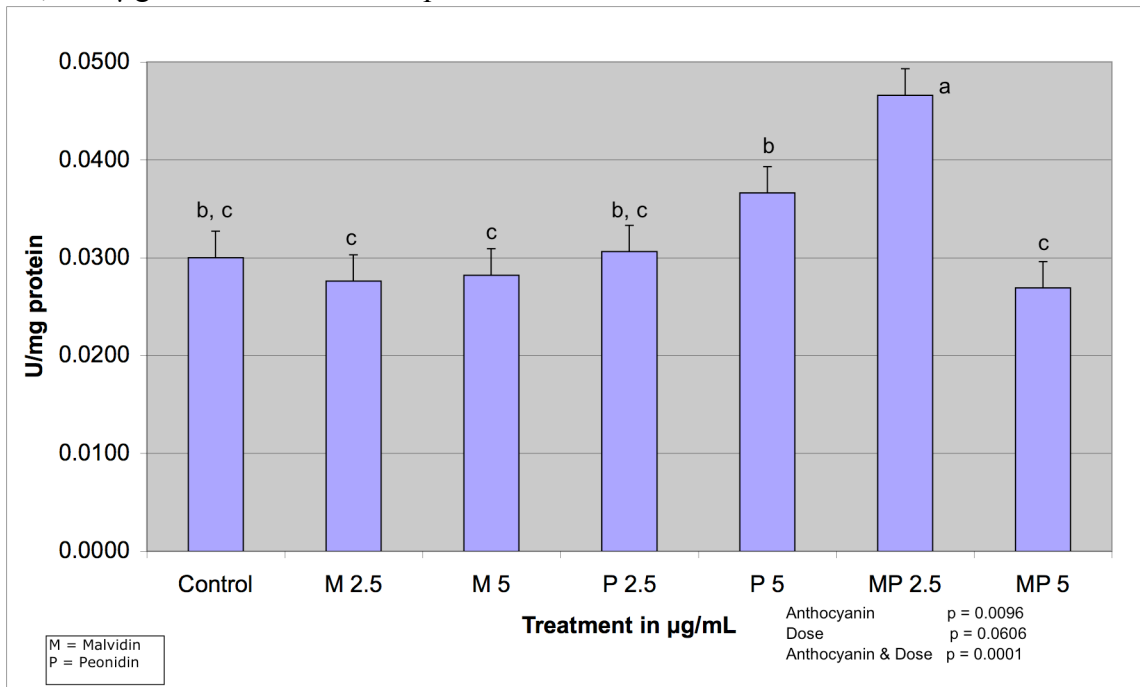
In conclusion, GPx, GST, and GR activities were significantly increased by the anthocyanins malvidin and peonidin. The lower concentrations showed a greater impact on enzyme activity. The combination of the two anthocyanins at 2.5 µg/mL had the

greatest effect in all three enzymes and proved to be synergistic. There was less of an effect from dose than anticipated, and it seems that the interaction between dose and anthocyanin type was more indicative of effect than dose alone. It is always important to remember that results from *in vitro* studies cannot be directly applied to dietary recommendations. For that reason, more *in vivo* research, both animal and human, needs to be done to confirm results from *in vitro* studies and to further examine the relationship between anthocyanin type and dose.

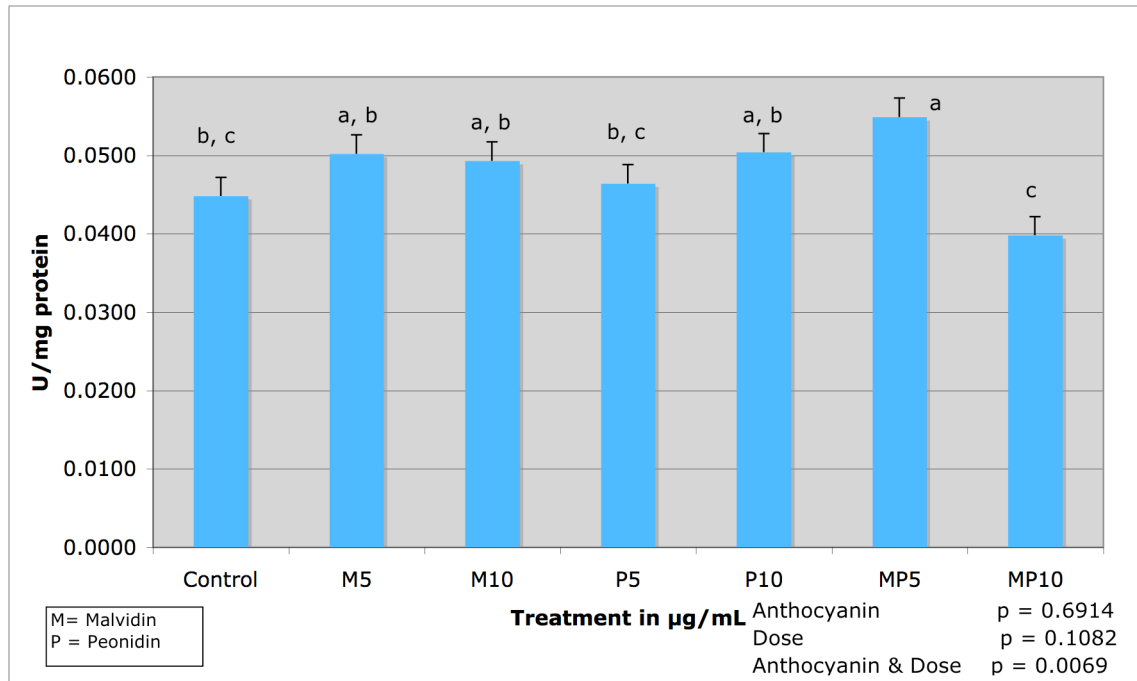
**Figure 3.** Glutathione reductase activity (U/mg protein) in HT-29 cells treated with 0, 5, or 10  $\mu\text{g/mL}$  malvidin and/or peonidin.



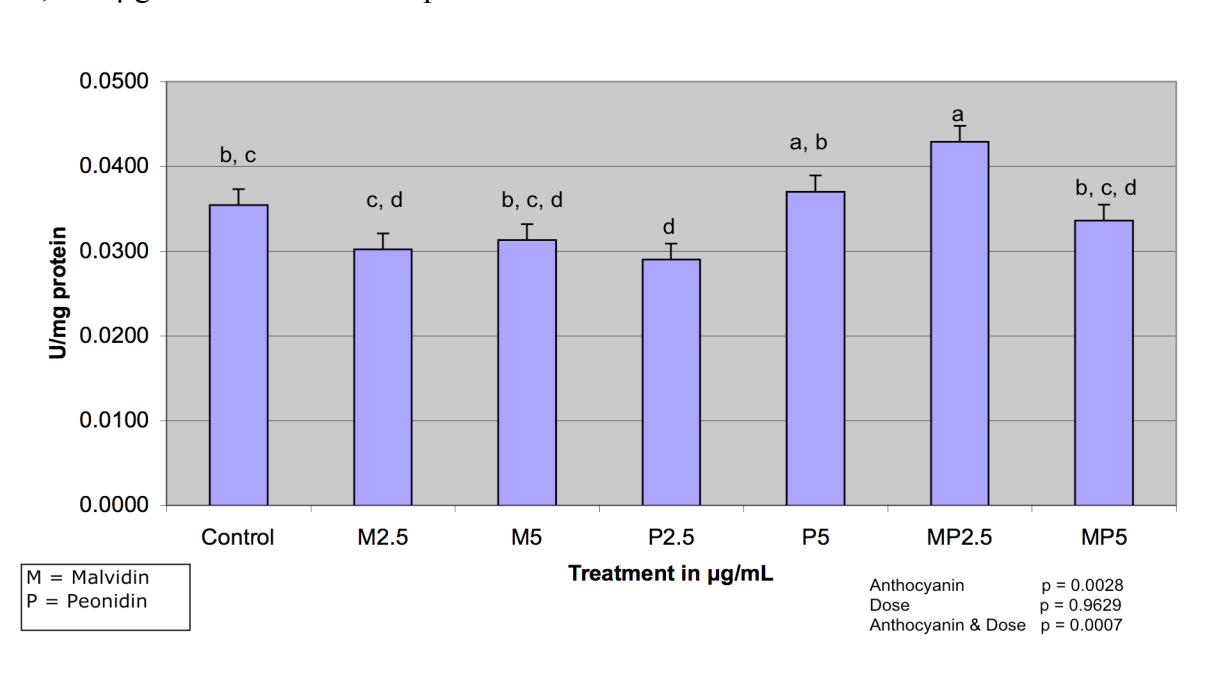
**Figure 4.** Glutathione reductase activity (U/mg protein) in HT-29 cells treated with 0, 2.5, or 5  $\mu\text{g/mL}$  malvidin and/or peonidin.



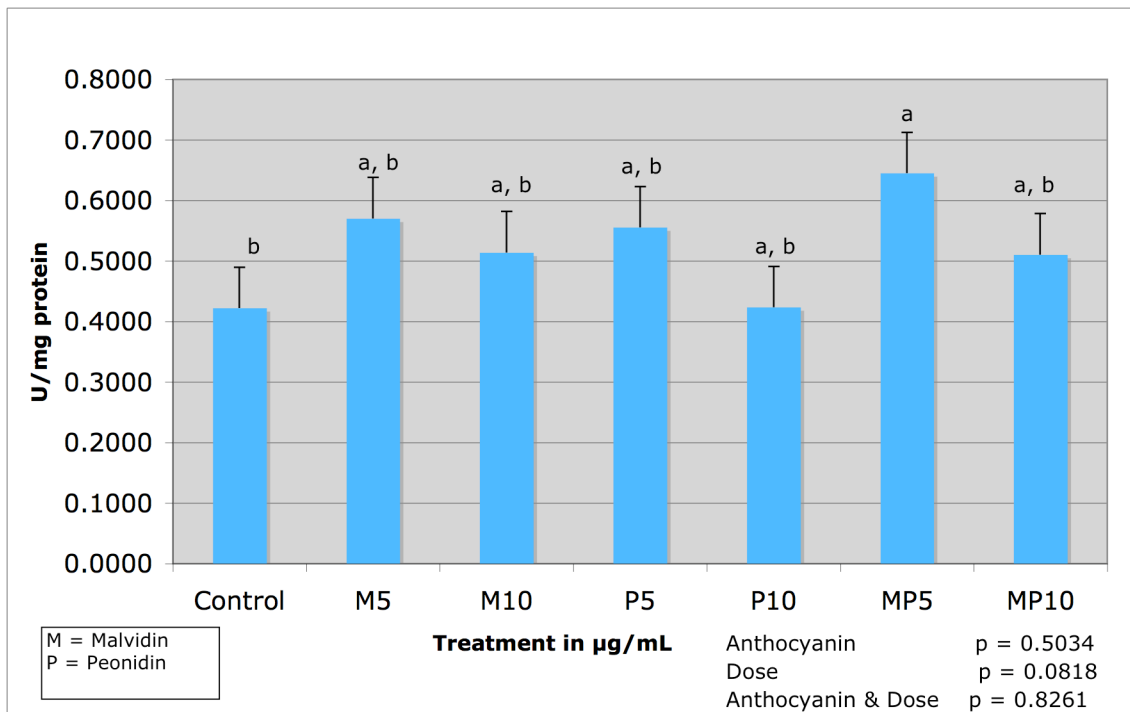
**Figure 5.** Glutathione peroxidase activity (U/mg protein) in HT-29 cells treated with 0, 5, or 10 µg/mL malvidin and/or peonidin.



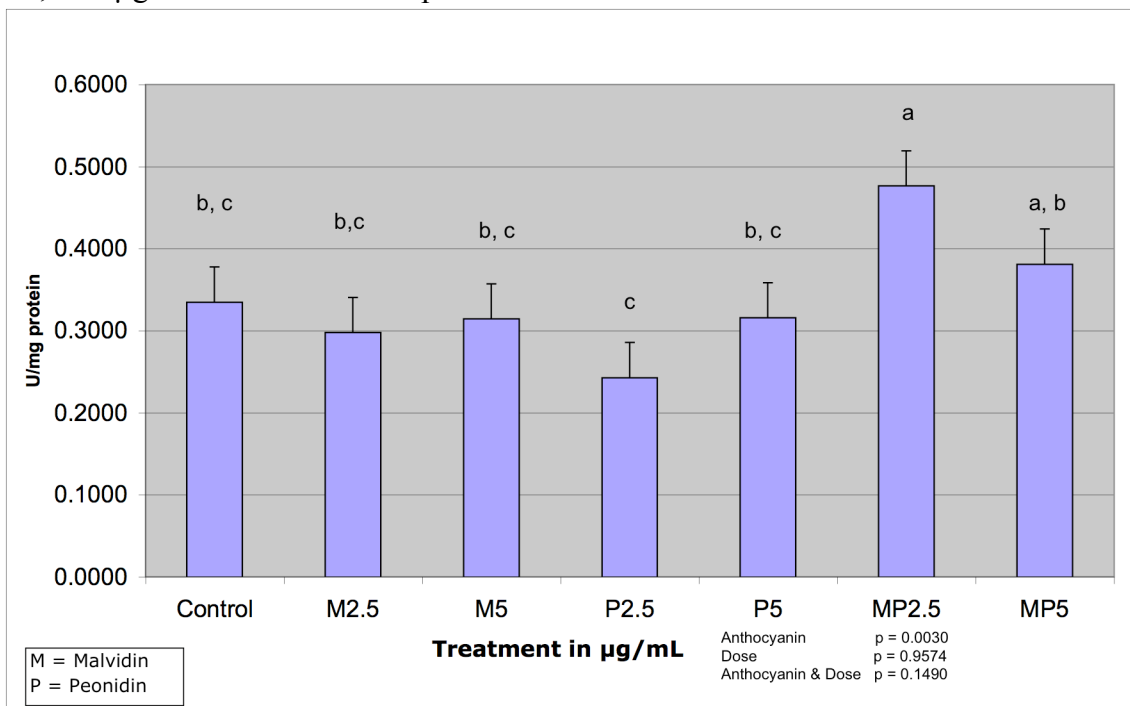
**Figure 6.** Glutathione peroxidase activity (U/mg protein) in HT-29 cells treated with 0, 2.5, or 5 µg/mL malvidin and/or peonidin.



**Figure 7.** Glutathione-S-transferase activity (U/mg protein) in HT-29 cells treated with 0, 5, or 10  $\mu\text{g/mL}$  malvidin and/or peonidin.



**Figure 8.** Glutathione-S-transferase activity (U/mg protein) in HT-29 cells treated with 0, 2.5, or 5  $\mu\text{g/mL}$  malvidin and/or peonidin.



**TABLE 2**

Antioxidant enzyme activity in HT-29 cells treated with 0, 5, or 10 µg/mL malvidin and/or peonidin.<sup>1,2</sup>

	GR	GPx	GST
		U/mg protein	
Control	0.009 ± 0.002 <sup>d</sup>	0.045 ± 0.002 <sup>b,c</sup>	0.0422 ± 0.050 <sup>b</sup>
Malvidin 5	0.027 ± 0.003 <sup>a,b</sup>	0.050 ± 0.002 <sup>a,b</sup>	0.570 ± 0.073 <sup>a,b</sup>
Malvidin 10	0.016 ± 0.002 <sup>c,d</sup>	0.049 ± 0.003 <sup>a,b</sup>	0.514 ± 0.045 <sup>a,b</sup>
Peonidin 5	0.017 ± 0.004 <sup>c,d</sup>	0.046 ± 0.001 <sup>b,c</sup>	0.555 ± 0.045 <sup>a,b</sup>
Peonidin 10	0.036 ± 0.003 <sup>a</sup>	0.050 ± 0.002 <sup>a,b</sup>	0.424 ± 0.071 <sup>a,b</sup>
Malvidin + Peonidin 5	0.021 ± 0.003 <sup>b,c</sup>	0.055 ± 0.002 <sup>a</sup>	0.645 ± 0.075 <sup>a</sup>
Malvidin + Peonidin 10	0.019 ± 0.005 <sup>b,c</sup>	0.040 ± 0.006 <sup>c</sup>	0.511 ± 0.115 <sup>a,b</sup>
<b>ANOVA (p-value)</b>			
Anthocyanin	NS	NS	NS
Dose	NS	NS	NS
Anthocyanin & Dose	0.001	0.01	NS

<sup>1</sup> Values are mean ± SE (n= 6, except peonidin 10, where n=5).

<sup>2</sup> Means within a given column with different superscript letters differ significantly (P<0.05).

**TABLE 3**

Antioxidant enzyme activity in HT-29 cells treated with 0, 2.5, or 5 µg/mL malvidin and/or peonidin.<sup>1,2</sup>

	GR	GPx	GST
	U/mg protein		
Control	0.030 ± 0.003 <sup>b,c</sup>	0.035 ± 0.003 <sup>b,c</sup>	0.335 ± 0.056 <sup>b,c</sup>
Malvidin 2.5	0.028 ± 0.003 <sup>c</sup>	0.030 ± 0.002 <sup>c,d</sup>	0.298 ± 0.049 <sup>b,c</sup>
Malvidin 5	0.028 ± 0.031 <sup>c</sup>	0.031 ± 0.002 <sup>b,c,d</sup>	0.314 ± 0.039 <sup>b,c</sup>
Peonidin 2.5	0.031 ± 0.003 <sup>b,c</sup>	0.029 ± 0.001 <sup>d</sup>	0.243 ± 0.037 <sup>c</sup>
Peonidin 5	0.037 ± 0.002 <sup>b</sup>	0.037 ± 0.002 <sup>a,b</sup>	0.316 ± 0.038 <sup>b,c</sup>
Malvidin + Peonidin 2.5	0.047 ± 0.004 <sup>a</sup>	0.043 ± 0.003 <sup>a</sup>	0.476 ± 0.044 <sup>a</sup>
Malvidin + Peonidin 5	0.027 ± 0.003 <sup>c</sup>	0.034 ± 0.001 <sup>b,c,d</sup>	0.381 ± 0.037 <sup>a,b</sup>
<b>ANOVA (p-value)</b>			
Anthocyanin	0.01	0.01	0.01
Dose	NS	NS	NS
Anthocyanin & Dose	0.0001	0.001	NS

<sup>1</sup> Values are mean ± SE (n= 6).

<sup>2</sup> Means within a given column with different superscript letters differ significantly (P<0.05).



## 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 to increase the activity of antioxidant enzymes *in vitro*. Three antioxidant enzymes of interest, GST, GR, and GPx, have not been sufficiently examined.

#### **Purpose**

The purpose of this research was to study the effect of the blueberry anthocyanins, malvidin and peonidin, on HT-29 adenocarcinoma cell antioxidant enzyme activity. The study examined whether or not the anthocyanins increased the activity of the antioxidant enzymes, GST, GR, and GPx. It attempted to determine at what concentration an increase was displayed and whether combining the anthocyanins would have a synergistic effect or not.

## **Findings**

In this study, we hypothesized that the blueberry anthocyanins, malvidin and peonidin, would increase the activity of the antioxidant enzymes, glutathione-S-transferase, glutathione peroxidase, and glutathione reductase, in human HT-29 adenocarcinoma cells. We anticipated that the single anthocyanins would individually increase the activity of the antioxidant enzymes and that combining the anthocyanins would have a synergistic effect on the enzymes.

Individual anthocyanins have been shown to have an effect on antioxidant enzymes, but there may be unknown factors within the metabolism of the anthocyanins that cause them to have a synergistic effect. Our study found that significant effects from individual anthocyanins, even at low concentrations, were plausible. However, the synergistic effect found between malvidin and peonidin was most notable. It suggests that although individual anthocyanins do have an effect, a combination of anthocyanins or maybe even a whole berry extract, would have a greater effect. And while it is essential to study individual anthocyanins to determine mechanisms of action, studying whole berries is also important because it can be more directly correlated to blueberries and other high anthocyanin foods in the diet.

Also of importance to note, this study did not find the anthocyanin dose to have a consistent effect on enzyme activity. A more consistent dose response was expected. However, this draws attention to the fact that the mechanism of action by which anthocyanins increase enzyme activity is still largely speculative. The various

concentrations could affect the enzymes differently, at one dose having an antioxidant effect, at another dose having a prooxidant effect, and at a third dose have no effect.

Our study demonstrated that malvidin and peonidin do significantly increase antioxidant enzyme activity. The results varied based on anthocyanin type and on anthocyanin and dose together, but not dose alone.

### **Limitations**

While *in vitro* studies are an excellent means for determining mechanisms of action in various processes, they do have some innate limitations. The first is that the growing environment of an incubator, while intended to be similar, is not exactly the same as the environment within the human body. There are many other factors in an *in vivo* model that must be taken into account and cannot be emulated in an *in vitro* model. However, the environment is conducive to cell growth and is at this time the best possible model to be used in an *in vitro* study.

Cell culture studies are also limited in their ability to be applied to broad recommendations. This research cannot be used to extrapolate any conclusions regarding dietary intake of anthocyanins. Results cannot be applied to other cell types, or other anthocyanins. However, these can be used to explain mechanism of action.

Little is known about the form anthocyanins are in once they reach the colon. Therefore, the anthocyanins used here may not be the same as those experienced by the

colon during normal digestion. The concentration of anthocyanins in the colon is also estimated. However, using colon cancer cells and the low anthocyanin concentration makes it much more likely to be a realistic situation. Furthermore, in the diet, blueberries may vary in anthocyanin concentration depending on growing and processing conditions. Also, many other phytochemicals are present, as well as vitamins, that could change the effects of the malvidin and peonidin. This variation could be additive, competitive, or synergistic.

## **Implications**

The results of the study showed mixed findings. The anthocyanins did impact the antioxidant enzyme activity in the cells, although the results were not entirely consistent. The lower doses of anthocyanins tended to show a greater increase in antioxidant activity. This implies that low, physiologically realistic concentrations of anthocyanins are all that is needed to show a significant increase in enzyme activity. Also, the combination of anthocyanins appeared to increase activity more. This is an important point to note since when eaten as whole berries, the anthocyanins will be present as a mixture.

## **Future Research**

As with all research, the results from this study elucidate other questions and areas of research that should be explored in future studies. This study showed that the two anthocyanins examined did impact the antioxidant enzyme activities. Expanding on these results and exploring other anthocyanins and anthocyanin mixtures, or even complete blueberry portions, would be helpful to show an effect that would more closely resemble blueberries in the human diet. Further studies should be done to determine if there is a dose response and at what concentrations. Previous studies have shown prooxidant effects of anthocyanins at high levels. Studies should be conducted using very high levels of anthocyanins to see if there is an inhibitory effect on antioxidant enzyme activity at very high levels. Human and animal in vivo studies that examine these same effects are greatly needed. It is important to see if these enzymes are similarly affected by anthocyanins in the body and whether or not similar results can be replicated in a dietary model and not simply in cell culture. This would allow the findings to be applied to dietary recommendations.

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## APPENDICES

## APPENDIX A

### Anthocyanidin Serial Dilution

#### NOTES:

- Solutions must be tightly covered with foil and lights must remain off during entire procedure to prevent degradation of compounds
- Beakers must be labeled before starting dilution to keep track of every thing.
- Use caution when opening the extract bottle as contents are powder and vacuum sealed. Opening too quickly will pull some of the powder out.
- Add DMSO to anthocyanins first as it will not dissolve in media.
- All steps must be done aseptically, under the hood.

#### PROCEDURE:

- Aseptically transfer 350 mL media into a beaker under the hood
- Make 150 ml of 0.1% dimethylsulfoxide (DMSO) = 0.15ml DMSO and 149.85ml media
- Make anthocyanin mixtures as follows:

#### First Run:

M

Make 10 µg/mL Malvidin

Mix 0.1 ml DMSO + 1 mg Malvidin

Mix 99.9 ml media + 0.1 ml DMSO/Malvidin

Make 5 µg/mL Malvidin

Mix 40 ml of 10 µg/mL Malvidin  
+ 40 ml 0.1 DMSO media

Make 2.5 µg/mL Malvidin

Mix 12.5 ml 5 µg/mL Malvidin  
+ 12.5 ml 0.1 DMSO Media

P

Make 10 µg/mL Peonidin

Mix 0.1 ml DMSO + 1 mg Peonidin

Mix 99.9 ml media + 0.1 ml DMSO/Peonidin

Make 5 µg/mL Peonidin

Mix 40 ml of 10 µg/mL Peonidin  
+ 40 ml 0.1 DMSO media

Make 2.5 µg/mL Peonidin

Mix 12.5 ml 5 µg/mL Peonidin  
+ 12.5 ml 0.1 DMSO Media

M&P

Make 10 µg/mL Malvidin + Peonidin

Mix 22.5 ml of 5 µg/mL Malvidin+ 22.5 ml 5 µg/mL Peonidin

Make 5 µg/mL Malvidin + Peonidin

Mix 22.5 ml 2.5 µg/mL Malvidin + 22.5 ml 2.5 µg/mL Peonidin

Second Run:

M

Make 10 µg/mL Malvidin

Mix 0.1 ml DMSO + 1 mg Malvidin

Mix 99.9 ml media + 0.1 ml DMSO/Malvidin

Make 5 µg/mL Malvidin

Mix 45 ml of 10 µg/mL Malvidin  
+ 45 ml 0.1 DMSO media

Make 2.5 µg/mL Malvidin

Mix 40 ml 5 µg/mL Malvidin  
+ 40 ml 0.1 DMSO Media

P

Make 10 µg/mL Peonidin

Mix 0.1 ml DMSO + 1 mg Peonidin

Mix 99.9 ml media + 0.1 ml DMSO/Peonidin

Make 5 µg/mL Peonidin

Mix 45 ml of 10 µg/mL Peonidin  
+ 45 ml 0.1 DMSO media

Make 2.5 µg/mL Peonidin

Mix 40 ml 5 µg/mL Peonidin  
+ 40 ml 0.1 DMSO Media

Make 5 µg/mL Malvidin + Peonidin

Mix 22.5 ml of 2.5 µg/mL Malvidin+ 22.5 ml 2.5 µg/mL Peonidin

Make 2.5 µg/mL Malvidin + Peonidin

Mix 11.25 ml 2.5 µg/mL Malvidin + 11.25 ml 0.1 DMSO media  
+ 11.25 ml 2.5 µg/mL Peonidin + 11.25 ml 0.1 DMSO media