THE EFFECT OF ANTHOCYANIN ACYLATION ON THE INHIBITION OF
HT-29 COLON CANCER CELL PROLIFERATION.

THESIS

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By

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ABSTRACT

Anthocyanins are the red, purple, and blue pigments responsible for the beautiful color of many fruits and vegetables. Previous research suggests a high correlation between diets rich in brightly colored fruits and vegetables and a lower incidence of certain types of cancers. Acylated anthocyanins have been found to exhibit increased stability in food matrices as compared to non-acylated anthocyanins. With the addition of acylation comes added protection from environmental conditions, but do these acylated anthocyanins also offer important chemoprotection?

The objective of this study was to evaluate the role of acylation on anthocyanin chemoprevention properties. To determine this objective four predominately acylated anthocyanin sources containing the cyanidin aglycone were evaluated: red onion, purple corn, red cabbage, and black carrot. The red onion and purple corn were acylated with aliphatic acids and the red cabbage and black carrot were acylated with cinnamic acids.

Anthocyanins were extracted and purified using acetone, chloroform, methanol and water. The extracts were used to treat human adenocarcinoma cells (HT29 colon cancer cells). Growth inhibition was measured using the sulforhodamine B assay. Chemopreventative properties of the four anthocyanin extracts were measured before and after removal of acylating groups (saponification).
Saponification of anthocyanins decreased or slightly (but not significantly, p>0.05) increased the inhibitory effect of the starting anthocyanin material. Purple corn anthocyanin extract’s percent inhibition values, with and without aliphatic acid acylation for concentrations of 100µg cyanidin-3-glucoside equivalent/ml, were 67.7 percent and 79.7 percent respectively. Red cabbage anthocyanin extract, acylated with cinnamic acids, showed a growth inhibition of 20.4 percent as compared to 24.7 percent (not significantly increased) for the saponified red cabbage at a treatment dose of 100µg cyanidin-3-glucoside equivalent/ml. However, cinnamic acid acylated black carrot showed a growth inhibition of 50.8 percent, significantly higher that the 24.1 percent inhibition obtained with non-acylated black carrot anthocyanins at a treatment dose of 50µg cyanidin-3-glucoside equivalent/ml. These results suggest that glycosylation pattern also plays an important role in anthocyanin chemopreventative activity. The differences in the anthocyanins composition accounted for the differences in chemopreventative properties.

Both the acylated and the non-acylated anthocyanin extracts displayed inhibition properties during the treatment of human adenocarcinoma cells (HT29). However, due to their increased stability, the acylated anthocyanin extracts may be able to impart a higher level of chemoprevention than the non-acylated anthocyanin extracts and also may be more widely used by the food industry in the production of functional foods.
Dedicated to Mom and Dad. I love you both dearly.
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FIELD OF STUDY

Major Field: Food Science and Nutrition
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Anthocyanins are flavonoid compounds responsible for the brightly colored and attractive red, purple and blue pigments of many fruits, vegetables and flowers. Anthocyanins have been found to be powerful antioxidants and may exert chemopreventative properties; therefore, anthocyanins have been the focal point of many recent biological studies. However, the chemical structure of the anthocyanin as it relates to its biological function is currently not well understood.

Anthocyanins are reported to play a significant role in the cancer cell growth inhibitory effect of anthocyanin-rich fruits and vegetables (Jing et al., 2006). Many anthocyanin-rich extracts from berries and vegetables have been reported to slow down the growth of HT-29 colon cancer cells in vitro, each of them to a different extent (Jing et al., 2006; Zhao et al., 2004). In addition, purified acylated anthocyanin fractions from red onion, purple corn, red cabbage, and black carrot, were found to effectively inhibit the growth of the human colon cancer cell line, HT-29. Several in vivo studies have shown that anthocyanin-rich foods and anthocyanin pigments from food inhibited carcinogenesis in the colon (Lala et al., 2006; Aoki et al., 2004; Hagiwara et al., 2001; Harris et al., 2001; Kang et al., 2003).
Both the acylated and the non-acylated anthocyanin extracts displayed inhibition properties during the treatment of human adenocarcinoma cells (HT29). However, due to their increased stability, the acylated anthocyanin extracts may be able to impart a higher level of chemoprevention than the non-acylated anthocyanin extracts and also may be more widely used by the food industry in the production of functional foods.

Our objective was to increase the understanding of the role of the anthocyanin chemical structure on their chemopreventative properties. Anthocyanins were extracted and purified using acetone, chloroform, methanol and water, from four sources: red onion, purple corn, red cabbage and black carrot. All four anthocyanin sources predominately contained the cyanidin aglycone. The red cabbage and black carrot were acylated with cinnamic acids and the red onion and purple corn were acylated with aliphatic acids. The extracts were used to treat human adenocarcinoma cells (HT-29). Growth inhibition was measured using the sulfurhodamine B assay. Chemopreventative properties of the four anthocyanin extracts were measured before and after saponification.
CHAPTER 2

LITERATURE REVIEW

2.1 Polyphenols

Polyphenols are common chemical compounds which contain multiple phenol groups; they can be found in a wide variety of fruits, vegetables and flowers. In nature these compounds are the secondary metabolites of plants and are involved in plant growth and reproduction, provide resistance from pathogens and predators and protect crops from disease and preharvest seed germination (Ross and Kasum, 2002).

Polyphenols play an important functional role in our diet and may impart many health benefits by way of their antioxidant activities; such activities have been suggested to be the mechanisms for inhibitory affects against carcinogenesis (Yang et al., 1998).

There are different classes of polyphenols known as tannins, lignins and flavonoids. Each class of polyphenols possesses chemical characteristics that set them apart from one another. Not only do they carry a multiplicity of phenolic groups, each encompasses a wide range of molecular weights (Haslam et al., 1988). Flavonoids are the most widely occurring polyphenol and are present in almost every form of human
consumed vegetation. Consumers and manufacturers have become interested in flavonoids due to their health promoting chemical activities, especially their potential role in the prevention of carcinogenesis.

### 2.2 Flavonoids

With over 9000 natural compounds identified in nature the flavonoid family is the largest group of polyphenolic compounds (Whiting et al., 2001). All flavonoids share a common skeletal backbone consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C) (Figure 2.1).

![Figure 2.1](image)

**Figure 2.1**: General structure and numbering pattern for common food flavonoids.

Flavonoids have been found to be the most abundant polyphenols in our diets. The biosynthesis of flavonoids is stimulated by sunlight (ultraviolet radiation), so higher concentrations of flavonoids can typically be found in the outer most layers of fruits and vegetables i.e. the skins. Flavonoids can be divided into six subclasses according to the
degree of oxidation of the oxygen heterocycle: flavones, flavonols, isoflavones, anthocyanins, flavanones and flavonols (catechins and anthocyanidins) (Schalbert and Williamson, 2000) (Figure 2.2).

![Chemical structures of the common flavonoid sub-classes](image)

**Figure 2.2:** Chemical structures of the common flavonoid sub-classes (Source: Ross and Kasum 2002).

According to Ross and Kasum, biogenetically, the A-ring arises from a molecule of resorcinol or phloroglucinol synthesized from the acetate pathway and has a characteristic hydroxylation pattern at the 5 and 7 position. The B ring comes from the shikimate pathway and is usually hydroxylated at the 4’, 3’4’, or 3’4’5’ positions. There are many factors which affect the formation of flavonoids in plants including ultraviolet
exposure, plant genetics, surrounding environment, germination, degree of ripeness and food processing and storage. With increased ultraviolet exposure comes increased biosynthesis of specific flavonoids (Vanderauwera et al., 2005). Increased biosynthesis of flavonoids help protect the plant from radiation that can cause DNA damage (Manach et al., 2004). Such plant protection is associated with the antioxidant capabilities of flavonoids.

Flavonoids have been shown to impart protection from oxidizing species, such as the superoxide anion, hydroxyl radical and peroxy radicals; they have also been proven to be quenchers of singlet oxygen (Harborne, J.B. et al., 1998). The antioxidant activity of flavonoids is thought to present many of the health benefits associated with consumption of fruits and vegetables to humans. Due to the increased interest in the health benefits of flavonoids, there have been an increasing number of biological studies in hopes to better understand these powerful antioxidants. In fact, the United States Department of Agriculture (USDA) recently began documenting the flavonoid content of commonly consumed fruits and vegetables (USDA 2.1, 2007).

2.3 Anthocyanins

Anthocyanins are a diverse group of colorful pigments responsible for the beautiful red, purple and blue pigments found in many fruits, vegetables, flowers, leaves and roots. The word anthocyanin was derived from two Greek words: anthos and kyanos, anthos meaning flower and kyanos meaning dark blue (Delgado-Vargas and O., 2003). Anthocyanins are water soluble pigments, which facilitate their incorporation into aqueous food systems, and have been consumed for centuries with no noted adverse
health affects (Giusti and Wrolstad, 2003). To date there are over 600 anthocyanins identified in nature (Anderson and Jordheim, 2006).

In nature anthocyanins are important pathogen and predator protectors to plants. They are also important to plants because their beautiful colors are thought to attract insects and animals for pollination and seed dispersal. Like flavonoids, anthocyanins, protect plants from ultraviolet radiation and the damage it can cause to the plants DNA, proteins and membranes (Vanderauwera et al., 2005). Biosynthesis of anthocyanins increases with increased ultraviolet radiation damage. Therefore, it can be said that with varying ultraviolet exposure anthocyanin concentration greatly varies. Besides their role as secondary metabolites, anthocyanins, are gaining interest as functional compounds for coloring foods, and as potential antioxidants, phytoalexins and antibacterial agents (Kang et al., 2003).

2.3.1 Anthocyanin Chemical Structure

Anthocyanins belong to the group of polyphenolics named flavonoids, which are secondary metabolites synthesized by higher plants. Anthocyanin molecules are very sensitive to their surrounding environment. When anthocyanins are exposed to light, heat, pH and other environmental factors they tend to degrade. In fact, anthocyanin degradation during food processing is a major limiting factor when applying anthocyanins to a food matrix. The aglycones of anthocyanins share a C-6 (A-ring)-C-3 (C-ring)-C-6 (B-ring) carbon skeleton (Harborne 1998). The basic anthocyanin structure (Figure 2.3) consists of the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl
groups. Different combinations of these side groups results in the six anthocyanidin chromophores most commonly found in nature. These anthocyanidin aglycones include cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), malvidin (Mv) and peonidin (Pn) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007). The color of the anthocyanin varies among aglycones (Table 2.1): the more hydroxyl groups the more bluish in color; the more methoxyl groups the more red in color (Heredia, Francia-Aricha et al., 1998; Delgado-Vargas and O., 2003).
Figure 2.3 Basic chemical structure of a common anthocyanidin
(Source: He, 2008)

<table>
<thead>
<tr>
<th>Name</th>
<th>Substitution</th>
<th>R$_1$</th>
<th>Color</th>
<th>λ$_{\text{max}}$ (nm)</th>
<th>Visible Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>Orange</td>
<td>494</td>
<td></td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>Orange-Red</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>OMe</td>
<td>H</td>
<td>Orange-Red</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>Red</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>Petunidin</td>
<td>OMe</td>
<td>OH</td>
<td>Red</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>OMe</td>
<td>OMe</td>
<td>Purple</td>
<td>510</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Differences in chemical structure, color and λ$_{\text{max}}$ of the six anthocyanidins most commonly found in nature (Source: He, 2008).
2.3.2 The Effect of Varying pH on Anthocyanin Chemical Structure

Anthocyanin chemical structures, unlike other flavonoids, can undergo reversible structural changes when exposed to varying pH conditions. According to Brouillard and Delaporte, there are four major anthocyanin forms that exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase and the colorless chalcone. The environmental pH of the anthocyanin has a major effect on the color produced by the anthocyanin pigment. In highly acidic, pH 1.0, environmental conditions an oxonium form of the anthocyanin produces a bright red to purple color, very desirable for natural colorants to be used in a food system. At a pH of 4.5, the anthocyanin is in its hemiketal state and is colorless. At a pH of 7.0 the anthocyanin pigment displays a blue to green color and is in its quinonoidal base form. It is important to note that anthocyanins are most stable in pH 1.0 environments where they provide their maximum coloration. Figure 2.4 shows the structural change of an anthocyanin in varying pH environments.
2.3.3 Glycosylation and Acylation

Stability of anthocyanin molecules is known to be increased by the complex pattern of glycosylation and acylation as well as storage at optimum pH (Chandra et al., 2001; Dao, Takeoka, Edwards, & Berrios, 1998; Giusti & Wrolstad, 2003). The hydroxyl groups of the anthocyanin aglycone may be linked to sugar moieties through glycosidic bonds and further linked to more sugar moieties or acylated with organic acids (cinnamic
and aliphatic acids) through ester bonds. The aglycone form of an anthocyanin is rarely found in nature due to its poor stability. Anthocyanins are commonly found in nature with the attachment of one or more glycosylations, as well as, acylating groups. Due to these glycosylation and acylation groups many anthocyanins, while in plants are resistant to light, pH, heat and oxidation conditions (Manach et al., 2004). With increased glycosylation and acylation anthocyanin pigments can show more resistance to degradation. Both glycosylation and acylation changes the chemical properties of an anthocyanins structure by changing both the molecules size and polarity. Increased glycosylation increases the anthocyanins polarity and water solubility and increased acylation reduces the polarity and water solubility. It has been shown that glycosylation and acylation improves anthocyanin stability by forming an intramolecular H-bonding network within the anthocyanin molecule (Borkowski et al., 2005). Anthocyanins can have many different kinds of sugar and acyl substituents and therefore many derivatives exist in nature.

Glycosydic substitution most commonly occurs at the 3 and 5 positions (Wrolstad et al., 2004). However, in some anthocyanins, there are additional glycosylating groups attached in other positions. The most commonly found sugar moieties attached to an aglycone are glucose and rhamnose, but other glycosylating groups, such as, galactose, xylose, arabinose are found as well (He, 2008). It has been found that the most prevalent anthocyanins in nature are the glucosides of cyanidin, delphinidin and pelargonidin, which are distributed among 80% of pigmented leaves, 69% of fruits and 50% of flowers (Borkowski et al., 2005). Research has shown that aglycones linked with sugar moieties at the 3 and 5 positions have a major impact on the spectral characteristics of the
anthocyanin (Harborne, 1967; Hong and Wrolstad, 1990; Giusti and Wrolstad, 1996), in addition to shifting their color to blue. That same research also reported that glycosylation at the 3 position caused an increased spectral absorbance as compared to glycosylation at the 5 position and both the 3 and 5 positions.

There are also many different types of acylating groups found attached to glycosylated aglycones. Anthocyanins are acylated with two types of acids, cinnamic and/or aliphatic. Cinnamic acids contain an aromatic ring and include $p$-coumaric, caffeic, ferulic, gallic and sinapic acids. Unlike cinnamic acids, aliphatic acids do not contain an aromatic ring; instead they contain straight chains, branched chains or non-aromatic rings, called alicyclic rings. Aliphatic acids include malonic, acetic, malic, succinic and oxalic acids. These acyl groups are commonly bound at the C-3 sugar, esterified to the 6-OH or less frequently at the 4-OH position of the sugars. It should be noted that very complex acylating patterns attached on different sugar moieties have been reported, however these acylation patterns are exceptionally less common in nature. For example, Figure 2.5 shows the very complicated acylation pattern of an anthocyanin found in blue flowers.
Figure 2.5: Complex chemical structure of an acylated anthocyanin (Giusti et al., 1998).

Acylation has also been shown to play an important role in the color of the pigment. Cinnamic acylations are known to cause a bathochromic shift on the wavelength of maximum absorbance, in addition to a hyperchromic effect (von Elbe and Schwartz, 1996; Giusti et al., 1999). It has also been found that acylation improves the color and tinctorial strength, as well as, stability of anthocyanins in environmentally unfavorable conditions, such as pH, heat and light (Andersen et al., 2004). Therefore, acylated anthocyanins are of interest for use as natural colorants for food applications. Examples of fruits and vegetables with a high amount of acylated pigments are as follows: red onion, red cabbage, red potatoes, black carrots, radishes, purple sweet potatoes and grapes. Among these fruits and vegetables it has been found that radishes and red potatoes have excellent color stability and stand out as potential alternatives for use of FC&C Red No. 40 (Giusti and Wrolstad, 2002). Figure 2.6 displays a bathochromic shift and hyperchromic effect.
Anthocyanin structural variation, including glycosylation, hydroxylation and methoxylation of the B-ring, as well as, the presence of acylating groups has been associated with increased color stability (Giusti et al., 1999). Glycosylation is noted for increasing molecular stability due to intramolecular H-bonding network within the sugar moiety and between the sugar moiety and the anthocyanin molecule (Borkowski et al., 2005). Acylation of the sugar substitution also increases the stability of the anthocyanin through intermolecular and intramolecular interactions called copigmentation (Harborne and Williams, 2000). During copigmentation of anthocyanins the charged C-ring can react with nucleophiles as an electrophilic center (Jing, 2006). An illustration of

**Figure 2.6:** Bathochromic and hyperchromic shifts produced by acylation of purple carrot anthocyanins at 520 nm. Cy-3-gal-xyl-glu (---) Cy-3-gal-xyl-glu + p-coumaric acid (—). Bathochromic shift indicates an increase in the visible absorption maximum (nm). Hyperchromic shift indicates an increase in the peak intensity (mAU).
(Source: Giusti and Wallace 2008).
intermolecular and intramolecular stacking is pictured below. Giusti and Wrolstad found that spatial configuration and stacking of acylated anthocyanins provided increased chemical stability. Anthocyanins may be acylated with one or more acylating groups and therefore may include a broad variety.

**Figure 2.7**: Intermolecular and Intramolecular stacking of Acylated Anthocyanins (Giusti and Wrolstad, 2003).

### 2.3.4: Flavonoids and Anthocyanin Distribution in the Human Diet

Flavonoids are the highest consumed polyphenol in the human diet (Manach et al., 2004). Scientists have reported varying data regarding the dietary intake of flavonoids but in 1976 it was reported by Kuhnau that the dietary intake of flavonoids was roughly 1g/day and consisted of the following: 16% flavonols, flavones and flavanones; 17% anthocyanins; 20% catechins; and 45% biflavones. Anthocyanins are an
important component of the flavonoids ingested during the consumption of fruits and vegetables due to their antioxidant affects in animals and humans. Studies conducted on the consumption of anthocyanins in the United States population were estimated to be an intake of 185-215mg per day, which was higher than all other flavonoids (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001).

Anthocyanins are widely distributed in all tissues of edible flowers, plants and tubers. They are produced in high concentrations in fruits and vegetables such as berries, red onion, red radish, purple corn, red cabbage, red potato and purple carrot. Anthocyanin concentration varies among the same species of plants according to environmental factors such as ultraviolet radiation and heat. Increased exposure to ultraviolet radiation allows the plant to produce more anthocyanins as a means to restore cellular function by quenching free radicals produced during cellular stress (Vanderauwera et al., 2005). Prior et al, 1998 found that in the same blueberry species anthocyanin content could range from 300-808 mg/100 mg fresh weight. Maximizing the anthocyanin content of fruits and vegetables could increase the consumption of these healthy antioxidants in the consumer’s diet. Horticulturists have discovered a way to manipulate the DNA of some anthocyanin pigments and thus produce fruits and vegetables with higher quality and quantities of anthocyanins, as well as, more attractive anthocyanin pigments for commercial use (Courtney-Guterson et al., 1994). **Table 2.2** lists the anthocyanin content of some commonly consumed foods.
<table>
<thead>
<tr>
<th>Source</th>
<th>Scientific Name</th>
<th>Anthocyanin mg/100mg</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td><em>Malus pumila</em> P. Mill.</td>
<td>1~17</td>
<td>(Wu et al., 2006)</td>
</tr>
<tr>
<td>Bilberry</td>
<td><em>Vaccinium myrtillus</em> L.</td>
<td>300~808</td>
<td>(Prior et al., 1998; Maatta-Riihinen et al., 2004)</td>
</tr>
<tr>
<td>Blackberry</td>
<td><em>Rubus spp.</em></td>
<td>72~1221</td>
<td>(Clarke et al., 2002)</td>
</tr>
<tr>
<td>Black Raspberry</td>
<td><em>Rubus occidentalis</em> L.</td>
<td>145~607</td>
<td>(McGhie et al., 2002; Moyer et al., 2002)</td>
</tr>
<tr>
<td>Blueberry</td>
<td><em>Vaccinium corymbosum</em> L.</td>
<td>63~430</td>
<td>(Prior et al., 1998; Moyer et al., 2002)</td>
</tr>
<tr>
<td>Cranberry</td>
<td><em>Vaccinium macrocarpon</em> Aiton</td>
<td>20~360</td>
<td>(Prior et al., 2001; Wang and Strech, 2001)</td>
</tr>
<tr>
<td>Grape</td>
<td><em>Vitis vinifera</em></td>
<td>27~120</td>
<td>(Wu et al., 2006)</td>
</tr>
<tr>
<td>Strawberry</td>
<td><em>Fragaria ananassa</em> D.</td>
<td>13~55</td>
<td>(Cordenunsi et al., 2002)</td>
</tr>
<tr>
<td>Red Onion</td>
<td><em>Allium cepa</em> L.</td>
<td>~49</td>
<td>(Wu et al., 2006)</td>
</tr>
<tr>
<td>Purple Carrot</td>
<td><em>Daucus carota</em></td>
<td>38~98</td>
<td>(Lazcano et al., 2001)</td>
</tr>
<tr>
<td>Red Radish</td>
<td><em>Raphanus sativus</em> L.</td>
<td>11~60</td>
<td>(Giusti et al., 1998)</td>
</tr>
<tr>
<td>Purple Corn</td>
<td><em>Zea mays</em> L.</td>
<td>~1640</td>
<td>(Cevallos-Casals and Cisneros-Zevallos, 2003)</td>
</tr>
<tr>
<td>Red Cabbage</td>
<td><em>Brassica oleracea</em> L.</td>
<td>~322</td>
<td>(Wu et al., 2006)</td>
</tr>
</tbody>
</table>

Table 2.2: Anthocyanin Content (mg/100mg fresh weight) of Commonly Consumed Fruits and Vegetables (adapted from Jing, 2006).

2.3.5: Bioavailability and Metabolism of Anthocyanins

The functions of anthocyanins are well understood in plants; however, there is much to be learned about the metabolism and bioavailability of anthocyanins in the human body. Diets high in fruits and vegetables are associated with decreased risk of cardiovascular disease, the formation of certain cancers, such as colon cancer and obesity (Hung et al., 2004 and Tsuda et al., 2003). Fruits and vegetables are high in essential vitamins and minerals that may help enhance health benefits. The Joint Expert
Committee on Food Additives (JECFA) approved an acceptable daily intake (ADI) for anthocyanins of 150-175 mg per day for an adult weight between 60-70 kg (Clifford, 2000). There are currently no upper limits for the daily intake of anthocyanins. The research of Prior 2001, found that the consumption of anthocyanins in the United States varied according daily diet patterns.

Anthocyanins have been shown to occur in the plasma and urine in small amounts after large ingestions of anthocyanin-rich berries (He, J., 2008). Anthocyanin-rich berries have been shown to have more of an inhibitory effect on tumorigenesis in the gastrointestinal tract as compared to other organs according to animal studies (Carlton et al., 2000; Carlton et al., 2001; Kresty et al., 2001; Casto et al., 2002). Individual anthocyanin concentration of different berry extracts where different in urine, suggesting that absorption of anthocyanins depended on the chemical structure of the molecule (McGhie et al., 2003). Anthocyanins have shown decreased uptake into the tissue when heavily glycosylated and acylated. It is thought that smaller anthocyanin molecules are more easily up taken and absorbed into the surrounding gastrointestinal tissue than the larger glycosylated and acylated anthocyanins. This indicates that glycosylated and acylated anthocyanins have lower bioavailability and in turn low bioactivity (Jing, 2006).

The complete understanding of why anthocyanins have such low bioavailabilities is not completely understood at the present time. However, a couple theories have been suggested. First, it is thought that anthocyanins are not able to be hydrolyzed by β-glucosidase in the gastrointestinal tracts, which in turn results in lowered absorption into the plasma (Nemeth et al., 2003). Secondly, it is thought that the varying pH conditions of the gastrointestinal tract affect the stability of the anthocyanins upon consumption. In
addition to these theories, it has been speculated that the surrounding food matrix can affect anthocyanin stability after consumption. For example proteins are thought to enhance the anthocyanin stability in vivo (Jing, 2006).

2.3.6: Anthocyanin Limitations

The structural instability of anthocyanins presents an intrinsic limitation when studying the biological activity of anthocyanins in vitro and in vivo. Introducing anthocyanin rich extracts into the cell culture environment may lead to rapid degradation (Wu et al., 2006b; Kern et al., 2007). Spontaneous and irreversible degradation may occur with the formation of aldehyde and phenolic acid derivatives at neutral and alkaline conditions (pH = 7-8) (Fleschhut et al, 2006; McGhie et al, 2007). Wu and others reported that anthocyanins from various berry extracts degraded at different rates in Delbecco’s Modified Eagle’s Media (DMEM) with HEPES buffer. Half-lives ranged from less than one hour for delphinidin to about nine hours for cyaniding-3-sambubioside-5-glucoside. In general, glycosylated anthocyanins were more stable than mono-glycosylated anthocyanins (Wu et al., 2006b). Furthermore, Kern and others (2007) assessed stability of purified anthocyanidins in DMEM containing 10% fetal calf-serum. The anthocyanidins all degraded significantly within the first 30min. They also showed that delphinidin was less stable than all other anthocyanidins. The most stable anthocyanidins were peonidin and pelargonidin, but more than 85% of these were degraded at 3 hours.
2.4 Colon Cancer

2.4.1 The Prevalence of Colon Cancer

More than 20% of death in the United States is caused by cancer (Smith, Marks and Lieberman, 2005). Colorectal cancer is the cancer of the colon or rectum. Colon cancer is cancer that forms in the tissue of the colon, the longest section of the large intestine. The majority of colon cancer arises from preexisting adenomas (Toribara and Sleisenger, 1995). Adenocarcinoma cells are cells that produce and excrete mucus and other fluids. Rectal cancer is cancer that forms in the tissue of the rectum, the last several inches of the large intestine closest to the anus. In 2004 colorectal cancer was ranked the third most prevalent cancer in both men and women (American Cancer Society). It was reported that more than 145,083 people were diagnosed with colorectal cancer and 53,580 people died from colorectal cancer in 2004 (American Cancer Society). Colorectal cancer affects both men and women and all races, however, men, African Americans and Alaskan Natives have an exceptionally high colorectal cancer incidence and mortality rate (American Cancer Society).

The treatment options for colorectal cancer depend on the stage of the cancer. According to the Food and Drug Administration (FDA) there are five stages of colorectal cancer: stage 0 (Carcinoma in situ), stage I colon cancer, stage II colon cancer, stage III colon cancer and stage IV and recurrent colon cancer. Pictured below in Figure 2.8 are the different stages of colon cancer.
2.4.2 Five Stages of Colorectal Cancer

According to the American Cancer Society there are five stages of colorectal cancer: Stage 0 (carcinoma in situ) refers to abnormal cells that have been found in the inner most tissue of the colon or rectum and have the potential to spread to other areas of the colon or rectum. This stage of colon or rectum cancer is also known as Dukes A Colorectal Cancer. Stage 0 colon cancer is usually treated by localized surgery.

Stage I colon cancer indicates that cancer cells have spread beyond the first layer of the colon or rectum into the second and third layers, but has not yet spread to the outside wall of the colon or rectum. Treatment for stage I colon cancer is surgical.
procedure known as a resection or anastomosis which removes the diseased section of the colon or rectum and connects healthy sections together.

Stage II colon cancer indicates that cancer cells have spread outside the colon or rectum to the close by tissue but has not yet spread to the lymph nodes. This type of colon or rectum cancer is also known as Dukes B Colorectal Cancer. Stage II colon cancer is also treated with a resection or anastomosis to remove the diseased section of the colon or rectum and connect healthy sections together.

Stage III colon cancer is a more severe stage of colon cancer. It indicates that tumor cells have moved to lymph nodes and organs near the colon or rectum. This stage of colon cancer is also known as Dukes C Colorectal Cancer. Treatment for stage III colon cancer includes a resection/anastomosis with chemotherapy and/or radiation therapy. Chemotherapy is a procedure which attempts to kill the colon or rectum cancer by the use of drugs. Radiation therapy is the use of high energy radiation from x-rays, gamma rays, neutrons, protons and other sources to kill cancer cells and shrink tumors.

The final stage of colorectal cancer is stage IV and recurrent colon cancer. Stage IV colon cancer indicates that the cancer has spread to nearby lymph nodes, as well as, to other organs in the body such as the liver. Recurrent colon cancer means that the colon or rectum cancer has returned from previous treatment. The cancer may or may not return to its previously occurring location. Stage IV and recurrent colon cancer is also known as Dukes D Colorectal Cancer. Treatment for stage IV and recurrent colon cancer includes: resection/anastomosis surgery, chemotherapy, radiation therapy, surgery to remove parts of other organs where cancer might have spread, clinical trials, biological
therapy and some patients may even be offered palliative therapy to improve their quality of life and relieve any discomfort they may be experiencing.

2.4.3 Risk Factors Related to Developing Colon Cancer

There are many risk factors which increase the chance of developing colorectal cancer such as, age, sex, race, obesity due to lack of exercise, consuming alcohol and smoking. Some of these risk factors including age, being a male and race are uncontrollable but others can be controlled. Certain actions can be taken to decrease the risk of developing colorectal cancer. Such actions include eating a well rounded diet with an abundance of fruits and vegetables, exercising regularly as to avoid becoming over weight and obese, limiting alcoholic beverage consumption and quitting smoking.

There are also several preventative measures that can be taken to help avoid the development of colorectal cancer. Recent research has shown that hormone replacement therapy of both estrogen and progesterone has decreased the risk of colorectal cancer in postmenopausal women (American Cancer Society). Men and women over the age of 50 are advised to have a colonoscopy to examine the inside of the rectum and colon for the early signs of colorectal cancer such as, polyps and other abnormal cell proliferation. Most colorectal polyps are adenomas which may eventually become cancer, so removing these polyps in the early stages may reduce the risk of developing colon cancer.

2.4.4 Strategies for Chemoprevention:

Chemoprevention is a process by which intervention steps are performed within the premalignant stages of cancer with specific chemical agents to reverse carcinogenesis
and prevent the development of invasive cancer (Lippman et al., 1993). Multistep carcinogenesis and field carcinogenesis are the two biological concepts for chemoprevention. Epidemiological studies strongly suggest a correlation between human diet and the prevention of carcinogenesis in humans. The fundamental understanding of multistep carcinogenesis is that preinvasive carcinogenesis will be reversible in its earlier stages. Field carcinogenesis is described by Boone and Lippman as the effect a carcinogen has on multiple and diffused primary premalignant foci, such as, the effect of the exposure to tobacco smoke has on the entire upper aerodigestive tract and lungs. Chemoprevention is thought to play a crucial role in controlling cancer initiated in this manner.

Cancer chemoprevention involves pharmacolonic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis (Singletary, 2000). However, diets rich in fruits and vegetables shows considerable scientific evidence, both epidemiologic and experimental, indicating that modifications in lifestyle and diet can show major reduction in the risk of developing numerous cancers (Martinez and Giovanucci, 1997). Generally speaking, cancer chemoprevention aims to inhibit or reverse carcinogenesis or to prevent the development of invasive cancer.

2.4.5: Chemoprevention with Anthocyanins

Multiple research trials demonstrate that anthocyanins have the ability to prevent cancer cell proliferation or carcinogenesis and interests in anthocyanins greatly intensified after the recognition of their potential health benefits (Schalbert and
Williamson 2000). Since this discovery many research experiments have been done on the biological effects of anthocyanins using *in vivo* and *in vitro* models.

Carcinogenesis is abnormal uncontrolled cell growth; it occurs in a multistage process involving initiation, promotion and progression (Hong and Sporn, 1997). Anti-cancer activities of anthocyanins have been established largely on *in vitro* evidence. Fruits and vegetables with high concentrations of anthocyanin content have been shown to reduce the risk of many cancers, including colon cancer (Zhao et al., 2004). There are several mechanisms for anti-carcinogenesis activities of anthocyanins including: anti-inflammatory effect, antioxidant activity, antiproliferation and detoxification activity.

Anthocyanin compounds have been shown to be stable in the acidic stomach region of the gastrointestinal tract; however these compounds begin to degrade in the more neutral pH conditions of the small and large intestines (Perez-Vicente et al., 2002; McDougall et al., 2005a, 2005b). In the gastrointestinal tract anthocyanins are in direct contact with the epithelial layer and largely available. There are two suggested mechanisms for the development of colon cancer. The first suggests that the cancer is initiated by a local irritation that produces an inflammatory response and the second suggests that the cancer is initiated by an electrolyte imbalance (Bruce et al., 2000). In both cases there is a problem with the epithelial barrier and lead to elevated ROS and COX-2 levels in the epithelial cells (He, 2008). Anthocyanins are powerful antioxidants and selective COX-2 inhibitors (He, 2008) and are thought to help these imbalances return to normal.
2.5 Cellular Models as a Mechanism to Study Colon Cell Carcinoma:

The use of human cell lines to study the inhibitory affects of anthocyanins against carcinogenesis is advantageous because they allow for complex systems to be studied in a rather simplistic model. It is well accepted that any model cancer system is an inadequate representation for the real human disease; however, the degree of which the system mimics the real disease is an important determinant of its usefulness (Brattain et al., 1981). It is important to understand that different colon cancer cell models reflect the tumor of which it came from to a different degree. There are several human colon cancer cell lines available as colon cancer cell models, including: Caco-2, HCT-116, SW-480 and HT-29).

The research of Chantret and others determined that Caco-2 cells have been shown to undergo spontaneously an enterocytic differentiation which is characterized by a polarization of the cell layer. Both HCT-116 and SW-480 cell line have been shown to organize into multilayers without showing epithelial polarity or enterocytic differentiation (Chantret et al., 1988). The HT-29 cell line has the ability to undergo differentiation and is currently the only cell line of intestinal origin that can be induced to display structural and functional features of well-differentiated intestinal cells when grown in a glucose free environment (Huet et al., 1987).

For the purpose of this study HT-29 colon cancer cells will be the focus of this discussion. Human colorectal cancer cells are of interest for this research because, unlike other organs, the cells lining the gastrointestinal tract are affected by compounds in direct contact not necessarily by compounds which are absorbed. HT-29 cells are derived from human colorectal carcinoma cells; they are widely used as a simple and rapid *in vitro*
screening method to determine chemopreventative compounds (Hirsh et al., 2000; Miranda et al. 2002).

2.6 Sulforhodamine-B Colorimetric Assay

The Sulforhodamine-B colorimetric assay is used for cell proliferation and chemosensitivity testing and substitutes for the older tetrazolium-based assays (Papazisis et al., 1997). The MTT colorimetric assay was the first of the tetrazolium-based assays to be developed, others included: MTS, XTT and WST-1. The tetrazolium-based assays where replaced by the sulfurhodamine-B colorimetric assay due to it having a number of advantages, including: better linearity, higher sensitivity, a stable end-point that doesn’t require time-sensitive measurement and lower cost (Perez et al., 2002). The sulfurhodamine-B colorimetric assays dye binds to the amino acids of cellular proteins and through colorimetric evaluation an estimation of the cell proliferation can be made as it relates to total protein mass.

2.7 Present study

The present study was designed to examine the chemopreventative affects of four anthocyanin samples: red onion, red cabbage, purple corn and black carrot on the HT-29 colorectal cancer cell line. Our objective was to increase the understanding of the role of the anthocyanin chemical structure on their chemopreventative properties. Anthocyanins were extracted and purified using acetone, chloroform, methanol and water, from four sources: red onion, purple corn, red cabbage and black carrot. All four anthocyanin sources predominately contained the cyanidin aglycone. The red cabbage and black carrot
were acylated with cinnamic acids and the red onion and purple corn were acylated with aliphatic acids. The extracts were used to treat human adenocarcinoma cells (HT-29). Growth inhibition was measured using the sulfurdhodamine B assay. Chemopreventative properties of the four anthocyanin extracts were measured before and after saponification.

Aim 1: Characterize anthocyanin rich extracts from red onion, purple corn, red cabbage and black carrot before and after saponification.

Aim 2: Measure the monomeric, polymeric and total phenolic content of the anthocyanin rich extracts from red onion, purple corn, red cabbage and black carrot before and after saponification.

Aim 3: Determine the ability of anthocyanin rich extracts from red onion, purple corn, red cabbage and black carrot to inhibit proliferation in HT-29 colorectal cancer cell lines.
CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

Red onions (U.S. No. 1) were purchased from a local grocery store in Columbus, Ohio. The red onions were fully ripe showing a deep intense purple coloration. Commercially standardized purple corn, 7.5% (Artemis International, Inc., Fort Wayne, IN) in powder form, as well as, commercially available crude extracts of red cabbage (Food Ingredient Solutions, Llc., Blauvelt, NY) in liquid form and black carrot (Artemis International, Inc., Fort Wayne, IN) in liquid form were donated by their manufacturers. Certified ACS grade HCl (12N), LCMS grade 99.9% methanol and 99.9% acetonitrile, as well as, certified ACS grade ethyl acetate and potassium hydroxide, and HPLC grade 88% formic acid, 99.9% acetone, and 99.9% chloroform were purchased from Fisher Scientific (Fair Lawn, NJ). Reagent grade gallic acid and Folin-Ciocalteau reagent were purchased from MP Biomedical (Aurora, OH). HT-29 colon cancer cells were purchased from American Type Culture Collection. Sterile McCoy’s 5A Medium with L-glutamine (Fisher Scientific, Florence, KY), fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA), Delbucco’s Phosphate Buffered Saline solution (DPBS) (Lonza, Walkersville, MD), and 2.5% Trypsin (Lonza, Walkerville, MD) were purchased from
their manufacturers. All other chemicals were certified ACS grade from Fisher Scientific.

3.2 Methodology

3.2.1 Extraction of Anthocyanins and other Phenolics from Plant Materials:

The skins of each layer of the red onions were peeled and quickly frozen using liquid nitrogen. The frozen red onion skins were then finely powdered using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT) with a 500 mL stainless steel container (Giusti and Rodriguez-Soana et al., 1999) in the presence of liquid nitrogen. Red onion skin powder was stored at -18°C until used for sample extraction.

Red onion skin anthocyanins were extracted by blending the powders with 30 mL of 70% acetone and 30% 0.1% HCl acidified water (v / v) for 5 minutes using a Waring Commercial Laboratory Blender (Waring Laboratories, Torrington, CT). The liquid was then passed through Whatman No. 4 filter paper (Whatman Inc., Florham, NJ) using a Buchner Funnel (Fisher Scientific, Fair Lawn, NJ). This process was repeated until the filtrate was clear and all of the pigment had been extracted from the powdered red onion skin particles (Rodriguez-Soana & Wrolstad, 2001). The anthocyanin solution was then placed into a separatory funnel (Fischer Scientific, Fair Lawn, NJ) and partitioned with 2 volumes of chloroform. The solution was mixed and left to stand over night at 4°C to ensure adequate separation. The top acetone/water layer containing anthocyanins and other phenolics was collected and residual acetone in the sample was evaporated using a Buchi rotary evaporator (Fisher Scientific, Fair Lawn, NJ). The solution was brought to
25 mL in a volumetric flask using LCMS grade water. Commercially standardized purple corn, 7.5% (Artemis International, Inc., Fort Wayne, IN) in powder form, as well as, commercially available crude extracts of red cabbage (Food Ingredient Solutions, Llc., Blauvelt, NY) in liquid form and black carrot (Artemis International, Inc., Fort Wayne, IN) in liquid form were diluted in acidified water (0.01% HCl).

3.2.2 Fractionation and Concentration of Acylated Anthocyanin Samples:

Anthocyanin phenolics from red onion, red cabbage, purple corn and black carrot extracts were concentrated by using Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corp., Milford, MA). About 0.5 g of each commercial anthocyanin extract was dissolved in 25 mL of HPLC grade water with 0.01% HCl, sonicated for 10 minutes and filtered by use of a Whatman No. 1 filter (Whatman Inc., Florham, NJ) on a Buchner Funnel (Fisher Scientific, Fair Lawn, NJ). The anthocyanin filtrates were passed through the Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corp., Milford, MA) 10 mL at a time. Anthocyanins and other phenolics were bound to the C_{18} cartridge, while sugars and other polar compounds were removed with 30 mL of 0.01% HCl acidified water, followed by 10 mL of hexane to remove the residual water. The non-anthocyanin phenolics were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin phenolic fraction was eluted with 30 mL of 0.01% HCl acidified methanol followed by 10 mL of 0.01% HCl acidified HPLC grade water. Excess methanol in the anthocyanin phenolic filtrate was evaporated in a Buchii rotovapor at 40°C and the anthocyanin fraction was redissolved in 0.01% HCl acidified HPLC grade water.
Red onion and red cabbage anthocyanins contain more than 90% acylated anthocyanin pigments. However, purple corn and black carrot acylated anthocyanins were concentrated to achieve >90% acylation by removing non-acylated anthocyanins present. For this purpose the Sep-Pak® C18 Vac solid cartridge (20cc, 5g sorbent; Waters Corp., Milford, MA) procedure described above was used, with one additional step. After removal of non-anthocyanin phenolics with ethyl acetate, a mixture of water and acetonitrile was used to remove the more polar non-acylated pigments (13% acetonitrile for purple corn or 15% acetonitrile for black carrot). The acylated anthocyanin extract was then eluted with 20 mL of 0.01% HCl acidified HPLC grade methanol followed by 0.01% HCl acidified HPLC grade water. Methanol was evaporated in a Buchi rotovapor at 40°C and the anthocyanins were then redissoved in 0.01% HCl acidified HPLC grade water and brought to 10 mL. The acylated anthocyanin extract was then frozen using liquid nitrogen followed by lyophilization (Labconco 4.5, Kansas City, MO).

### 3.2.3 Alkaline Hydrolysis of Anthocyanin Samples:

Anthocyanin samples were saponified in a screw-cap test tube with 10 mL of 20% aqueous KOH for 8 min at room temperature in the dark (Durst and Wrolstad, 2001; Giusti et al., 1999). The anthocyanin / KOH solution was neutralized and acidified by the addition of about 20 mL of HCl (2 N) when a bright red coloration was achieved. The anthocyanin fraction was then applied to an activated C-18 Sep-Pak 5g cartridge (Waters Corp., Milford, MA) where they were: rinsed twice with 5 mL of 0.01% HCl acidified water, rinsed with 10 mL of ethyl acetate to remove liberated acid groups, rinsed with
0.01% HCl acidified methanol to collect the purified anthocyanin fraction, and then roto evaporated to remove acidified methanol (Giusti et al., 1999).

3.2.4 Monomeric and Polymeric Analysis:

Monomeric anthocyanin content was determined using the pH differential method as described in detail by Giusti and Wrolstad, 2001. This method uses the spectral difference that anthocyanins exhibit at different pH values. Dilution factor was determined by diluting each extract in 0.025 M potassium chloride buffer, pH 1.0, until the absorbance was within the appropriate spectrophotometer range (for most spectrophotometers absorbance should be less than 1.2). Each anthocyanin extract was then appropriately diluted using pH 1.0 and pH 4.5 buffer. Solutions were allowed to sit at room temperature for at least 15 minutes in the dark. Using a UV-Visible Spectrophotometer 2450 (Shimadzu, Columbia, MD) absorbance were read at 520 nm and 700 nm with 1 cm pathlength disposable cells. Monomeric anthocyanin content was calculated using the following equation:

\[
\text{Anthocyanin} = \left[ (A_{520\text{nm}} - A_{700\text{nm}}}_{\text{pH1.0}} - (A_{520\text{nm}} - A_{700\text{nm}}}_{\text{pH4.5}} \times \text{DF} \times 1000 \times \text{MW}) / \varepsilon \right]
\]

Where DF is the dilution factor; MW is the molecular weigh; and \( \varepsilon \) is the molar absorptivity coefficient. MW and \( \varepsilon \) are calculated as cyanidin-3-glucoside, where MW = 449.2 and \( \varepsilon = 26,900 \text{ cm}^{-1} \text{ mg}^{-1} \). Absorbance was determined in replicates of three for each sample.
Polymeric anthocyanin content was determined using the polymeric method described by Wrolstad, 1999. Appropriate dilutions for each sample was determined using pH 1.0 buffer and then 1-cm cuvettes were prepared with 2.8 mL of diluted sample in water to each of two cuvettes. Next, 0.2 mL of water was added to one of the cuvettes and 0.2 mL of bisulfite solution to the other and allowed to equilibrate for 15 minutes before reading at 420nm, 520nm and 700nm. Polymeric anthocyanin content was calculated using the following equations:

\[
\text{Color Density} = [(A_{420\text{ nm}} - A_{700\text{ nm}}) + (A_{\lambda \text{ vis max}} - A_{700\text{ nm}})] \times DF
\]

\[
\text{Polymeric Color} = [(A_{420\text{ nm}} - A_{700\text{ nm}}) + (A_{\lambda \text{ vis max}} - A_{700\text{ nm}})] \times DF
\]

\[
\text{Percent Polymeric Color} = \frac{(\text{Polymeric Color} / \text{Color Density}) \times 100}{0}
\]

Where DF is the dilution factor and absorbance was determined in replicates of three.

3.2.5 Total Phenolics:

Total phenolics were measured using a microscale protocol for Folin-Ciocalteu Colorimetry (Singleton and Rossi, 1965). 20µl of sample, a gallic acid calibration standard, a blank were placed into separate 1-cm, 2-ml plastic cuvettes. Then, 1.58 ml of water, followed by 100 µl of Folin-Ciocalteu reagent were added to each cuvette and thoroughly mixed and incubated for 1-8 minutes. Next, 300 µl of sodium carbonate solution was added and mixed and allowed to incubate for 2 hours at room temperature. Absorbance of samples and standards at 765 nm were measured on the UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Finally, total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve.
3.2.6 HPLC-MS Analysis of Samples:

Samples were analyzed using a Shimadzu high performance liquid chromatography (HPLC) (Shimadzu Scientific Instruments, Inc., Columbia, MD) system equipped with LC-20AD pumps and SIL-20AC autosampler coupled with an SPD-M20A photodiode array (PDA) detector and a single quadrupole electron spray ionization (ESI) Mass Spectrometer (MS) detector. LCMS Solutions Software (Version 3, Shimadzu, Columbia, MD) was used to analyze and graphically represent the analyzed data. A reverse phase Symmetry C-18 column with a 3.5 micron size and a 4.6 x 150 mm column (Waters Corp. MA, USA) and a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp. MA, USA) were used. Solvents and samples were filtered through 0.45µm GE Magna nylon membrane filter (Fisher Scientific, Fair Lawn, NJ). Separation of all samples was achieved using a linear gradient from 5% to 20% B, 25 min; 20% to 25% B, 15 min; 25% to 25% B, 10 min. Solvent A was 4.5% (v / v) formic acid in water and B was 100% acetonitrile. The flow rate was set at 0.8 mL / min and an injection volume of 50µL was used. Spectral data of the anthocyanins was collected between 250-700 nm.

A 0.25 mL / min flow was diverted to the mass spectrometer. Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu, Columbia, MD, USA). Mass spectrometry analysis were performed under positive ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200°C; focus lens, -2.5 V; entrance lens, -50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu / sec. A full scan (Total Ion Count, TIC) was performed with a
mass range from 200-1500 \( m/z \) and Selective Ion Monitoring (SIM) was used to search for the molecular ions of the common anthocyanins throughout the analysis.

3.2.7 HT-29 Colon Cancer Cell Line:

The HT-29 colon cancer cell line derived from human colorectal adenocarcinoma cells (HTB 38; American Type Culture Collection, VA, USA) were grown at 37°C in a Nuaire\textsuperscript{TM} IR Autoflow CO\textsubscript{2} Water-Jacketed Incubator at a modified atmosphere (5% CO\textsubscript{2}, 95% O\textsubscript{2}) in McCoy’s 5A Medium with L-glutamine (Fisher Scientific, Florence, KY) and supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA). Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco’s Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of HT-29 cell line was determined by microscopic examination. Cell cultures were maintained in 75 cm\textsuperscript{2} Flasks with canted necks (Fisher Scientific, Florence, KY). All cell culture experiments were performed with the initial 20 passages.

3.2.8 Determining HT-29 Cell Concentration using a Hemocytometer:

HT-29 colon cancer cells were seeded at a volume of 1 x 10\textsuperscript{4} per well in a 96 well plate. Suspended cells were counted using a Nikon Eclipse 50i Brightfield Microscope and a Reichert Bright-Line hemocytometer (Hausser Scientific; Horsham, PA) to estimate total viable cell concentration. Using the 10x lens of the microscope, cells were counted on 5 primary squares of the hemocytometer using a hand counter. To calculate HT-29 cell concentration the following formula was used:
Cells/mL = (Total # cells counted)/(Number of primary squares counted) x 1 x 10^4

HT-29 colon cancer cells were seeded at 10^4 cells/ml and allowed to grow for 24 hours. At 24 hours the HT-29 colon cancer cells were treated anthocyanin rich extract from red onion, saponified onion, red cabbage, saponified red cabbage, black carrot, saponified black carrot, purple corn and saponified purple corn (100µg, 50 µg, 25 µg and 12.5 µg cyanidin-3-glucoside equivalents/mL of media) for 48 hours.

3.2.9 Anthocyanin Treatments:

Monomeric anthocyanin content was determined for each anthocyanin sample using the detailed method explained by Giusti and Wrolstad, 2001. According to the monomeric anthocyanin content, 100µg ACN / 100g of dry weight for each sample was determined and a corresponding treatment value was given in grams of sample weight.

3.2.10 Sulforhodamine-B Assay:

HT-29 colon cancer cell media was removed and cells were fixed by addition of 150 µL of 50% trichloroacetic acid (TCA) at 4°C for 1 hour. TCA was removed and wells were washed with tap water 5 times and allowed to air dry at room temperature. 75 µL of Sulforhodamine-B (0.4% in water containing 0.01% acetic acid) was added to each well and allowed to stain cells at room temperature for 30 minutes. Each well was washed with 1% acetic acid 5 times to remove any residual unbound dye and dried at room temperature. The protein bound dye was solubilized with 150 µL of 10 mM Tris
base for 5 minutes at room temperature on a plate shaker. The absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). KC4 version 3.03 with PowerReports™ Bio-Tek® software was used to analyze the data.

3.2.11 Cell Growth Inhibition:

HT-29 colon cancer cells were plated at 10,000 cells/well in 96 well cell culture flat bottom plates (Fisher Scientific, Florence, KY) using McCoy’s 5A Medium with L-glutamine (Fisher Scientific, Florence, KY) containing 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA). Cells were allowed to grow for 24 hours to attain log phase growth at the time of sample addition (time 0). Semi-purified samples were compared for their inhibitory effects at concentrations of 100, 50, 25 and 12.5 µg/mL of growth media. HT-29 colon cancer cell growth inhibition was determined after 48 hours of incubation at the different anthocyanin concentrations by using the sulforhodamine B assay (SRB) at 490 nm. Each treatment had 4 replicates and every treatment was repeated at least 4 times. The percentage growth inhibition is calculated as:

\[
\% \text{ Growth Inhibition} = 100 - \frac{(T_{\text{trt}} - T_0) \times 100}{(T_{\text{ctr}} - T_0)}
\]

Where \( T_0 \): Time zero. The absorbance of sample at 490 nm after first 24 hour incubation period prior to anthocyanin treatment. \( T_{\text{trt}} \): Absorbance of sample at 490 nm after anthocyanin treatment. \( T_{\text{ctr}} \): Absorbance of sample at 490 nm after total incubation (72 hours) without anthocyanin treatment.
3.2.12 Statistical Analysis:

The percentage of cell growth inhibition was subjected to Analysis of Variance (ANOVA) with Tukey’s post-hoc comparisons (JMP.8 software) and an alpha level of $p<0.05$ acceptance of the null hypothesis was used to determine significant variables.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of Anthocyanin Samples:

Red onion anthocyanins were identified by comparing retention times order of elution and UV-visible spectra to previous literature (Wu and Prior., 2005). Eight major anthocyanins identified in red onion (Figure 4.1 and Table 4.1) include: peak 1 cyanidin-3-glucoside (~4%), peak 2 cyanidin-3-laminaribioside (~4%), peak 3 cyanidin-3-(3-malonylglicoside) (~1.7%), peak 4 peonidin-3-glucoside (<1%), peak 5 cyanidin-3-(6-malonyl-glucoside) (~63.3%), peak 6 cyanidin-3-(6-malonyl-laminaribioside) (~22.6%), peak 7 peonidin-3-(malonyl) glucoside (~2.7%), and peak 8 cyanidin-3-(malonyl)(acetoxyl)-glucoside (< 1%). Acylated anthocyanins comprised approximately 91% of total red onion anthocyanins. The chromatogram at 280 nm revealed the presence of a variety of phenolic compounds, in addition to the anthocyanins (Figure 4.1). This is an indication that non anthocyanin phenolic compounds were not removed by the fractionation procedure used with a C-18 cartridge and ethyl acetate. Previous reports that show that the ethyl acetate fractionation procedure has some limitations and is not effective with all plant materials (He, 2008). Red onion anthocyanins were
saponified to remove the acylating groups. Three major anthocyanins were identified in saponified red onion (Figure 4.2 and Table 4.2): peak 1 cyanidin-3-glucoside (~68.4%), peak 2 cyanidin-3-laminaribioside (~27.5%), and peak 3 peonidin-3-glucoside (~4.1%). Acylating acids were successfully removed through saponification and no acylated anthocyanins were detected by HPLC / PDA. Similar to the starting material, other phenolics materials were present in significant amounts, as seen in the 280 nm chromatogram, (Figure 4.2) of saponified red onion.
Figure 4.1: Chromatogram of fractionated red onion extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-laminaribioside, peak 3 cyanidin-3-(3-malonylglucoside), peak 4 peonidin-3-glucoside, peak 5 cyanidin-3-(6-malonyl-glucoside), peak 6 cyanidin-3-(6-malonyl-laminaribioside), peak 7 peonidin-3-(malonyl) glucoside, and peak 8 cyanidin-3-(malonyl)(acetoxyl)-glucoside. The phenolic profile for red onion was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area @ 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin-3-glucoside</td>
<td>9.4</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>cyanidin-3-laminaribioside</td>
<td>10.4</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>cyanidin-3-(3-malonylglucoside)</td>
<td>12.9</td>
<td>1.7</td>
</tr>
<tr>
<td>4</td>
<td>peonidin-3-glucoside</td>
<td>14.4</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>Cyanidin-3-(6-malonyl) glucoside</td>
<td>15.8</td>
<td>63.3</td>
</tr>
<tr>
<td>6</td>
<td>cyanidin-3-(6-malonyl) laminaribioside</td>
<td>17.3</td>
<td>22.6</td>
</tr>
<tr>
<td>7</td>
<td>peonidin-3-(malonyl) glucoside</td>
<td>20.7</td>
<td>2.7</td>
</tr>
<tr>
<td>8</td>
<td>cyanidin-3-(malonyl)(acetoxyl) glucoside</td>
<td>21.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 4.1: Fractionated Red Onion Anthocyanins.
Figure 4.2: Chromatogram of saponified red onion extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-laminaribioside, peak 3 peonidin-3-glucoside. The phenolic profile for red onion was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area at 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin-3-glucoside</td>
<td>10.4</td>
<td>68.4</td>
</tr>
<tr>
<td>2</td>
<td>cyanidin-3-laminaribioside</td>
<td>11.7</td>
<td>27.5</td>
</tr>
<tr>
<td>3</td>
<td>peonidin-3-glucoside</td>
<td>14.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Table 4.2: Saponified Red Onion Anthocyanins.
The nine major anthocyanins were identified in purple corn (Jing, 2006) (Figure 4.3 and Table 4.3): peak 1, cyanidin-3-glucoside (<1%), peak 2, pelargonidin-3-glucoside (<1%), peak 3 cyanidin-3-(malonyl) glucoside (48.5%), peak 4 peonidin-3-glucoside (7.0%), peak 5 cyanidin-3-(malonyl) glucoside (27.1%), peak 6 pelargonidin-3-(malonyl)glucoside (9.5%), peak 7 pelargonidin-3-(malonyl)glucoside (<1%), peak 8 peonidin-3-(malonyl)glucoside (2.8%), and peak 9 peonidin-3-(malonyl) glucoside (3.6%). Acylated anthocyanins comprised approximately 92.2% of total purple corn anthocyanins. A variety of phenolic compounds, including the identified anthocyanins, can be seen on the chromatogram at 280 nm (Figure 4.3). This is an indication that non anthocyanin phenolic compounds were not removed by the fractionation procedure used with a C-18 cartridge and ethyl acetate. Previous reports that show that the ethyl acetate fractionation procedure has some limitations and is not effective with all plant materials (He, 2008). Purple corn anthocyanins were saponified to remove acylation groups. Three major anthocyanins were identified in saponified purple corn (Figure 4.4 and Table 4.4): peak 1 cyanidin-3-glucoside (71.07%), peak 2 pelargonidin-3-glucoside (8.1%), peonidin-3-glucoside (20.8%). Acylated anthocyanins comprised approximately 0% of total purple corn anthocyanins, making up a significant minority of the anthocyanin pigments. The identified anthocyanins appeared to represent a majority of the phenolic profile (280 nm) (Figure 4.4) of purple corn.
Figure 4.3: Chromatogram of fractionated purple corn extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 pelargonidin-3-glucoside, peak 3 cyanidin-3-(malonyl) glucoside, peak 4 peonidin-3-glucoside, peak 5 cyanidin-3-(malonyl) glucoside, peak 6 pelargonidin-3-(malonyl) glucoside, peak 7 pelargonidin-3-(malonyl) glucoside, peak 8 peonidin-3-(malonyl) glucoside, and peak 9 peonidin-3-(malonyl) glucoside. The phenolic profile for cabbage onion was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area at 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin-3-glucoside</td>
<td>11.4</td>
<td>51.3</td>
</tr>
<tr>
<td>2</td>
<td>pelargonidin-3-glucoside</td>
<td>13.8</td>
<td>6.0</td>
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<tr>
<td>3</td>
<td>peonidin-3-glucoside</td>
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<tr>
<td>4</td>
<td>cyanidin-3-(malonyl)glucoside</td>
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<td>9.5</td>
</tr>
<tr>
<td>5</td>
<td>pelargonidin-3-malonylglucoside</td>
<td>21.2</td>
<td>0.7195</td>
</tr>
<tr>
<td>6</td>
<td>peonidin-3-malonylglucoside</td>
<td>23.0</td>
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</tr>
<tr>
<td>7</td>
<td>peonidin-3-malonylglucoside</td>
<td>23.3</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Table 4.3: Fractionated Purple Corn Anthocyanins.
**Figure 4.4:** Chromatogram of saponified purple corn extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 pelargonidin-3-glucoside, and peak 3 peonidin-3-glucoside. The phenolic profile for cabbage onion was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area @ 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin-3-glucoside</td>
<td>11.6</td>
<td>71.1</td>
</tr>
<tr>
<td>2</td>
<td>pelargonidin-3-glucoside</td>
<td>14.1</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>peonidin-3-glucoside</td>
<td>16.1</td>
<td>20.8</td>
</tr>
</tbody>
</table>

**Table 4.4:** Saponified Purple Corn Anthocyanins.
Anthocyanins in the red cabbage extract were identified by comparing retention times order of elution and UV-visible spectra and previous literature (Wu and Prior., 2005). The twelve major anthocyanins identified in red cabbage (Figure 4.5 and Table 4.5) include: peak 1 cyanidin 3-diglucoside-5-glucoside (3.00%), peak 2 cyanidin 3-(sinapoyl)diglucoside-5-glucoside (2.89%), peak 3 cyanidin 3-(caffeoyl)(p-coumaroyl)diglucoside-5-glucoside (1.54%), peak 4 cyanidin 3-(glycopyranosyl-feruloyl)diglucoside-5-glucoside (1.68%), peak 5 cyanidin 3-(sinapoyl)diglucoside-5-glucoside (1.44%), peak 6 cyanidin 3-(sinapoyl)(p-coumaroyl)diglucoside-5-glucoside (1.29%), peak 7 cyanidin 3-(p-coumaroyl) diglucoside-5-glucoside (29.43%), peak 8 cyanidin 3-(p-coumaroyl) diglucoside-5-glucoside (36.07%), peak 9 cyanidin 3-(sinapoyl) diglucoside-5-glucoside (0.98%), peak 10 cyanidin-3 (feruloyl)(feruloyl) diglucoside-5-glucoside (6.77%), peak 11 cyanidin 3-(sinapoyl)(feruloyl) diglucoside-5-glucoside (6.22%), and peak 12 cyanidin 3-(sinapoyl)(sinapoyl) diglucoside-5-glucoside (8.64%). Acylated anthocyanins comprised approximately 96.95% of total red cabbage anthocyanins. The anthocyanins represented a large amount of the phenolic profile (280 nm) (Figure 4.5).

Red cabbage anthocyanins were saponified to remove acylating groups. Saponified red cabbage anthocyanins were extracted from lyophilized saponified red cabbage powder with 0.01% HCl acidified HPLC grade water. Anthocyanins in the saponified red cabbage extract were identified by comparing retention times and UV-visible spectra. The three major anthocyanins identified in saponified red cabbage (Figure 4.6 and Table 4.6) include: peak 1 cyanidin-3-diglucoside-5-glucoside (71.7%), peak 1b cyanidin-3-diglucoside-5-xyloside (new peak) (24.9%), peak 2 and cyanidin-3-
(sinapoyl) diglucoside-5-glucoside (3.4%). Acylated anthocyanins comprised approximately 3.4% of total red cabbage anthocyanins, indicating saponification was successful at removing most of the acylated anthocyanin compounds. The identified anthocyanins appeared to represent a majority of the phenolic profile (280 nm) (Figure 4.6) of saponified red cabbage.
**Figure 4.5:** Chromatogram of fractionated red cabbage extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin 3-diglucoside-5-glucoside, peak 2 cyanidin 3-(sinapoyl)diglucoside-5-glucoside, peak 3 cyanidin 3-(caffeoyl)\((p\text{-coumaroyl})\)diglucoside-5-glucoside, peak 4 cyanidin 3-(glycopyranosyl-feruloyl)diglucoside-5-glucoside, peak 5 cyanidin 3-(sinapoyl)diglucoside-5-glucoside, peak 6 cyanidin 3-(sinapoyl)\((p\text{-coumaroyl})\)diglucoside-5-glucoside, peak 7 cyanidin 3-(p-coumaroyl)diglucoside-5-glucoside, peak 8 cyanidin 3- \((p\text{-coumaroyl})\)diglucoside-5-glucoside, peak 9 cyanidin 3-(sinapoyl)diglucoside-5-glucoside, peak 10 cyanidin-3-(feruloyl)\((feruloyl)\)diglucoside-5-glucoside, peak 11 cyanidin 3-(sinapoyl)\((feruloyl)\)diglucoside-5-glucoside, and peak 12 cyanidin 3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside. The phenolic profile for red cabbage was also observed at 280 nm.
<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area @ 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin 3-diglucoside-5-glucoside</td>
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<td>2</td>
<td>cyanidin 3-(sinapoyl)diglucoside-5-glucoside</td>
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<tr>
<td>3</td>
<td>cyanidin 3-(caffeoyl)(p-coumaroyl)diglucoside-5-glucoside</td>
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<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>cyanidin 3-(glycopyranosyl-feruloyl)diglucoside-5-glucoside</td>
<td>16.9</td>
<td>1.7</td>
</tr>
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<td>17.2</td>
<td>1.4</td>
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<td>20.1</td>
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<td>cyanidin 3-(p-coumaroyl)diglucoside-5-glucoside</td>
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<td>cyanidin 3-(p-coumaroyl)diglucoside-5-glucoside</td>
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<td>10</td>
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<td>6.8</td>
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<tr>
<td>11</td>
<td>cyanidin 3-(sinapoyl)(feruloyl)diglucoside-5-glucoside</td>
<td>27.4</td>
<td>6.2</td>
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<tr>
<td>12</td>
<td>cyanidin 3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside</td>
<td>27.8</td>
<td>8.6</td>
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</table>

**Table 4.5**: Fractioned Red Cabbage Anthocyanins.
**Figure 4.6:** Chromatogram of saponified red cabbage extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-diglucoside-5-glucoside, peak 1b cyanidin-3-diglucoside-5-xyloside, peak 2 cyanidin-3-(sinapoyl) diglucoside-5-glucoside. The phenolic profile for saponified red cabbage was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>Percent Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyanidin 3-diglucoside-5-glucoside</td>
<td>6.754</td>
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<tr>
<td>1b</td>
<td>cyanidin 3-diglucoside-5-xyloside</td>
<td>7.858</td>
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<tr>
<td>2</td>
<td>cyanidin 3-(sinapoyl)diglucoside-5-glucoside</td>
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<td>3.3762</td>
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**Table 4.6:** Saponified Red Cabbage Anthocyanins.
Black carrot anthocyanins were identified by comparing retention times, order of elution and UV-visible spectra to previous literature (Krammerer et al., 2003) (Figure 4.7 and Table 4.7). The major peaks include: peak 1 cyanidin-3-xylosyl-glucosyl-galactoside (5.2%), peak 2 cyanidin-3-(caffeoyl) xylosyl-glucosyl-galactoside (<1%), peak 3 cyanidin-3-xylosyl-galactoside (23.2%), peak 4 cyanidin-3- (p-hydroxybenzoyl) xylosyl-glucosyl-galactoside (38.4%), peak 5 cyanidin-3-(sinapoyl) xylosyl-glucosyl-galactoside (17.0%), peak 6 cyanidin-3-(feruloyl) xylosyl-glucosyl-galactoside (3.9%), and peak 7 cyanidin-3-(p-coumaryl) xylosyl-glucosyl-galactoside (8.0%). Acylated anthocyanins comprised approximately 67.9% of total black carrot anthocyanins. The identified black carrot anthocyanins appeared to represent a majority of the phenolic profile (280 nm) (Figure 4.7).

Black carrot anthocyanins were saponified to remove acylation groups. Anthocyanins in the saponified black carrot extract were identified by comparing retention times and UV-visible spectra. The three major anthocyanins identified in saponified black carrot (Figure 4.8 and Table 4.8) include: peak 1 cyanidin-3-xylosyl-glucosyl-galactoside (54.5%), peak 3 cyanidin-3-xylosyl-galactoside (43.7%), and peak 6 cyanidin-3-(sinapoyl) xylosyl-glucosyl-galactoside (1.7%). Acylated anthocyanins comprised approximately 1.7% of total black carrot anthocyanins, making up a significant minority of the anthocyanin pigments and suggesting that saponification was successful at removing most acylated anthocyanins. The identified anthocyanins appeared to represent a majority of the phenolic profile (280 nm) (Figure 4.8) of saponified black carrot.
Figure 4.7: Chromatogram of fractionated black carrot extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-xylosyl-glucosyl-galactoside, peak 2 cyanidin-3-(caffeoyl)xylosyl-glucosyl-galactoside, peak 3 cyanidin-3-xylosyl-galactoside, peak 4 cyanidin-3-(p-hydroxybenzoyl)xylosyl-glucosyl-galactoside, peak 5 cyanidin-3-(sinapoyl)xylosyl-glucosyl-galactoside, peak 6 cyanidin-3-(feruloyl)xylosyl-glucosyl-galactoside, peak 7 cyanidin-3-(p-coumaryl)xylosyl-glucosyl-galactoside. The phenolic profile for black carrot was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>% Area @ 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
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<td>cyanidin-3-xylosyl-glucosyl-galactoside</td>
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<td>cyanidin-3-(caffeoyl)xylosyl-glucosyl-galactoside</td>
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<tr>
<td>7</td>
<td>cyanidin-3-(p-coumaryl)xylosyl-glucosyl-galactoside</td>
<td>16.0</td>
<td>8.0</td>
</tr>
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</table>

Table 4.7: Fractionated Black Carrot Anthocyanins.
Figure 4.8: Chromatogram of saponified black carrot extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-xylosyl-glucosyl-galactoside, peak 3 cyanidin-3-xylosyl-galactoside, and peak 6 cyanidin-3-(sinapoyl) xylosyl-glucosyl-galactoside. The phenolic profile for saponified black carrot was also observed at 280 nm.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Anthocyanin</th>
<th>Retention Time</th>
<th>Percent Area</th>
</tr>
</thead>
<tbody>
<tr>
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<td>cyanidin-3-xylosyl-glucosyl-galactoside</td>
<td>8.991</td>
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<td>cyanidin-3-(sinapoyl)xylosyl-glucosyl-galactoside</td>
<td>13.032</td>
<td>1.7467</td>
</tr>
</tbody>
</table>

Table 4.8: Saponified Black Carrot.
Below is a representation of the main acylated anthocyanin in red onion, purple corn, red cabbage and black carrot (Figure 4.9). The figure shows the varying glycosylation patterns and common acylating groups found in each sample.

**Figure 4.9:** Representative chemical structures of main acylated anthocyanins in red onions (A), purple corn (B), red cabbage (C) and black carrots (D)
4.2 Growth Characteristics of HT-29 Cell line:

Before treating HT-29 colon cancer cells with anthocyanin extracts, the growth characteristics were examined. Proliferation rates for HT-29 colon cancer cells seeded at $10^4$ cells/ml in a 24 well plate were determined by manually counting cells using a hemocytometer at 24, 48 and 72 hours. The 72 hour growth pattern for HT-29 colon cancer cells can be seen below (Figure 4.10). HT-29 colon cancer cells exhibited lag phase growth between 0-24 hours and log phase (exponential) growth between 24-72 hours. Cells also achieved 80-90% confluence at the end of 72 hours, which is an appropriate confluence for HT-29 colon cancer cell treatment.

![HT-29 Cell Growth Pattern](image)

**Figure 4.10**: HT-29 colon cancer cell 72 hour growth pattern. Each point represents mean ± SD for n=9 cultures.
4.3 **HT-29 Colon Cancer Cell Growth Inhibition:**

The effects of growth inhibition of HT-29 colon cancer cells varied according to sample treatment and dose. The SRB assay was used throughout this study to determine the effects of anthocyanin treatments on the proliferation of HT-29 colon cancer cells. The sulfurhodamine-B colorimetric assays dye binds to the amino acids of cellular proteins and through colorimetric evaluation an estimation of the cell proliferation was made as it relates to total protein mass.

Cells were incubated in medium with anthocyanin rich extract from fractionated red onion at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. Fractionated red onion extract appeared to be cytotoxic to HT-29 cells above 50 µg cyanidin-3-glucoside equivalents/ml. This can be inferred from the inhibition levels of over 100%. The overall observed growth inhibition of HT-29 cells when treated with fractionated red onion extract at concentrations of 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 15.53, 68.64, 119.23, and 120.28 percent respectively and can be seen in figure 4.11A below.

Cells were also incubated in medium with anthocyanin rich extract from saponified red onion at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. In contrast to the red onion extract, anthocyanin rich saponified red onion extract had lower HT-29 cell inhibition at all concentration levels evaluated. Removal of acylation significantly decreased (p<0.05) the degree of HT-29 cell growth inhibition at concentration levels of 50 and 25 ug anthocyanin/mL media. However, the saponified red onion extract did show greater than 50 percent inhibition at
concentrations of 50µg cyanidin-3-glucoside equivalents/ml and higher. The overall observed growth inhibitions of HT-29 cells when treated with saponified red onion extracts at 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 10.84, 20.98, 52.64, and 92.52 percent respectively and can be seen in figure 4.11B below. This data suggests that fractionated red onion extracts when used to treat HT-29 cells at concentrations 100 µg cyanidin-3-glucoside equivalents/ml and below have a higher chemopreventative effect than non-acylated red onion extracts.
**Figure 4.11:** Growth inhibition of (A) acylated red onion anthocyanins and (B) saponified red onion anthocyanins at 12.5, 25, 50 and 100µg cyanidin-3-glucoside equivalent/ml media.
Cells were incubated in medium with anthocyanin rich extract from fractionated and concentrated purple corn at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. The fractionated and concentrated purple corn extract appeared to have greater than 40 percent inhibition at 50µg cyanidin-3-glucoside equivalents/ml and above. The overall observed growth inhibitions of HT-29 cells when treated with fractionated and concentrated purple corn extracts at 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 3.57, 16.23, 41.10, and 67.75 percent respectively and can be seen in figure 4.12A below.

Anthocyanin rich purple corn extract was saponified and cells were incubated in medium with the anthocyanin rich extract at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. Saponified purple corn extract appeared to have greater than 50 percent inhibition at 50µg cyanidin-3-glucoside equivalents/ml and above. The overall observed growth inhibitions of HT-29 cells when treated with saponified purple corn extracts at 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 1.15, 27.36, 53.72, and 79.66 percent respectively and can be seen in figure 4.12B below. The anthocyanin rich saponified purple corn extract appeared to show more inhibition than the fractionated and concentrated purple corn extract, suggesting that the non-acylated form of purple corn has greater levels of chemoprevention when compared to acylated purple corn at concentrations of 100 µg cyanidin-3-glucoside equivalents/ml and below. However, the increase in HT-29 cell inhibition was not found to be statistically significant at the 0.05 significance level.
Figure 4.12: Growth inhibition of (A) acylated purple corn anthocyanins and (B) saponified purple corn anthocyanins at 12.5, 25, 50 and 100µg cyanidin-3-glucoside equivalent/ml media.
Anthocyanin rich red cabbage extract was used to treat HT-29 cells and the cells were allowed to incubate in medium with the anthocyanin rich extract at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. The anthocyanin rich red cabbage extract appeared to show less inhibition than afore mentioned anthocyanin rich extracts. Red cabbage extract appeared to have its highest inhibition at a concentration of 100µg cyanidin-3-glucoside equivalents/ml. The overall observed growth inhibitions of HT-29 cells when treated with anthocyanin rich red cabbage extracts at 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 0, 0, 7.62, and 20.37 percent respectively as seen in figure 4.13A below.

Anthocyanin rich saponified red cabbage extract was also used to treat HT-29 cells and the cells were allowed to incubate in medium with the anthocyanin rich extract at concentrations of 12.5, 25, 50, and 100µg cyanidin-3-glucoside equivalents/ml for 48 hours. The saponified red cabbage extract also appeared to show less inhibition than afore mentioned anthocyanin rich extracts; however, at a concentration of 100µg cyanidin-3-glucoside equivalents/ml it appeared to exhibit slightly more inhibition than the red cabbage. The overall observed growth inhibitions of HT-29 cells when treated with anthocyanin rich saponified red cabbage extracts at 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 0, 0.6, 5.97, and 24.74 percent respectively as seen in figure 4.13B below. This suggests that at concentrations of 100µg cyanidin-3-glucoside equivalents/ml or higher non-acylated red cabbage anthocyanins have a greater chemopreventative effect on HT-29 colon cancer cell proliferation.
**Figure 4.13:** Growth inhibition of (A) acylated red cabbage and (B) saponified red cabbage anthocyanins at 12.5, 25, 50 and 100µg cyanidin-3-glucoside equivalent/ml media.
Anthocyanin rich fractionated and concentrated black carrot extract was used to treat HT-29 cells and the cells were allowed to incubate in medium with the anthocyanin rich extract at concentrations of 12.5, 25, 50, and 100 µg cyanidin-3-glucoside equivalents/ml for 48 hours. The black carrot extract appeared have greater than 50 percent HT-29 cell growth inhibition at concentrations of 50 µg cyanidin-3-glucoside equivalents/ml or higher. The overall observed growth inhibitions of HT-29 cells when treated with anthocyanin rich fractionated and concentrated black carrot extracts at concentrations of 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 18.80, 36.67, 50.75, and 65 percent respectively and can be seen in figure 4.14A below.

Saponified black carrot extract was used to treat HT-29 cells and the cells were allowed to incubate in medium with the anthocyanin rich extract at concentrations of 12.5, 25, 50, and 100 µg cyanidin-3-glucoside equivalents/ml for 48 hours. Compared to fractionated and concentrated black carrot extracts the saponified black carrot extract appeared have less of an inhibitory effect on the proliferation of HT-29 cells. However, the saponified black carrot extract had greater than 40 percent cell growth inhibition at a concentration of 100 µg cyanidin-3-glucoside equivalents/ml and above. The overall observed growth inhibitions of HT-29 cells when treated with anthocyanin rich saponified black carrot extracts at concentrations of 12.5, 25, 50 and 100 µg cyanidin-3-glucoside equivalents/ml were 4.61, 8.57, 24.10, and 47.66 percent respectively and can be seen in figure 4.14B below. This suggests that acylated black carrot anthocyanins have a higher chemopreventative effect than the non-acylated black carrot anthocyanins when used to treat HT-29 cells at concentrations of 100 µg cyanidin-3-glucoside equivalents/ml and lower.
Figure 4.14: Growth inhibition of (A) acylated black carrot and (B) saponified black carrot anthocyanins at 12.5, 25, 50 and 100µg cyanidin-3-glucoside equivalent/ml media.
The results show that anthocyanin extracts from red onion, purple corn, red cabbage and black carrot provide different inhibitory effects to the proliferation of HT-29 cells at a concentration of 100µg cyanidin-3-glucoside equivalent/ml and lower. Our data suggests that the observed anti-proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the normal HT-29 cell growth pattern. The proliferation of HT-29 cells was inhibited by the anthocyanin extracts in a dose dependent manner.

The samples used in this study contained different glycosylation and acylation patterns. Red onion and purple corn anthocyanins contain aliphatic acid groups (malonic). Acylation with aliphatic acids donates electrons to the anthocyanins chemical structure and causes a bathochromic shift and a hyperchromic effect (Von Elbe and Schwartz, 1996; Giusti et al., 1999). Acylation also contributes to the stabilization of the anthocyanin via intermolecular interaction (Giusti and Wrolstad., 2003). Aliphatic acid acylation is an important structural character to consider when studying the chemopreventative effects of anthocyanins.

The results of this study show that the growth of HT-29 colon cancer cells were significantly decreased when treated with acylated and non-acylated red onion and purple corn extracts. The growth inhibition of HT-29 cells was greater than 100 percent when treated with acylated red onion at concentrations of 50 µg and 100µg cyanidin-3-glucoside equivalent/ml, which indicates that the extract exhibited a cytotoxic effect. The non-acylated red onion extract was also a potent HT-29 colon cancer cell inhibitor; however, it did not necessarily exhibit a cytotoxic effect. When the aliphatic acids were removed from the purple corn extract the inhibition of HT-29 cells was increased from
67.75 percent to 79.66 percent at a concentration of 100µg cyanidin-3-glucoside equivalent/ml. These finding have also been confirmed by the research of Jing et al., 2006, where deacylated anthocyanins and their counterparts were found to exhibit a dose-dependent inhibitory effect on the growth of HT-29 colon cancer cells. The results suggest that removal of aliphatic acid groups in anthocyanin rich red onion and purple corn extracts can maintain or increase the cell growth inhibitory effect to levels comparable to the acylated anthocyanin starting material.

Anthocyanin rich red cabbage and black carrot extracts contain cinnamic acid acylation (sinapic, ferulic, caffeic, p-coumaric). Cinnamic acids are known for their aromatic ring chemical structure. Previous research suggests that anthocyanin acylation with cinnamic acids may negatively correlate to the inhibition of HT-29 colon cancer cell proliferation (Jing et al., 2006).

The HT-29 colon cancer cell growth inhibition was increased when treated with non-acylated red cabbage anthocyanins at a concentration of 100µg cyanidin-3-glucoside equivalent/ml; increasing HT-29 cell inhibition from 20.37 percent to 24.74 percent. These findings suggest that acylation has an important impact on the chemoprotective effect of anthocyanins. However, the opposite was found for the removal of cinnamic acids from black carrot anthocyanins. The results showed that HT-29 cell growth inhibition was higher when treated with acylated black carrot anthocyanins. It was found that acylated black carrot anthocyanins had a 65 percent growth inhibition as compared to 47.66 percent growth inhibition with non-acylated anthocyanins at a concentration of 100µg cyanidin-3-glucoside equivalent/ml. These findings suggest that the type of acylation is not the only important factor when comparing the chemoprotective effect of
anthocyanins. Previous research showed that the saponification of peonidin-3-(caffeylferuly) sophoroside-5-glucoside, greatly increased chemoprotection as compared to its acylated counterpart; while saponification of cyanidin-3-(caffeylferuly) sophoroside-5-glucoside did not great increase the chemoprotection as compared to its acylated counterpart (Yoshimoto et al., 2001); suggesting that both acylation and aglycone have a major impact on the chemoprevention of anthocyanins. More specifically, a previous study compared the inhibition of HT-29 cell proliferation of monoglucosylated and a try-glycosylated pelargonidin and cyaniding derivatives (Jing et al., 2006). This study found that more sugars attached to the molecule caused a lower growth inhibition in the HT-29 cell line. Red cabbage and black carrot both have three glycosylations (Figure 4.9). The results of this research project strongly support the hypothesis that three sugar substitutions significantly decrease anthocyanin chemopreventative properties. Comparing our anthocyanin sources and their chemical structures (Figure 4.9) it is also possible and likely that the position of the glycosylation will have an impact on the ability of the extract to inhibit HT-29 colon cancer cell proliferation.
CHAPTER 5

CONCLUSIONS

5.1 Conclusion:

The results of this project provided evidence that chemical structure plays an important role in HT-29 colon cancer cell growth inhibition. Saponification of aliphatic acids in purple corn and red onion extracts increased or maintained their chemoprotective abilities. Anthocyanins acylated with cinnamic acids exhibited a different chemoprotective effects depending on the aglycone and glycosylation patterns. Tri-glycosylated anthocyanins are shown to have a negative correlation in HT-29 colon cancer inhibition, as well as their structural position. The in vitro activity of anthocyanins may be relevant to in vivo because when ingested anthocyanins are in direct contact with the epithelial cell layer of the colon. However, further research is needed to better understand the impact of acylation and glycosylation on anthocyanin chemoprevention.
LITERATURE CITED


