Extraction of Bioactive Compounds from Whole Red Cabbage and Beetroot using Pulsed Electric Fields and Evaluation of their Functionality

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EXTRACTION OF BIOACTIVE COMPOUNDS FROM WHOLE RED CABBAGE AND BEETROOT USING PULSED ELECTRIC FIELDS AND EVALUATION OF THEIR FUNCTIONALITY

by

Valli Kannan

A THESIS

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Lincoln, Nebraska

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ABSTRACT

EXTRACTION OF BIOACTIVE COMPOUNDS FROM WHOLE RED CABBAGE AND BEETROOT USING PULSED ELECTRIC FIELDS AND EVALUATION OF THEIR FUNCTIONALITY

Valli Kannan, MS
University of Nebraska, 2011

Adviser: Harshavardhan Thippareddi

Application of high voltage pulsed electric fields (PEF) to plant tissues increases porosity of the plant cells, enhancing juice extraction from fruits and vegetables. Red cabbage and beetroot are rich sources of bioactive compounds anthocyanins and betalains, respectively. Pulsed electric field (red cabbage: 1 kV/cm electric field strength, 0.66 µF and 20 pulses; red beets: 1.5 kV/cm electric field strength, 0.66 µF and 20 pulses) was applied to whole red cabbage and beetroot to enhance juice extraction. Total polyphenols, anthocyanins (red cabbage), betalains (beetroot) and total antioxidant activity (ABTS and DPPH) of the extracted juice was determined. The bioactivity of the juices on colon cancer cell line HCT-116 (p53 +/- and p53 -/-) were analyzed. The anti-proliferative activity, apoptosis and cytotoxicity were determined. Pulsed electric field treatment increased (p ≤ 0.01) juice extraction, total phenolics, anthocyanins (red cabbage), betalains (beetroots) and antioxidant activity of both red cabbage and beetroot juice compared to non-PEF extracted samples. The juices reduced colon cancer cell proliferation, increased apoptosis and did not exhibit cytotoxicity. There was no difference between the PEF extracted and non-PEF extracted juice with respect to its bioactivity on cancer cells.
Keywords: pulsed electric field, extraction, red cabbage, beetroot, bioactivity
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CHAPTER 1
INTRODUCTION

Cancer is one of the leading causes of death all over the world ranked after cardiovascular disease. Cancer is caused both by internal and external factors. The internal factors include inherited mutations, hormones, immune conditions and mutations due to metabolism. The external factors are tobacco, infectious organisms, chemicals and radiation (Cancer facts and figures, 2009). In any cancer type, six essential alterations occur in cells that lead to malignant growth (Figure 1.1; Hanahan, 2000). The six alterations are:

i. Self-sufficiency in growth signals.
ii. Insensitivity to growth-inhibitory (antigrowth) signals.
iii. Evasion of programmed cell death (apoptosis).
iv. Limitless replicative potential.
v. Sustained angiogenesis, and
vi. Tissue invasion and metastasis.

Figure 1.1 Acquired capabilities of Cancer (Hanahan, 2000).
Many epidemiological studies have consistently shown that a high dietary intake of fruits, vegetables and whole grains is strongly associated with reduced risk of developing chronic diseases like cancer and cardiovascular diseases. The National Academy of Sciences in a report on diet and health recommended that consuming five or more servings of fruits and vegetables for reducing the risk of both cancer and heart disease (National Academy of Sciences Report, 1989). Phytochemicals are nonnutrient plant compounds derived from fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Liu, 2004).

Anthocyanins, a group of polyphenols present in fruits and vegetables, are the most abundant antioxidants in our diet (Scalbert, 2000; Francis, 1989). The interest in anthocyanins increased because of the recognition of their potential health benefits (He, 2010). Several epidemiological studies have suggested associations between the consumption of anthocyanin-rich foods and prevention of chronic diseases like, cancer (Reddivari et al., 2007; Zhao et al., 2004), cardiovascular disease (Gracia et al., 1997), and others. Red cabbage is a rich source of acylated anthocyanins which are stable to pH, temperature, light and acidic gastric digestion conditions (Tanchev, 1969; Dyrby et al., 2001; Walkowiak-Tomczak, 2007; McDougall et al., 2007). Red cabbage color has been found to suppress colorectal cancer in mice (Hagiwara et al., 2001).

Betalains are a class of water-soluble pigments which is classified into betacyanins and betaxanthins. Beetroot red is a widely permitted natural food colorant (E162; Azeredo et al., 2009; Henry, 1996).
Betalains contribute to the antioxidant activity of beetroots, thus preventing against cancer (Cao et al., 1996; Kapadia et al., 2003).

Studies have shown that anthocyanins and betalains are extracted using an organic solvent or water (Metivier et al., 1980; Kanner et al., 2001). Several novel extraction technologies have also been used to extract anthocyanins (Corrales, 2008; Sun et al., 2007). Pulsed electric field is a non-thermal processing technology used to extract juice and other value-added components from fruits and vegetables (Bouzrara, 2000; Gachovska et al., 2010). To extract juice or intracellular components from a plant cell, a sample preparation step such as homogenizing is required. We implemented a method, where PEF pre-treatment is given to red cabbages and beetroots without any sample preparation step.
Figure 1. 2 Flowchart for juice extraction using conventional method and method implemented in this study.

The objective of this study was to:

i. Extract juice from whole red cabbage and beetroot using pulsed electric field without a sample preparation step.

ii. Quantify the bioactive compounds in the extracted juice and

iii. Evaluate the anti-carcinogenic activity of the extracted juice using colon cancer cell lines HCT-116 (p53+/+ and p53 -/-).
1.1 References


natural food anthocyanins, purple sweet potato color and red cabbage color, of 2-
amino-1-methyl-6-phenylimidazo[4,5-b]Pyridine (PhIP)-associated colorectal
carcinogenesis in rats initiated with 1,2-dimethylhydrazine. *The Journal of
toxicological sciences, 27*(1), 57-68.


11. He, J. & Giusti, M. M. (2010). Anthocyanins: Natural colorants with health-
promoting properties. *Annual Review of Food Science and Technology, 1*(1), 163-
187.

Houghton JD (Eds) Natural Food Colourants (pp. 40-79). London: Chapman &
Hall.

cationized antioxidants. *Journal of Agricultural and Food Chemistry, 49*(11),
5178-5185.

Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract.
*Cancer letters, 100*(1-2), 211-214.


from red cabbage-Stability to simulated gastrointestinal digestion.
*Phytochemistry, 68*(9), 1285-1294.


24. Yangzhao Sun, Xiaojun Liao, Zhengfu Wang, Xiaosong Hu and Fang Chen (2007). Optimization of microwave-assisted extraction of anthocyanins in red raspberries and identification of anthocyanin of extracts using high-performance

2.1 Phenolics

Plants synthesize a variety of compounds for growth and are classified as primary and secondary metabolites. The primary metabolites include carbohydrates, proteins and lipids, which play an important role in the basic life functions such as cell division and growth, respiration, storage and reproduction. The secondary metabolites are synthesized from lipid precursors and aromatic amino acids and play an important role in the adaptation of plants to their environment (Bourgaud, 2001).

Based on their biosynthetic pathways, the plant secondary metabolites are classified into three large families (Bourgaud, 2001): (i) phenolics, (ii) terpenes and (iii) steroids. Within the three families, the phenolic compounds or polyphenols are the most numerous with more than 8,000 phenolic structures currently identified (Harborne, 1986).

Natural polyphenols have a wide variety of structures. They range from simple compounds containing an aromatic ring with one or more hydroxyl moieties to highly complex polymeric substances such as tannins (Dragsted, 1993; Harborne, 1994). Most of the polyphenols are conjugated with one or more sugar residues. Commonly, glucose or rutinose is attached, although other sugars galactose, rhamnose and arabinose are also found. Conjugation with organic acids, lipids and other phenols are also observed (Bravo, 1998).
The polyphenols can be classified into ten classes depending on their basic chemical structure (Table 2.1; Harborne, 1989). Flavonoids constitute the most common and widely distributed group of plant phenolics and have a skeleton of diphenylpropanes (C6-C3-C6) and consist of two benzene rings (A and B) linked through three carbons forming a closed pyran ring (C) with the benzene A ring (Figure 2.1). Biosynthesis of the flavonoids starts with the A ring derived from a molecule of resorcinol or phloroglucinol synthesized in the acetate/malonate pathway and the B ring derived from the carbons of phenylalanine in the Shikimate pathway (Figure 2.1; Bravo, 1998).

Flavonoids are further subdivided into 13 classes, with more than 5,000 compounds. Most of the flavonoids are low molecular weight and are soluble depending on their polarity and chemical structure (Table 2.1; degree of hydroxylation, glycosylation, acylation, etc.; Bravo, 1998).
Table 2.1. Classification of flavonoids (Bravo, 1998).

<table>
<thead>
<tr>
<th>Chalcones</th>
<th>Dihydrochalcones</th>
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<tr>
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<td><strong>Aurones</strong></td>
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<tr>
<td><strong>Flavandiol or leucoanthocyanidin</strong></td>
<td><strong>Anthocyanidin</strong></td>
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<td><strong>Isoflavonoids</strong></td>
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<td>[Image]</td>
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<tr>
<td><strong>Proanthocyanidins or condensed tannins</strong></td>
<td></td>
</tr>
</tbody>
</table>
2.2 Flavonoids

2.2.1 Distribution of Flavonoids in Plants

Flavonoids are present in various plant groups and are more abundant in the angiosperms. Animals and fungi except marine coral lack the flavonoids. The most primitive plant species, in which the flavonoids have been found, is green algae (Markham, 1969). They are present universally in leaves and petals and also frequently found in other organs: fruits, roots, sepals, etc. Flavonoids also occur in leaf waxes, bud excretions and in heartwood of trees (Harborne, 1977). Specifically, flavonoids are present in the vacuoles of guard cells, epidermal cells as well as sub-epidermal cells and shoots of plants (Hutzler, 1998).

2.2.2 Functions of Flavonoids in Nature

The main function of flavonoids in plants is to attract insects to help in pollination and in seed dispersion because of their bright color and patterns. Many of the scientific breakthroughs in the past 150 years have used flavonoids as an important factor. Besides providing pigmentation in flowers, fruits, seeds, and leaves, flavonoids have key roles in signaling between plants (allelopathy), and microbes, in male fertility of some species, in defense as antimicrobial agents (phytoalexins) and feeding deterrents, and in UV protection (Shirley, 2001). The epidermal layer of plants absorbs 90-99% of the incident ultraviolet radiation (Robberecht, 1978; Caldwell, 1983), acting as a shield against the potentially harmful UV-A and UV-B radiation (Schnabl, 1986; Hutzler, 1998).
2.2.3 Human Uses of Flavonoids

Flavonoids have applications in food industry as well as cut flower industry. Flavonoids are generally responsible for color, taste, prevention of fat oxidation and protection of vitamins and enzymes in foods (Yao, 2004).

The flavonoids are considered as bioactive compounds, the “extra-nutritional” constituents that are typically naturally occurring in small quantities in plant products and lipid rich foods. The bioactive compounds of plant origin include phenolic compounds (flavonoids, resveratrol and phytoestrogens) and are present in tea, red wine; lycopene; organosulfur compounds; plant sterols; dietary fibers; isothiocyanates; and monoterpenes. The bioactive compounds have beneficial health effects (Kris-Etherton, 2002), and contribute to the antioxidant properties of green vegetables, fruits, olive and soybean oils, red wine, chocolate and tea. Some flavonoids are antiallergic, anti-inflammatory, antiviral, antidiabetic, antiproliferative, anticarcinogenic and have effects on mammalian metabolism. Flavanoids act as antioxidants in the prevention of cancer and cardiovascular diseases. They also protect from ulcers, allergies, vascular fragility and viral and bacterial infections (Yao, 2004).

Several epidemiological studies have reported an inverse association between flavonoid intake and the risk of coronary disease and cancer.
2.3 Anthocyanins

2.3.1 Introduction

Anthocyanins constitute the most conspicuous group of flavonoids. Anthocyanins (from the Greek *anthos*, a flower; and *kyanos*, dark blue), best known of the natural pigment are responsible for the blue, purple, violet, magenta, red and orange colors of a majority of plant species and their products (Delgado-Vargas, 2000; Mazza, 1993). Anthocyanins are found in flowers, leaves, stems, fruits, seeds, and the root tissue of plants.

2.3.2 History

The name anthocyanin was first coined by Marquart in 1835 and is still retained to the present day. Vegetable pigments served as a matter for investigation dating back to 1664. In “Experiments and Considerations Touching Colours” by Robert Boyle discusses the red and green color the plant pigments produce when treated with an acid or a base. In 1849, Morot isolated a blue pigment from cornflower and found that it contained carbon, hydrogen and oxygen. Wigand in 1862, suggested that anthocyanin “arises by the oxidation of a colorless tannin-like chromogen, a substance widely distributed in plants”. As to the chemical processes involved in anthocyanin formation, Palladin in 1905, put forward a hypothesis that “anthocyanins are formed from a flavone by the action of an oxidising enzyme or oxidase”.

Willstatter (1913) stated that “all natural anthocyanins are present in the plant in the condition of glucosides, and that many of them are very unstable in water solution, and readily change in these circumstances to a colorless isomer”. The change can be prevented by adding certain neutral salts, and also acids, to the pigment solution. The explanation, according to Willstatter, of these phenomena lies in the fact that anthocyanin is an oxonium compound having a quinonoid structure and containing tetravalent oxygen. The quinonoid structure is rendered stable by the formation of oxonium salts with acids or with neutral salts, such as sodium nitrate or chloride. The pigment itself, in the neutral state, is purple in color, and has the structure of an inner oxonium salt. With acids the pigment forms red oxonium salt, and with alkaline blue salts, the position of the metal being undetermined.

2.3.3 Evolution of Anthocyanins

The anthocyanins pelargonidin and delphinidin types occur more frequently in advanced plants, replacing cyanidin which is considered to be the simplest and most primitive pigments. This trend was found to be related to natural selection for bright orange and scarlet colors (pelargonidin) in tropical climates where humming birds pollinate the flowers and for deep blue colors (delphinidin) in temperate climates where bees are the major pollinators (Harborne, 1977).

The anthocyanins are widespread in higher plants, except for the 9 caryophyllalles families, i.e., Aizoaceae, Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didiereaceae, Nyctaginaceae, Phytolaccaceae and Portulacaceae except Caryophyllaceae
and Molluginaceae. Instead, the members of these families synthesize the betalain pigments (Piattelli, 1964).

2.3.4 Anthocyanin Structure

Anthocyanins consist of an aglycone or anthocyanidin bound to one or more sugar moieties (glycosylated derivatives of 3, 5, 7, 3’-tetrahydroxy-flavylium cation). While the sugar at position-3 is always present, additional sugars at positions-5 and 7 are often attached. Free anthocyanidins are rarely formed in plants as the electron deficiency of the flavylium cation makes the free anthocyanidins highly reactive and thus, the molecule is very unstable. Since sugar stabilizes the anthocyanin molecule, glycosidic structures are more stable than the anthocyanidins. The sugar moieties can be a mono or disaccharide unit or acylated with a phenolic or aliphatic acid but the sugar molecules are commonly glucose, rhamnose, galactose or arabinose. Further, modification of the glycosides through acylation and through complexation with non-cyanic flavonoids and metal ions can also occur. Subtle differences in cell pH, along with the possibilities of copigmentation and ionic complexation lead to a virtually unlimited degree of color variation.
There are a total of 539 anthocyanins reported to be isolated from plants (Andersen, 2005) and all of this variation is based on a limited number of fundamental flavonoids. The commonly occurring anthocyanidins in foodstuffs are cyanidin (Cy; 30%), delphinidin (Dp; 22%), pelaragonidin (Pg; 18%), peonidin (Pn; 7.5%), malvidin (Mv; 7.5%) and petunidin (Pt; 5%; Nyman 2001; Francis 1999; Figure 2.2). Cyanidin, delphinidin and pelaragonidin are non-methylated anthocyanidins and are widespread in nature compared to the three methylated anthocyanidins, peonidon, malvidin and petunidin.

2.3.5 The Biosynthesis of Anthocyanins

There are several points in the flavonoid pathway where the product of one reaction provides the substrate for two or more subsequent steps (Figure 2.3). The first
step in the biosynthesis of flavonoids is the formation of chalcones. The enzyme chalcone synthase (CHS) catalyses the reaction between \( p \)-coumaroyl CoA and malonyl CoA to yield chalcone. \( p \)-coumaroyl CoA and malonyl CoA is synthesized from L-phenylalanine in the phenyalanine/hydroxycinnamate pathway (Strack, 1993) catalysed by phenylalanineammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CY4H), and hydroxycinnamatecoenzyme A ligase (C4L; Hahlbrock, 1989).

Chalcone isomerase (CHI), the second enzyme in the flavonoid pathway catalyzes the cyclization of a chalcone to the corresponding flavanone isomers (Naringenin). The next enzyme is the Flavanone 3-hydroxylase (FN3H) which catalyzes the stereospecific-flavanones to the dihydroflavonols (dihydrokaempferol).

Stafford (1982) detected the dihydroflavonol reductase (DFR) activity and identified the product as a flavan-3, 4-diol. The reaction involves the reduction of dihydrokaempferol to flavan-3, 4-diol. Flavan-3, 4-diols, which are also called leucoanthocyanidins, are very unstable molecules and are likely converted into the corresponding anthocyanidin. Adequate demonstration of this conversion in a cell-free system has not been accomplished, although Stafford and others use the designation ANS (anthocyaian synthase) to provide a name that can identify the overall reaction (Stafford, 1990). Other workers refer to the enzyme as leucoanthocyanidin dioxygenase (LDOX; Sparvoli, 1994). The conversion of leucoanthocyanidin to anthocyanin is likely both very fast and closely associated with a UDP glucose flavonoid 3-glucosyltransferase (UF3GT) that converts the newly formed anthocyanidin into the corresponding 3-O-glucoside (the anthocyanin).
Anthocyanins are present in the vacuole, to be more specifically, in anthocyanoplasts, the membrane bound spherical vesicles present inside the vacuole (Pecket, 1980) or in the cytoplasm (Harborne, 1988). Anthocyanoplasts are formed during pigment synthesizes, and are dispersed to produce a pigmented vacuole. In flowers, anthocyanins are exclusively located in epidermal cells, and only occasionally in the mesophyll cells (Delgado-Vargas, 2000). The flavonoids (anthocyanins) in grape berry are accumulated
in the central vacuole of three to six sub-epidermal cell layers at high concentrations in an acidic environment (Moskowitz, 1981).

### 2.3.6 Anthocyanins Sources

Natural sources of anthocyanins include a variety of colored fruits, vegetables, spices and nuts. These include berries, grapes, plums, peaches, pomegranate, red onion, red cabbage, egg plant, purple corn, purple sweet potato, black bean, pistachios and dates (Wu, 2006). The concentration and type of anthocyanins present in each fruit and vegetable vary considerably. For example, strawberry or raspberry contains cyanidin and pelaragonidin derivatives and red cabbage contain only cyanidin derivatives, while grapes and blueberries have almost all the anthocyanidin derivatives. In the U.S., the average daily intake of anthocyanins has been estimated to be 215 mg during the summer and 180 mg during the winter (Kuhnau, 1976). The common dietary sources include a variety of colored fruits and vegetables as well as fruit based processed foods and beverages like jelly, juices and red wine (Table 2.2).

### 2.3.7 Anthocyanins as a Food Colorant

Consumers are increasingly associating natural colorants to health benefits. Interest in the use of natural pigments in the food in place of synthetic food dyes has increased since the ban of FD&C Red Nos. 2 and 4 and Red No. 40 (Sapers, 1981). Pigments from natural sources are generally considered safe, display a wide range of colors and in addition, they possess health promoting benefits. Some of the natural colors
used in food include carotenoids from carrots, betalains from beets and anthocyanins from grapes, red cabbage and other fruits and vegetables.

Table 2. 2 Common sources of anthocyanins (Clifford, 2000).

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Content (mg litre(^{-1}) or mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackberry</td>
<td>1,150</td>
</tr>
<tr>
<td>Blueberry</td>
<td>825 – 4,200</td>
</tr>
<tr>
<td>Boysenberry</td>
<td>1,609</td>
</tr>
<tr>
<td>Cherry</td>
<td>20 – 4,500</td>
</tr>
<tr>
<td>Chokeberry</td>
<td>5,060 – 10,000</td>
</tr>
<tr>
<td>Cranberry</td>
<td>600 – 2,000</td>
</tr>
<tr>
<td>Cowberry</td>
<td>1,000</td>
</tr>
<tr>
<td>Currant (black)</td>
<td>1,300 – 4,000</td>
</tr>
<tr>
<td>Elderberry</td>
<td>2,000 – 10,000</td>
</tr>
<tr>
<td>Grape (red)</td>
<td>300 – 7,500</td>
</tr>
<tr>
<td>Loganberry</td>
<td>774</td>
</tr>
<tr>
<td>Orange, Blood (juice)</td>
<td>2,000</td>
</tr>
<tr>
<td>Plum</td>
<td>20 – 250</td>
</tr>
<tr>
<td>Raspberry (black)</td>
<td>1,700 – 4,277</td>
</tr>
<tr>
<td>Raspberry (red)</td>
<td>100 – 600</td>
</tr>
<tr>
<td>Raspberry (red) single strength juice</td>
<td>4 – 1,101</td>
</tr>
<tr>
<td>Sloe</td>
<td>1,600</td>
</tr>
<tr>
<td>Strawberry</td>
<td>150 – 350</td>
</tr>
<tr>
<td>Cabbage (red)</td>
<td>250</td>
</tr>
<tr>
<td>Eggplant</td>
<td>7,500</td>
</tr>
<tr>
<td>Onion</td>
<td>Up to 250</td>
</tr>
<tr>
<td>Rhubarb</td>
<td>Up to 2,000</td>
</tr>
<tr>
<td>Wines (red)</td>
<td>240 – 350</td>
</tr>
<tr>
<td>Wines (port)</td>
<td>140 – 1,100</td>
</tr>
</tbody>
</table>

According to the numbering system used by the *Codex Alimentarius Commission*, anthocyanins (any anthocyanin-derived colorant) are listed as natural colorant by the European Union (EU) legislation as product E163. In the U.S., the FDA (Food and Drug Administration) has a different list of “natural” colors that do not require certification (without any FD & C numbers), and anthocyanins can be obtained either from “grape
color extract”, “grape skin extract”, or “fruit juices or vegetable juices”. In addition to grapes, other common sources include red cabbage, blackcurrants, radishes, elderberries, chokeberries, blackberries, black raspberries, and black currants. Espin (2000) studied the antiradical capacity (DPPH) of anthocyanin based fruit extract (black chokeberry, black-thorn and strawberry) and commercial colorant Ponceau 4R. The fruit based natural color exhibited antioxidant activity comparable to that of butylated hydroxyanisole whereas the synthetic colorant Ponceau 4R did not. Espin (2000) suggested that natural extracts can be used as potential colorants and as antiradicals.

Enocyanin, an anthocyanin was extracted from grape skin and used as a food colorant in Italy as early as 1879 and was initially used to color red wine. But later, it was used as a food colorant and a number of countries started producing the colorant. Other sources, elderberries, blueberries, cherry plums, black currants, red raspberries, red sweet potatoes are also used for the extraction of anthocyanins to be used as food colorant. When Imbert (1966) compared pigments of commercial blackberry extract, enocyanin and red sweet potato, the sweet potato pigments (acylated cyanidin and peonidin based anthocyanins) were more stable than the commercial blackberry extract and enocyanin.

2.3.7 The Properties and Reactions of Anthocyanins

pH

Anthocyanins co-exist in aqueous solution in equilibrium between four species depending on pH: red flavylium cation, blue quinonoidal base, colorless carbinol
pseudobase and the colorless chalcone (Brouillard and Delaporte, 1977; Brouillard and Dubois, 1977; Brouillard, 1990; Brouillard, 1982).

Below pH 2, anthocyanins exist predominantly in the red flavylium cation form. As the pH increases, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the thermodynamic proton transfer reaction related to the acidic hydroxyl groups of the aglycone. Rapid hydration of the flavylium cation occurs at the C-2 position to generate the colorless carbinol pseudobase at pH values ranging from 3 to 6 which can further undergo ring opening to a chalcone pseudo-base (Fossen, 1998). At any pH, the chalcone present is much lower than the carbinol form. The formation of chalcone can be increased with an increase in temperature. Deprotonation of the flavylium cation to generate the quinonoidal bases occurs at slightly acidic to neutral condition, and this reaction is extremely fast (Brouillard, 1977). Further, deprotonation of the quinonoidal bases takes place with the formation of purplish resonance-stabilized quinonoid anions between pH values of 6 and 7.

The reverse transition from carbinol pseudobase to flavylium cation is almost instant upon acidification (Brouillard, 1977). Reversion of chalcone to flavylium cation is a very slow process and takes hours to reach completion (Francis, 1989).

Temperature

Foods containing anthocyanins are thermally processed before consumption and this process greatly influences the anthocyanin content in the final product (Giusti, 2003).
Generally, temperatures higher than 70°C cause rapid degradation and discoloration of anthocyanins (Cemeroglu, 1994; Kirca, 2003). Opening of the pyrylium ring with a loss of the B-ring and chalcone formation which further decompose to coumarin glucoside derivatives was considered as the first anthocyanin degradation step (Markakis, 1957; Hrazdina, 1971). Hydrolysis of the glycosidic moiety and formation of aglycon even at acidic pH as the initial degradation reaction, has been reported (Adams, 1973). Tanchev (1976) detected seven compounds following thermal degradation of anthocyanins, among which quercetin, phloroglucinaldehyde and protocatechuic acid were identified by paper chromatography. It is believed that the thermal degradation of anthocyanins leads to two end products: aldehyde and benzoic acid derivatives or chalcone and coumaric acid (Markakis, 1974; Jackman, 1992; Piffaut, 1994; Seeram, 2001). In case of acylated anthocyanins, acyl-glycoside moieties were first split off from the flavylium backbone and finally ended up with phenolic acids and phloroglucinaldehyde (Figure 2.4; Sadilova, 2007). Anthocyanin degradation is generally correlated to a loss or change in color and a decline in the antioxidant activity. Sadilova (2007) studied the structural changes of anthocyanins extracted from black carrot, elderberry and strawberry heated over 95°C. The antioxidant activity of anthocyanins from strawberry had lower antioxidant activity compared those from black carrots had the maximum antioxidant activity compared among the three extracts. The loss of anthocyanin bioactivity could not be compensated by the antioxidant activity of the newly formed phenolics. The degradation of black currant anthocyanins was studied using black currant nectar for a period of six months.
Iversen (1999) reported a loss of 50% of the anthocyanins after six months stored at 20°C.

Figure 2. 4 Thermal degradation anthocyanins (Sadilova, 2007).

**Light**

Light is a significant factor accelerating anthocyanin degradation similar to temperature and oxygen. While temperature effects predominate in degradation of cranberry anthocyanins, at higher temperature (55°C), fluorescent light effects predominate at lower temperature (40°C). Also, molecular oxygen is required for degradation of anthocyanins by light. The degradation of anthocyanins by light follows first order kinetics, similar to degradation at higher temperatures (Attoe, 1981). Photodegradation of anthocyanins proceeds from flavylium cation via carbinol pseudo-base to the chalcone (Maccarone, 1985), although direct photodegradation of the
flavylium cation can also occur (Furtado, 1993). Acylation of anthocyanins reduces their susceptibility to light (Giusti, 1996).

Other factors like oxygen, hydrogen peroxide, ascorbic acid, and enzymes like polyphenoloxidase and peroxidase can also degrade anthocyanins. Sugar degraded anthocyanins at a faster rate compared to thermal degradation. The degradation products of sugar - furfural and 5-hydroxymethylfurfural greatly accelerate the pigment destruction (Daravingas, 1968).

2.3.8 Anthocyanins and Health

Interest in anthocyanins intensified after the recognition of their potential health benefits (Scalbert, 2000). Epidemiological studies have suggested a reverse association between consumption of polyphenols and incidence of chronic diseases cancer and cardiovascular diseases. Anthocyanins are among the most abundant polyphenols in fruits and vegetables and possess potent antioxidant activity and have been incorporated into the human diet for several centuries. The anthocyanins were components of traditional herbal medicines used by North American Indians, the Europeans and the Chinese and were normally derived from dried leaves, fruits (berries), storage roots or seeds. Anthocyanin-rich mixtures and extracts (though not purified compounds) have been used historically to treat conditions as diverse as hypertension, pyrexia, liver disorders, dysentery and diarrhoea, urinary problems including kidney stones and urinary tract infections, and the common cold. Consumption of anthocyanins have been reported
to improve vision and blood circulation (Konczak, 2004), antiulcer activity (Cristoni, 1987) and UV protection (Sharma, 2001).

**Anthocyanins and Prevention of Cardiovascular Diseases**

Oxidation of low-density lipoprotein (LDL) triggers accumulation of macrophage white blood cells in the artery wall. Rupture of the plaque deposits oxidized cholesterol onto the artery wall, leading to atherosclerosis and eventually cardiovascular diseases (Aviram, 2000). Mortality from coronary heart disease can be reduced by moderate consumption of red wine. The primary mechanisms for this reduced risk include reduced platelet coagulation and higher circulatory high-density lipoprotein cholesterol (HDL). Other effects, inhibition of lipoprotein oxidation, free-radical scavenging and modulation of eicosanoid metabolism are presumed to play a role in the reduction of atherosclerosis (Mazza, 2007). Gracia et al. (1997) evaluated the antioxidant activity of several anthocyanins *in vitro* on human LDL and in a lecithin-liposome system and reported inhibition of LDL oxidation increases with increase in concentration of the anthocyanin. Malvidin exhibited greater inhibition of oxidation followed by delphinidin, cyanidin and pelarogonidin.

**Antioxidant Activity**

Reactive oxygen species (ROS), including free radicals (O$_2^-$), singlet oxygen (¹O$_2$), hydrogen peroxides (H$_2$O$_2$) and reactive nitrogen species such as nitric oxide are generated in animals and humans as a result of metabolic reactions. The cells utilize ROS as biological stimuli. They influence biochemical and molecular processes and cause
changes in cells during differentiation, aging and transformation. They influence the expression of several genes, signal transduction pathways and act as sub-cellular messengers for certain growth factors (Allen, 2000). Thus, they are important to the immune system, cell signaling, and several normal body functions (He, 2010). However, overproduction of ROS may exceed the physiological antioxidant capacity of the antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase) and antioxidant compounds (glutathione, tocopherol or ascorbic acid). This may lead to damage and dysfunction of enzymes, cell membranes and genetic material (Stintzing, 2004). Eventually, such sustained damage can lead to degenerative diseases like inflammation, cardiovascular disease, cancer and aging (Allen, 2000). Antioxidants can reduce oxidative molecular and cellular damage by preventing the damage to the biomolecules by free radicals or by interrupting the perpetuation of the free radical species.

Anthocyanins are potent antioxidants in vitro, quench free radicals and terminate the chain reaction that is responsible for the oxidative damage. Renis (2008) demonstrated the antioxidant effects of anthocyanins in vitro using human colon cancer cell line (CaCo2). Cyanidin and Cyanidin-3-glucoside treatment reduced cell growth and cell proliferation in a dose-dependent way, decreased ROS level by any concentration of CY and only at the lowest concentration, by CY3G and increased cell cycle/stress proteins expression. Both the compounds affected DNA fragmentation, highlighting their antioxidant activity. The authors concluded that anthocyanins exhibit antioxidant properties and could therefore be useful in the treatment of pathologies where free radical
production plays a key role (Acquaviva, 2003). The antioxidant activity of anthocyanins is largely because of the presence of hydroxyl groups in position 3 of ring C and also in the 3’, 4’ and 5’ positions in ring B of the molecule. Anthocyanidins have superior antioxidant activity compared to their respective anthocyanins and the activity decreases as the number of sugar moieties increase (Wang, 2008).

Ramirez-Toetosa (2001) investigated the antioxidant activity of anthocyanins in vitamin-E depleted rats. The rats were fed with rations containing highly purified anthocyanin-rich extract (3-glucopyranoside of delphinidin, cyanidin, petunidin, peonidin and malvidin) for 12 weeks. Consumption of anthocyanin rich diet significantly improves plasma antioxidant activity and decreases vitamin E deficiency enhanced lipid peroxidation and DNA damage. The antioxidant activity of many anthocyanins was comparable to the commercial antioxidants such as tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and vitamin E (Seeram, 2001).

**Anticarcinogenic Activity**

The anticancer activity of anthocyanins has been established largely based on *in vitro* evidence. The anticancer activities of anthocyanins are attributed to multiple mechanisms: anti-oxidative activity, phase II enzyme activation, anti-cell proliferation, induction of apoptosis, anti-inflammatory effects, anti-angiogenesis, anti-invasiveness, and induction of differentiation (Figure 2.5). Several *in vivo* studies in animals have shown the effect of anthocyanins in multiple cancer types. Most of the observed cancer
preventive effects were on gastrointestinal tract (GIT)-related organs including oral cavity, esophagus and the colon. In the GIT lumen, anthocyanins are largely available and are in direct contact with the epithelial layer (He, 2005), whereas their availability to non-GIT organs requires delivery through blood (He, 2010).

Figure 2. 5 Potential mechanisms of cancer prevention by anthocyanins (Hou, 2003).

**Phase II Enzymes**

The human body is continually exposed to potential carcinogens in the environment. The body deals with these compounds through a system of xenobiotic-metabolizing enzymes called phase I and phase II enzymes. Phase I enzymes oxidize xenochemicals into electrophilic intermediates, that can induce DNA damage and mutations. These mutations are responsible for the carcinogenic activity of many chemicals. Phase II detoxification enzymes are responsible for metabolizing products of the phase I metabolic reactions and degrade reactive electrophilic intermediates through
conjugation or reduction reactions, protecting cells from oxidative damage (Srivastava, 2007). Activation of phase II detoxifying and antioxidant enzymes is considered a major protective strategy against carcinogenesis. Activation of phase II detoxifying enzymes such as UDP-glucuronyl transferase (UGT), glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase (QR) results in the detoxification of carcinogens and hence reducing their anticarcinogenic effect. Anthocyanins can induce phase II antioxidant and detoxifying enzymes in clone 9 liver cells (Shih, 2007). Among the ten anthocyanins tested, cyanidin, kuromanin, delphinidin and malvidin showed higher efficacy in their antioxidant capacity and induction of phase II enzymes.

**Antiproliferative Activity**

Anthocyanins and anthocyanin rich extracts from fruits and vegetables have exhibited anti-proliferative activity towards multiple cancer cell types in vitro. Cell proliferation was inhibited by the ability of anthocyanins to block various stages of the cell cycle via effects on cell cycle regulator proteins (e.g. p53, p21, p27, cyclin D1, cyclin A, etc.; Wang, 2008). Zhang (2005) evaluated the anti-proliferated activity of anthocyanidins (cyanidin, delphinidin, pelargonidin, petunidin and malvidin) and anthocyanins (cyanidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-galactoside and pelargonidin-3-galactoside) against human cancer cell lines (stomach, colon, breast, lung, and central nervous system). The anthocyanins did not inhibit cell proliferation whereas the anthocyanidins showed antiproliferation activity. Malvidin showed the highest inhibitory activity, owing to the presence of a free hydroxyl group at 3-position in the C-ring, the hydroxyl groups at 3 and 4’and methoxy groups at 3’ and 5’ positions in
the B ring in malvidin is responsible for its higher activity. In anthocyanins, the hydroxyl group at 3-position is substituted by various sugar moieties and hence prevented it from being inhibitory to cancer cell proliferation.

**Apoptosis**

Apoptosis or programmed cell death plays a key role in the development and growth regulation of normal cells, and is often dys-regulated in cancer cells. Some of the most effective chemopreventive agents are strong inducers of apoptosis in premalignant and malignant cells (Wang, 2008). Apoptosis is characterized by chromatin condensation, cytoplasmic blebbing, and DNA fragmentation. The cells that have undergone apoptosis are rapidly recognized and engulfed by macrophages before cell lysis, and subsequently removed without inducing inflammation (Hou, 2003). Several anthocyanins, anthocyanidins and anthocyanin-rich extracts have exhibited pro-apoptotic effects in multiple cancer cell lines *in vitro*. They induce apoptosis through both intrinsic (mitochondrial) and extrinsic (FAS) pathways. Srivastava (2007) studied the effect of anthocyanin fractions from four high bush blueberry cultivars and reported that the anthocyanin fractions increased apoptosis by increasing the DNA fragmentation and caspase-3 activity when compared to the control cells.

**Inflammation**

Inflammation is a complex biological in response to tissue injury. Several cancers occur at sites of inflammation as inflammatory cells provide a microenvironment favorable for tumor development (He, 2010). Abnormal up-regulation of inflammatory proteins cyclooxygenase-2 (COX-2), which convert arachidonic acid to various
prostaglandins, prostacyclin, thromboxane A₂ and nuclear factor-kappa B (NF-κB) is a common occurrence in many cancers. Anthocyanins inhibit the mRNA and/or protein expression levels of COX-2, NF-κB and various interleukins, thus exhibiting their anti-inflammatory effect (Wang, 2008). Seeram (2001) investigated several cherry and berry anthocyanins for their cyclooxygenase inhibitory and antioxidant activities. The anti-inflammatory activity of anthocyanins from raspberries and sweet cherries were comparable to that of commercial anti-inflammatory drugs like ibuprofen and naproxen (Seeram, 2001).

**Angiogenesis**

Angiogenesis is the process of new blood vessel formation from the existing vascular network and is a key event that feeds tumor growth and cancer metastasis. Vascular endothelial growth factors (VEGF) play a crucial role in angiogenesis, and VEGF expression is frequently enhanced in developing tumors. Anthocyanins have been shown to suppress angiogenesis through several mechanisms such as: inhibition of H₂O₂ - and tumor necrosis factor alpha (TNF-α)-induced VEGF expression in epidermal keratinocytes, and by reducing VEGF and VEGF receptor expression in endothelial cells (Wang, 2008). Matsunaga (2009) showed that bilberry anthocyanins (delphinidin, cyanidin and malvidin) inhibited VEGF-induced tube formation in a coculture of human umbilical vein endothelial cells and fibroblasts in a concentration dependent manner. Bilberry was also shown to decrease vessel proliferation in chick chlorioallantoic membrane angiogenesis model *in vivo* (Ozgurtas, 2009).
Metastasis

The progression of a tumor from being in situ to invasive is a major prerequisite for spread of cancer and involves the acquisition of cell motility, surface adhesion properties and activity of extracellular proteases like serine proteinase, matrix metalloproteinases (MMPs) and cathepsins. These proteases degrade the basement membrane collagen and eventually result in metastasis (Chen, 2006). Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside exerted a dose-dependent inhibitory effect on the migration and invasion of highly metastatic A549 human lung carcinoma cells (Chen, 2006). The two cyanidins were shown to decrease the expression of matrix metalloproteinase-2 (MMP-2) and urokinase-plasminogen activator (u-PA) in a dose-dependent manner and enhance the expression of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and plasminogen activator inhibitor (PAI).

The number of sugar and hydroxyl groups present on the B ring plays an important role in determining the bioactivity of anthocyanin. The ortho-dihydroxyphenyl structure on the B-ring is also essential for the activity. Many authors who studied the activity of cyanidin and cyanidin-glucosides or cyanidin-diglucosides report that cyanidin showed better activity at much lower concentration compared to the antioxidant activity of commercial antioxidants like BHA, BHT. The biological activities of anthocyanins increase with decreasing number of sugar units and/or increasing number of hydroxyl groups on the flavylium cation (Hou, 2003).
2.3.9 Anthocyanin Absorption and Metabolism

Most of the research on the health benefits of anthocyanins has been performed *in vitro*. To validate the prominent health promoting benefits, it is important to consider the bioavailability of anthocyanins *in vivo*. Bioavailability is generally defined as the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Anthocyanins have a range of molecular structures as a result of biosynthesis and accumulation in plant tissues. The naturally occurring anthocyanin can be modified before consumption, particularly during the processing and storage/shelf life (McGhie, 2007). Anthocyanins experience numerous environments and physiochemical conditions which may also modify them once they enter the body. Within the gastrointestinal tract there are distinct environments, stomach, small intestine, and colon, is characterized by different pH and microbial populations; both of which can modify the anthocyanins. The pH of the stomach is very low (pH 1-2), which maintains the anthocyanins in their stable form, the flavylium cation (McDougall, 2005). In contrast, the pH of the small and large intestine is largely neutral, where anthocyanins are much less stable and undergo multiple modifications (Seeram, 2001). And the microbial populations especially in the colon are likely to modify the molecular structures of anthocyanins. Deglycosylated and demethylated to the corresponding aglycones occurs on exposure of the anthocyanins to gut microflora (Keppler, 2005). The aglycones are unstable at neutral pH and are rapidly degraded to their corresponding phenolic acids, aldehydes through cleavage of the C-ring.
Anthocyanins are large, highly water-soluble molecules which cannot be absorbed by the cells or into the circulatory systems of animals and humans. Despite their high water solubility, the anthocyanins can cross the cell membrane and enter the cell. However, this absorption varies between different anthocyanidins and sugar moieties. For example, comparison between delphinidin-3-glucoside and malvidin-3-glucoside showed that malvidin had higher transport efficiency than delphinidin. Malvidin has two methyl groups which make them slightly non-polar compared to delphinidin which has all the hydroxyl groups. Similarly, a comparison between glucosides and galactosides showed that glucosides had higher transport efficiencies. Absorption of the anthocyanins could start from the stomach (Piskula, 1999) or the small intestine. When absorbed by stomach, they appear rapidly in the circulatory system. Bilitranslocase have been shown to interact with the absorbed anthocyanins and transfer it from the stomach to the liver. Some studies also suggest that anthocyanins were significantly absorbed by jejunum and slightly absorbed by duodenal tissue. Once absorbed by the jejunum or duodenum, activity of aglycones from primary flavonoid-glucoside occurs due to endogenous β-glucosidase and to a lesser extent the galactosides, xylosides and arabinosides. Free anthocyanidins are more hydrophobic and are much smaller than their corresponding anthocyanin, thus can easily penetrate the cell membrane passively. Intact anthocyanins are also absorbed by the small intestine either by inefficient passive diffusion or by the sodium-dependent glucose transporter (SGLT1). Acylated anthocyanins are generally recognized as non-absorbable in the small intestine due to their larger molecular size and lack of a sugar moiety for transporter binding. Non-absorbed anthocyanins and
anthocyanin-rhamnose and anthocyanin-rutinose travel down to the colon, where tremendous amount of microorganisms (≈10^{12}/cm^3) reside to provide catalytic and hydrolytic potential (Scheline, 1973). Humans do not have endogenous esterases to release the phenolic acids from these anthocyanidin-rhamnose and rutinose (Scalbert, 2000). So, the esterase activity of colonic microflora is required for the metabolism of these compounds and the acylated anthocyanins (Plumb, 1999). The released anthocyanidins undergo spontaneous ring fission to some extent to generate simple compounds such as phenolic acids. Anthocyanidins taken up from the GIT lumen are subsequently metabolized by phase II drug-metabolizing enzymes in the liver to glucuronides, sulfates and methylates. These conjugated metabolites are likely to be excreted in urine but alternatively a portion of them may re-enter the jejunum with the bile and later either being absorbed by the colon entering the enterohepatic circulation (Ichiyanagi et al., 2005). The absorbed intact anthocyanins and anthocyanidins are largely excreted in urine whereas the non-absorbed anthocyanins are excreted through feces.

Anthocyanin distribution in tissues has recently been evaluated in blackberry extract fed rat and blueberry fed pig models. In rats, the anthocyanin fractions were found in jejunum, stomach, kidney, liver and brain (Talavera, 2005), whereas in some, the anthocyanins were found mainly in liver and also in eyes, cortex and cerebellum (Kalt, 2008). These results suggest that the anthocyanins cross the blood-brain barrier and the blood-retinal barrier to potentially provide protection for brain and eye tissues.
2.3.10 Extraction of Anthocyanins

As anthocyanins are located inside the plant cell, organic solvents are used to extract them from homogenized plant material. In conventional extraction procedures, acidified organic solvents like methanol, ethanol or acetone are used. These solvents, along with the acid can denature the cellular membrane and release of intracellular anthocyanins into the solvent and maintain the anthocyanins in their native form. Use of excess acid may cause hydrolysis of the sugar residues into furfural and hydroxymethylfurfural, which have been shown to favor pigment decay. Elevated temperatures are used for the extraction of anthocyanins because of the increased diffusion rate and solubility of analytes in the solvent (Ju, 2003). However, temperatures above 70°C have been shown to degrade anthocyanins at a higher rate (Markakis, 1957). Thus, conventional extraction technologies are time consuming, require large volumes of solvent and additional process steps to remove the solvent for use of anthocyanins as additive in foods.

Novel extraction methods like accelerated solvent extraction, ultrasound assisted extraction, microwave assisted extraction, high hydrostatic pressure and pulsed electric field assisted extraction are used instead of the conventional Soxhlet extraction technique. In accelerated solvent extraction, mostly water is used as a solvent and high temperature and pressure gives the water a non-polar character. Moreover, the viscosity and the surface tension of the water decreases and the diffusion rate and solubility of the target compound increases. But as accelerated solvent extraction employs high temperature, anthocyanins may degrade during extraction. Petersson (2010) extracted red onion
anthocyanins using accelerated solvent extraction at 110°C, and reported that the extraction effect dominates at first, but soon, the degradation of anthocyanins takes over.

Ultrasound assisted extraction is another novel technique for the extraction of bioactive compounds. Sound waves with frequencies higher than 20 kHz are involves in expansion and compression cycles when it travels through a medium. The expansion creates bubbles in the liquid producing negative pressure. The collapse of a bubble near a solid surface pushes solvents into cellular materials at high speed, improving mass transfer. When Corrales (2008) compared ultrasonics, high hydrostatic pressure and pulsed electric field assisted extraction of anthocyanins from grape by-products, the ultrasonics had the lowest extraction compared to the other techniques. Compared to conventional extraction at 70°C for 1 hour, ultrasonics has two-fold more extraction. The major disadvantage of the ultrasonics is that the waves are restricted to a zone located near to the ultrasound emitter. And also with increase in the solid particles, the ultrasound intensity goes down. For a uniform treatment of the sample, continuous stirring of the sample is required (Wang, 2005). Microwave assisted extraction can be used to achieve higher extraction rate with high temperature and short time. Water which has high dielectric constant heats up rapidly and with increase in the temperature, the diffusion rate increases. The plant matrix can also act as a dielectric and exposure to microwaves results in heating the matrix, leading to expansion and rupture of the cell wall, liberating the intracellular compounds. For extractions using microwaves, polar solvents like water or acetone are required as non-polar solvents like hexane cannot be used. Microwave assisted extraction is a good alternative to conventional extraction because of the low
consumption of solvent and shorter extraction time. Sun (2007) reported an extraction efficiency of 98.3% of the total red pigments recovery from red raspberries using microwaves. However, this method requires the presence of a solvent with similar dielectric constant as water. And also an increase in temperature of the solvent is another factor to be considered even though literature on the degradation of anthocyanins during microwave extracted is lacking. High hydrostatic pressure works by deprotonating the charged groups, disrupting the salt bridges and hydrophobic bonds of the proteins present in the cell membrane. Because of these changes, the proteins lose their conformational structure and denature. Hence, the compounds present inside the cell can easily move out into the solvent. Corrales (2008) extracted anthocyanins from grape skins using high hydrostatic pressure. With extraction conditions of 100% ethanol, 50°C and 600 MPa, an improvement (23%) in extraction was reported. When the high pressure processing and thermal processing on total antioxidant activity, phenolic, ascorbic acid and anthocyanins content of strawberry and blackberry purees were studied, high pressure processing did not have any significant impact on the above properties. However, thermal processing reduced ascorbic acid, the anthocyanin concentration went down and also the antioxidant activity reduced when compared to the control (Patras, 2009). Extraction of bioactive components using high pressure processing requires packaging of the product prior to high pressure treatment. The sample has to be in vacuum packages and this limits the amount and quantity of samples which can be treated.

Compared to other extraction technologies, pulsed electric field has been used widely for extraction of pigments, sugar and other value added metabolites. The major
advantage of pulsed electric field, the temperature increase is negligible (< 1°; Gachovska, 2010). Previous research on extraction of intracellular compounds from plant cells using pulsed electric field employed a treatment chamber which can treat only limited quantity of sample and also they require the presence of a solvent like water or any organic solvent suitable for extraction. In this study, we treated the samples as a whole without any shredding and also without any solvent.

2.4 Pulsed Electric Field

2.4.1 History

Interest in electric field application for treatment of food and agricultural raw materials has increased over the past two decades. In 1940s, the electric fields were used in food processing for purposes other than inactivation of microorganisms. Flaumenbaum successfully used pulsed electric fields in a process that increases the permeability of plant tissues, facilitating extraction of cellular fluid. Many applications of PEF focus on increasing the efficiency of juice extraction from fruits using PEF as pretreatment.

The advantages of electrical treatment applications in the food industry are as follows:

1. simple and does not require any complex and expensive equipment
2. short processing time
3. possible application of AC electrical fields with industrial parameters
4. material processing without any food quality deterioration, in particular, as compared with traditional thermal processing methods;
5. can be easily applied in a combined mode, as supplementary to any pressing, thermal or microwave treatment.

2.4.2 Fundamental Aspects of Pulsed Electric Field Treatment

PEF technology is based on a pulsing power delivered to the product placed between two electrodes. The equipment consists of a high voltage pulse generator and a treatment chamber and necessary monitoring and control devices. Food product is placed in the treatment chamber, where two electrodes are connected together with a non-conductive material to avoid electrical flow from one to the other. Generated high voltage electrical pulses are applied to the electrodes, which then conduct the high intensity electrical pulse to the product placed between the two electrodes. The food product experiences a force per unit charge (the electric field), which is responsible for the irreversible cell membrane breakdown (Benz, 1980).

System Components

The pulsed electric field processing system consists of a high voltage source, capacitor bank, switch, treatment chamber, voltage and current measuring devices. Generation of pulsed electric fields requires a fast discharge of electrical energy within a short period of time. This is accomplished by the pulse-forming network (PFN), an electrical circuit consisting of one or more power supplies with the ability to charge voltages (up to 60 kV), switches (ignitron, thyratron, tetrode, spark gap, semiconductors),
capacitors (0.1-10 µF), inductors (30 µH), resistors (2 Ω-10 MΩ), and treatment
chambers (Gongora-Nieto, 2002).

**Power Supply**

High voltage pulses are supplied to the system via a high voltage pulse generator
at the required intensity, shape, and duration. The high voltage power supply for the
system can either be an ordinary source of direct current (DC) or a capacitor charging
power supply with high frequency alternating current (AC) inputs that provide a
command charge with higher repetitive rates than the DC power supply (Zhang, 1997).
The simplest PFN is an RC (resistance-capacitance) circuit in which a power supply
charges a capacitor that can deliver its stored energy to a resistive load (treatment
chamber) in a couple of microseconds, by activation of a switch (Gongoro-Nieto, 2002).
Total power of the system is limited by the number of times a capacitor can be charged
and discharged in a given time. The electrical resistance of the charging resistor and the
number and size of the capacitors determine the power required to charge the capacitor,
wherein a small capacitor will require less time and power to be charged than a larger
one. The capacitance $C_0$ (F) of the energy storage capacitor is given by:

$$C_0 = \frac{\tau R}{\sigma A} \frac{\tau \sigma A}{d}$$

Where $\tau$ (s) is the pulse duration, $R$ (Ω) is the resistance, $\sigma$ (S/m) is the conductivity of
the food, $d$ (m) is the treatment gap between electrodes, and $A$ ($m^2$) is the area of the
electrode surface. The energy stored in a capacitor is defined by the mathematical expression:

\[ Q = 0.5C_0V_2 \] ..........................(2.2)

Where \( Q \) is the stored energy, \( C_0 \) is the capacitance, and \( V \) is the charge voltage.

The second component of the PFN is the high voltage switching device needed to discharge the stored energy through the PFN circuit instantaneously. The switch plays an important role in the efficiency of the PEF system and is selected on its ability to operate at a high voltage and repetition rate. There are two main groups of switches currently available: ON switches and ON/OFF switches. ON switches provide full discharging of the capacitor but can only be turned off when discharging is completed.

For a pulse-forming network system, the relative electrical value of each component determines the shape of the pulse. In a capacitance-resistance circuit, the pulse generated is exponentially decaying where the voltage across the treatment chamber as a function of time is defined as

\[ V(t) = V_0 e^{\frac{t}{\tau}} \] ..........................(2.3)

Where \( V_0 \) is the voltage charged in the capacitor of the PFN, \( t \) is the pulse duration time, and \( \tau = RC \) is the time constant where in an RC circuit pulse duration equals approximately five time constants (Cogdell, 1999).
2.4.3 Mechanisms of Membrane and Cell Damage in External Electric Fields

The main purpose of PEF is to disrupt the plant cell membrane, to increase the release of intracellular compounds. The PEF treatment removes the cellular turgor component of the texture and exerts an effect on the viscoelastic properties of plant tissue (Finčan and Dejmek, 2003; Lebovka, 2003). The damage degree of materials depends on the electric field treatment strength, \( E \) and the treatment time. Electric field selectively influences the structure of the biological membranes inside the solid-water saturated biological materials. Because of the very low electrical conductivity of the membrane as compared to the surrounding liquid inside the biological tissue, the electric field appears to be concentrated mainly on the membranes.

For an ideal biological cell which is spherical, the transmembrane potential depends on the angle ‘\( \theta \)’ between the external electrical field \( E \) and the radius-vector on the membrane surface (Schwan, 1957):

\[
u_m = 0.75 f d_c E \cos \theta \]

Here, \( d_c \) is the cell diameter, \( f \) is a parameter depending on electrophysical and dimensional properties of the membrane, cell and surrounding media. Intensity of the electric field generated inside the membrane is \( E_m = u_m/d_m \) and the electric field enhancement factor \( k_m \) may be defined as
Because of the selective concentration of the electric field on membranes, the membrane structure alteration and damage may occur. The critical transmembrane potential of the membrane damage is of order $u_m \sim 1$ V (Weaver and Chizmadzhev, 1996) which corresponds to the critical electric field intensity generated inside the membrane of order $E_m \sim 10^8$ V/m. The PEF treatment with short pulses may cause damage of biological cells without noticeable heating of the media.

Some of the theories put forward to explain the selective damage of membrane is electroporation theory, electromechanical, electro-hydrodynamical, visco-elastic, electrothermal and electro-osmotic instability.

The electromechanical instability theory proposed by Zimmerman (1974) is widely accepted and proposes the formation of pores in the membrane. This theory considers the cell membrane as a capacitor that is filled with a dielectric material of a very low dielectric constant compared to the inside the cell and the surrounding environment. Because of the difference in the dielectric charges between cell membrane and inside of cells, free charges accumulate on both sides of the membrane generating a transmembrane potential of about 10 mV. A normal cell maintains this transmembrane potential for a variety of energy-linked processes like maintainence of intracellular pH, generation of ATP, active transport of solutes etc. Application of an external electric field results in accumulation of more charges causing membrane compression. The charges attract each other and more compression takes place and the membrane thickness is
reduced and the viscoelastic forces oppose this electrocompression of the membrane. When the transmembrane potential reaches approximately 1 V, the compressive force takes over the viscoelastic properties of the membrane and causes electrical membrane breakdown occurs.

During Joule overheating of the membrane surface, the electrical current flowing in a conductive media generates the Joule heat and warms up the medium. Here the membranes are the main Joule heating elements and the local temperature rise on a membrane is very high and can cause thermal damage of the membrane. Tsong (1991) proposed that exposure of a biological membrane to an electric field causes the formation of hydrophobic and hydrophilic pores. Hydrophilic pores conduct electricity generating localized Joule heating and hence the damage of the semi-permeable membrane of the cell. The cell membrane also has protein channels, pores and pumps. Joule overheating can also occur on these protein channels and permanently denature them resulting in the formation of pores. Electromechanical theory is based on the calculation of balance between the electric compressive forces and the elastic restoring forces of the membrane. Breakdown of a membrane occurs when electric compressive forces dominate. This model predicts thinning of the membrane by 30% of its original thickness.

Most of the theories are not yet fully understood, as numerous of discrepancies between the theoretical and experimental results exist, partly because of the complexity of the biological membrane.
2.4.4 Applications

The application of PEF for extraction of juice, pigment and other compounds from food materials is not new. The research on extraction using PEF started in 1949 when Flaumenbaum reported an increase in juice yield from prunes, apples and grapes using an alternating electric field of 220 V. PEF have been used as a pre-treatment step prior to juice pressing or as an intermediate treatment after pressing the juice. PEF has also been used for the extraction of sugar from sugar beets, anthocyanins from a large number of fruits, vegetables and also from agricultural waste, betalains from red beets and pectin from orange peel.

Juice Extraction

Pulsed electric field has been largely used in the extraction of juice and anthocyanins from grapes and grape byproducts. Expression and characteristics of juice extracted from white grapes was studied by Praporscic (2007) and reported an increase in juice yield from 49% to 76% with a significant improvement in absorbance and turbidity, indicating concentration. Juice extraction from Chardonnay white grape using pulsed electric field with two pressure conditions was studied. A PEF treatment of 400 V/cm was applied. The PEF pretreatment increased the juice yield by 67 - 75% compared to the control sample without any treatment (Grimi, 2009).

PEF was used to increase the extraction of juice from alfalfa mash. The mash was subjected to a PEF treatment of 200 pulses at 1 Hz in two successions. The juice yield
increased 38% and also the protein, mineral content and the dry matter increased significantly compared to non-PEF treated samples (Gachovska et al., 2006).

Bouzrara and Vorobiev (2000) showed an improvement in juice yield from sugar beet slices treated at 215, 300 and 427 V/cm at 500 pulses. The yield increased 43%, 68% and 79% for the above electric fields.

PEF was used as an intermediate in the cold juice extraction from sugar beet cossettes using a pilot scale multi-plate and frame pressing equipment and a pulse generator. A yield of about 80% in juice per initial mass of cossettes was achieved before washing. Purity of juices was higher following PEF treatment compared to those juices without to PEF treatment (96-98% and 90-93% respectively; Jemai and Vorobiev, 2004).

The influence of PEF treatment on apple mash (Royal Gala) prior to pressing was investigated by Toepfl et al. (2005), using a field strength of 0.5 to 5 kV/cm, applying 10-40 pulses. After a treatment at 3 kV/cm and 20 pulses a juice yield of 83.0% was obtained after pressing in comparison to 80.1% after enzymatic maceration and 75.4% for the untreated control.

**Anthocyanin Extraction**

Corrales (2008) compared extraction of anthocyanins from grape by-products using ultrasonics, high hydrostatic pressure and pulsed electric field, and reported an improvement of 10% in extraction compared to HHP and 17% more compared to conventional extraction, which minimal increase was observed with ultrasonics. They used electric field strength of 3 kV/cm and 30 pulses and further extraction was carried
out at 70°C using ethanol and water as solvents. Lopez (2008) studied the effect of PEF on the extraction of phenolic compounds during the fermentation of Tempranillo grapes. A treatment of 5 kV/cm and 10 kV/cm was used. The concentration of anthocyanin increased 21.5% and 28.6% for 5 kV/cm and 10 kV/cm respectively compared to control where there was no pretreatment of the must with PEF. PEF was used to extract anthocyanins from red raspberry. The extraction of anthocyanins increased with an increase in the number of treatment pulses (Zhang et al., 2006). Toepfl (2006) extracted anthocyanins from red grapes using PEF at 1.3 kV/cm and 120 pulses prior to pressing. Increased anthocyanin extraction was achieved compared to the control sample. In terms of the phenolic content, highest recovery was obtained for PEF treated sample, 3% lower yield was obtained for high pressure liquid extraction and 35% lower yield was obtained for conventional extraction method. A PEF treatment of 3 kV/cm and 30 pulses increased the anthocyanin extraction up to 10% in comparison to high pressure liquid extraction and 30% compared to convention extraction method.

The advantage of using PEF for the extraction of juice is increase in the yield as well as purity compared to mechanical pressing of juice. Mechanical pressing breaks the cells with weaker cell walls and extracts only that juice, sometimes highly turbid needing further processing such as centrifugation and filtration.

Normal juice extraction procedures do not extract all of the juice from the plant material. A pretreatment step which will break a majority of the cells in the plant material and results in higher juice yield is required. Pulsed electric field has been reported to efficiently extract juice from a variety of plant materials. Previous reports on pulsed
electric field treat a limited quantity of sample placed in a treatment chamber at a time. In industrial scale, treating small quantities is not feasible, more time consuming and requires design of large treatment chambers. To avoid these problems, the samples were treated directly by placing them between the electrodes without any pre-preparation step. This way, more samples can be treated continuously. Many of the papers reporting higher extraction of juice with PEF analyzed only for their total phenolics, anthocyanin or betalain concentration and their antioxidant activity. The effect of PEF on the compounds of the juice and if PEF affects the bioactivity against cancer cells was not tested. In this experiment, the extracted juice was analyzed for its phenolics and anthocyanin or betalain concentration, antioxidant activity and their bioactivity was tested against colon cancer cell lines.

2.5 References


56. López, N., Puértolas, E., Condón, S., Álvarez, I., & Raso, J. (2008). Effects of pulsed electric fields on the extraction of phenolic compounds during the fermentation of must of Tempranillo grapes. *Innovative Food Science & Emerging Technologies, 9*(4), 477-482.


CHAPTER 3
EXTRACTION OF JUICE FROM WHOLE RED CABBAGE BY PULSED ELECTRIC FIELD AND DETERMINING THEIR BIOACTIVITY

Abstract

Red cabbage is a rich source of anthocyanins which have health promoting properties and potential applications as natural food color. Pulsed electric field (PEF) technology used to enhance juice extraction from plant material by causing irreversible breakdown of the cell membrane. Red cabbage (whole floret) was treated without preparation (shredding or mashing), by placing it between two electrodes and 1 kV/cm electric field of 0.66 µF capacitance and 20 pulses were applied. The treated red cabbage was pressed to express the juice and the juice was analyzed for total phenolics, anthocyanin concentration and total antioxidant activity (2, 2’-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH)). Anti-proliferative and pro-apoptotic activities of the components of the extracted juice were evaluated on colon cancer cell line HCT-116 (p 53 +/- and p53 -/-). The juice volume, total phenolics, anthocyanin concentration and total antioxidant activity increased (p ≤ 0.01) for the PEF extracted red cabbage compared to non-PEF red cabbage. The red cabbage juice, irrespective of PEF treatment, reduced (p ≤ 0.05) HCT-116 cell proliferation and increased the pro-apoptotic activity. Application of PEF improved juice extraction, total phenolics and anthocyanin content, and did not affect bioactivity of the extracted juice.

Keywords: pulsed electric field, extraction, red cabbage, bioactivity, cancer
3.1. Introduction

Anthocyanins, responsible for the red, orange and purple colors of many flowers, fruits and vegetables also provide several health-promoting benefits (Markakis, 1982). Common sources of anthocyanins include blueberry, strawberry, grapes, sweet potato, black beans, red radish, purple corn and red cabbage. Red cabbage is a rich source of anthocyanins, mainly acylated anthocyanins: cyanidin 3, 5-diglucoside, cyanidin 3-sophoroside-5-glucoside and cyanidin3-sophoroside-glucoside acylated with sinapic acid (Tanchev, 1969).

Red cabbage belongs to the *Cruciferae* family, is inexpensive and is easy to grow, harvest and store (Pliszka *et al.*, 2009). Red cabbage color can be used as a natural food color. In general, acylated anthocyanins contain two sugar molecules (glucose and sophorose) and several aromatic acids (Dyrby, 2001). Other common sources of acylated anthocyanins are red radishes, red potatoes, black carrots and purple sweet potatoes (Giusti, 2003). While anthocyanin concentration is reduced after pancreatic digestion (McDougall, 2007), acylated anthocyanins are stable under acidic gastric digestion conditions. Acylated anthocyanins are very stable to pH, light and temperature as an extract, in non-carbonated beverages and pharmaceutical formulations compared to non-acylated anthocyanins (Dyrby *et al.*, 2001; Walkowaik-Tomczak, 2007; Chigurupati, 2002). Red cabbage color is being marketed commercially as a solution and as a spray dried powder for use as a food colorant. The red cabbage anthocyanins transition from purple-red to pink-red to blue-green between pH levels of 3 and 6, respectively. This red cabbage color can be widely used in wines, beverages, fruit sauces, candies and cakes.
While anthocyanins have been traditionally used as food colorants, they also possess health promoting benefits including anticarcinogenic activity. The effect of anthocyanins on different cancers have been studied, including colon, pancreatic, esophageal, lung and skin cancers.

Colon cancer is the third most common frequent cause of cancer-related death and the fourth most common malignancy in the United States (WHO, 2009). Diets high in fat are believed to cause colon cancer in humans (Chao, 2005) and diets rich in carbohydrates, fruits, vegetables and fiber may have a protective effect on cancer development (Terry et al., 2001). Red cabbage color available in the market was found to suppress colorectal carcinogenesis in mice (Hagiwara et al., 2002). Red cabbage extract has been reported to possess anti-diabetic and anti-inflammatory effects (Kataya, 2007; Igarashi, 2000).

Juice from fruits and vegetables is normally extracted using a hydraulic press. During this process, only a portion of the plant tissue cells are ruptured while some cells remain intact, resulting in inefficient extraction of juice. Pre-treatment steps such as heating, freezing/thawing, alkaline and enzymatic treatments are used to increase cellular disruption. However, some of the pre-treatment steps may destroy the bioactivity of the resultant juice and may affect its freshness (Chalermchat, 2004). Application of pulsed electric irreversible damage to cell walls and other structures and improves juice yield with minimal impact on nutritional quality (Chalermchat, 2004; Bouzrara, 2000; Bazhal, 2001; Ade-Omowaye, 2001). Gachovska (2010) used PEF treatment to enhance anthocyanin extraction from red cabbage samples and reported a 2.15 times increase in
anthocyanin extraction compared to non-treated red cabbage. However, the tissue samples had to be prepared (mashed) to fit the treatment chamber. Similarly, high juice yield from sugar beets, apple and paprika were reported (Bouzrara, 2000; Bazhal, 2001; Ade-Omowaye, 2001). However, most of the experimental treatments were done on small sample sizes (40 g). However, commercially it will be advantageous to have minimal preparatory steps and scale up to a continuous process.

The objectives were to evaluate

i. juice yield, total phenolics, total monomeric anthocyanins and total antioxidant activity of juice from PEF extracted and non-PEF extracted red cabbage and

ii. the effect of red cabbage juice on anti-proliferative and pro-apoptotic activities of colon cancer cell lines.

3.2. Materials and Methods

All reagents and chemicals were purchased from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich (St. Louis, MO), unless otherwise stated. The red cabbages were purchased from a local market and were used immediately for the experiment. The outer soiled leaves were removed, weighed, the height and diameter across the stem were measured. The red cabbages were then randomly assigned to control and treatment groups based on their weights.
The pulsed electric field treatment circuit consisted of an exponential decay pulse generator (CF60/25-12C, Hipotronics, Inc., Brewster, NY), a capacitor (General Atomics Electronic systems, San Diego, CA) and a spark gap switch. The generator contained a DC power supply with a maximum voltage of 60 kV and maximum power of 10 mA. The energy stored in the capacitor was discharged directly to the red cabbage placed between two stainless steel electrodes. The distance between the electrodes was the height of the red cabbage placed along its diameter (11 cm). One electrode was a circular stainless steel plate which was placed on one end of the red cabbage (stem). The ground electrode was a rectangular plate. Aqueous sodium chloride solution (7% w/w) was applied to the ground electrode to improve electrical conduction. The insulation material for the two electrodes was purchased from Deorin (American Plastics Supply & Fabrication, Omaha, NE). PEF treatment parameters were chosen based on preliminary experiments (not reported). The voltage applied to the sample was measured with a voltmeter (Model, P6015 A, Tektronix, Inc., Beaverton, OR). To select the number of pulses, the electrical impedance, an indirect measure of cell permeability was measured after each pulse treatment of the sample.

The PEF treatment was given to whole red cabbage by placing it between the two electrodes at different positions (diagonally not along the stem, along the stem and cutting into two halves and treating each half separately). The contact between the electrode and the red cabbage was better only when the red cabbage was placed along its stem (Figure 3.1). The distance between the electrodes was 12 cm and a voltage of 12 kV was applied (electric field strength of 1 kV/cm and capacitance 0.66µF). The impedance
was measured every 10 pulses, to evaluate the tissue rupture. Impedance vs. pulse number was plotted and a pulse number of 20 were selected (Figure 3.2). The pulse width was approximately 15 µs and the frequency of the pulse was 1 Hz. Treatment time is the product of pulse width and the number of pulses and therefore the treatment time was 0.3 ms. Negligible (<1°C) increase in temperature was observed.

The red cabbages were shredded after treatment using a domestic food processor (MFP 200, Minipro™, Black & Decker, Baltimore, MD) for 1 min to obtain a homogenous mash without any addition of water.

The homogenized mash (approximately 40 g) were placed in a juice press as reported by Gachovska et al. (2010) and pressed (3000 N) using an Instron universal testing machine (Bluehill2 Software, Norwood, MA).

A portion (5 mL) of the extracted juice was filtered (0.22 µm) and stored at -20°C to evaluate the bioactivity. The other part was refrigerated and used for spectrophotometric analysis.

3.2.1 Total Phenolics

Total phenolics were determined using Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). Briefly, Folin-Ciocalteu reagent (FC; 150 µL of 0.2 M) was added to diluted sample (35 µL). The samples were thoroughly mixed, held for 5 min at room temperature and sodium carbonate (115 µL ; 7.5%) was added to the sample and incubated for 30 min at room temperature (25°C). Absorbance was then measured at 765
nm (Synergy 2, BioTek, Winooski, VT) using gallic acid as a standard. The results were expressed as mg GAE/100 g fresh weight. All samples were analyzed in triplicate.

3.2.2 Total Monomeric Anthocyanins by pH-differential Method

Anthocyanins undergo reversible structural transformations with pH changes, showing a striking difference in the absorption spectra. The red colored oxonium form and the colorless hemi-ketal form predominates at pH 1.0 and 4.5, respectively. The pH-differential method permits accurate and rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds (Giusti and Wrolstad, 2001). Degraded pigments do not change color with pH changes and they are not included in the measurement as they absorb light at both pH values (1.0 and 4.5; Durst, 2005).

Two dilutions of the sample with potassium chloride buffer (pH 1.0; 0.025 M) and sodium acetate buffer (pH 4.5; 0.4 M) were prepared. An aliquot (0.1 mL) of the juice was transferred to a volumetric flask (10 mL) and made up to 10 mL with the corresponding buffer and absorbance was measured at 520 and 700 nm using a spectrophotometer (Shimadzu, UV-1800 Spectrophotometer, Columbia, MD). The absorbance (A) of the sample was calculated using the following equation:

\[ A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5} \]  

(3.1)

The monomeric anthocyanin concentration in the original sample was calculated using the following formula and expressed as mg Cy3gl equivalents/L:

\[ \text{Monomeric anthocyanin pigment (mg Cy3gl eq./L)} = \frac{(A \times MW \times DF \times 1000)}{(\varepsilon \times l)} \]
Where:

A is the absorbance calculated using the previous equation

MW is the molecular weight for cyanidin-3-O-glucoside (449.2).

DF is the dilution factor.

ε is the molar absorptivity of cyanidin-3-O-glucoside (26900).

l is the path-length (1 cm) of the cuvette.

The final results were expressed as mg cy3glu eq/100 g FW.

3.2.3 Total Antioxidant Activity by ABTS Assay

The total antioxidant capacity of the red cabbage juice was determined using 2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS⁺⁺) and was performed as described by Re (1999). Pre-formed radical mono-cation ABTS⁺⁺ was generated by oxidation of ABTS with potassium persulfate. The ABTS⁺⁺ working solution was generated by mixing two stock solutions (8 mM ABTS and 3 mM potassium persulfate) and allowed to react for at least 12 h at room temperature (25°C) in the dark. The solution (5 mL) was then diluted with phosphate buffer (45 mL of pH 7.4) containing 150 mM NaCl (405 mL of 0.2 M Na₂HPO₄, 95 mL of 0.2 M NaH₂PO₄ and NaCl (8.77 g) made up to 500 mL with deionized water and pH adjusted to 7.4 with 1 M NaOH). To 10 μL of sample (10 times diluted), 290 μL of ABTS⁺⁺/phosphate buffer solution was added. The samples were mixed and allowed to react for 30 min and measured at 734 nm (Synergy 2, BioTek, Winooski, VT), using trolox (concentration 0 μg/mL to 500 μg/mL) was used as
the standard. The results were expressed as µg trolox equivalents/100 g fresh weight. All
the samples were conducted in quadruplicate.

3.2.4 Total Antioxidant Activity by DPPH Assay

The total antioxidant activity of the beetroot juice was measured using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method described by Brand-Williams (1995). A DPPH stock solution (24 mg DPPH in 100 mL 80% ethanol) was diluted with ethanol (80% aqueous) to obtain an absorbance of 1.1 at 515 nm using a spectrophotometer. The diluted sample (15 µL) was allowed to react with the diluted DPPH (285 µL) solution for 30 min. After 30 min, the samples were read at 515 nm (Synergy 2, BioTek, Winooski, VT), using trolox as a standard. All samples were measured in quadruplicate. The results were expressed as µg trolox equivalents/100 g fresh weight.

3.2.5 Cell Lines

Human colon carcinoma cells HCT-116 (p53 +/- and p53 -/-) were grown in McCoy’s 5A medium containing phenol red (Sigma, St. Louis, MO) and maintained at 37°C in 5% CO₂ jacketed incubator. The media was supplemented with of bovine serum albumin (100 mL/L; Hyclone, Fisher, Pittsburg, PA), sodium bicarbonate (2.2 g/L; Sigma, St. Louis, MO) and streptomycin/penicillin solution (10 mL/L; Fisher, Pittsburgh, PA).
3.2.6 Anti-Proliferation Assay

HCT-116 cells were seeded (6,000 cells/well) and incubated for 24 h at 37°C. The medium was replaced with Dulbecco’s modified Eagle’s medium (DMEM) F-12 media without phenol red containing 0.25% charcoal stripped serum. Filtered red cabbage juice was added at concentrations ranging from 5 to 25 µg of GAE/mL of media. Medium (DMEM without phenol red) and deionized sterile water (pH 4.5) were used as controls. The cells were incubated for 48 h at 37°C and cell proliferation was measured using the water soluble tetrazolium assay (WST-1, Roche Molecular Biochemicals, Indianapolis, IN). WST-1 (10 µL) was added to each well and the cells were incubated for 2 h and the absorbance of the medium was measured at 480 nm (Synergy 2, BioTek, Winooski, VT).

The HCT-116 cells were plated (5 × 10^4 cells/mL per well) and incubated for 24 h at 37°C. Different concentrations of the red cabbage juice were added to the cells. The medium and deionized water pH 4.5 served as controls. The cell population was enumerated using a Cellometer Automated Cell Counter (Nexcelom, Lawrence, MA). All experiments were performed in triplicate and the results were expressed as means ± SE.

3.2.7 Pro-Apoptotic Assay

The HCT-116 cells were plated (5 × 10^4 cells/mL per well) incubated for 24 h at 37°C. Different concentrations of the beetroot juice were added to the cells. After 48 h of incubation, cell number was determined using a Cellometer (Nexcelom, Lawrence, MA) and 2 × 10^4 cells from each well were transferred to a 96-well opaque plate (Nunc 96 MicroWell Plate White, Thermo Scientific, Rochester, NY). The volume of each well
was made to 200 µL with DMEM stripped fetal bovine serum medium without phenol red. Caspase-Glo® 3/7 reagent (Caspase-Glo® 3/7 buffer and Caspase-Glo® substrate; 100 µL; Promega, Madison, WI) was added to each well. The plate was incubated for 30 min at room temperature (25°C) and the luminescence was measured (Synergy 2, BioTek, Winooski, VT). The samples were measured in triplicate and the results were expressed as means ± SE.

3.2.8 Lactate Dehydrogenase (LDH) Enzyme Assay (Cytotoxicity)

The cytotoxicity of the red cabbage juice was determined using a cytotoxicity detection kit (Roche Molecular Biochemicals, Indianapolis, IN). Cell-free supernatant (100 µL) from treated cells were collected. DMEM medium (200 µL) containing stripped fetal bovine serum (100 mL/L) was used as background control. Triton-X 100 (100 µL, 2%) treated cells were used as high control and supernatant from the cells treated with medium alone was used as low control. The reaction mixture (Diaphorase/NAD⁺, Iodotetrazolium chloride and sodium lactate) was also used as a control. The reaction mixture (100 µL) was added to the supernatant (100 µL) and high control (100 µL) and incubated for 30 min in dark at room temperature (25°C) and the absorbance was measured at 492 nm. All samples were processed in triplicate. The background control absorbance was subtracted from the absorbance value of each of the sample to obtain the expected value. The cytotoxicity (%) was calculated as

\[
\text{Cytotoxicity (％)} = \frac{\text{expected value} - \text{low control}}{\text{high control} - \text{low control}} \times 100 \quad \text{..........................(3.2)}
\]

The cytotoxicity (%) was expressed as mean.
3.2.9 Experimental Design

Red cabbages were purchased from a local market. The height and weight were measured. Ten red cabbages were selected based on their weights. The ten red cabbages were then separated into two groups randomly. The weight in both the groups was similar (800 g). First group containing five red cabbages were given PEF treatment. The second group of five red cabbages was not given any treatment and was called non-PEF treated red cabbages. For spectrophotometric analysis, juice from each red cabbage was analyzed in triplicate (total phenolics) and quadruplicate (total antioxidant activity). For cell culture studies, juice from one PEF-treated and non-PEF treated red cabbage was selected randomly. The juice was analyzed at 5 µg GAE/mL (WST-1 assay) and 15 and 25 µg GAE/mL (cell count, pro-apoptotic activity and cytotoxicity). These concentrations were selected because this the maximum concentration at which the colon cancer cells can be treated in vitro. The PEF-extracted and non-PEF extracted juice at each concentration was done in duplicate (pro-apoptotic activity) and triplicate (WST-1 assay, cell count, cytotoxicity).

3.2.10 Statistical Analyses

The amount of extracted juice, total phenolics, anthocyanins and antioxidant activity were analyzed by independent t-test. Cell proliferation and apoptosis were analyzed by general linear model SPSS Statistics Software (SPSS Inc., Chicago, IL).
3.3. Results and Discussion

3.3.1 Amount of Juice

PEF treatment of red cabbages resulted in drip of juices with brighter blue color within 20 min compared to the control samples (Figure 3.3). PEF treatment of plant tissue reduces turgor in the cells and the elastic modulus of the plant membrane, resulting in breakdown of the plant membrane and dripping of water (Bazhal, 2004). Gachovska et al. (2006) reported juice drip from PEF treated alfalfa mash within 20 min, indicating disruption of the cells due to electroplasmolysis.

Greater juice volume and yield (2.56 times) was obtained from PEF treated red cabbage compared to non-treated control (Figure 3.4). Geulen (1994) reported an increase in juice yield from 30% to 70% in carrot mash with particle size 3 mm and treatment of 2.6 kV/cm and 50 pulses. Similar improvement in juice yield (29%) was observed in sugar beets, after first pressing. A PEF treatment following the first press increased the juice yield to 80% of sugar beets (Jemai et al., 2006). Gachovska et al. (2006) reported a 38% increase in juice extraction for PEF treated alfalfa mash (1.5 kV/cm, 1 µF and 200 pulses) compared to control samples. Extensive sample (fruits or vegetables) preparation was performed for application of PEF in all previous reports. This preparation requires an additional unit operation(s) for the extraction of juices. Treatment of whole fruit or vegetable will simplify the process and also may reduce energy costs for juice production.
3.3.2 Total Phenolics

Application of PEF to red cabbage improved the total phenolics concentration by 2.47 times compared to the control (Figure 3.4). The PEF extracted red cabbage juice had 149.21 mg GAE/100 g fresh weight (FW) while the non-PEF extracted red cabbage juice had 60.33 mg GAE/100 g FW. Podsdek (2006) reported a total phenolics concentration of 171.36 mg/100 g FW for Kissendrup cultivar of red cabbage and 134.73 mg/100 g FW for the Koda variety. Singh (2006) reported a total phenolics (hydroxybenzoic, hydrocinnamic acids and anthocyanins) concentration of 101.30 mg/100 g for red cabbage. As we were only pressing out the juice, it was possible that some of the phenolics were trapped in the solid material, resulting in lower amount of total phenolics.

Guyot and others (2003) reported that only 42% of the total phenolics were extracted into the apple juice while over half of the total phenolics were trapped in the apple pomace. The total phenolics concentration of a solvent extracted sample is higher, as solvent extracts the majority of the components from the solid material. However, when a solvent is used for extraction, additional steps are needed to remove the solvent, which require additional time and energy. The method of extraction as well as the application of pressure (constant vs. progressively increasing) affect the release of the polyphenols from the plant tissue (fruit or vegetable; Grimi, 2009). Application of compressive force (3,000 N) for 2 min on control red cabbage resulted in extraction of 24.13 mg GAE/100 g FW of polyphenols. The lower amount of polyphenols probably was due to the application of the constant force, thus retaining the polyphenols in the tissue. However, in our experiment,
the total phenolics concentration for juice extracted from PEF treated cabbage increased compared to control red cabbage samples.

### 3.3.3 Total Monomeric Anthocyanins

The total monomeric anthocyanins in the extracted red cabbage juice were determined by a pH differential method. The major anthocyanins of red cabbage were cyanidin based molecules and hence, cyanidin-3-glucoside was used as a standard. Greater concentrations (1.84 times; p ≤ 0.01) of cyanidin-3-glucoside equivalents of anthocyanins were extracted from PEF treated red cabbage (23.72 mg/100 g FW) compared to non-treated red cabbage (12.89 mg/100 g FW; Figure 3.4). Timberlake et al. (1988) reported 25 mg/100 g FW of total monomeric anthocyanin content in red cabbage extracted using methanol. Mazza (1993) reported a wide range of anthocyanin concentration in red cabbage from 25 to 495 mg/100 g FW. The variations in the anthocyanin concentration depended largely on the cultivar selected and the extraction technology used. For example, the Koda cultivar of red cabbage had 40.53 mg/100 g FW whereas the Kissendrup cultivar had 76.16 mg/100 g FW of total anthocyanins (Podsedek, 2006). Further, total phenolics were extracted using 70% methanol and the anthocyanins concentration was determined using an HPLC, which may have resulted in the determination of higher concentration of anthocyanins. McDougall et al. (2007) reported that red cabbage contained 137.5 mg/100 g FW of total anthocyanins extracted using 0.5% acetic acid in water as solvent. It is possible that the solvent used for extraction resulted in determining different concentration of anthocyanins. Gachovska et al. (2010) extracted anthocyanins from red cabbage mash using pulsed electric field, and
water extraction was used followed by HPLC to quantitate the individual anthocyanin concentration. They reported a 2.15 times increase in anthocyanin extraction in PEF treated compared to non-treated red cabbage. Solvents extract more anthocyanins from solid materials than application of pressure alone. It is possible anthocyanins could have been trapped within the solid particles, resulting in lower concentration extracted in the juice. Metivier et al. (1980) reported that methanol extracted 20% more than ethanol and 73% more than water. When PEF is used as a pre-treatment to extract anthocyanins, use of organic solvents is not necessary and hence, further concentration techniques are not required. And also as red cabbage juice was further analyzed for its anti-carcinogenic activity, the presence of a solvent may interfere with the analysis. In this case, the solvent is removed without affecting the activity of the compounds present in the juice.

3.3.4 Total Antioxidant Activity: ABTS and DPPH Assays

The juice from PEF treated red cabbage contained 3.65 mMol Trolox/L and non-treated red cabbage juice had 1.42 mMol Trolox/L (Figure 3.4). Pliszka (2009) reported 2.59 to 3.19 mMol Trolox/L for three cultivars of red cabbage. It is possible that other components with antioxidant activity also were extracted and determined by ABTS*+ method. This method determines the antiradical activity of the polyphenols and other compounds with antioxidant activity in the extract such as anthocyanin condensate products, vitamins, amino acids, minerals and synergistic effects. Also, PEF can possibly inactivate enzymes. That also may be the reason for higher antioxidant activity (Corrales, 2008).
The DPPH assay measures a decrease in absorbance which is caused by reduction in free radicals in the presence of an antioxidant. The PEF treated red cabbage juice exhibited greater antioxidant activity (0.96 μMol Trolox/1 g FW) compared to the juice from non-PEF treated red cabbage (0.37 μMol trolox equivalents/1 g FW; Figure 3.4). Antioxidant activity of red cabbage juice using DPPH method was reported to be 6.76 - 9.19 μMol trolox/1 g of FW (Podsedek, 2006). The author reported that the red cabbage had higher antioxidant activity and ranked several vegetables based on their antioxidant capacity as red cabbage > Brussels sprouts > savoy cabbage > white cabbage.

3.3.5 Anti-Proliferation Activity

The anti-proliferative effect of red cabbage juice on p53 +/- and p53 -/- HCT-116 colon cancer cell lines was investigated at two concentrations (15 and 25 μg of GAE/mL) of the red cabbage juice. In both p53 +/- and p53 -/-cell lines, the red cabbage juice (PEF and non-PEF extracted) inhibited the proliferation of cells compared to controls (media and water pH 4.5) at both concentrations. With both cell lines, 15 μg GAE/mL did not inhibit cell proliferation (p ≤ 0.05) compared to 25 μg GAE/mL, indicating that the inhibition was dose dependent (Figure 3.5). Anthocyanins are potential inhibitors of cancer cell proliferation (Kang et al., 2003; Zhao et al., 2004; Yi et al., 2005; Reddivari et al., 2007). Tart cherry anthocyanins significantly decreased the proliferation of colon cancer cell line HT-29 and HCT 116 (Kang, 2003). Similarly, Zhao (2004) showed that chokeberry anthocyanin rich extract inhibited HT-29 cells more effectively than grape and bilberry anthocyanin rich extract. In both cases, cyanidin or cyanidin based derivatives
were more inhibitory than other anthocyanidin derivatives. Hagiwara et al. (2002) reported the effect of red cabbage color on 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhiP)-associated colorectal carcinogenesis in rats initiated with 1, 2-dimethylhydrazine. The anthocyanins inhibited promotion of colon tumors. Roy et al. (2006) showed that white cabbage juice had the highest anti-proliferative activity in HL 60 cells. Literature on anti-proliferative activity of red cabbage juice is lacking. As red cabbage juice is rich in acylated cyanidin derivatives, it may possess anti-cell proliferation properties. The PEF treatment did not affect (p ≤ 0.05) compounds in the red cabbage, as we did not observe differences between the juice from PEF-treated and non-PEF treated red cabbage.

The cell proliferation also was measured using a water soluble tetrazolium (WST-1) assay to confirm the results obtained in the cell count assay. Tetrazolium (slightly red) is cleaved to formazan (yellow) by the cellular enzymes. When the population of cells increases, the mitochondrial dehydrogenases in the sample increase, resulting in an increase in the amount of formazan dye. The formation of formazan is directly proportional to the number of metabolically active cells in the culture. The juice from PEF-treated as well as non-PEF treated red cabbages showed difference (p ≤ 0.05) in proliferation compared to media and water. At 5 µg GAE/mL, the treatments (PEF and control) did not show any difference in proliferation compared to controls (media and water pH 4.5). Largest concentration evaluated (25 µg GAE/mL) showed a decrease in cell proliferation compared to other two concentrations (5 and 15 µg GAE/mL; Figure 3.6). PEF has been reported to extract more bioactive compounds from a plant material
and may be responsible for the difference between the control and PEF treated samples. The results were similar to the cell count data. Reddivari et al. (2007) reported that, when using WST-1 assay, the purple potato showed a reduced cell proliferation and their data were similar to that obtained from the cell count data.

**3.3.6 Pro-Apoptotic Activity**

A reduction in apoptosis is required for survival of the cancer cells. Caspases are the primary enzymes involved in apoptosis as they cleave a number of intracellular substrates that trigger cell dissolution and eventually, cell death. The juice from both PEF-treated as well as non-PEF treated red cabbages increased (p < 0.05) apoptosis at 25 µg GAE/mL compared to 15 µg GAE/mL (Figure 3.7). We did not observe specific differences in apoptotic activity between the red cabbage juice from PEF and non-PEF treated red cabbage at the concentrations evaluated. Srivastava (2007) reported an increase in caspase-3 activity when HT-29 cells were treated with anthocyanin fraction from different blueberry cultivars. They reported that the highest activity was observed at 150 µg/mL. Apoptosis was more pronounced in the p53 +/+ cell line compared to the p53 -/- cell line, indicating that a functional p53 was required for its apoptosis enhancing activity. Resveratrol and other bioactive compounds require a functional p53 gene to enhance apoptosis of tumor cells (Bhat, 2001; Hastak, 2005). The cells lacking the p53 gene continued to grow without undergoing apoptosis even upon treatment with red cabbage juice.
3.3.7 Lactate Dehydrogenase (LDH) Enzyme Assay (Cytotoxicity)

The LDH assay measures the cytoplasmic enzyme lactate dehydrogenase which is rapidly released into the cell culture supernatant upon damage of the plasma membrane. Treatment of the cells with Triton-X 100, a surfactant damages plasma membrane, releasing LDH. The red cabbage juice, irrespective of the treatment (PEF and non-PEF), did not show toxicity to the cells compared to the Triton-X 100 treated sample (Figure 3.8). Similarly, tart cherry anthocyanins did not exhibit cytotoxic effect on HT 29 and HCT 116 cells even at the highest concentration of 1000 µM evaluated (Kang, 2003). Other reports also suggest that anthocyanins from other fruits did not have cytotoxic effect on colon cancer cells (Zhao, 2004).

3.4. Conclusions

Pulsed electric field treatment can be applied to the whole red cabbage without pre-treatment preparation such as mashing or size reduction. The PEF treatment resulted in an increase in juice expression (64 %), total phenolics, and anthocyanin concentrations and had greater antioxidant activity. The red cabbage juice reduced (p ≤ 0.05) the proliferation of human colon cancer cells and increased pro-apoptotic activity of the cells. The red cabbage juice makes an attractive alternative to artificial food colorants used in the food industry in addition to its potential health benefits.
3.5 References


3.6 Legend to Figures

Figure 3.1 Experimental set-up.

Figure 3.2 Measurement of impedance to determine pulse number.

Figure 3.3 Red cabbage after PEF treatment.

Figure 3.4 Effect of pulsed electric field treatment of whole red cabbage on the extraction (juice) volume, total phenolics and anthocyanin concentration in the juice and antioxidant activity (ABTS and DPPH assay) of the extracted juice.

Juice volume (g), total phenolics (mg GAE/100 g FW), anthocyanins (mg cyanidin-3-glucoside eq./100 g FW) and antioxidant activity (mg Trolox/100 g FW); *significant difference between PEF and non-PEF extracted red cabbage juice (p ≤ 0.01).

Figure 3.5 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) determined using cell counter.

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 3.6 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) determined using WST-1 assay.

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 3.8 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the pro-apoptotic activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 3.9 Cytotoxicity (%) of juice extracted from red cabbage treated with pulsed electric field treated and non-pulsed electric field on colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).
Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics.
Figure 3. Experimental set-up.
Figure 3. 2 Measurement of impedance to determine pulse number.
Figure 3. 3 Red cabbage after PEF treatment.
Figure 3.4 Effect of pulsed electric field treatment of whole red cabbage on the extraction (juice) volume, total phenolics and anthocyanin concentration in the juice and antioxidant activity (ABTS and DPPH assay) of the extracted juice.
Figure 3.5 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) determined using cell counter.
Figure 3. 6 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/- and p53 -/-) determined using WST-1 assay.
Figure 3.7 Effect of pulsed electric field treated and non-pulsed electric field treated red cabbage juice on the pro-apoptotic activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).
Figure 3. 8 Cytotoxicity (%) of juice extracted from red cabbage treated with pulsed electric field treated and non-pulsed electric field on colon cancer cell line HCT-116 (p53 +/- and p53 -/-).
CHAPTER 4
EXTRACTION OF BETALAINS FROM BEETROOTS USING PULSED ELECTRIC FIELD AND DETERMINING THEIR BIOACTIVITY

Abstract

Red beet color is a universally permitted food color and is a rich source of the betalains (betacyanins and betaxanthins). Increasing consumer demand for healthy and natural foods has resulted in searches for colorants that have health benefits and meet the regulatory requirements. Majority of the pulsed electric field (PEF) applications for extraction of bioactive components from plant materials require extensive preparation such as grinding and size reduction. Whole beetroots were treated with a 1.5 kV/cm electric field, 0.66 µF capacitance and 20 pulses. The beetroots were pressed for juice (3,000 N) and analyzed for total phenolics, betalains (betacyanins and betaxanthins) and total antioxidant activity (2, 2’-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS++) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH)). The juice was analyzed for its anti-proliferative and pro-apoptotic activity on colon cancer cell line HCT-116 (p53 +/+ and p53 -/-). The juice volume, total phenolics, betacyanins, betaxanthins concentrations and antioxidant activity were increased (p ≤ 0.01) for the juice from PEF treated compared to non-PEF treated beetroot. The red beet juice, irrespective of treatment (PEF and non-PEF) reduced (p ≤ 0.05) HCT-116 cell proliferation and increased apoptosis.

Keywords: pulsed electric field, extraction, beetroots, colon cancer
4.1. Introduction

Betalains are water-soluble pigments present in most families of the order Caryophyllales, Ranunculaceae (Cronquist, 1988) and can be classified as yellow betaxanthins and violet betacyanins. Similar to anthocyanins, they are present in the vacuoles of both vegetative tissue and the reproductive organs, mostly epidermal and/or sub-epidermal tissues (Jackman, 1996).

Betalains are derived from betalamic acid (Wohlpert and Mabry, 1968), with tyrosine as precursor via L-DOPA. The condensation of cyclo-DOPA (derivative of L-DOPA) and betalamic acid results in red betacyanin (betanidin) whereas the condensation of betalamic acid with an amino acid gives yellow betaxanthin (indicaxanthin; Stafford, 1994; Fig. 4.1).

![Figure 4.1 Structure of betalamic acid, betacyanin (betanidin) and betaxanthin (indicaxanthin).]
4.1.1 Biosynthesis of Betacyanins and Betaxanthins

The biosynthesis of betalains starts with two molecules of tyrosine. The tyrosine molecule is hydroxylated to L-DOPA by tyrosinase and L-DOPA is oxidized further and rearranged to form cyclo-DOPA by the same enzyme. L-DOPA formed from the other tyrosine undergoes a ring opening reaction at position 4 and 5 to form an unstable product seco-DOPA which spontaneously closes itself to form the betalamic acid. This is catalyzed by the enzyme DOPA 4, 5 dioxygenase (Fischer, 1972; Terradas, 1991 and Schliemann, 1998). Condensation of betalamic acid with cyclo-DOPA results in betacyanin. The betacyanin undergoes further glucosylation and acylation steps carried out by glucosyltransferases and acyltransferases. Betaxanthins are formed by the condensation of an amino acid to the aldehyde group of the betalamic acid to form Schiff’s base. Indicaxanthin is formed from the condensation of proline and betalamic acid (Zryd, 2004). The betaxanthins has an absorption maximum at 480 nm and the betacyanins at 538 nm, where the aromatic structure of cyclo-DOPA is responsible for the absorbance (Wyler and Dreiding, 1961; Figure 4.2).

The edible sources of betalains are red and yellow beetroot, colored Swiss chard, grain or leafy amaranth and cactus fruits of Opuntia and Hylodereus genera (Azeredo, 2009). The beetroot belongs to the Chenopodiaceae family of the Caryophyllales kingdom (Moreno, 2008). The two major pigments in red beet are the red-violet betanin, isobetaninn betanidin and isobetanidin and the yellow vulgaxanthin I and vulgaxanthin II (Azeredo, 2009). Depending on the cultivar, the betacyanin and the betaxanthin content of the red beetroots varies from 0.04-0.21% and 0.02-0.14%, respectively (Nilsson,
Beetroots are cultivated widely for their betanin compound for use as a natural food colorant (E162). Beetroot red is permitted widely in Europe and North America as a natural food colorant in dairy products like ice cream, sherbet and yogurt, dry soft drink mixes, confectionary and also used in soups as well as tomato and bacon products (Henry, 1996).

Figure 4. 2 Biosynthesis of betacyanin (betanidin) and betaxanthin (indicaxanthin; Stafford, 1994).

**4.1.2 Health Benefits of Betalains**

Beet root is one of the ten most potent vegetable for antioxidant activity, and betalains are the main components contributing to the antioxidant activity (Cao, 1996;
Stintzing, 2004). The antiradical activity of betacyanins was greater than that of betaxanthins, the two main components of betalains. The antioxidant activity of the betalains increases with an increase in the number of hydroxyl/imino groups and the position of hydroxyl groups and glycosylation of aglycones in the betalain molecules (Cai, 2003). Acylation increase the antioxidant activity while glycosylation reduces the activity.

The beet root extract has a greater anticancer activity compared to caspanthin, cranberry, red onion skin and short and long red bell peppers (Kapadia, 1996). Beetroot extracts induce phase II enzymes like quinone reductase in murine hepatoma cells in vitro (Wettasinghe, 2002) that detoxify potential carcinogens. Beetroot products inhibit neutrophil oxidative metabolism in a concentration-dependent manner and its pro-apoptotic effects were observed in stimulated neutrophils (Zielinska-Przyjembska, 2009).

4.1.3 Juice Extraction

Beetroot juice is normally extracted by pressing or aqueous extraction (Herbach, 2004; Kanner, 2001). Pulsed electric field (PEF) is a non-thermal processing technology used to enhance juice extraction from plant materials. PEF have been used to extract bioactive added compounds from plant cells including anthocyanins (Corrales, 2008; Tanya, 2010), sugar (Bouzrara, 2000; Belghiti, 2004) and betalains (Chalermitchat, 2004; Fincan, 2004; Lopez, 2009). PEF was used previously to extract betalains from beet slices by subjecting them in a treatment chamber. To avoid pre-preparation steps, in this study we subjected whole beetroots without peeling the skin to electric field.
The objectives of this study were to evaluate

i. juice yield, total phenolics, betalains (betacyanins and betaxanthins) and total antioxidant activity of juice from PEF treated and non-PEF treated red beet and

ii. The anti-proliferative and pro-apoptotic activity of PEF-treated and non-PEF extracted beetroot juice on colon cancer cell lines.

4.2 Materials and Methods

All reagents and chemicals were purchased from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich (St. Louis, MO). The beetroot were purchased from a local market and were used immediately for the experiment. The beetroots were washed thoroughly to remove the soil, weighed and the heights were measured. The beetroots were then assigned randomly to control or treatment groups based on their weights.

Pulsed electric field treatment circuit consisted of an exponential decay pulse generator (CF60/25-12C, Hipotronics, Inc., Brewster, NY), a capacitor (General Atomics Electronic systems, San Diego, CA) and a spark gap switch. The generator contained a DC power supply with a maximum voltage of 60 kV and maximum power 10 mA. The energy stored in the capacitor was discharged directly to the red beet placed between two stainless steel electrodes. The distance between the electrodes was the height of the beetroot (6 cm). An electrode, a small circular stainless steel plate was placed on one end of the beetroot and the ground electrode was a rectangular plate. Aqueous sodium chloride solution (7% w/w) was applied to the ground electrode to improve electrical
conduction. The insulation material for the two electrodes was purchased from Deorin (American Plastics Supply & Fabrication, Omaha, NE). PEF treatment parameters were chosen based on preliminary experiments and literature review (Fincan, 2004). The voltage applied to the sample was measured with a voltmeter (Model, P6015 A, Tektronix, Inc., Beaverton, OR). The pulse number was selected based on measurement of electrical impedance, an indirect measure of cell permeability (Figure 4.3).

The beetroot was placed between the two electrodes and electric field strength of 1.5 kV/cm, 0.66 µF and 20 pulses were applied. The pulse width was approximately 15 µs and the frequency of the pulse was 1 Hz. Treatment time was the product of pulse width and the number of pulses and therefore the treatment time was 0.3 ms. Temperature of the red beetroot after PEF treatment was measured using a temperature probe. The temperature increase was negligible.

Subsequent to PEF treatment, both the control and treated beetroot were shredded using a domestic food processor (MFP 200, Minipro™, Black & Decker, Baltimore, MD) for 1 min to obtain a homogenous mash without any addition of water.

The homogenized mash (approximately 60 g) was placed in a juice press. The press had a sample chamber, a plunger and a juice container. The juice was pressed using an Instron universal testing machine (Bluehill 2 software, Norwood, MA). A force of 3,000 N was used to press the juice out of the beetroots.
A portion (5 mL) of the extracted juice was filtered (0.22 μm) and stored at -20°C to evaluate the bioactivity. The other part was refrigerated and used for spectrophotometric analysis.

4.2.1 Total Phenolics

Total phenolics were determined using Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). Briefly, Folin-Ciocalteu reagent (FC; 150 μL of 0.2 M) was added to the diluted sample (35 μL). The samples were thoroughly mixed, held for 5 min at room temperature and sodium carbonate (115 μL ; 7.5%) was added to the sample and incubated for 30 min at room temperature (25°C). Then, the absorbance was then measured at 765 nm (Synergy 2, BioTek, Winooski, VT) using gallic acid as a standard. The results were expressed as mg GAE/100 g fresh weight. All samples were analyzed in triplicate.

4.2.2 Betalain Analysis

The betalain content was quantified by a method proposed by Nilsson (1970) with few modifications. The red beet juice was diluted (75 times) with deionized water and the absorbance of the diluted juice was read at 538 nm and 480 nm using a spectrophotometer (Shimadzu, UV-1800 Spectrophotometer, Columbia, MD). The betalain content was calculated using an equation proposed by Cai (1999)

\[
\text{Betalain content (mg/L)} = \frac{A \times DF \times MW \times 1000}{\epsilon \times L} \]

\[ \text{..................................(4.1)} \]
where,

A is the final absorbance after correcting the absorbance at 700 nm

DF is the dilution factor

MW is the molecular weight. 550 g/mol for betanin and 308 g/mol for indicaxanthin

ε is the molar extinction coefficient. 60000 l/mol for betanin and 48000 l/mol for indicaxanthin, and

L is the pathlength of the 1-cm cuvette.

All were measured in triplicate and results were expressed as means ± SE.

### 4.2.3 Total Antioxidant Activity by ABTS Assay

The total antioxidant capacity of the beetroot juice was determined using 2, 2’-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS*) and was performed as described by Re (1999). Pre-formed radical mono-cation ABTS* was generated by oxidation of ABTS with potassium persulfate. The ABTS* working solution was generated by mixing two stock solutions (8 mM ABTS and 3 mM potassium persulfate) and allowing them to react for at least 12 h at room temperature (25°C) in the dark. The solution (5 mL) was then diluted with phosphate buffer (45 mL of pH 7.4) containing 150 mM NaCl (405 mL of 0.2 M Na₂HPO₄, 95 mL of 0.2 M NaH₂PO₄ and NaCl (8.77 g) made up to 500 mL with deionized water and pH adjusted to 7.4 with 1 M NaOH). To 10 µL of sample (10 times diluted), 290 µL of ABTS*/phosphate buffer solution were
added. The samples were mixed and allowed to react for 30 min and was measured at 734 nm (Synergy 2, BioTek, Winooski, VT) using trolox (concentration 0 µg/mL to 500 µg/mL) as standard. The results were expressed as µg trolox equivalents/100 g fresh weight. All the samples were conducted in quadruplicate.

4.2.4 Total Antioxidant Activity by DPPH Assay

The total antioxidant activity of the beetroot juice was measured using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method described by Brand-Williams (1995). A DPPH stock solution (24 mg DPPH in 100 mL 80% ethanol) was diluted with ethanol (80% aqueous) to obtain an absorbance of 1.1 at 515 nm using a spectrophotometer. The diluted sample (15 µL) was allowed to react with of the diluted DPPH (285 µL) solution for 30 min. After 30 min, the samples were read at 515 nm (Synergy 2, BioTek, Winooski, VT) using trolox was used as a standard. All samples were measured in quadruplicate. The results were expressed as µg trolox equivalents/100 g fresh weight.

4.2.5 Cell Lines

Human colon carcinoma cells HCT-116 (p53 +/- and p53 -/-) were grown in McCoy’s 5A medium containing phenol red (Sigma, St. Louis, MO) and maintained at 37°C in 5% CO₂ jacketed incubator. The medium was supplemented with of bovine serum albumin (100 mL/L; Hyclone, Fisher, Pittsburg, PA), sodium bicarbonate (2.2 g/L; Sigma, St. Louis, MO) and streptomycin/penicillin solution (10 mL/L; Fisher, Pittsburg, PA).
4.2.6 Anti- Proliferation Assay

HCT-116 cells were seeded (6,000 cells/well) and incubated for 24 h at 37°C. The medium was replaced with Dulbecco’s modified Eagle’s medium F-12 media (DMEM) without phenol red containing 0.25% charcoal stripped serum. Filtered beetroot juice was added at concentrations ranging from 5 to 25 µg of GAE/mL of media. Medium (DMEM without phenol red) and deionized sterile water (pH 2.5) were used as controls. The cells were incubated for 48 h at 37°C in an incubator and cell proliferation was measured using the water soluble tetrazolium assay (WST-1, Roche Molecular Biochemicals, Indianapolis, IN). WST-1(10 µL) was added to each well, incubated for 2 h and the absorbance of the medium was measured at 480 nm (Synergy 2, BioTek, Winooski, VT).

The HCT-116 cells were plated (5 × 10^4 cells/mL per well) and incubated for 24 h at 37°C. Different concentrations of the beetroot juice were added to the cells. The medium and deionized water (pH 2.5) served as controls. The population of cell was enumerated using a Cellometer Automated Cell Counter (Nexcelom, Lawrence, MA). All the experiments were performed out in triplicate and the results were expressed as means ± SE.

4.2.7 Pro-Apoptotic Assay

The HCT-116 cells were plated (5 × 10^4 cells/mL per well) incubated for 24 h at 37°C. Different concentrations of the beetroot juice were added to the cells. After 48 h of incubation, cell number was determined using a Cellometer (Nexcelom, Lawrence, MA) and 2 × 10^4 cells from each well were transferred to a 96-well opaque plate (Nunc 96
MicroWell Plate White, Thermo Scientific, Rochester, NY). The volume was made to 200 µL with DMEM stripped fetal bovine serum medium without phenol red. Caspase-Glo® 3/7 reagent (Caspase-Glo® 3/7 buffer and Caspase-Glo® substrate; 100 µL; Promega, Madison, WI) was added to each well. The plate was incubated for 30 min at room temperature (25°C) and the luminescence was measured (Synergy 2, BioTek, Winooski, VT). The samples were measured out in triplicate and the results were expressed as mean ± SEs.

4.2.8 Lactate Dehydrogenase (LDH) Enzyme Assay (Cytotoxicity)

The cytotoxicity of the beetroot juice was determined using a cytotoxicity detection kit (Roche Molecular Biochemicals, Indianapolis, IN). Cell-free supernatant (100 µL) from treated cells were collected and transferred to a 96-well plate. DMEM medium (200 µL) containing stripped fetal bovine serum (100 mL/L) was used as background control. Triton-X 100 (100 µL, 2%) treated cells were used as high control and supernatant from the cells treated with medium alone was used as low control. The reaction mixture (Diaphorase/NAD⁺, Iodotetrazolium chloride and sodium lactate) was also used as a control. The reaction mixture (100 µL) was added to the supernatant (100 µL) and high control (100 µL) and incubated for 30 min in the dark at room temperature (25°C) and the absorbance was measured at 492 nm. All the samples were processed in triplicate. The background control absorbance was subtracted from the absorbance value of each of the sample to obtain the expected value. The cytotoxicity (%) was calculated as:
Cytotoxicity (%) = \frac{\text{expected value} - \text{low control}}{\text{high control} - \text{low control}} \times 100 \hspace{1cm} (4.2)

The cytotoxicity (%) was expressed as mean.

4.2.9 Experimental Design

Beetroots were purchased from a local market. The height and weight were measured. Twelve beetroots were selected based on their weights. The Twelve beetroots were then separated into two groups randomly. The weight in both the groups was similar (90-100 g). First group containing six beetroots were given PEF treatment. The second group of six beetroots was not given any treatment and was called non-PEF treated beetroots. For spectrophotometric analysis, juice from each beetroot was analyzed in triplicate (total phenolics) and quadruplicate (total antioxidant activity). For cell culture studies, juice from one PEF-treated and non-PEF treated beetroot was selected randomly. The juice was analyzed at 5 µg GAE/mL (WST-1 assay) and 15 and 25 µg GAE/mL (cell count, pro-apoptotic activity and cytotoxicity). These concentrations were selected because this the maximum concentration at which the colon cancer cells can be treated \textit{in vitro}. The PEF-extracted and non-PEF extracted juice at each concentration was done in duplicate (pro-apoptotic activity) and triplicate (WST-1 assay, cell count, cytotoxicity).

4.2.10 Statistical Analyses

The amount of extracted juice, total phenolics, anthocyanins and antioxidant activity were analyzed by independent t-test. Cell proliferation and apoptosis were analyzed by general linear model SPSS Statistics Software (SPSS Inc., Chicago, IL).
4.3 Results and Discussion

4.3.1 Amount of Juice

PEF treatment of beetroots resulted in drip of juices immediately after treatment compared to the control samples (visual observation). Similar observations were reported by Gachovska et al. (2006) from PEF treated alfalfa mash.

PEF has been reported to increase juice yield in several fruits and vegetables (Bouzrara, 2000; Bazhal, 2001; Schilling, 2007; Praporscic, 2007). PEF treatment of whole beetroots resulted in an increase (38.5%; $p \leq 0.01$) in the juice yield compared to the control (10%; Figure 4.4). Schilling (2007) reported an increase in apple juice yield from 1.7 to 7.7% for an electric field treatment of 1, 3 and 5 kV/cm of apple mash. Bouzrara (2000) reported a three fold increase in juice extraction from sugar beet cossettes. An increase (80%) in juice yield was reported by Jemai (2005) for a treatment which involved two initial pressing steps with an intermediate PEF treatment. In all of these reports, extensive sample preparation steps were performed, which requires an additional unit operation in the extraction of juices. For scaling up, treatment of whole fruit or vegetables would speed up the process and may also reduce energy costs for juice production.

4.3.2 Total Phenolics

The concentration of total phenolics increased ($p \leq 0.01$) by 55% for the PEF extracted sample compared to the non-PEF extracted (15%) beetroots (Figure 4.4). The total phenolics concentrations reported in literature are phenolics extracted using an
organic solvent or water. Kujala (2000) reported a total phenolics concentration of 15.5 mg GAE/g of dry material. As we were only pressing out the juice, it was possible that some of the phenolics were trapped in the solid material, resulting in lower amount of total phenolics yields in the juice. Kujala (2002) detected three phenolics [5, 5’, 6, 6’-tetrahydroxy-3, 3’-biindolyl, feruloylglucose and β-D-fructofuranosyl-α-D-(6-O-(E)-feruloylglucopyranoside)], two phenolic amides (N-Trans-feruloyltymamine and N-trans-feruloylhomovanillylamine) and four flavonoids (betagarin, betavulgarin, cochliophulin A and dihydroisorhamnetin) in aqueous methanol (80%) extracts from beetroot. These compounds were observed in the peel, crown and flesh of the beetroot with the flesh containing the least amount.

### 4.3.3 Betacyanins and Betaxanthins

The major betacyanin pigments are betanin and isobetanin while the major betaxanthin pigments are vulgaxanthin I and vulgaxanthin II (Azeredo, 2009). The application of PEF to beetroots increased betacyanin content from 9.29 to 26.61 mg/100 g FW and betaxanthin content from 4.38 to 9.18 mg/100 g FW (Figure 4.5). The red-violet betacyanin was the major pigment compared to the yellow betaxanthin. Fincan (2003) subjected thin disks of red beetroot to 1 kV/cm electric field strength and reported extraction of approximately 90% of total red pigment and ionic content were released following 1 h aqueous extraction compared to the untreated beetroots (5%). Aqueous extraction of shredded beetroots extracted 45-70% of the betalain pigments while from PEF extracted (7 kV/cm) beetroots five-fold more betalain pigments were extracted.
(Lopez et al., 2009). The authors also reported that PEF in combination with pressing shortened the extraction time 18-fold.

4.3.4 Total Antioxidant Activity: ABTS and DPPH Assays

Beetroots are among the most potent vegetables for of their antioxidant activity (Cao et al., 1996) which include garlic, kale, spinach, brussels sprouts, alfalfa sprouts and broccoli flowers. Green pepper, spinach, purple onion, broccoli, beet and cauliflower are the leading sources of antioxidant activities (Ou et al., 2002). In ABTS$^{++}$ assay, the antioxidant activity increased (p≤0.01) from 0.054 mMol trolox equivalents/100 g FW in non-PEF extracted beetroot juice to 0.223 mMol trolox equivalents/100 g FW in PEF extracted beetroot juice (Figure 4.4). Betalains that contain imino and hydroxyl groups as well as phenolics contribute to the antioxidant activity (Wu et al., 2005). Escribano (1998) reported that the antioxidant activity of betacyanins was greater than that of betaxanthins. Wettasinghe (2002) reported 210 µg/mL antioxidant activity for beetroots, similar to concentration observed in this study. The antioxidant activity was also determined by DPPH assay. The antioxidant activity increased from 0.079 mMol trolox equivalents/mL in non-PEF extracted sample to 0.293 mMol trolox equivalents/mL for PEF extracted samples (Figure 4.4). Literature on the antioxidant activity of red beet juice determined by DPPH assay is lacking. In this experiment, the PEF treatment of beetroot increased the antioxidant activity of the juice determined both by ABTS and DPPH assay. Since, more juice was extracted using PEF, it resulted in more antioxidant activity.
4.3.5 Anti-Proliferation Activity

The anti-proliferative of the beetroot juice on colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) was determined by counting the cells and WST-1 assay. A set of random PEF and non-PEF extracted samples were selected and concentrations of 15 and 25 µg GAE/mL were used. The PEF extracted and the non-PEF extracted juice reduced (p ≤ 0.05) cell proliferation compared to the controls (water and pH 2.5) using the cell count as well as the WST-1 assays. The number of cells decreased in both p53 +/+ and p53 -/- cell line (Figure 4.6 and 4.7).

Addition of beetroot juice from non-PEF extracted beetroot did not affect (p ≤ 0.05) p53 +/+ cell proliferation. Similarly, differences between the two concentrations of juice used (15 and 25 µg GAE/mL) were minimal for cell line. In the WST-1 assay, there was a difference between the PEF and non-PEF extracted juice, with reduced (p ≤ 0.05) cell concentration in the PEF extracted juice compared to the non-PEF extracted juice.

Reduced (p ≤ 0.05) cell proliferation were observed with PEF and non-PEF extracted beetroot juice compared to controls (media and water pH 2.5). The cell proliferation activity were similar for PEF and non-PEF extracted juice was similar (p≤ 0.05). Higher concentration (25 µg/mL) of beetroot juice reduced cell proliferation when compared to 15 µg/mL in both PEF and non-PEF extracted juice. Cell proliferation determined by WST-1 assay showed similar results with PEF extracted and non-PEF extracted juice reducing the cell growth compared to the controls (media and water pH 2.5).
Literature on the anti-proliferative activity of red beet on cancer cells is not available. However, Chavez-Santoscoy (2009) reported that prickly pear juice diminished cell viability of four cancer cells evaluated (prostate, colon, mammary and hepatic cancer cell lines). An increase in the total phenolic content from 22.3 to 226.3 µg GAE/g was reported from prickly pear. The betacyanin content ranged from 3.1 to 189 µg/g and the betaxanthin content ranged from 1.6 to 300.5 µg/g. Similarly, Wu (2005) reported that the peel of red pitaya prevented proliferation of melanoma cells. Red pitaya a source of betalains (10.3 mg/100 g FW of betacyanins), had an influence on the anti-proliferative effect. And also the flavonoids (myricetin, baicalein and gallic acid) present in red pitaya would have had an effect on cell viability. It is suggested that the presence of a C2-C3 double bond and ortho-dihydroxyphenyl structure in the flavonoid A- or B-ring confer greater antiproliferative activity to the flavonoid (Martinez, 2003).

4.3.6 Pro-Apoptotic Activity

Apotox- GLO Triplex assay was used to determine the pro-apoptotic effect of red beet juice on colon cancer cell line. The juice from PEF extracted red beet at 25 µg/mL showed pro-apoptotic activity while the non-PEF extracted juice did not show any pro-apoptotic activity in the p53 +/- cell line (Figure 4.8). That showed that the beetroot juice required a functional p53 gene to induce apoptosis. In the p53 -/- cell line, there was no difference in apoptosis between the controls and the beetroot juice.

Betanin from Opuntia ficus-indica induced apoptosis in chronic myeloid leukemia cells. The author showed that betanin induced apoptosis after cell entry through
intrinsic pathway mediated by the release of cytochrome c from mitochondria into the cytosol and PARP cleavage (Sreekanth, 2007).

**4.3.7 Lactate Dehydrogenase (LDH) Enzyme Activity (Cytotoxicity)**

The LDH assay measures the cytoplasmic enzyme lactate dehydrogenase which is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The beetroot juice tested was not cytotoxic to the cells, as indicated by the low LDH activity (Figure 4.9). The juice inhibited cell proliferation by not being cytotoxic to the cells.

**4.4 Conclusion**

Pulsed electric field treatment of whole beetroots (1.5 kV/cm) resulted in greater juice extraction compared to non-PEF extracted beetroots. The juice from PEF extracted beetroots contained higher concentration of polyphenols, betalains and antioxidant activity compared to non-PEF extracted juice. The beetroot juice decreased cell proliferation in both the cell lines (p53 +/- and p53 -/-) and increased apoptosis in the p53 +/- cell line. There were no significant differences between the juices from PEF extracted beetroot and non-PEF extracted beetroot on colon cancer cell line proliferation and apoptosis. Thus, PEF treatment improved juice extraction from beetroots and did not affect their functional and beneficial health promoting properties.
4.5 References


dihydroxyphenylalanine in a model ssay System. *Phytochemistry, 49*(6), 1593-
1598.

phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology
and Viticulture, 16*(3), 144-158.

V., & Reddanna, P. (2007). Betanin a betacyanin pigment purified from fruits of
Opuntia ficus-indica induces apoptosis in human chronic myeloid leukemia cell


33. Terradas, F. & - Wyler, H. (- 1991). 2,3- and 4,5-secodopa, the biosynthetic
intermediates generated from L-Dopa by an enzyme system extracted from the fly
agaric, Amanita Muscaria L., and their spontaneous conversion to muscaflavin
and betalamic Acid, respectively, and betalains. *Helvetica Chimica Acta, 74*(1),
124-140.

34. Wettasinghe, M., Bolling, B., Plhak, L., Xiao, H., & Parkin, K. (2002). Phase II
enzyme-inducing and antioxidant activities of beetroot (*Beta vulgaris* L.) extracts
from phenotypes of different pigmentation. *Journal of Agricultural and Food Chemistry*, 50(23), 6704-6709.


4.6 Legend to Figures

Figure 4.3 Measurement of impedance to determine pulse number.

Figure 4.4 Effect of pulsed electric field treatment of whole beetroot on the extraction (juice) volume, total phenolics concentration in the juice and antioxidant activity (ABTS and DPPH assay) of the extracted juice.

Juice volume (g), total phenolics (mg GAE/100 g FW), and antioxidant activity (mg Trolox/100 g FW); *significant difference between PEF and non-PEF extracted beetroot juice (p ≤ 0.01).

Figure 4.5 Effect of pulsed electric field treatment of whole beetroot on the extraction of betalains (betacyanins and betaxanthins) of the extracted juice.

Betalains (mg/100g FW); *significant difference between PEF and non-PEF extracted beetroot juice (p ≤ 0.01).

Figure 4.6 Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) determined using cell counter.

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 4.7 Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-) determined using WST-1 assay.

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 4.8 Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the pro-apoptotic activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics; Different letters represent significant difference (p ≤ 0.05).

Figure 4.9 Cytotoxicity (%) of juice extracted from whole beetroot treated with pulsed electric field treated and non-pulsed electric field on colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).

Designations 5, 15 and 25 represent 5, 15 and 25 µg GAE/mL of total phenolics.
Figure 4.3 Measurement of impedance to determine pulse number.
Figure 4.4 Effect of pulsed electric field treatment of whole beetroot on the extraction (juice) volume, total phenolics concentration in the juice and antioxidant activity (ABTS and DPPH assay) of the extracted juice.
Figure 4.5 Effect of pulsed electric field treatment of whole beetroot on the extraction of betalains (betacyanins and betaxanthins) of the extracted juice.
Figure 4.6 Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/- and p53 -/-) determined using cell counter.
Figure 4. Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the anti-proliferative activity of colon cancer cell line HCT-116 (p53 +/- and p53 -/-) determined using WST-1 assay.
Figure 4.8 Effect of pulsed electric field treated and non-pulsed electric field treated whole beetroot juice on the pro-apoptotic activity of colon cancer cell line HCT-116 (p53 +/+ and p53 -/-).
Figure 4. Cytotoxicity (%) of juice extracted from whole beetroot treated with pulsed electric field treated and non-pulsed electric field on colon cancer cell line HCT-116 (p53 +/- and p53 -/-).
CHAPTER 5

SUMMARY AND CONCLUSION

Pulsed electric field was used to treat red cabbages and beetroots for juice extraction without any sample preparation step. The PEF treatment increased ($p \leq 0.01$) the juice volume for both red cabbage and beetroot compared to non-PEF extracted vegetables. The PEF-treated juice was evaluated for total phenolics, anthocyanin (red cabbage), betalains (beetroot) and antioxidant activity. For the red cabbage juice, the total phenolics and anthocyanin concentration increased thus increasing the antioxidant activity of the juice. Similarly, for the beetroot juice, the antioxidant activity increased with increase in the total phenolics and betalains concentration. The juices were tested for their activity on colon cancer cells (HCT 116: p53 +/- and p53 --). The juices reduced cancer cell growth and increased apoptosis in the cancer cells. The juices showed increased apoptosis in the p53 +/- cell line compared to the p53 -- cell line. This finding suggests that the red cabbage anthocyanins and beetroot betalains require a functional p53 gene to induce apoptosis. Otherwise, the PEF extracted and non-PEF extracted juice did not have any difference with respect to their activity on cancer cell growth and apoptosis. This suggests that PEF did not affect the compounds present in the juice. Overall, PEF resulted in better extraction of juice and the PEF-treated and non-PEF extracted juices showed anti-carcinogenic activity on colon cancer cells. Higher yield obtained with pulsed electric field is of major interest from industrial point of view, since solvent amounts might be reduced and extraction times shortened. We have shown that
PEF can be applied to whole fruits and vegetables, this reduces the overall extraction time.
Reccomendations for Future Research

Recommendations for the future of this study includes, developing a continuous process, where the fruits and vegetables are treated with pulsed electric fields and pressed for juice. This requires designing electrodes appropriately so that any size of fruit and vegetable can be treated. The ground electrode can be designed to hold the fruit/vegetable and the positive electrode can be designed to have a spring mechanism to adjust the height according to the fruit/vegetable. This process if made automatic may not need any trained personnel.

Since both red cabbage and beetroot juice showed anticarcinogenic activity, further experiments are needed to evaluate their performance in vivo. First, the mechanism by which the juices increases apoptosis needs to be studied. As apoptosis increased in p53+/+ cell line, it suggests that the juices need a functional p53 gene. So when a p53 inhibitor such as α-pithifrin is added to the cells treated with juice, we can see how the p53 +/+ cells behave in the presence of both the juice and the p53 inhibitor. If the apoptosis is reduced in this case, we can confirm that the juices require a functional p53 gene for apoptosis. Further experiments can be designed to carry out in animal models. A diet can be designed which will contain the red cabbage and beetroot juice and fed to animals having colon cancer and studied for the anticarcinogenic activity of the juice.

As PEF parameters for whole vegetable treatment has been optimized on a batch-scale, the focus should be on making this a continuous process. And also, based on the in vitro cell culture experiments, further research on animal models is required to evaluate the anti-carcinogenic activity of red cabbage and beetroot juice in vivo.
Appendix

Procedures

A.1 Total Phenolics

Preparation of Gallic acid Standards

- Accurately prepare fresh gallic acid stock standard of ~1mg/mL in 80% acetone. Prepare 25mg in 25mL volumetric flask for best accuracy.
- Record exact weight and adjust standard curve accordingly.
- Dilute stock standard to appropriate range for the types of samples being assayed.

Standard: For samples with higher TP concentrations, use less dilute standards. 7.0 mL stock GA + 3.0 mL 80% acetone. Then prepare GA standard curve dilutions using volumes as follows for the best accuracy.

<table>
<thead>
<tr>
<th>µg/mL in assay</th>
<th>µL of Std</th>
<th>µL of distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>350</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>330</td>
</tr>
<tr>
<td>80</td>
<td>40</td>
<td>310</td>
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<tr>
<td>120</td>
<td>60</td>
<td>290</td>
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<td>160</td>
<td>80</td>
<td>270</td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>250</td>
</tr>
</tbody>
</table>
On plate, set up standard curve as above. In triplicate, pipette 35µL of each standard concentration into the well.

**Assay Procedure**

1. Parameters used
   - End point assay with 10 second mixing
   - Wavelength – 765nm
   - Incubator - 45°C
   - Standard curve constructed
   - Concentrations and statistics reported

2. Dilute 100µL juice with 900µL distilled water and then vortex. In triplicate microplate wells, pipette 35µL of dilute sample. *(Note: Use different pipette tips for different samples)*

3. Add 150µL 0.2M Folin-Ciocalteu (FC) reagent (freshly dilute strength of SIGMA reagent 1/10 with distilled water) to all wells. 2.0mL FC Reagent + 18mL distilled water is sufficient for one 96-well microplate.

4. Mix plate on a platform vortex. Shake at 400rpm for 30 seconds then hold for exactly 5 minutes at room temperature.

5. After 5 minutes, add 115µL 7.5% (w/v) Na₂CO₃ (7.5g/100mL distilled water) to all wells.

6. Mix again as above on a platform vortex.
7. Place the microplate in an incubator at 45°C for exactly 30 minutes. Cool to room temperature for 1 hour.

8. Read plate absorbance at 765nm.

Results

1. Use absorbance values form the microplate reader at concentrations expresses as µg GAE/mL in assay for calculations.

2. When calculating linear equation for GA standard curve, force line through zero (0mg/ml std is “blank” in TP microplate protocol).

A.2 Total Monomeric Anthocyanin Concentration

Prepare pH 1.0 and 4.5 buffer solutions.

Potassium chloride buffer, 0.025 M, pH 1.0: Mix 1.86 g KCl and 980 ml of distilled water in a beaker. Measure the pH and adjust to 1.0 with concentrated HCl. Transfer to a 1 liter volumetric flask and fill to 1 liter with distilled water.

Sodium acetate buffer, 0.4 M, pH 4.5: Mix 54.43 g CH₃CO₂Na ⋅ 3 H₂O and ~960 ml distilled water in a beaker. Measure the pH and adjust to 4.5 with concentrated HCl. Transfer to a 1 liter volumetric flask and fill to 1 liter with distilled water.

1. Take 0.1 mL of juice and prepare two dilutions of the sample, one with 10 mL of potassium chloride buffer, pH 1.0, and the other with 10 mL of sodium acetate buffer, pH 4.5.
2. Calculate the absorbance of the diluted sample (A) as follows:

\[ A = (A_{520} - A_{700}) \text{pH}_{1.0} - (A_{520} - A_{700})\text{pH}_{4.5} \]

3. Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

Monomeric anthocyanin pigment (mgCy3glu eq./L) = \( \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \)

Where:

- A is the absorbance calculated using the previous equation
- MW is the molecular weight for cyanidin-3-O-glucoside (449.2).
- DF is the dilution factor.
- \( \varepsilon \) is the molar absorptivity of cyanidin-3-O-glucoside (26900).
- l is the path-length (1 cm) of the cuvette.

**A.3 Betalain Concentration**

1. Dilute the juice 75 times.

2. Mix the diluted juice with 10 mL of water at pH 6.5.

3. Measure the absorbance at 438 nm, 510 nm and 700 nm using a spectrophotometer.

4. Calculate the concentration of betacyanins and betaxanthins using:

\[ \text{Betalain content (mg/L)} = \left\lfloor \frac{A \times DF \times MW \times 1000}{\varepsilon \times l} \right\rfloor \]

where,

- A is the final absorbance after correcting the absorbance at 700 nm
DF is the dilution factor

MW is the molecular weight. 550 g/mol for betanin and 308 g/mol for indicaxanthin

ε is the molar extinction coefficient. 60000 l/mol for betanin and 48000 l/mol for indicaxanthin, and

L is the pathlength of the 1-cm cuvette.

A.4 Total Antioxidant Activity: ABTS Assay

1. **Mother Solution:** Prepare 8 mM of ABTS (44 mg/10 ml) and 3 mM of potassium persulfate K2S2O8 (8 mg/10 ml) solutions using distilled deionized water. Mix equal volumes of the 2 and let react in the dark for atleast 12 hr at room temperature.

2. **Working Solution:** Add 5 ml of Mother Solution to 145 ml of phosphate buffer solution.
   - Prepare 500 mL of phosphate buffer containing 0.2 M Na2HPO4 (12.1 g) and 0.2 M NaH2PO4.H2O (2.62 g). 8.77 g of NaCl was added and the pH was adjusted to 7.4 using 1 Mol NaOH.

3. **Trolox standard:** Add 290 µl of the working solution to 10 µl serial trolox dilutions (0 µg/ ml, 50 µg/ ml, 100 µg ml, 200 µg/ml, 300 µg/ml, 400 µg/ ml, 500 µg/ ml), shake and let react for 15 min. Measure absorbance at 734 nm.
   
   Absorbance readings should fall between 0.1-1.6. R² should be atleast 0.995.

   **Trolox:**
Standard solution: 10000 µg/ml, which is 10 mg/ml. Make 100mg of trolox and add 20 ml 200 proof ethanol. Now the concentration is 5 mg/ml but a working solution of 1 mg/ml is required.

Working solution: from the 5 mg/ml standard solution, take 1 ml and dilute with 4 ml of 200 proof ethanol. So this makes 1 mg/ml.

Trolox dilutions: 0 µg/ml: 1000 µl 10% ethanol
50 µg/ml + 950 µl 10% ethanol
100 µg/ml + 900 µl 10% ethanol
200 µg/ml + 800 µl 10% ethanol
300 µg/ml + 700 µl 10% ethanol

4. Sample Analysis: add 290 µl working solution to 10 µl sample extract (dilute 10 times with deionized water), shake and let react for exactly 30 min. Measure absorbance at 734 nm.

A.5 Total Antioxidant Activity: DPPH Assay

1. Prepare DPPH solution by dissolving 24 mg DPPH in 100 ml 80% ethanol.

2. Dilute this stock solution ~10:55 with 80% ethanol until the spectrophotometer reads 1.1 at 515 nm.

3. Pipette out juice of 15 µl in to a scintillation vial and add 285 µl of diluted DPPH solution.

4. Allow the reaction for 30 min. after 30 min measure the absorbance at 517 nm using a spectrophotometer.
5. Prepare a standard curve of known concentrations of trolox. Use regression equation to convert the antioxidant activity into equivalents of trolox.

A.6 Anti-Proliferation Assay: WST-1 Assay

1. Seed 6,000 cells/well in microplates to a final volume of 200 µL culture medium and incubate in a humidified atmosphere (37°C, 5% CO₂).

2. After 24 h, replace the medium in the cells with fresh DMEM medium containing 0.25% stripped serum. Add 5, 15 and 25 µg GAE/mL of juice to the cells. Incubate at 37°C for 24 h.

3. Add 10 µL/well WST-1 reagent after 24 h.

4. Incubate the cells for 2 h in a humidified atmosphere (37°C, 5% CO₂).

5. Measure the absorbance using a microplate reader at 480 nm.

A.7 Anti-Proliferation Assay: Cell Count

1. Seed 5× 10⁴ cells in 12-well plates. Allow the cells to grow for 24 h in a humidified atmosphere (37°C, 5% CO₂).

2. After 24 h, replace the medium with fresh DMEM medium containing 0.25% stripped serum and add 15 and 25 µg GAE/mL of juice to the cells. Let the cells to react for 24 h at 37°C in an incubator.

3. 24 h later, count the cells using a cell counter. Add 20 µL of the trypsinized cells from the plate to a disposable cell counting slide. The cell counter gives the results as cells/mL.
A.8 Pro-Apoptotic Assay: Caspase-Glo

1. Seed $5 \times 10^4$ cells in 12-well plates. Allow the cells to grow for 24 h in a humidified atmosphere (37°C, 5% CO$_2$).

2. After 24 h, replace the medium with fresh DMEM medium containing 0.25% stripped serum and add 15 and 25 µg GAE / mL of juice to the cells. Let the cells to react for 24 h at 37°C in an incubator.

3. 24 h later, count the cells using a cell counter. Transfer $2 \times 10^4$ cells to a opaque microplate used for reading luminescence and make up the volume to 200 µL using DMEM medium containing 0.25% charcoal stripped serum.

4. Add 100 µL of Caspase-Glo 3/7 reagent to all wells and briefly mix by shaking.

5. Incubate for 30 min at room temperature.

6. Measure the luminescence.

A.9 Cytotoxicity: LDH Assay

1. Seed 6,000 cells /well in microplates to a final volume of 200 µL culture medium and incubate in a humidified atmosphere (37°C, 5% CO$_2$).

2. After 24 h, replace the medium in the cells with fresh DMEM medium containing 0.25% stripped serum. Add 5, 15 and 25 µg GAE / mL of juice to the cells. Incubate at 37°C for 24 h.

3. Next day, transfer 100 µL of supernatant from the cells to a 96-well microplate. Remove the supernatant without disturbing the cells. Supernatant is taken before WST-1 assay or cell count is done.
4. 100 µL of the LDH reagent was added and was incubated for 30 min in dark at room temperature.

5. After 30 min, the absorbance was measured at 492 nm.


   Low control: supernatant from cells not treated with juice.

   High control: cells treated with Triton-X 100.

7. Cytotoxicity (%) was calculated by using the following equation. The absorbance value was subtracted from its background control to obtain the expected value.

\[
\text{Cytotoxicity (\%)} = \frac{\text{expected value} - \text{low control}}{\text{high control} - \text{low control}} \times 100
\]