ABSTRACT

Title of Thesis **EFFECTS OF ANTHOCYANIN RICH EXTRACTS ON MULTIPLE BIOMARKERS OF COLON CANCER**

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The aim of this study was to investigate the chemoprotective activity of anthocyanin-rich extracts (AREs) from bilberry (*Vaccinium myrtillus*), chokeberry (*Aronia meloncarpa)* and grape (*Vitis vinifera*) by assessing multiple biomarkers of colon cancer in rats treated with a colon carcinogen, azoxymethane. Male F344 rats (n = 40) were fed AIN-93 diet (control) or AIN-93 diet supplemented with AREs for 14 weeks. Biomarkers evaluated included total aberrant crypt foci (ACF) that were reduced in ARE diet groups as compared to the control group. The number of large ACF and colonic cellular proliferation were decreased in rats fed bilberry and chokeberry ARE diets. Rats fed bilberry and grape ARE diets had lower COX-2 mRNA levels expression. Increased levels of fecal anthocyanins, fecal mass and moisture occurred in ARE-fed rats. Significant reduction of fecal bile acids was observed in ARE-fed rats. The results from this study suggest a protective role of AREs in colon carcinogenesis, and indicate multiple mechanisms of action are involved.

EFFECTS OF ANTHOCYANIN RICH EXTRACTS ON MULTIPLE BIOMARKERS OF COLON CANCER

By

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1.1. Introduction

Cancer, a disease resulting from deregulated cell growth control, is caused by the interaction of dietary, genetic, and environmental risk factors. Dietary factors are considered to play a major role in cancer etiology. It has been estimated that potential for cancer prevention by a healthy diet and lifestyle is excellent and might reduce the burden of frequently occurring cancers of the breast, prostrate and colon by 33-55%, 10-20% and 66-75%, respectively (1).

Colorectal cancer is the third most common and the third leading cause of cancer related mortality in the United States (2). It is estimated that approximately $8.4 billion is spent in the United States each year on treatment of colorectal cancer (3). In Maryland even though the knowledge regarding colorectal cancer screening is high (92%) colon cancer is still second leading cause of cancer (4). Several epidemiological and laboratory studies suggest a strong relationship between colon cancer risk and dietary factors (5-7). There is increasing evidence that risk is increased by high intakes of meat and fat, and that risk is decreased by high intakes of fruits and vegetables, and dietary fiber (8, 9). This field of investigation is nevertheless very confusing, particularly because longstanding hypotheses, such as the presumed protective effects of fruits, vegetables, and fiber, have recently been challenged by well-designed prospective trials. The search for individual components in the diet that convey protection persists (10).

Anthocyanins are natural pigments that provide intense purple to red color in many fruits and vegetables such as blueberries, grapes, red cabbages and purple corn. Epidemiological investigations have indicated that moderate consumption of

anthocyanins through intake of products such as red wine (11) or bilberry extract (12) is associated with lower risk of cardiovascular disease and improvement of visual functions. In recent years, considerable studies have exhibited the ability of anthocyanins to inhibit oxidative stress (13, 14) and to induce apoptosis in malignant cells (15, 16) which both suggest that anthocyanins may prevent carcinogenesis.

Anthocyanin fractions extracted from different sources, including flower petals (17), grape rinds and red rice (18), red soybeans and red beans (19), *Vaccinium* species (20), different cherry and berry extracts (14), have demonstrated anticancer activity. *In vitro,* anthocyanin fractions more effectively inhibited growth of human intestinal carcinoma HCT-15 cells than did flavonoids (19). HCT116 colon cancer cells were inhibited by anthocyanin-containing berry extracts including cowberry, strawberry, blueberry, and bilberry extracts (21). Similarly, tart cherry anthocyanins and their aglycon cyanidin were shown to inhibit the growth of human colon cancer cell lines HT-29 and HCT116 (22). *In vivo*, the tart cherry extract inhibited the intestinal tumor development in Apc(min) mice (22), suggesting that anthocyanins may reduce the risk of intestinal cancer. Freeze-dried black raspberries (23), purple corn (24), purple sweet potato and purple cabbage (25) have been shown to inhibit azoxymethane-induced colon tumors in rats.

To further understand the role of anthocyanins as potential chemopreventive agents, Zhao *et al.* (26) previously investigated the chemopreventive activity of commercially available anthocyanin-rich extracts (AREs) of bilberry, chokeberry and grape *in vitro* in colon cancer cell lines. It was reported that AREs from bilberry, grape

and chokeberry exhibited different anthocyanin profiles and were able to significantly inhibit growth of human colon cancer cells, HT-29 with little effect on growth of non-transformed colon epithelial cells, NCM460 (26, 27). Further investigation demonstrated that chokeberry ARE inhibited growth and cell cycle progression in colon carcinoma cells mainly through up regulation of $p21^{WAF}$ and $p27^{kip1}$ genes and down regulation of cyclin A and cyclin B1 genes (27).

1.2. Colorectal Cancer

1.2.1. Introduction

Intestinal tumors develop from epithelial cells lining the crypts and villi. It is considered through several observations that food passes through the small intestine much faster than large intestine. This may account, in part, for the 50 times lower incidence of tumors in jejunum and ileum than in colon and rectum. The recto sigmoid area, where feces are retained the longest, is the most common site of intestinal cancer in humans (28).

Other risk factors that have been correlated with the etiology and pathogensis of colon cancer are increasing age, family history, genetic factors such as Familial Adenomatous Polyposis (FAP) or Gardner's syndrome, inflammatory bowel disease like ulcerative colitis, and Crohns disease (28).

Death rates for colorectal cancer for each race/ethnicity are approximately 40% higher among men than women. Blacks had the highest death rate for colorectal cancer, followed by whites. Death rates for colorectal cancer have decreased 2.2% per year for white men and 1.8% per year for white women. Declines in death rates among black men

and women were approximately 50% less than that for whites (2), Until age 50, men and women have similar incidence of mortality rates, after age 50 men are more vulnerable (3).

Almost 95 percent of malignant colorectal tumors are adenocarcinoma, and remaining 5 percent compromise of carcinoids, sarcomas and lymphomas (28). Adenomas are lumps of epithelial dysplasia, which are classified into three major histological types: tubular, villous and tubulovillous. An adenoma is considered malignant and becomes an adenocarcinoma, when there is evidence that neoplastic cells pass through the muscularis mucosae and infiltrate the submucosa (28). After crossing the submucosa, the local invasion of neoplastic cells and potential for metastasis is possible. Adenomas show different grades of dysplasia, degree of severity and these abnormalities are graded into mild, moderate and severe dysplasia. Colorectal carcinoma can be graded into well-differentiated, moderately differentiated and poorly differentiated lesions. The more poorly differentiated lesions are more advanced and have poorest prognosis (28,29).

1.2.2. Multistage Progress of Colon Carcinoma

Colorectal carcinogenesis is a complex multistage process. The multistage progression requires years and possibly decades to develop from normal tissue to invasive cancer. The process is accompanied by a number of genetic and cellular alterations. The genetic pathway model for the pathogenesis of sporadic colorectal cancer was proposed by Fearon and Vogelstein (30,31). This model of colorectal carcinogenesis

was proposed over 10 years ago. Since that time, other mutations that occur at a high frequency in colorectal cancer have been identified, and the original model has been considerably elaborated to take in account the alternative pathways for the development of cancer that are now known to exist (29).

Mutations in two classes of genes, tumor-suppressor genes and proto-oncogenes, are thought to impart a proliferative advantage to cells and contribute to development of the malignant phenotype. Inactivating mutations of both copies (alleles) of the adenomatous polyposis coli (APC) gene, a tumor-suppressor gene on chromosome 5q mark one of the earliest events in colorectal carcinogenesis (32). Germline mutation of the APC gene and subsequent somatic mutation of the second APC allele cause the inherited FAP syndrome. Mutation leading to dysregulation of the K-ras protooncogene is also thought to be an early event in colon cancer formation (32). After initiation, the transition from adenoma to carcinoma or high-grade dysplasia (HGD) appears to involve tumor suppressor genes, like tumor protein 53 (TP53), that are considered guardians of the genome. Once HGD occurs it has been suggested that genetic chaos ensues setting the stage for malignant transformation (33). The goal of cancer prevention studies is to either prevents the initiation of, or to slow or stop the process of, cancer development at as early a stage as possible, as the more progressed the tumor, the less responsive to prevention or treatment strategies. Therefore, the use of early biomarkers of colon cancer in prevention studies will be reviewed.

The multistage process of carcinogenesis has also given rise to the idea that the real target for initiation is in a stem cell population (34). Stem cells are considered to

have innate capacity for long-term proliferation and the ability to differentiate along several directions. In the large intestine, the stem cell population is thought to exist near the base of crypts (35). The progeny of stem cells migrate up the crypt, continuing to divide until they reach the mid portion where the cells stop dividing and instead begin to differentiate to mature cells. When differentiated cells reach the top of the crypt, they undergo apoptosis and are engulfed by stromal cells or shed into the lumen. It was considered that neoplastic cells within early neoplasm are derived from stem cells at the base crypts and such cells should give rise to new, completely dysplastic crypts that branch as the lesions expand. However the development and morphogensis of the adenomatous polyp is still a topic of further research.

Regarding successful strategies of diet intervention studies, it has been suggested that animal studies are important as a necessary preliminary stage before expensive human studies are attempted (36). This is especially relevant for colorectal cancer prevention due to the multistage nature of carcinogenesis. It is imperative to know and understand the point of action in the carcinogenesis process, as well as to identify appropriate dosing of cancer preventive agent before designing definitive human intervention trials where cancer or biomarkers of cancer are used as outcome measures.

1.3. Biomarkers of colon cancer in animal model

Chemical carcinogen- induced rodent models are well accepted and widely used for the study of potential colon cancer chemopreventive agents. Macroscopic colon tumors in rodents have been considered an endpoint marker for many years but due to some practical drawbacks of these studies such as requiring a large group of rodents, the long time period required for tumors to develop (up to 8-9 months) and time-consuming histological procedure for confirmation of the tumors, alternative markers for colon cancer are now being developed and validated (37).

1.3.1 Aberrant crypt foci

Abberant crypt foci (ACF) are considered precancerous lesions of colorectal cancer, and are recognized in the colonic surface of rats treated with colon specific carcinogens like azoxymethane (AOM) (38). ACF have an increased proliferative activity and some reveal histopathological dysplasia. They are defined as crypts that have (i) altered luminal openings, (ii) exhibit thickened epithelia, (iii) are larger than adjacent normal crypts and (iv) are microscopically elevated (39) (Fig.1A and B). The use of the ACF system to study modulators of colon carcinogenesis has accelerated for the last 10 years, for it provides a simple and economical tool for preliminary screening of potential chemopreventive agents. In a systematic review of ACF and tumor data**,** Corpet *et al.* reported significant correlation of the potencies of 57 chemopreventive agents to inhibit ACF and to inhibit colon tumors. The presence of ACF has also been shown to correlate with colon cancer risk and adenoma size and number in humans (40). Therefore ACF are now regarded as a surrogate end point for tumors in rats and human intervention trials.

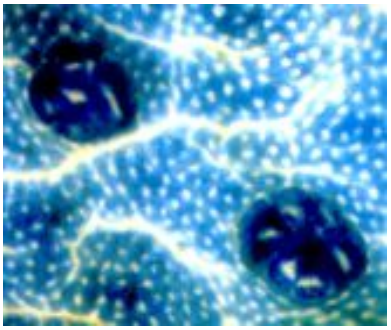

Fig. 1A. Topographic view of two ACF in a rat exposed to AOM. Each Focus has 6-7 crypts, which are much larger than the surrounding normal crypts. Methylene blue staining of colon facilitates identification.

(Adapted from H. Mori et al 2004.)

Fig 1B. Endoscopic features of ACF in patients with adenomas. Methylene blue staining reveals a small focus consisting of three crypts with semicircular or oval lumens). The aberrant crypts stain more darkly, are larger, and have a thicker epithelial lining

(Adapted from T.Takayamya et al. 1998)

1.3.2. Cell proliferation

Cell proliferation is considered to play an important role in different stages of multistage carcinogenesis. In proliferating tissues, various genetic changes progressively accumulate and clonal proliferation of genetically damaged cells leads to the formation of foci, nodules and then to tumors that can escape from the control of neighboring cells (41). Abnormal patterns of cell proliferation in the entire colonic mucosa of patients with colonic adenoma or carcinoma and also in patients with heredity disposition to colorectal cancer (e.g. FAP) have been reported (42). In the colon, the mucosal cell proliferation, which is normally restricted to the stem cell zone, extends to the entire crypt during carcinogenesis and leads to formation of ACF, polyps, adenomas and carcinomas. In a review of different dietary endpoint markers, Rafter *et al.* (37) consider nutritional inhibition of cell proliferation as a valuable surrogate marker. The inhibition is considered to be beneficial in both colon cancer prevention and tumor therapy. Measurement of proliferation biomarkers utilizes immunohistochemistry. These methods are considered relatively simple, reliable and suited for large number of specimens (43).

Two commonly used antibodies are Ki67 and proliferating cell nuclear antigen (PCNA). They both are nuclear proteins whose level varies as a function of the cell cycle. PCNA, which has been used in this study, is an auxillary protein to polymerase delta and is expressed during G1 phase of cell cycle (44).

1.3.3. *Cyclooxygenase 1 and 2*

Cycoloxygenase (COX) catalyzes a key step in conversion of arachidinoic acid to prostaglandins (PGs) (45). PGs have important functions in almost every organ system and regulate such diverse physiological processes as immunity, reproduction, maintenance of vascular integrity and bone metabolism (46). There are two known isoforms of COX that differ in their tissue distribution and regulation. COX-1 is responsible for housekeeping PG biosynthesis and is constitutively expressed in most tissues in the body. COX-2 is not normally expressed in most tissues but is induced by a wide spectrum of growth factors and proinflammatory cytokines in specific pathophysiological conditions (46). COX-2 is over-expressed in many cancers including esophagus, stomach, colon, lung, pancreas head and neck (47).

Direct evidence implicating COX-2 in colorectal carcinogenesis comes from a mouse model ApcΔ716, in which there is a knockout of the COX-2 gene. The number and size of intestinal polyps was reduced in these mice compared with COX wild type mice (48). Ebhart *et al.* (49) documented for the first time significant elevation in COX-2 expression in 85% of human colorectal carcinomas and approximately 50% of colorectal adenomas. COX-2 is also over-expressed in adenomas of Apc^{min} mice (50) and carcinoma samples from the colon of AOM – treated rats (51). Inhibition of COX-2 activity is considered a valuable surrogate marker of chemoprevention. There is significant evidence from non-steroid inflammatory drugs (NSAIDS) that COX-2 inhibitors are effective at suppressing the growth of established colorectal tumors (52). Although the effect of diet on COX-2 inhibition is less explored, it is considered that

dietary components that can inhibit the expression of COX-2 may be important for inhibiting tumor development in the colon (37).

1.3.4. Fecal bile acids

Evidence drawn from a large number of observations in animal and human trials support an important role of fecal bile acids (FBA) in colon carcinogenesis. In animal models, the bile acid, cholic acid acts in the promotion phase of carcinogenesis. Furthermore, bile acid increases colonic epithelial proliferation (53). The bile acid, deoxycholic acid was proposed to be carcinogen in 1939 and 1940 (54). On the basis of later work with rodent models, bile acids came to be regarded as cancer promoters rather than carcinogens (55). However, considerable evidence obtained more recently again supports the view that bile acids are carcinogens in humans for gastrointestinal cancer (54). Epidemiological studies have found that fecal bile acid concentrations are elevated in populations with a high incidence of colon cancer (55).

The mechanisms by which bile acids promote colon carcinogenesis are not completely understood. The secondary bile acids deoxycholic acid and chenodeoxycholic acid have been reported to be cytotoxic to colonic epithelial cells, moderately mutagenic, associated with dysplastic changes (56), and to be antiapoptotic (57). Dietary intake of calcium lowers the incidence of cancer. Calcium supplements in the presence of phosphate removes bile acid from solution and decrease their interaction with colon epithelium (58). In human studies, high intake of dietary fat that correlates with increased colon cancer risk, results in increased FBA concentration whereas wheat barn, which

negatively correlates with colon cancer, reduces FBA concentration (37, 59, 60). However, to date there is no literature reporting the effect of dietary anthocyanins on fecal bile concentrations.

1.4. Dietary intervention in prevention of colon cancer

Different stages of colorectal cancer can be modulated by dietary factors, which directly interact with gene expression or modulate key enzymes activities involved in cell proliferation and differentiation (37). A large number of studies have provided evidence that there is an inverse relationship between colorectal cancer and consumption of plant foods (61). One explanation for this relationship is that vegetables, fruits and whole grains contain a wide variety of phytochemicals (like terpenes, isothiocyanates, caretenoids, and flavanoids), which have potential to modulate cancer development (10). Flavonoids are plant secondary metabolites, present in all terrestrial vascular plants, with a wide variety of beneficial biological properties such as anticarcinogenic and antioxidative modes of action (62). In this study, one class of flavonoids, anthocyanins, were evaluated. Therefore, the anthocyanins will be the focus of the next section of this review.

1.5. Anthocyanins

Anthocyanins are one class of flavonoid compounds that are truly pigments and produce great range of colors from scarlet to blue (63) Flavonoids are plant secondary metabolites, present in all terrestrial vascular plants with a wide variety of beneficial biological properties such as anticarcinogenic and antioxidative modes of action (62). Anthocyanins are also considered powerful dietary antioxidants. It has been estimated that human intakes of anthocyanins are between 180 and 215 mg/day in the US, which is much higher than the intake of other known flavonoids. The daily intake of flavanoids including quercetin, myricetin, and kaempferol, luteolin and apigenin is estimated to be only 20-22 mg/day in the US (64).

1.5.1 Chemical structure

Anthocyanins show high diversity and are an important group of water-soluble pigments in nature (63, 65). The word anthocyanin is derived from two Greek words *anthos,* which means flower, and *kyanos,* which means dark blue, revealing their important characteristic as natural colorants. Their contribution to the colorful appearance of fruits, vegetables and flowers help them to attract animals, leading to seed dispersal and pollination. There diversity in nature is based on a basic anthocyandin structure (63).

1.5.2. Basic Structure

The range of Anthocyanin is represented by infinity of natural colors produced by the chemical combination of the basic C-6-C-3-C-3 (phenyl-2-benzopyrilium) anthocyanindin structure (Fig. 2). Anthocyanin structure is complemented by one or more sugar molecules bonded at different hydroxylated, positions of basic structure. Thus anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrillium (anthocyanidins). Their diversity is associated with number of sugar molecules in their structure and also by chemical combination of these sugars with organic acids. Moreover the number of hydroxyl and methoxyl groups attached to the basic 3 ring structure affects color of anthocyanins, *i.e.* if more hydroxyl groups are present, then the color is more toward bluish shade and if more methoxyl groups are present, then redness is increased (63, 65).

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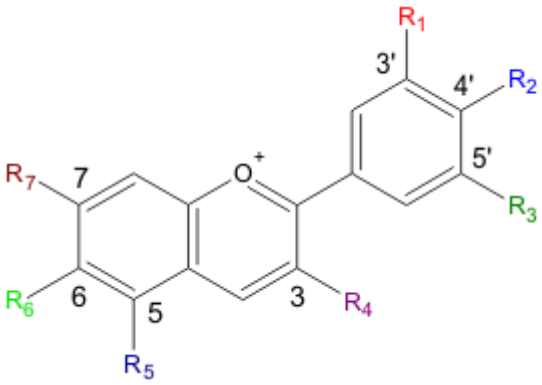

Figure 2.: Basic structure of anthocyanidin pigment (flavylium cation). Rx could be H, OH or OCH_3 depending on considered pigment

1.5.3. Glycosylation and Acylation

To obtain an anthocyanin, the anthocyanidin (also called aglycon) is glycosylated by one or more sugar molecule. Thus anthocyanins are also known by the number of sugar molecules in their structures (e.g. monosides, biosides, triosides) (63). Glucose, galactose, rhamnose and arbinose are the sugars most commonly encountered (66). The stability of anthocyanins is enhanced by one or more sugar molecules bonded at different hydroxyl positions. With a few exceptions anthocyanins are always glycosylated at C-3 (67). Diversity is further increased by the chemical combination of these sugars with organic acids to produce acylated anthocyanins (63). Common acylating agents include cinnamic acid derivatives such as caffeic, p-coumaric, ferulic and sinapic acid as well as a range of aliphatic acids such as acetic, malic, malonic, oxalic, and succinic acid. Since each anthocyanidin may be glycosylated and acylated by various sugars and acids at different positions, a great number of chemical combinations exist. (63)

Based on several reviews, it was estimated that more than 600 anthocyanins had been found in nature (68). Such tremendous variations, together with the pH-dependent and chelating metal ion-dependent color change (69), are responsible for the amazing array of natural colors from anthocyanins.

1.5.4. Daily intake, absorption, and metabolism

In US, average daily intake of anthocyanins has been estimated at 215 mg during summer and 180 mg during winter. It has been suggested that regular consumers of red

wine are likely to have significantly higher intakes since the concentration of anthocyanins in red wines is in the range of 240-350 mg/litre (66, 70).

The various studies done in humans have suggested low bioavailability of anthocyanins as indicated by very low recovery in the plasma and urine after ingestion (71, 72). Single doses ranging from 150 mg to 2 g total anthocyanins were given to the volunteers generally in the form of berries, berry extracts or concentrates. After such intakes, concentrations of anthocyanins measured in plasma were very low, in the order of 10-50 nmol/l and in some studies they were below the detection limit. The mean time to reach C_{max} was 1.5 h (range: 0.75–4 h) for plasma and 2.5 h for urine. Most studies reported low relative urinary excretions, ranging from 0.004% to 0.1% of the intake, although Lapidot *et al.* (73) and Felgines *et al.* (74) measured higher levels of anthocyanin excretion (up to 5%) after red wine or strawberry consumption. The time course of absorption was consistent with absorption in the stomach, as described for animals (75,76). The prominent feature of these studies is considered to be that anthocyanins are very rapidly absorbed and eliminated, and that they were absorbed with poor efficiency (77).

Recent studies (78) in rats measured plasma levels of anthocyanins after oral supplementation with blueberry skin extract containing cyandin-3 (Cy-3) galactoside, cyandin-3 glucoside (C3G), Cy-3 arbinoside and the aglycon, cyandin. The results indicated that cyandin glycosides are absorbed from the digestive tract to the blood stream in their intact glycosylated forms. These results were in agreement with a pervious report by Tsuda *et al*. (79) on cyandin –3-O-β-glucosides (C3G). In this study rats were

orally administered C3G (0.9 mmol/kg body weight), and C3G rapidly appeared in the plasma. However, the aglycon of C3G (cyanidin; Cy) was not detected, although it was present in the jejunum. Protocatechuic acid (PC), which may be produced by degradation of Cy, was present in the plasma and the concentration was 8-fold higher than that of C3G. In the liver and kidney, C3G was metabolized to methylated C3G (methyl-C3G), suggesting that C3G and/or methyl-C3G act as antioxidants in the tissues.

Miyazawa (80) and Matsumoto *et al.* (81) also showed that cyandin glycosides were detected in unaltered forms in blood of both rats and humans after oral administration of black currant (mainly containing C3G and Cy-3,5-diglycoside) or elderberry juices (primarily composed by Cy-3-*O*-β-rutinoside).

In two studies with rats, Tsuda and co-workers (13) also suggested that C3G is partly hydrolyzed by the β-glycosidase reaction in the intestines, thus explaining the detection of the aglycon in the jejunum. No detection of the aglycon in the plasma was related to cyanidin instability in plasma and its consequent massive degradation to protocatechuic acid.

Seeram *et al.* (82) confirmed that protocatechuic acid was the predominant degradation product of cyanidin in a cell culture study.

Another study to assess the metabolism of C3G was performed by Stumpf *et al.* (83). They used an isolated perfused rat small intestinal preparation. Results confirmed that C3G was absorbed into the vascular effluent and incorporated into intestinal tissue,

whereas the aglycone cyanidin was not detectable. They also reported detection of glucuronate and sulfate conjugates of C3G as intestinal metabolites.

Animal studies have identified some metabolites of anthocyanins but in a recent review, Manach *et al.* (77) suggested that based on our current knowledge, there are important differences in the metabolism of anthocyanins, as compared with other polyphenols. Therefore further research for the analysis of metabolites of anthocyanins should be performed.

1.6. Role of anthocyanins in maintaining health

Anthocyanins are implicated in many biological activities that may impact positively on health (84). Their use for therapeutic purposes has long been supported by both anecdotal and epidemiological evidence, but only in recent years some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been conclusively verified by rigorously controlled *in vitro*, *in vivo*, or clinical research trials (84).These pigments may reduce the risk of coronary heart disease through modulation of arterial vasomotion (85) inhibition of platelet aggregation (86) or endothelial protection (87). In addition, anthocyanins could exert anticarcinogenic activities, reduce inflammatory insult and also modulate immune response. All these effects might be mediated by their antioxidant activity (69).

In many other cases, the exact roles of the anthocyanins in human health maintenance versus other phytochemicals in a complex mixture from a fruit extract or whole food have not been completely sorted out. In fact, some reports suggest that anthocyanin activity potentates when it is delivered in mixtures (88, 89).

Visual acuity can be markedly improved through administration of anthocyanin pigments to animal and human subjects, and the role of these pigments in enhancing night vision or overall vision has been particularly well documented (84). Oral intake of anthocyanoside from black currants resulted in significantly improved night vision adaptation in human subjects (90) and similar benefits were gained after administration of anthocyanins from bilberries (91).

Tsuda *et al*. (92) recently provided evidence that anthocyanins extracted from purple corn, when provided to mice in combination with a high-fat diet, effectively inhibited increases in both body weight and adipose tissue. Typical symptoms of hyperglycemia, hyperinsulinemia, and hyperleptinemia provoked by a high-fat diet did not occur when mice also ingested isolated anthocyanins. The experiments suggest that anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes.

Anthocyanins have been credited with capacity to modulate cognitive and motor function, to enhance memory, and to have a role in preventing age-related declines in neural function. Cho *et al.* (93) reported that administration of isolated semipurified anthocyanins from purple sweet potato enhanced cognitive performance as assessed by passive avoidance tests in ethanol-treated mice, and also effectively inhibited lipid peroxidation in rat brain tissues. By administering blueberry extracts with significant anthocyanin content (but not purified pigments), it was noted that the blueberry-supplemented diets led to effective reversal of age-related deficits in various neural and behavioral parameters (memory and motor functions) (94). Further investigations by this laboratory team demonstrated that anthocyanins (in particular, cyanidin-3-sambubioside-

5-glucoside and cyanidin-3, 5-diglucoside) were highly bioavailable in endothelial cells, which was linked to their roles in prevention of atherosclerosis and neurodegenerative disorders (78, 94).

Anthocyanins exerted multiple protective effects against pleurisy in a rat model and were capable of attenuating inflammation. Anthocyanin treatment also downregulated expression of enzymes involved in the inflammation of the lung (95). The antimicrobial activity of anthocyanins in general has been well established, including significant inhibition of aflatoxin biosynthesis (96). The experimental evidence demonstrating anthocyanin benefits for diabetes and pancreatic disorders has also accumulated in recent years, and again the efficacy is attributed to the multiple, simultaneous biological effects these pigments cause in the body, including prevention of generation of free radicals, decreased lipid peroxidation, reduced pancreatic swelling, and decreased blood sugar concentrations in urine and blood serum (97, 98).

1.7. Antioxidant properties of anthocyanin

The antioxidant capacity of anthocyanins has been demonstrated with a wide variety of assay methods. Berry extracts demonstrated potent antioxidant activities that correlated with anthocyanin content (99, 100). Ferric reducing ability of plasma (FRAP) method used to reflect antioxidant activity showed that the 3-glucosides of delphidin, petunidin and malvidin were 2-2.5 times more potent antioxidants than ascorbic acid (101). The antioxidant capacity measured with oxygen radical absorbing capacity (ORAC) tests in Trolox equivalents absorbing capcity (TAEC) found anthocyanins to be 3-6 fold that of standard antioxidant trolox (101,102) . According to Cooke *et al.* the structure-antioxidant pattern emerging from different studies indicates that potent

antioxidant activity is associated with presence of hydroxyl groups in the anthocyanin B-ring. The antioxidant capacities of cyandin and cynadin 3- glucosides were similar to α – tocopherol in assay systems of linolenic acid, liposomes, rabbit erythrocyte membranes and rat liver microsomes (103).

1.8. Anthocyanins and Colon Cancer

Anthocyanidins and anthocyanins have been shown to inhibit the growth of embryonic fibroblasts and of cancer cells derived from malignant human tissues. Tissues originating from the central nervous system (CNS), lung, breast, prostrate uterus, vulva and colon have been investigated for the evidence of chemopreventive activity by anthocyanins. (104, 105). The studies linking the specific role of anthocyanins and colon cancer are small in number, but those conducted to date have provided consistent and convincing evidence of a protective effect.

1.8.1 Effects of anthocyanin on colon cancer *in vitro*

In vitro growth inhibition of human colon cancer cell lines HCT 15 (106) , HT-29 (22) and HCT–116 (22) has been demonstrated by anthocyanin fractions. Tart cherry anthocyanins and cyandin reduced cell growth of human colon cancer cell lines, HT29 and HCT 116. The IC 50 (inhibitory concentration 50%) is the concentration required for 50% inhibition of cell growth was measured for anthocyanins and cyandin. It was 780 μM for tart cherry anthocyanins and 63 μM for cyandin in HT 29 cells. IC50 for HCT 116 cells was 285 μM for tart cerry anthocyanin and 85 μM for cyandin respectively (22).

Olsson *et al.* (99) investigated the effects of 10 different extracts of fruits and berries on cell proliferation of HT 29 colon cancer cells. The fruits and berries used were rosehips, blueberries, black currant, black chokeberries, apple, sea buckthorn, plum, lingonberries, cherries, and raspberries. The extracts decreased the proliferation of HT 29 cells. The effect of inhibition was in range of 48-74% (average=62%) for HT 29 cells. The anthocyanin fraction of the extract of black chokeberries, apple, rosehips, raspberries and lingonberries decreased cell proliferation, whereas blueberries and cherries increased cell proliferation at the highest concentration. HT 29 cells were inhibited to lower extent than HCT 116 colon cancer cells. They also investigated other antioxidants present in these fruits and concluded that the synergistic effect of anthocyanin, vitamin C and to a certain extent, some caroteniods control cancer cell proliferation. The authors have also stated that sugar-conjugated anthocyanins are less effective than the aglycon, cyandin, in inhibiting the growth of HT 29 cells.

In contrast to the above results, recent study done by Yi *et al.* (15) concludes that anthocyanins from blue berries can inhibit colon cancer cell proliferation. This study systematically evaluated the bioactivities of phenolic compounds in rabbiteye blueberries and assessed their potential antiproliferation and apoptosis induction effects using two colon cancer cell lines, HT 29 and Caco-2. Polyphenols in three blueberry cultivars, Briteblue, Tifblue, and Powderblue, were extracted and freeze-dried. The extracts were further separated into phenolic acids, tannins, flavonols, and anthocyanins. They found that the greatest antiproliferation effect among all four fractions was from the anthocyanin fractions. Both HT 29 and Caco-2 cell growth was significantly inhibited by >50% by the anthocyanin fractions at concentrations of 15-50 μg/mL. Anthocyanin

fractions also resulted in 2-7 fold increases in DNA fragmentation, indicating the induction of apoptosis. The effective dosage levels were close to the reported range of anthocyanin concentrations in rat plasma.

The growth inhibitory effect of anthocyanins extracted from flower petals in HCT-15 cells colon carcinoma cells was greater than other flavonols and flavanones (17). Among ethanol extracts of 10 edible berries, bilberry extract was found to be the most effective at inhibiting the growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells *in vitro*. Only pure delphinidin and the delphinidin glycoside isolated from the bilberry extract, but not malvidin and the malvidin glycoside, inhibited the growth of HCT116 cells (21).

In an earlier study, Kamei *et al.* (106) investigated the anti-tumor effect of crude methanol extracts of red and white wines on colon and gastric cancer cells. Extracts were added to diethyl ether in order to divide them into the anthocyanin fraction (insoluble in diethyl ether) and fractions containing other flavonoids and their derivatives (soluble in diethyl ether). The white wine did not contain anthocyanins (all of the methanol extract was soluble in diethyl ether). HCT-15 cells, derived from human colon cancer, or AGS cells, derived from human gastric cancer, were cultured with these fractions. The anthocyanin fraction from the red wine and the non-anthocyanic substances extracted from red and white wines suppressed the growth of the cells, and the suppression rate by the anthocyanin fraction was significantly higher than that of the other fractions.

Anthocyanin fractions from red soyabeans and red beans inhibited growth of HCT–15 human colon carcinoma cells (19). In a recent study by Yi *et al*. (107), phenolic compounds were extracted from muscadine grape skins and were further separated into

phenolic acids, tannins, flavonols, and anthocyanins. The greatest inhibitory activity was also found in the anthocyanin fraction, with a 50% inhibition at concentrations of approximately 200 μg/mL in HT-29 and 100-300 μg /mL in Caco-2 cells respectively. Anthocyanin fractions also resulted in 2-4 times increase in DNA fragmentation, indicating the induction of apoptosis. These studies demonstrate anthocyanin potential usefulness as colon cancer chemopreventive agent.

1.8.2 Effects of anthocyanin on colon cancer *in vivo*

In vivo, anthocyanin-containing mixtures have shown colon cancer chemopreventive activity in rats and Apc ^{Min} mice. Apc ^{Min} mice carry an APC gene mutation. They develop adenomas in the small intestine tract and are considered to reflect, in many respects, human familial adenomatous polyposis coli (FAP) syndrome (108). In a comparative investigation done by Kang *et al.* (22), Apc ^{Min} mice received either a mixture of anthocyanins at 800 mg/l or pure cyandin at 200 mg/l with drinking water or tart cherries added to the diet (200 g/kg diet). These amounts correspond to doses of approximately 2.4 and 0.6 mg anthocyanins/animal/day or 600 mg of tart cherries/animal/day, respectively based on average daily intake of diet and water. Mice that received these interventions had significantly fewer and smaller cecal adenomas than mice consuming the control diet or non-steroidal inflammatory drug sulindac. However, no significant effect was observed on colon tumors, possibly due to the low number of colon tumors that develop in this model.

A recent study by Cooke *et al.* (104) suggested a moderate but significant reduction in small intestinal adenoma number in Apc ^{Min} that received either an anthocyanin-containing blueberry extract or pure cyandin –3-glucoside at 0.1% (w/w) in

their diet. This concentration was considered to be approximately a dose of 3.0 mg anthocyanin/mouse/day.

Purple sweet potato (PSP), red cabbage and purple corn color (PCC), which are rich in anthocyanins, have also been shown to inhibit chemically-induced carcinogenesis in male F344/DuCrj rats, initially treated with 1,2-dimethylhydrazine (DMH) (24, 25). After DMH initiation, rats ingested PSP, red cabbage or PCC colorings at doses approximately 490, 620 and 233 mg anthocyanins/rat/day, respectively along with 0.02% 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in the diet until week 36. The incidence and multiplicity of colonic adenomas and adenocarcinomas were significantly decreased in all dose groups as compared to control rats. The PCC and red cabbage also inhibited PhIP -induced aberrant crypt formation in rats that didn't receive DMH.

Harris *et al.* (23) showed the effect of consumption of lyophilized black raspberries (BRB) on azoxymethane (AOM)-induced aberrant crypt foci (ACF), colon tumors, and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in male Fischer 344 rats. The BRB were consumed at doses of 1.5, 0.75 or 0.38 g/animal/day. ACF multiplicity was decreased by 36%, 24%, and 21%, respectively. Adenocarcinoma incidences were decreased by 28-80%. The protection effect was paralleled by a significant reduction in urinary levels of 8- hydroxy –2'deoxyguanosine, a marker of oxidative DNA damage. The *in vivo* studies summarized above suggest a remarkable chemopreventive potential of anthocyanin for colon cancer.

1.8.3 Mechanisms

Based on accumulating evidence that anthocyanins are chemopreventive, other studies have investigated the mechanism(s) of anthocyanins interference with biochemical activities from promotion to progression of malignancies such as those mediated by COX, tyrosine kinases and phosphodiesterases. Seeram *et al.* (109) found that cyanidin and malvdin showed higher COX inhibitory activities compared with commercial non-steroidal anti- inflammatory drugs like ibuprofen, naproxen, vioxx and celebrex.

Anthocyanin extracts from bilberry or purified delphidin inhibited lipid polysaccharide (LPS) – induced COX-2 expression at protein and transcriptional levels in mouse RAW24 macrophage cells. This study also demonstrated that the blockage of NF-κB signaling pathway is involved in the inhibition of COX-2 gene expression by anthocyanin (69).

Delphidin, cyandin and malvidin inhibited activity of epidermal growth factor receptor (EGFR) tyrosine kinase obtained from human epidermoid cells A431 with IC50 values of 18, 42 and 61 μM respectively (110). Studies done by Marka *et al.* (111) showed that abilites of anthocyandins to inhibit EGFR tyrosine kinase in decreased in the order of delphidin=cyandin> pelargonidin> peonidin> malvidin. They suggested that the potency might be positively correlated with the hydroxyl groups in position 3' and 5' of the ring B of the anthocyanin molecule and inversely with the presence of methoxy groups in these positions. In contrast, the inhibition of phosphodiesterase activity in HT-29 cells by anthocyandins demonstrated an inverse molecular structure activity relationship (111). Inhibitory potency in decreasing order was malvidin> peonidin> pelargonidin= cyanidin > delphidin. This observation suggested that phosphodiesterase

inhibitory potency is positively correlated with the number of methoxy moieties and inversely with the number of hydroxyl groups in possible 3' and 5' positions in the ring B (104).

Anthocyanins may also affect carcinogenesis through cell cycle regulation. Anthocyandins from grape rinds and red rice arrested colon cancer HCT -15 cells in the S phase of the cell cycle (18). Anthocyanin fractions extracted from rose petals, and red and white wines arrested the cells at various stages including G1, S and G2 /M phase (106, 112) . Malik *et al.* (27) showed dual blockage at G1/G0 and G2/M phases in HT-29 colon cancer cells by chokeberry ARE. The blockage in cell cycle was not observed in NCM460 normal colon cells. An additional mechanism that has been reported for anthocyanin inhibition of carcinogenesis is inhibition of nitric oxide (NO) production. NO is generated in excess during viral and bacterial infections and promotes oxidative stress and cancer. anthocyanins have shown strong inhibitory effects on NO production n LPS/IFN-gamma-activated RAW 264.7 macrophages (69).

1.9. Current Study

To further understand the role of anthocyanins as potential chemopreventive agents, commercially available anthocyanin-rich extracts (AREs) of bilberry, chokeberry and grape prepared for the food industry were investigated. Although some purified anthocyanins are commercially available, they are too expensive for animal studies. The advantage of using commercially prepared AREs is that unlike crude extracts of anthocyanin-rich foods, these extracts are produced in large volumes to have a consistent standardized composition.

Zhao *et al.* (26) showed that AREs from bilberry, grape and chokeberry have different pigment profiles, and were able to significantly inhibit growth of human colon cancer cells, HT-29 with little effect on growth of non-transformed colon epithelial cells, NCM460 (26, 27). Further investigation by Malik *et al.* (27) demonstrated that chokeberry ARE inhibited growth and cell cycle progression in colon carcinoma cells mainly through up regulation of $p21^{WAF}$ and $p27^{kip1}$ genes and down regulation of cyclin A and cyclin B1 genes (27).

The aim of the present study was to determine if these AREs would be effective *in vivo* by assessing their effect on a number of biomarkers in F344 rats treated with the colon carcinogen, azoxymethane (AOM). Biomarkers that were evaluated included the number and multiplicity of colonic aberrant crypt foci (ACF), colonic cell proliferation, fecal bile acids, and urinary levels of oxidative DNA damage. To assess the bioavailability, levels of anthocyanins in serum, urine and feces were evaluated. The specific anthocyanins and anthocyanin metabolites detected in these samples are reported

in the paper by He *et al.* (113). We further wanted to determine if the *in vivo* mechanisms of action would be similar to those observed in the human cell lines by evaluating the expression of cyclooxygenase genes.

Chapter 2. Materials and Methods

2.1. Chemicals and Materials

Food grade AREs of bilberry (*Vaccinum myrtillus L.)* and chokeberry (*Aronia meloncarpa E.)* were supplied by Artemis International, Inc. (Fort Wayne, IN). Grape (*Vitis vinifera*) extract was supplied by Polyphenolics, Inc. (Madera, CA). AOM was obtained from Sigma Chemical (St. Louis, MO).

2.2 Measurement of ARE Properties

2.2.1. Monomeric Anthocyanin Content and Total Phenolics. The monomeric anthocyanin content of extracts was determined by pH-differential method (114). A Shimadzu 1601 UV spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) and 1 cm path length disposable cuvettes were used for spectral measurements at 520 and 700 nm. Pigment content calculation was done as cyanidin-3-glucoside, using extinction coefficient (ε) of 26900L cm^{-1} mol^{-1} and molecular weight (MW) of 449.2 g mol^{-1}. The anthocyanin profiles of these extracts have been previously reported (26).

Total phenolics were measured by the Folin Ciacalteau method as described (26). The absorbance of the samples and standards was measured at 755 nm. Total phenols were calculated as gallic acid equivalents based on a gallic acid standard curve.

2.2.2. Oxygen Radical Absorbance Capacity (ORAC) Values. The ORAC values for AREs was measured based on procedure described by Prior and coworkers (115). The

automated assay was carried out on the COBAS FARA II centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ) with fluorescence detector. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a control antioxidant standard. *R*-phycoerythrin (*R*-PE) was used as a target of free radical attack with 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) as a peroxyl radical generator. The analyzer was programmed to record the fluorescence of *R*-PE every 2 min after addition of AAPH. The final ORAC values were calculated using a differences of areas under the quenching curve of R-PE decay curves between a blank and a sample expressed as micromoles of Trolox equivalents per g of compound.

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2.3. Animal and Experimental design

2.3.1. Preparation of Diets. Control diet was AIN-93 powdered diet (Dyets Inc., Bethlehem, PA). Diets containing 3.85g/kg monomeric anthocyanin from chokeberry, bilberry or grape ARE were prepared by supplementing AIN-93 powdered diet at the expense of cornstarch. All the three anthocyanin-rich extracts were added on the basis of their monomeric anthocyanin content (Table 1). All diets were prepared fresh on a weekly basis and stored at 4°C until use.

Table 1: Diet Composition

Ingredient gm/kg	AIN-93	AIN-93 with 5% Chokeberry ARE	AIN-93 with 3.5% Bilberry ARE	AIN-93 with 2.6% Grape ARE
Casein	200	200	200	200
L-Cystine	3.0	3.0	3.0	3.0
Sucrose	100	100	100	100
Corn starch	397.486	347.486	362.486	370.986
Soybean oil	70	70	70	70
Fiber (cellulose)	50	50	50	50
Mineral Mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
AREs	-	50	35	26

All ingredients are in g/kg

2.3.2 Animal Treatment and Housing. Forty 3-4 week old male specific pathogen free F344 rats were obtained from Harlan (Indianapolis, Indiana). In the first week of acclimatization, rats were slowly weaned from pellet rat chow to powdered AIN-93 diets. Chewing bones (Bio-Serv, Frenchtown, NJ) were also provided to prevent overgrowing teeth due to feeding of a powdered diet. Rats were randomly allocated in four groups of ten animals each. The animals were housed in pairs in suspended stainless steel cages with wire mesh floor and front. Powdered diet was provided in standard feeding cups.

Diet and tap water was available *ad libitum*. Artificial light was supplied from fluorescent tubes, in a 12 h light –12 h dark cycles. Relative humidity was maintained at 25% - 60%. Health signs were monitored daily and clinical signs for all the animals were recorded weekly. All animals randomly received one dose of a subcutaneous injection of AOM in saline at a dose of 20 mg/kg.

Body weight was recorded twice every week and food intake was recorded twice for 3 days/cage during the 14 weeks study. All animal experiments were conducted in accordance with U.S. Animal Welfare Act and U.S. Public Health Service Policy. The University of Maryland Institutional Animal Care and Use Committee (IACUC) approved experimental protocols.

2.4. Sample Collection

2.4.1. Urine and Feces Collection. One week before the end of the study, samples of urine and feces were collected from eight animals randomly selected from each group. Each animal was placed individually in nalgene metabolic cages (Mini Mitter Inc., Bend, OR). The powdered diet was not provided during this time to reduce any contamination of urine by diet spillage. Urine samples were collected during the dark cycle every 6 hrs for 12 hrs and divided equally into eppendorf tubes. To one set of samples, 20% trifluoroacetic acid (TFA) was added to preserve anthocyanins prior to freezing at -80°C. Fecal samples were collected at the end of 24 hrs and weighed. A portion of each sample was analyzed for moisture content and remaining was frozen at -80°C for further use.

2.4.2.Tissue and Blood Collection. To collect blood, light anesthesia by CO_2 exposure was used prior to decapitation. This was done to reduce stress in animal and enhance safety of personnel/ the researcher. Blood was collected into 10ml tubes without heparin and after clotting, the blood samples were ringed and centrifuged at 10,000g for 10 min at room temperature. The serum samples were quickly divided into several eppendorf tubes. To one aliquot, 20% TFA was added to preserve anthocyanins and to precipitate proteins. This sample was centrifuged for 5 min at 3000 rpm at 4°C prior to freezing at -80°C.

Colons were flushed with ice-cold saline to remove fecal material. The colon was slit open longitudinally and the proximal 4 cm was immersed and fixed in RNAlater (Ambion Inc., Austin, TX) for RNA isolation. The remaining colon was fixed in 10%

buffered formalin for evaluation of ACF and cell immunohistochemistry. Other tissues were flash frozen and stored at –80^{o}C for further use.

2.5. Biomarkers Evaluated

2.5.1. Fecal Moisture Content. Fecal pellets were well formed and diarrhea was not observed but visual observation suggested that feces from animals on ARE diet were distinctive from rats on control diet. The feces were moist and appeared darker and larger. This led us to measure the fecal wet weight and fecal dry matter by oven-drying method (105°C until weight constancy).

2.5.2. Fecal bile acids. Bile acids in freeze-dried feces were assayed by the method as described by Romero *et al.* (116) t-butanol/water (1:1) (4 mL) was added to 0.2 g freeze-dried fecal samples. The samples were homogenized and incubated for 15 min at 37°C with continuous shaking. The samples were centrifuged at 3000g for 10 min. and the supernatant was removed for analysis of total bile acid content by a enzymatic recycling method using total bile acid assay kit (Diazyme Laboratories, La Jolla, CA). Each sample was analyzed in at least duplicate.

2.5.3. Aberrant Crypt Foci. ACF were evaluated as previously described (117). ACF were highlighted by staining the colons with 0.1% methylene blue in PBS and identified

using light microscope at 40X magnification. Number, multiplicity (number of crypts per focus) and distribution of ACF were recorded.

2.5.4. Anthocyanin Profiles. The anthocyanin profiles of the AREs used in this study have been reported previously (26). In this study, the presence and concentration of anthocyanins were measured in the serum, urine and feces of rats.

Sample preparation. Analysis of urine and serum anthocyanin was performed as previously described (118). The urine and serum samples were applied to 6cc C18 Sep-pak cartridges (Waters Corp., Milford, MA) preconditioned with 5 ml methanol containing 1% TFA followed by 5ml 1% aqueous/TFA solution to remove the methanol. Loaded cartridges were washed with 5ml 1% aqueous/TFA solution, and anthocyanins eluted with 5ml 1% methanol/TFA solution. The elutant was evaporated, dissolved in aqueous 1% TFA solution and filtered through 0.45 μm polypropylene filters (Whatman Inc., Clifton, NJ). Fecal samples were prepared according to the procedure of He and colleagues (113, 119). Samples were ground to a powder and 0.1 g of feces extracted with 20 ml of methanol:water (60:40 v/v) solution containing 1 % TFA. Samples were homogenized and sonicated for 10 min. After centrifugation (3500 rpm, 15 min) the supernatant was removed and the pellet reextracted with 10 ml of the same solvent.

Combined supernatants were diluted with acidified water and applied to a C18 cartridge. To remove polar lipids, the column was washed with 5 ml of aqueous 1%TFA solution followed by a wash with 3 ml of dichloromethane/1%TFA solution. To further remove polar and neutral lipids, the column was washed with 3mL of hexane/1%TFA (benzene) and ethylacetate/1%TFA solution respectively. Anthocyanins were eluted from

the C18 column as described for urine and serum samples and 300 µl injected into the HPLC.

High Pressure Liquid Chromatography. A Waters Delta 600 HPLC equipped with a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and Millenium 32 software (Waters Corp.) was used for analyses of anthocyanins. The mobile phase was composed of the following solvents: A- 1% phosphoric acid, 10% acetic acid, 5% acetonitrile, 84% water, and B- 100% acetonitrile. Solvents and samples were filtered though a 0.45 µm polytetrafluoroethylene membrane filters (Pall Life Sciences, Ann Arbor, MI) and 0.45 µm polypropylene filters (Whatman Inc.), respectively.

The gradient system used for all samples was: 0-5 min 100% isocratic, 5-40 min linearly decrease to 65% A, 40-45 min linearly increase to 100% A. For quantification the total peak area under the peak at 520 nm was calculated. By comparing external standard of cya-3-galactoside, the peak area was converted to anthocyanin concentration expressed as cya-3-galactoside.

2.5.5. Measurement of Urinary 8-OHdG. Urinary 8-OHdG was measured using the Enzyme-linked Immunosorbent Assay (ELISA, Genox Corporation, Baltimore, MD), to assess the ability of the three ARE diets to reduce oxidative DNA damage. As there was variation in the total amount of urine collected from different rats, urine creatinine concentrations were measured. Analysis was performed using a creatinine colorimetric microplate assay kit (Oxford Biomedical Research Inc. Oxford, MI) according to the manufacturer's protocol.

2.5.6. Cyclooxygenase-1 and -2 mRNA Levels Expression. Changes in mRNA levels expression of cyclooxygenase genes were analyzed as described earlier (27). In brief, total cellular RNA was extracted from colonic mucosa lining using Trizol reagent (Invitrogen, Carlsbad, CA). Human COX-1 and COX-2 gene-specific Relative RT-PCR Kits (Ambion Inc.) were used with ribosomal gene 18S (498 bp) as an internal control. The following thermocycling conditions were used for PCR assays: one 2-min cycle at 92^0C followed by 26 cycles of denaturation for 30 s at 92^0 C, annealing for 30 s at 59^0C (COX-1) or 30 s at 60^0C (COX-2), and extension for 1 min at 72^0C. The final extension was given for 5 min at 72^0C. The PCR products from multiplex reactions were analyzed using DNA LabChip® and Agilent 2100 bioanalyzer according to the manufacturer's protocol. The changes in the gene expression were represented by the changing ratio between the area of bands representing gene of interest and the band representing 18S gene.

2.5.7. Cell Proliferation Measurements. Colonic cell proliferation was measured using PCNA immunohistochemistry as described previously (117) with slight modifications. Following quenching of endogenous peroxidase activity, slides were incubated with Dako Protein Block Serum Free (DakoCytomation, Carpinteria, CA) and mouse biotinylated PCNA monoclonal antibody clone PC10 at 1:200 dilution (Invitrogen, Carlsbad, CA). To visualize crypts, a light Mayer's hemotoxylin counter stain was used.

2.5.8. Data analysis. One-way analysis of variance (ANOVA) was conducted for the all data analyses using SAS software (8.1, SAS Institute Inc, Cary, NC) to determine if there

were significant differences between rats fed ARE and control diets. ANOVA with repeated measures was done to determine the significant difference in body weight. Duncan's test was used for multiple mean comparisons when the ANOVA was significant.

Chapter 3. Results

3.1. Food Consumption and Rat Body Weight.

There was no significant difference ($p>0.05$) in food consumption or body weights among groups throughout the 14-week study (Figure3). Average food consumption was 17 grams/day. Based on the average body weight of the rats and anthocyanin and phenolics concentrations in the diet, rats consumed approximately 26 mg anthoycanin/kg/day and 80 mg phenolics/kg/day. No adverse clinical signs were observed during the study.

3.2. Extract and Diet Characteristics.

Grape ARE had the highest percentage of monomeric anthocyanin (14.7%) and total phenolics (43.2%) followed by bilberry with 11.0% and 32.6%, and chokeberry ARE with 7.7% and 32.9%, respectively (Table 2). Grape ARE also had a higher ORAC value than bilberry and chokeberry ARE (Table 2). To achieve equal amount of anthocyanin per gram of diet, different amounts of ARE extracts were added to the diet at the expense of cornstarch. The resultant calculated total phenolics and ORAC values for the diets are shown in Table 2.

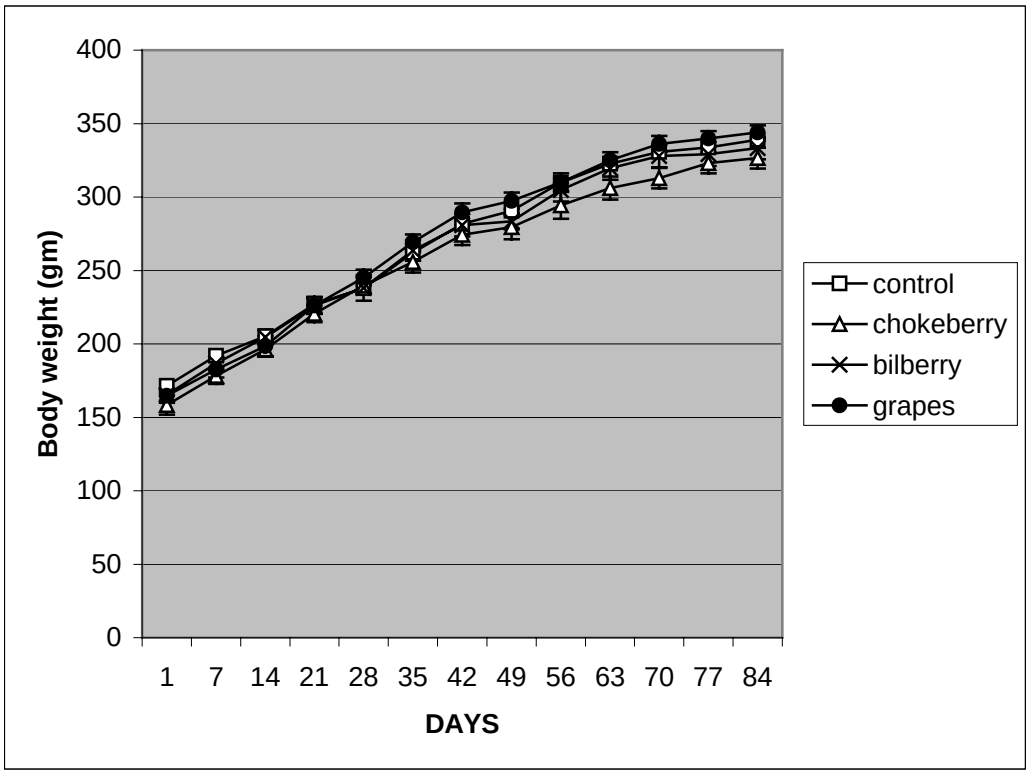

Figure 3. Average body weights of rat fed ARE or control diet. Values are the mean ± SEM of 10 rats per group.

Table 2: Total monomeric anthocyanin content, phenolic content and antioxidant activity (ORAC) in bilberry, chokeberry and grape anthocyanin rich extracts and diets

Extracts	**Monomeric anthocyanin**[a] (mg/g)	**Total phenolics** [b] (mg/g)	**ORAC** [c] (μmol TE/g)
Bilberry	11.0	32.6	3042.9
Chokeberry	7.7	32.9	3388.0
Grapes	14.7	43.2	4282.7
Diets	**Monomeric anthocyanin** (mg/g)	**Total phenolics** (mg/g)	**ORAC** (μmol TE/g)
Bilberry	0.385	1.143	1.065
Chokeberry	0.385	1.645	1.694
Grapes	0.385	1.132	1.122

[a] data expressed as milligrams of cyanidin-3 glucoside equivalents per gram of extract

[b] data expressed as milligrams of gallic acid equivalents per gram of extract

[c] data expressed as micromoles of trolox equivalents per gram of fresh weight

3.3. Aberrant Crypt Foci.

ACF were present in all animals. The total number of ACF was significantly ($p<0.05$) decreased in the colons of rat fed bilberry, chokeberry and grape ARE diets when compared to rats fed the control AIN-93 diet (Table 3). Large ACFs (≥ 5 multiplicity) were significantly reduced in rats fed bilberry ARE ($p=0.0003$), and chokeberry ARE ($p=0.002$) as compared to grape ARE or control group. Rats fed bilberry ARE had a striking 70% reduction in large ACF compared to controls. The number of medium ACFs (4-5 multiplicity) in bilberry ($p=0.005$) and grape ($p=0.02$) fed groups was significantly lower compared to either chokeberry ($p=0.08$) or control AIN-93 group. No significant difference ($p>0.05$) was observed among the small ACFs (2-3 multiplicity) among all groups. Most ACF were found in the distal portion of the colon and the ARE diets did not change the distribution of the ACF.

Table 3: Effect of AREs on total ACF and different categories of ACF multiplicity

Diet group	Total	Small (2-3)	Middle (4-5)	Large >5
AIN-93 (Control)	94 ± 12.2 [a]	46 ± 6.0	33 ± 4.9	15 ± 3.0 [a]
Bilberry	67 ± 9.1 [b]	43 ± 7.1	19± 2.8	4 ± 0.7 [b]
Chokeberry	70 ± 3.5 [b]	39 ± 2.8	25 ± 1.8	6± 1.4 [b]
Grape	69 ± 6.2 [b]	35 ± 4 .3	22 ± 2.3	11±1.7 [a]

[ab] Means (mean ± SE) with different letters are significantly different at $P < 0.05$. N= 10/group

3.4. Anthocyanin Concentrations in Animal Samples.

3.4.1 Urine. Anthocyanins were not detected in the urine of control rats, whereas anthocyanin peaks were observed in urine of all rats on the ARE diets at 520 nm (data not shown). Significant differences (*p=0.002*) were observed in the concentration of total monomeric anthocyanins among urine samples from bilberry, chokeberry and grape ARE-fed rats varying from 23.6 mg cy-3-gal/L urine in chokeberry ARE-fed rats to 7.8 mg/L in bilberry ARE-fed rats (Figure 4A).

3.4.2 Feces. The total monomeric anthocyanin concentrations in fecal extracts varied from 2.0 mg/L in bilberry ARE fed rats to 0.7 mg/L in grape ARE fed rats (Figure 4B). Bilberry and chokeberry ARE-fed rats had higher fecal anthocyanin concentrations as compared to grape ARE-fed rats (*p=0.029*). Anthocyanin specific peaks were not detected in the fecal extracts of rats on control diets.

3.4.3 Serum. Anthocyanins were detectable but below quantifiable levels in serum samples from all ARE-fed rats, and undetectable in serum from control diet rats.

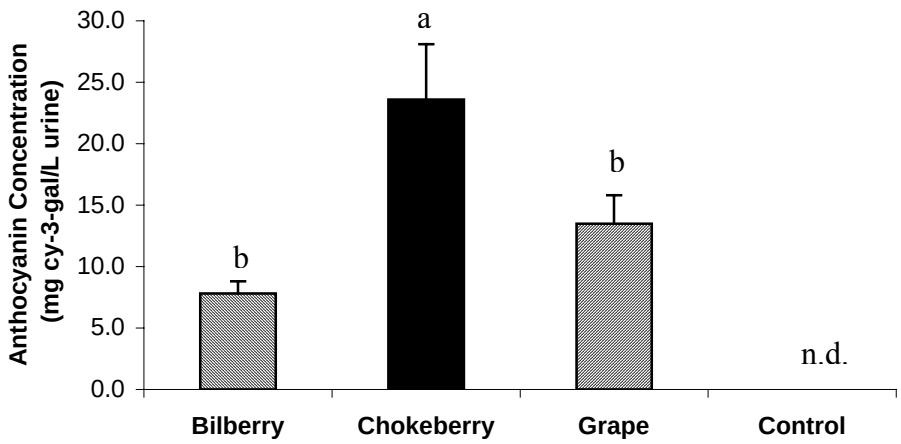

Figure 4A. Total anthocyanin concentration in urine of rats fed ARE or control diet. Values are the mean ± SEM of 6-8 rats per group. Bars with different letters are significantly different at $p < 0.05$. Anthocyanins were not detected (n.d.) in urine of rats fed the control diet.

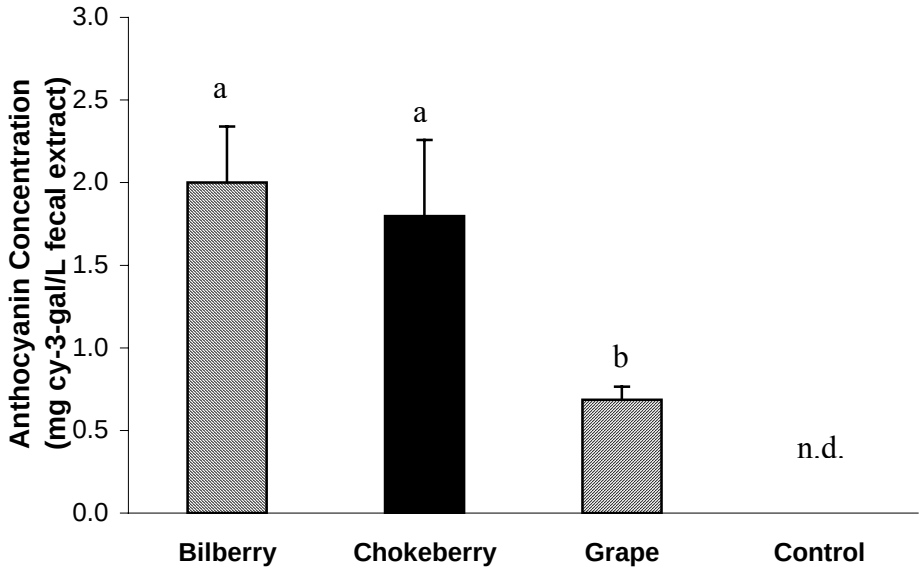

Fig. 4B. Total anthocyanins concentration in fecal extracts from rats fed ARE or control diet. Values are the mean ± SEM of n=7 for chokeberry and grape fed rats, n=5 for bilberry fed rats. Bars with different letters are significantly different at $p < 0.05$. Anthocyanins were not detected (n.d.) in feces of rats fed the control diet.

3.5. Fecal Moisture Content.

During the experiment, we observed that rats on ARE diets had larger feces. To determine whether the increase in the size was due to increased moisture content or increased fecal content, the moist and dry fecal weight from 24 hr collections at two different times were determined. As observed in Table 4, the moisture content of feces from ARE-fed rats was significantly higher than for control diet rats. Dry weight of feces was also significantly higher in rats fed both bilberry and grape ARE as compared to controls.

3.6. Fecal Bile Acids.

The concentration of total bile acids in fecal extracts was dramatically reduced from 385 μmol/L in control rats to 133 - 68 μmol/L in ARE-fed rats (Table 5). As the amount of feces excreted per day was increased in ARE-fed rats (Table 5), fecal bile acid values were converted to μmol/g feces and multiplied by the individual fecal output to determine if the ARE-diet altered the total daily bile acid excretion. The daily (24 hour) fecal excretion of bile acids in ARE fed-rats ranged from 0.5 to 2.0 μmol/day compared to 2.7 μmol/day by control diet-fed rats (Table 5).

Table 4: Effect of feeding AREs diets on 24-hour fecal collections

Diet group	Fecal weight (g/day)	Fecal dry weight (g/day)	Fecal moisture content (%)
Control	0.63 ± 0.07 [a]	0.38 ± 0.04 [a]	39.2 ± 0.97 [a]
Bilberry	1.39 ± 0.17 [b]	0.69 ± 0.07 [b]	48.9 ± 0.96 [c]
Chokeberry	0.86 ± 0.10 [a]	0.42 ± 0.04 [a]	49.3 ± 1.68 [c]
Grape	1.32 ± 0.16 [b]	0.71 ± 0.07 [b]	45.1 ± 0.99 [b]

[abc] Means (mean ± SE) with different letters are significantly different at $P < 0.05$. N=8/group

Table 5: Effect of feeding ARE diets on fecal bile acids concentration in 24-hour fecal collections

Diet group	Bile acids in fecal extract (µmol/L)	Bile acid concentration (µmol/g dry feces)	Total fecal bile acids (µmol/g day)
Control (n=5)	385.1 ± 111.4[a]	7.70 ± 2.23 [a]	2.68 ± 0.65 [a]
Bilberry (n=5)	67.7 ± 10.8 [b]	1.35 ± 0.22 [b]	1.26 ± 0.31 [b c]
Chokeberry (n=5)	83.2 ± 15.6 [b]	1.67 ± 0.31 [b]	0.55 ± 0.10 [c]
Grape (n=6)	133.1 ± 28.4 [b]	2.66 ± 0.57 [b]	2.00 ± 0.51[a b]

[abc] Means (mean ± SE) with different letters are significantly different at $P < 0.05$.

3.7. Urinary 8-OHdG Determination.

Urinary 8-OHdG is widely used as a measure of oxidative damage of DNA. 8-OHdG reflects DNA mutation potential (120). There was no significant difference in urinary 8-OHdG levels among any of the groups. Adjusting for urine creatinine levels did not change the results.

3.8. Cyclooxygenase mRNA.

The COX-2 gene mRNA levels were down regulated in colonic mucosa of rats fed bilberry and grape ARE diets compared to rats fed either control or chokeberry ARE diet (*p=0.009*) (Figure 5). COX-2 m-RNA levels expression in chokeberry ARE-fed rats did not differ from control rats (Figure 5). No significant difference was observed in the expression of COX-1 gene in the colonic mucosa among groups.

3.9. Colonic Cell Proliferation. A significant decrease in colonic cell proliferation in rats fed bilberry ARE (*p=0.008*) and chokeberry ARE (*p=0.015*) diets, compared to rats fed the control diet (Table 6). Rats fed grape ARE diet showed no change in the cellular proliferation compared to controls (Table 6). However, there was no significant difference in the crypt height among the diet groups (Table 6).

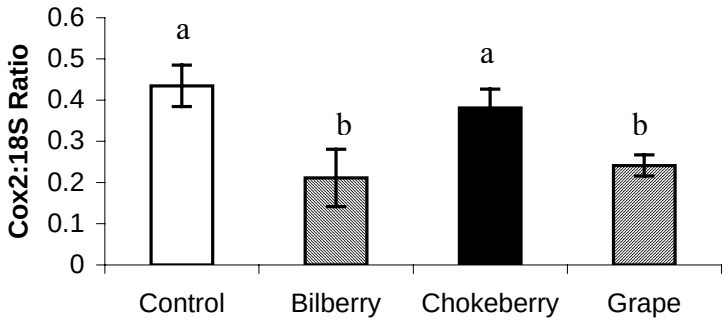

Fig 5. Effect of ARE diets on colonic mucosa cyclooxygenase 2 (COX2) mRNA levels expressed as relative to 18S mRNA levels. Bars with different letters are significantly different at $p < 0.05$.

Table 6: Colonic crypt height and proliferation index of rats fed anthocyanin-rich or control diet

Diet group	Crypt height	Proliferation index
AIN-93 (Control) (n=10)	31.7 ± 0.74	23.6 ± 2.85 [a]
Bilberry (n=5)	31.2 ± 0.54	12.1 ± 2.01 [b]
Chokeberry (n=5)	29.7 ± 1.24	16.0 ± 2.82 [b]
Grape (n=5)	32.7 ± 0.38	22.1 ± 3.02 [a]

[ab] Mean (mean ±SE) with different letters are significantly different at $P < 0.05$.

Chapter 4. Discussion

We report significant reduction of several biomarkers of colon cancer by dietary AREs in the azoxymethane animal model, providing *in vivo* confirmation of the chemopreventive activity observed *in vitro* in HT29 colon cancer cell line studies (26, 27). This *in vivo* study also identified multiple mechanisms for the chemopreventive activity of AREs, including several mechanisms of action predicted by cell lines studies. In addition, we report for the first time, that dietary AREs significantly alter the composition of the colonic contents resulting in a protective effect against colon carcinogenesis.

Rats on ARE diets had significantly fewer colonic ACF when compared to the control group, indicating that all three extracts may have chemopreventive activity. ACF are preneoplastic lesions of colon cancer (38, 121) widely accepted as a biomarker for risk of colon cancer development (37) . Rodent ACF, induced with chemical carcinogens such as AOM, have been utilized for screening chemopreventive agents (122) and also for risk assessment of chemicals (123). ACF rarely occur in untreated rats (117,124) but appear in colon and rectum within few weeks during post initiation phase after treatment. Quantification of these lesions in humans has recently been reported using magnifying endoscopies (119,125) and these lesions are being utilized as endpoints for chemoprevention trials.

The aberrant crypts of higher multiplicity, multicrypt foci, are a more consistent predictor of tumor outcome than the number of ACF (38, 126, 127). Rats fed bilberry

ARE had 70% fewer large ACF compared to rats fed the control diet, indicating significant chemoprevention. Chokeberry-fed rats had a 59% reduction in large ACF, whereas the reduction was only 27% in rats fed grape ARE. The type and number of glycosylations in the anthocyanins in these three extracts varied greatly, which markedly affected absorption as discussed in our report on the anthocyanin profiles of the plasma and urine samples from the rats in this study (113, 128). Others have reported inhibition of ACF and colonic tumors by addition of anthocyanin-rich extracts (24, 25) and anthocyanin-rich freeze-dried black raspberries (23). Anthocyanins isolated from tart cherries inhibited cecal adenomas but not colonic tumors in Apc^{Min} mice (22). The AREs used in this experiment contained not only anthocyanins, but also other phenolics. Although the chokeberry diet contained a higher amount of total phenolics, rats fed the chokeberry diet had similar ACF growth as rats fed the bilberry diet, suggesting inhibition did not depend on difference in total phenolics. These results are in agreement with our cell line work (26).

Greater inhibition of cell proliferation in rats fed bilberry and chokeberry AREs also agrees with our cell line studies in which greater inhibition of HT29 cell growth was observed with these extracts as compared to grape ARE. Zheng and coworkers (129) observed a correlation between the ability of retinoids to prevent ACF and decrease in PCNA labeling index. We reported that chokeberry ARE blocks HT29 cells at the G1/G0 and G2/M phases of the cell cycle, which coincided with increased expression of p21 and p27 genes, and decreased cyclin A and B gene expression (27). Although Lazze and coworkers (130) also reported that anthocyanins induced cell cycle blockage in CaCo2 and HeLa cells, their studies were conducted with anthocyaninadins, the aglycones of

anthocyanins, rather than with anthocyanins. Aglycone compounds are rarely, if ever, found in natural food components (131). Although deglycosylation of anthocyanins to aglycones by intestinal bacteria was initially suspected, direct absorption of glycosylated anthocyanins has been well documented (80, 118, 132), and aglycones are considered unstable at physiological pHs (79, 118). Hence, the findings of Lazze and coworkers (130) are not supportive of the effects of food-based anthocyanin extracts. Kang *et al.* (22) reported inhibition of cell proliferation in HT29 and HCT116 cells as a potential mechanism for the inhibition of intestinal tumors by tart cherry anthocyanins observed in APC^{min} mice. However, to our knowledge, this is the first report of inhibition of cell proliferation by AREs *in vivo*.

To determine if the antioxidant activity of the three extracts may have a role in their anticarcinogenic activity, we measured the ORAC values of the AREs and diets. The ORAC value of grape ARE was comparable to freeze-dried skins of highly pigmented red wine grapes (133). The chokeberry ARE ORAC value was 21 times higher than reported for wild fruits (134). Bilberry ARE had 23-161 times higher ORAC value compared to that reported from fresh bilberry fruit samples (135). Despite the high ORAC values, we found no effect of the ARE diets on urinary 8-OHdG levels. This is in contrast to Harris *et al*. (23) who reported significant reduction in urine 8-OHdG levels in rats treated with azoxymethane and fed freeze-dried black raspberries. The difference may be attributed to stage of carcinogenesis during which urine is collected. In our study, urine was collected from rats at the ACF stage, whereas the study by Harris (23) was longer-term and rats had advanced colonic tumors at the time of urine collection. Although we did not obtain evidence of an antioxidant effect of AREs *in vivo*, increased

serum antioxidant activity following consumption of anthocyanins has been reported (115). Perhaps serum antioxidant levels may have been a better parameter to measure in this study than urinary 8-OH deoxyguanosine. However, in our study, all serum was used for anthocyanin analyses.

One observation to note is that although bilberry diet had the lowest ORAC value, it was the most effective in inhibiting large ACF development and cell proliferation. This suggests that antioxidant activity of anthocyanin-rich extracts, as measured by ORAC, is not directly predictive of *in vivo* chemoprotective activity. Similarly, Lui and colleagues (136) reported that the total antioxidant activity of various raspberries was not correlated with their ability to inhibit the growth of HepG2 cells..

Inhibition of COX-1 and COX-2 enzymes has been demonstrated by anthocyanins from different berries including tart cherries, raspberries and blackberries (137, 138). Seeram *et al.* (138) demonstrated that cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside, anthocyanins from raspberries and sweet cherries inactivate COX-1 and COX-2 enzymes *in vitro*. However, we found that in the HT-29 cells, chokeberry ARE had a very transient inhibitory effect on COX-2 mRNA levels with no effect on COX-2 protein or prostaglandin E2 levels (27). In this study, rats fed bilberry and grape ARE, but not chokeberry, demonstrated a decreased expression of COX-2 mRNA levels. Epidemiological and preclinical studies have shown that several nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the COX-2 gene and enzyme, can reduce ACF and colon cancer both in animals and humans (139). However there is considerable concern about their adverse effects, which can be occasionally severe and life

threatening, and are at least partly attributed to inhibition of COX-1. None of the ARE diets inhibited COX-1 gene expression, and inhibition of COX-2 may have contributed to the chemopreventive activity of the bilberry and grape ARE diets.

One of the questions that arise when considering anthocyanin extracts as potential chemopreventive agents is the degree to which these compounds are bioavailable. To address this question, we measured the concentration of anthocyanins in serum, urine and feces. Cancers of the gastrointestinal tract are unique in that even compounds that are not absorbed can have direct contact with cells. As has been reported by other researchers (72, 118) serum of rats fed AREs contained only very low levels of anthocyanins (below the level of quantitation). However, the presence of anthocyanins in urine indicated that absorption did occur. We report for the first time, that high amounts of anthocyanins were found in the intestinal contents. Indeed, rats fed the bilberry and chokeberry extracts, had the fewest ACF and the highest fecal anthocyanin concentrations, suggesting that the chemopreventive activity of anthocyanins may be not via absorbed, but rather via unabsorbed anthocyanins. This hypothesis is supported by the observation that anthocyanin-rich diets have been effective in prevention of cancers in animal studies in the oral cavity, the esophagus (140), and colon (22-25) but not lung cancer (141).

In a review of mechanisms of colon cancer prevention (142), three mechanistic groups of agents were described: (1) agents that are confined to the intestinal lumen and function to reduce irritation of epithelium; (2) agents that inhibit COX2 and reduce inflammatory responses; and (3) agents that are antioxidants and reduce damage by

reactive oxygen intermediates. The data from this study suggest that AREs may be agents that act by all of these mechanisms. Rats fed the ARE diets had significantly increased fecal bulk and fecal moisture compared to rats fed the control diet. The increased fecal moisture content and fecal bulk would reduce the concentration of endogenous tumor-promoting compounds such as bile acids in the colon resulting in less irritation and this may have also contributed to the reduction in cell proliferation. In rodent models, bile acids have been well established as promoters of colon cancer (38, 143). Recently bile acids have been proposed as colon carcinogens in humans (54). Reduction of levels of fecal bile acids and fatty acids by dietary means such as calcium supplementation and high fiber diets is considered to be chemopreventive because the reduction of cytoxicity of the aqueous phase of the colonic contents is associated with a reduction of proliferative markers (144). Poly ethylene glycol 8000 (PEG), a highly effective colon chemopreventive agent, increased fecal bulk and reduced the bile acid concentration in feces of PEG- fed rats but the daily fecal excretion of bile acid was same as controls (145). In the present study, the concentration of 7.7 μmol bile acids/g dry feces in rats fed the control diet was similar to the levels of 3.3 μmol/g fresh feces reported by Corpet and Parnaud (1999) when the difference in moisture content is considered. ARE-fed rats showed a dramatic 65-82% reduction in the bile acid concentration in feces, and, unlike PEG, the daily total fecal excretion of bile acids was also reduced. The decrease in total bile acid concentration in feces with dietary supplementation of AREs indicates that reduced gastrointestinal irritation may be responsible for the decrease in cell proliferation as observed with PCNA

inmmunohistochemistry. Further investigation is required to establish the mechanism by which dietary AREs reduce fecal bile acids.

Chapter 5. Conclusion and Future research

In conclusion, the present *in vivo* study in F344 rats demonstrates that anthocyanin rich extracts from bilberry, chokeberry and grape significantly inhibited ACF formation induced by AOM. Our results clearly support the chemopreventive activity of these extracts reported in previous *in vitro* studies (26, 27). We have also conducted detailed analyses of the anthocyanins found in the serum, urine and feces as compared to the anthocyanins in the diets fed to the rats in this study (113, 128). These data indicate that the structure of the anthocyanins, specifically the degree of glycosylation, affects the degree to which the anthocyanins will be absorbed and excreted in the feces.

High levels of anthocyanins in the intestinal contents appear to act both directly on cells lining the gastrointestinal tract, and indirectly by altering the gastrointestinal environment to be less damaging to the mucosal surface. These data suggest that anthocyanins may play a significant role in prevention of gastrointestinal cancers by fruits and vegetables. Furthering our understanding of the effect of structure of anthocyanins on uptake and excretion will improve our ability to develop optimal anthocyanin profiles for chemoprevention of cancers of the gastrointestinal tract.

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