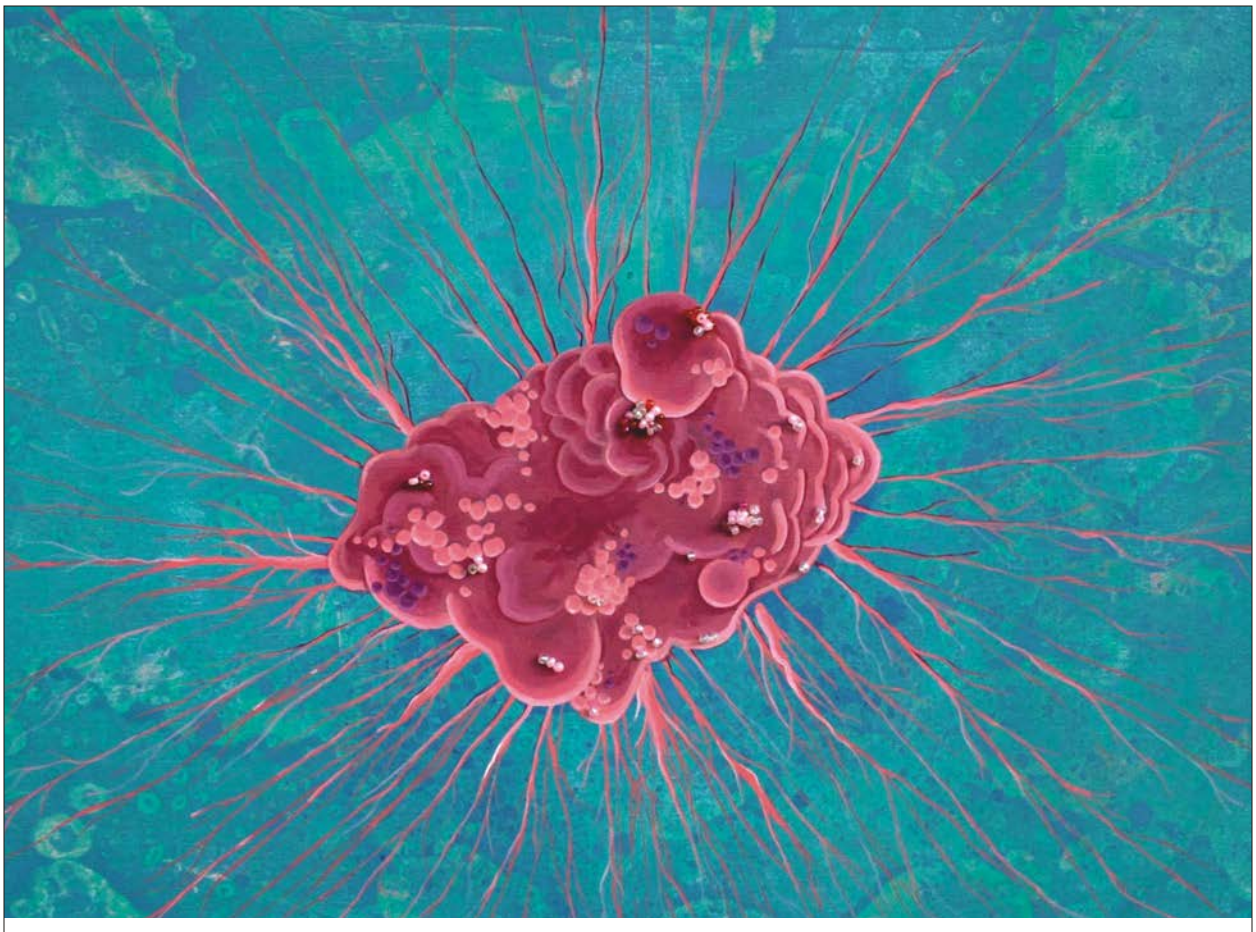

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Chemopreventive Potential of Cranberries on Azoxymethane Induced Aberrant Crypt Foci in Fisher 344 Male Rats

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Abstract: In this study, the chemopreventive potential of Cranberry was analyzed in reducing the Aberrant Crypt Foci (ACF) induced by Azoxymethane (AOM) in Fisher 344 male rats. After 1 week period of acclimatization, rats were divided into five different groups. Cranberry meal was mixed in an AIN 93G based diet at 5 and 10% and juice was provided at 2.5 and 5%. Daily feed intake and weekly body weights were recorded. At 17 week of age, rats were killed and samples were collected. Number of ACF and number of cypts/foci were enumerated in the colon. There were no significant differences in feed intake, weight gain, cecal weight and cecal pH among all groups. Total ACF incidence (119) was significantly ($p<0.05$) higher in control group than in treatment groups. Reduction in total ACF induction was higher in rats fed 10% Cranberry (65.75%) compared to control. A two to six fold increase in selected hepatic enzymes activities (units/mg enzyme) were seen in rats fed 5 and 10% treatment diets compared to control. Results of this study showed that administration of Cranberry meal and juice resulted in significant ($p<0.05$) reductions in the incidence of ACF in azoxymethane induced preneoplastic lesions.

Key words: Azoxymethane, aberrant crypt foci, cranberry, detoxification enzymes, antioxidative enzymes

INTRODUCTION

Colon cancer is a major health problem in the US. There will be 112,340 new cases of colon cancer and 56,180 deaths in the year 2007 which accounts for 10% of total cancer related deaths (American Cancer Society, 2005). The incidence of colorectal cancer is almost similar in men and women (10 and 11%) (Jemal *et al*., 2007). The American Cancer Society emphasizes the inclusion of whole grains, fruits and vegetables and limited consumption of red meats in the diet in order to reduce the risk of cancer. Epidemiological evidence suggests that a diet rich in vegetables and fruits can notably reduce the risk for diverse human cancers including colon cancer (Block *et al*., 1992).

Phytochemical content and the corresponding antioxidant activity of fruits and vegetables contribute to their protective effects against chronic and degenerative diseases. Anthocyanins are prevalent in fruits such as red grapes, raspberries, blueberries and cranberries, reaching concentrations in excess of 10 g kg^{-1} in some berry cultivars (Clifford, 2002). Anthocyanidins were reported to be more powerful antioxidants than vitamin C and E (Bagchi *et al*., 1997). Anthocyanidins can scavenge free radicals and singlet oxygen by donating phenolic hydrogens and may also be involved in molecular mechanisms such as cytotoxicity, inhibition of cell proliferation and apoptosis (Hou *et al*., 2003).

Cranberry, *Vaccinium macrocarpon* Ait. (Ericaceae), is one of the native fruits of North America and is widely grown in Wisconsin and Massachusetts. It is ranked first in its polyphenol content and sixth in its antioxidative potential among 20 commonly consumed fruits in the US (Vinson *et al*., 2001).

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The cranberry fruit and juice are reported to exhibit various health benefits including the prevention of microbial adhesion in the urinary tract and reduction of biofilm formation, potent antioxidant activity, cholesterol reduction, vasorelaxant effects and *in vitro* anticancer effects (Kandil *et al*., 2002). Many of these biological effects are due to presence of a wide variety of diverse phytochemical compounds in the fruit including flavonols, anthocyanins, proanthocyanidins, phenolic acids, terpenoids and sugars (Roy *et al*., 2002).

Many studies on assessing the chemopreventive potential of dietary or phytochemical components have used the Azoxymethane (AOM) Fisher-344 male rat model. AOM is a potent carcinogen and induces the formation of tumors and early preneoplastic lesions, Aberrent Crypt Foci (ACF), after two injections in rats. It offers the ability to screen different compounds as cancer preventive agents. Biotransformation of AOM occurs in the liver by phase I enzymes such as CYP2E1 and others convert it into methyldiazonium ions responsible for methylation of DNA (Fiala, 1977). Later, Phase II detoxification enzymes conjugate the methyl ion and eliminate them.

Induction of phase II, Glutathione-S-transferase/(GST), detoxification enzymes by fruits and vegetables may partially account for an inverse association between their consumption and the risk of colorectal cancer (Steinmetz and Potter, 1996). Antioxidants such as phenols and flavonoids in fruits and vegetables may increase the activity of antioxidative enzymes superoxide dismutase (SOD) and catalase (Nielsen *et al*., 1999; Castenmiller *et al*., 1999; Young *et al*., 1999). Additive and/or synergistic effects of phytochemicals in fruits can provide protective health benefits. Cranberry phytochemicals exhibited anti proliferative, (Murphy *et al*., 2003) and apoptotic activity, (Ranelletti *et al*., 2000) expression of ornithine decarboxylase (ODC) (Zhang *et al*., 2005) and induction of xenobiotic detoxification enzymes *in vitro* (Bomser *et al*., 1996). However, little is known about the *in vivo* chemopreventive potential of cranberry in order to correlate its anticancer properties to *in vitro* experiments. The objective of the study was to determine the effect of feeding Cranberry meal and Cranberry Juice on AOM-induced aberrant crypt foci in Fisher 344 male rats at nutritionally consumable levels.

MATERIALS AND METHODS

Animal Housing and Diets

All protocols involving rats were approved by the Institutional Animal Care and Use Committee of Alabama A and M University, (2006). Fisher 344 weanling rats (Harlan, IN) were housed in stainless steel wire cages at the rate of two rats per cage in a temperature- and humidity-controlled room (2°C and 50% relative humidity) with a 12 h light/dark cycle. Rats were given free access to AIN 93G diet and water. After a one week adaptation period, rats were divided into five groups and each group had six rats. Cranberry was given as meal at 5 and 10% and juice at 2.5 and 5% levels. Cranberries were obtained from the local market, freeze dried and mixed into the diet. Whole cranberry juice was also obtained from the local market and diluted to 2.5 and 5% levels. Diets were formulated and made isocaloric based on AIN 93G diets by modifying dextrose, corn starch and fiber. Dietary ingredients were obtained from M.P. Biomedicals (Costa Mesa, CA). All diets were prepared fresh each week and stored at 4°C until fed. Body weights were recorded biweekly, food and fluid intakes were monitored daily. After 17 week of age, feed was withheld overnight and the rats were killed by CO_2 euthanasia. The cecum from each rat was excised, split open, weight of cecal tissue and pH of the contents were noted.

Carcinogen Injection

For induction of colon ACF, all rats were given two subcutaneous injections of Azoxymethane (AOM), (NCI Repository, Kansas City, MO) in saline at the rate of 6 mg kg^{-1} body weight at seventh and eighth week of age.

Enumeration of Aberrant Crypt Foci

ACF in the proximal and distal sections as well as crypts per focus were counted as described by Bird (1987). Each segment was observed under microscope and total number of ACF and crypt multiplicity was scored.

Preparation of Microsomal Fractions of Liver Homogenate

The liver was excised, washed in ice cold 1.15% KCl solution, blotted, weighed and homogenized in 9 volumes of homogenizing buffer (pH 7.4). The resulting liver homogenate was centrifuged at 10,000 g for 30 min and a portion of supernatant was removed to determine antioxidative enzymes; SOD and catalase activities. The other portion was further centrifuged at 100,000 g for 60 min at 4°C. Microsomal pellets were suspended in equal volumes of homogenization buffer and stored at -80°C. The liver microsomes were used in the analysis of phase I and phase II enzyme activities. Total protein was determined by the method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as a standard, at 660 nm.

Determination of Antioxidative Enzyme Activity

Liver catalase was estimated in a UV recording spectrophotometer at 240 nm by monitoring the decomposition of H_2O_2 as described by Aebi (1984). Superoxide dismutase was assayed by the technique of Fridovich (1989).

Activity of Phase I and Phase II enzymes

Glutathione S-transferase activity was determined by the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene [CDNB] detected by a change in absorbance at 340 nm (Habig *et al*., 1974). Cyp 2E1 catalyzes the hydroxylation of p-nitrophenol to p-nitrocatechol (PNP). Enzyme activity was determined by hydroxylation of PNP spectrophotometrically at 600 nm (Koop *et al*., 1997).

Statistical Analysis

Data were analyzed by SAS, 9.1 (SAS Institute, Cary, NC, USA). One way Analysis of Variance (ANOVA) at $p<0.05$ was performed to test significant differences between the groups. Tukey's studentized range test was used to determine significant differences among the groups.

RESULTS

General Observations

Feeding cranberry meal (5 and 10%) and administration of cranberry juice (2.5 and 5%) via drinking water had no effect on weight gain, feed intake, cecal weight and cecal pH (Table 1). The mean daily feed intake ranged from 12.64 -14.67 g day^{-1} and body weight gain ranged from 195-226.40 g, indicating that feeding cranberry meal or juice did not cause any changes in weight gain compared to the rats fed the control (AIN 93G) diet. There were no significant differences in cecal weight or cecal pH among all the groups. The lowest cecal pH (7.62) was observed in rats fed with 10% CM.

Incidence of Aberrant Crypt Foci (ACF) in Colon of Fisher 344 Male Rats

The number of ACF was significantly ($p<0.05$) lower in all the groups fed cranberry juice or meal compared to the control group. Higher number of ACF was seen in the distal colon compared to the proximal colon of rats in all groups. In the proximal colon, the number of ACF was significantly lower in rats fed CM 10% (18.5) and CJ 5% (17.75) compared to the other groups. All the treatment groups had a lower ($p<0.05$) induction of ACF in the distal colon compared to the control group (Fig. 1).

Table 1: Effect of cranberries on weight gain, feed intake, Cecal weight and Cecal pH on AOM-induced Fisher 344 male rats

Groups	Feed Intake (g)	Weight gain (g)	Cecal weight (g)	Cecal pH
Control	12.64±0.72	226.40±10.54	1.03±0.14	7.83±0.06
CJ 2.5%	13.65±1.05	208.20±4.910	1.21±0.19	7.87±0.08
CJ 5%	13.20±0.94	223.40±9.910	1.25±0.20	7.79±0.09
CM 5%	14.21±1.12	195.40±10.85	1.09±0.06	7.90±0.05
CM 10%	14.67±1.78	201.30±8.160	1.10±0.19	7.62±0.02

CJ: Cranberry Juice; CM: Cranberry Meal, Values are means±SEM; n = 6

Table 2: Crypt multiplicity and Number of crypts in colon of rats

Groups	Crypt ≤3	Crypt ≥4	Total crypts
Control	90.25±5.02^{a}	28.75±2.75^{a}	330.50±16.78^{a}
CJ 2.5%	62.00±2.73^{b}	9.75±1.10^{b}	176.00±3.69^{b}
CJ 5%	48.50±2.87^{bc}	6.50±2.17^{b}	125.50±13.00^{bc}
CM 5%	51.75±5.87^{b}	9.75±1.65^{b}	161.50±12.11^{b}
CM 10%	33.25±0.85^{c}	7.50±1.25^{b}	105.00±8.17^{c}

CJ: Cranberry Juice; CM: Cranberry Meal, Values are means±SEM; n = 6. Values not sharing a common superscript are significantly different ($p<0.05$) with Tukey's Studentized Range Test in a column

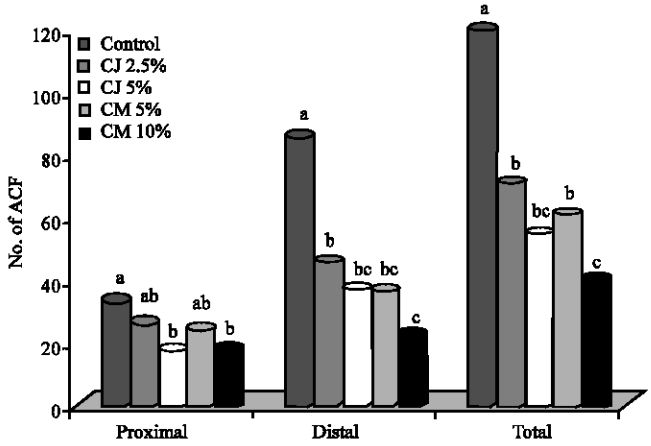

Fig. 1: Effect of cranberries on AOM-induced ACF in the colon of Fisher 344 male rats. CJ: Cranberry Juice; CM: Cranberry Meal, Values are means; n = 6. Bars not sharing a common superscript are significantly different ($p<0.05$) with Tukey's Studentized Range Test

Reductions in number of ACF in the distal colon were 47.07% ($p<0.05$) in rats fed CJ 2.5%, 56.43% in CJ 5%, 57.30% in CM 5% and 73.97% in CM 10% compared to the control fed rats. Rats fed CM 10% and CJ 5% had lower number of ACF in the distal colon compared to their counterparts (CM 5% and CJ 2.5%). A similar pattern was seen in number of total ACF induction. Rats fed cranberry meal had a lower ACF incidence compared to rats fed cranberry juice. However, total number of ACF was similar for all treatment groups except rats fed CM 10% (40.75) which had significantly lower ACF incidence compared to the CJ 2.5% and CM 5%. The incidence of ACF/colon was significantly higher in the rats fed the control diet (119) compared to the rats fed cranberry.

Crypt Multiplicity and Total Crypts

Rats fed treatment diets had a significantly lower number of ACF with ≤3 and ≥4 crypts/foci and total crypts compared to the control group (Table 2). The number of small ACF (crypts ≤3) was significantly ($p<0.05$) lower in rats fed CM 10% (33.25) compared to the rats fed CJ 2.5% (62.00),

Table 3: Activity of selected hepatic enzymes (CYP2E1 and GST)

Groups	CYP2E1 (nmol min^{-1} mg^{-1})	GST (mmol min^{-1} mg^{-1})
Control	0.72±0.02^{a}	10.35±1.87^{a}
CJ 2.5%	0.69±0.01^{a}	48.74±3.92^{b}
CJ 5%	0.62±0.03^{a}	71.05±6.14^{c}
CM 5%	0.67±0.02^{a}	56.23±4.29^{b}
CM 10%	0.71±0.01^{a}	75.81±5.68^{d}

CJ: Cranberry Juice; CM: Cranberry Meal; CYP: cytochrome; GST: Glutathione-S-Transferase, Values are mean±SEM; n = 6. Values not sharing a common superscript are significantly different ($p<0.05$) with Tukey's Studentized Range Test in a column

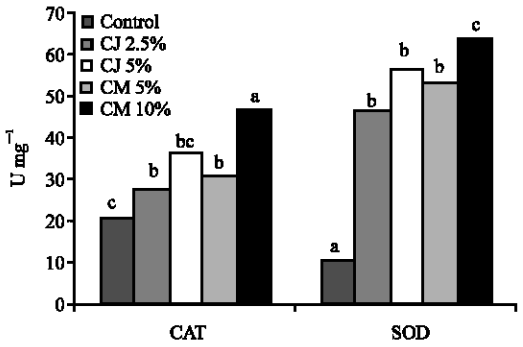

Fig. 2: Activity of Antioxidative enzyme in liver of rats. CJ: Cranberry Juice; CM: Cranberry Meal; CAT: Catalase; SOD: Superoxide Dismutase. Values are means; n = 6. Bars not sharing a common superscript are significantly different ($p<0.05$) with Tukey's Studentized Range Test

CM 5% (51.75) and control (90.25) diets. A similar trend was also found in the number of total crypts. Reductions in total crypts was highest (68.23%) in rats fed CM 10%, followed by CJ 5% (62.02%), CM 5% (51.13%) and CJ 2.5% (46.74%) compared to the control. A higher reduction (%) in the occurrence of large ACF (crypts ≥ 4) was seen in all rats fed treatment diets compared to the small ACF (crypts ≤ 3).

Activity of Phase I and Phase II Enzymes

The activity of hepatic CYP2E1 in the rats fed cranberry diets was not significantly different from the control fed rats. Liver GST activity was significantly ($p<0.05$) higher in rats fed cranberry (CM and CJ) compared to the control fed rats. Highest activity (75.81 mmol/min/mg) was found in the group fed CM 10% compared to the other treatment groups. GST activity was higher in rats fed CM 10% and CJ 5% compared to their counterparts (CM 5% and CJ 2.5%). GST activity (mmol/min/mg) ranged from a low of 10.35±1 in the control group to a high of 75.81±5.68 in rats fed CJ 10%. GST activity was 49-75% higher in rats fed treatment diets compared to the control (Table 3)

Activity of Antioxidative Enzymes

SOD and CAT activities in the liver of rats fed cranberry were significantly ($p<0.05$) higher compared to the control fed rats. Administration of cranberry meal at 10% had the greatest effect in increasing the enzyme (SOD and CAT) activities (U mg^{-1}) compared to other treatments. A four - five fold increase was seen in the activity (U mg^{-1}) of SOD in treatment groups except CM 10% (63.2) which had nearly six fold increase compared to the control (10.62). CAT activity (U mg^{-1}) ranged from a low of 20.65 in rats fed the control diet to 46.00 in rats fed CM 10%. The CAT and SOD (U mg^{-1}) activities were 23.51-55.10 and 77.01-83.2% higher in treatment groups compared to the control (Fig. 2).

DISCUSSION

Cranberry fruit is gaining the attention of researchers as a functional food due to its well documented protection against urinary tract infections (Santillo and Lowe, 2007), *in vitro* anticancer properties (Neto, 2007) and cholesterol lowering effects (Reed, 2002). The present study was conducted to evaluate the chemopreventive potential of cranberry meal and cranberry juice at selected concentrations reflecting nutritionally consumable levels on reduction of azoxymethane induced aberrant crypt foci formation in Fisher-344 male rats.

ACF, a biomarker for colon cancer, are precursors of colon tumors in chemically induced colon cancer. The reduction in the number of ACF in short term (17 weeks) studies with dietary administration of different compounds indicates its ability in modulating colon cancer. All rats fed cranberry either in meal or juice form had significantly ($p<0.05$) lower number of proximal, distal and total ACF, indicating that cranberry may have significant chemopreventive properties. Higher number of ACF was found in the distal portion compared to the proximal colon in all rats, which is also observed in humans with colon cancer. The number of ACF were lower in rats fed cranberry which is consistent with reductions in ACF also reported with feeding bilberry, grape and chokeberry anthocyanin rich extracts compared to the control, with feeding bilberry resulting in the greatest (70%) reduction in large ACF (Lala *et al*., 2006) compared to the control.

Phytochemicals in cranberry might have antiproliferative and apoptotic activities, which resulted in reducing the number of ACF. Total polyphenol extracts of cranberry showed higher antiproliferative activity in oral (KB, CAL 27), breast, colon (HT 29), prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines at 200 μg mL^{-1} compared to isolated individual phytochemical extracts (Seeram *et al*., 2004). Flavonol (quercetin) present in cranberry reduced the growth of breast, colon leukemia cell lines with GI50 (15-60 mg) (Murphy *et al*., 2003). Cranberry extracts also significantly inhibited the proliferation of breast cancer cells (MCF-7) in a dose dependent manner at concentrations of 2.5-30 mg mL^{-1}. Quercetin induced apoptosis by modulating Bax and Bcl-2 expression and also reduced ACF induction by 75% (Volate *et al*., 2005).

Anthocyanins have high antioxidative potential and may reduce oxidative stress in cancer. Although the cranberry anthocyanin extract did not have any effect in inhibiting the growth of cell lines, they exhibited anti-angiogenic and anti-inflammatory properties (Atalay *et al*., 2003; Bagchi *et al*., 2004; Roy *et al*., 2002).

The possible mechanisms of action in reducing aberrant crypt foci due to administration of cranberry might be the overlapping additive and or synergistic activities of various phytochemicals. Apoptosis and cell cycle arrest at G1 phase was reported with acetone extracts of cranberry (Sun and Hai Liu, 2006) and fractions from cranberry presscake (Ferguson *et al*., 2004).

A higher percentage reduction in ACF was found in rats fed cranberry meal compared to rats fed cranberry juice, which may be due to the availability of fiber and other phytochemicals present in the meal such as ursolic acid. Whole and flavonoid extracts of cranberry presscake exhibited growth inhibitory, apoptotic and metastatic acuities in different cell lines (Ferguson *et al*., 2004). Kondo *et al*. (2004) reported that cranberry fruits had high urosolic acid compared to the jelly and juice.

The number of crypts/focus was significantly lower in rats fed cranberry diets compared to the control. ACF with higher number of crypts (≥ 4 crypts/focus) are more likely to develop into tumors over a long period of time, whereas, those with smaller number of crypts (≤ 3 crypts/focus) may dissolve and disappear over time. Cranberry may have significant potential as a chemopreventive agent as there was a higher reduction of aberrant crypts with ≥ 4 crypts/focus compared to those with (≤ 3 crypts/focus.

Chemical carcinogens such as DMH and AOM decrease the activity of antioxidative enzymes in rats (Moghadasian *et al*., 1996). Antioxidative enzymes such as catalase, superoxide dismuatse

catalyzes the reactions of radical scavenging. Although the exact mechanisms are not yet known, dietary antioxidants have a potential role in reducing the cancer progression. One study (Sengottuvelan *et al*., 2006) reported that phytochemicals induced the activity of antioxidative enzymes. The activity of antoxidative enzymes was significantly higher in all treatment groups compared to the control group. The increased antioxidative potential as seen with increased antioxidative enzyme activity in the rats fed cranberry meal and juice might be due to the anthocyanin and polyphenols in cranberry. Extracts of cranberry which are high in flavonoids have shown strong radical scavenging activity in DPPH assay and also prevented lipoprotein oxidation (Yan *et al*., 2002). Cranberry juice consumption did not alter the plasma antioxidative enzyme activities in healthy individuals supplemented with 750 mL day^{-1} (Duthie *et al*., 2006). Resveratrol increased the hepatic antioxdative enzyme activity and reduced number of ACF in Wistar rats administered with DMH (Sengottuvelan *et al*., 2006).

Phase I CYP 450 enzymes are needed for the bioactivation of carcinogens. Hepatic CYP2E1 enzyme converts azoxymethane into Methylazoxymethane (MAM) through hydroxylation. There were no significant differences found in the activity of CYP2E1 among the groups in this study. Supplementation of garden cress also did not induce the activity of CYP1A2 in rats administered with 2-amino-3-methyl-imidazo Quinoline (IQ) (Kassie *et al*., 2002). The extract of cranberry induced the expression or activity of xenobiotic enzyme, quinone reductase *in vitro* (Bomser *et al*., 1996). Our data showed that both cranberry meal and juice induced the activity of a Phase II, detoxification enzyme GST in rats. A four-seven fold increase in the enzyme activity might be due to action of various phytochemicals in inducing enzyme activity. The increased activity of Phase II enzymes with no induction in the activity of Cyp2E1 enzyme might be a possible mechanism for the reduction of ACF in the rats fed cranberry.

CONCLUSION

Results of this study indicated that feeding cranberry meal and cranberry juice at selected concentrations significantly ($p<0.05$) reduced azoxymethane induced aberrant crypt foci formation in Fisher-344 male rats. To our knowledge this is the first study conducted to determine the chemopreventive potential of cranberry against colon cancer in rats. Therefore, further experiments, long term tumor model and mechanistic studies, need to be conducted for more conclusive evidence.

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