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Selected Cruciferous Vegetables and their Effects on Azoxymethane (AOM) Induced Aberrant Crypt Foci

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ABSTRACT

Cruciferous vegetables contain naturally occurring substances that are beneficial to health. The purpose of this study was to investigate the effects of selected cruciferous vegetables (Collard Green (CG), cabbage (CB), turnip green (TG) and canola green (CN) on Azoxymethane (AOM)-induced Aberrant Crypt Foci (ACF) on Fisher 344 male rats. Thirty-six rats were randomly assigned to 9 groups (n = 4). The control group was fed AIN-93G diet and the other eight groups were fed AIN-93G along with the selected vegetables at 5 and 10%. The rats were administered subcutaneous injections of AOM at 7 and 8 weeks of age at 16 mg kg⁻¹ body weight. At 17 weeks of age, rats were killed by CO₂ asphyxiation. Total ACF numbers in rats fed CB, TG, CG and CN at 5% were 55, 41, 47 and 59, while at the 10% level, ACF numbers were 54, 63, 54 and 46, respectively. Total ACF in rats fed 5 and 10% cruciferous vegetables were significantly (p<0.05) lower (41-63) than rats fed the control diet (151). Findings indicate that cruciferous vegetables, including canola reduced the incidence of ACF and could potentially be used as a dietary chemopreventive agent against colon cancer.

Key words: Cabbage, canola, collard greens, turnip green, cruciferous

INTRODUCTION

Numerous studies have shown a strong relationship between diet and the prevention of diseases such as cancer. Donaldson (2004) reported that approximately 30-40% of tumors may be prevented by modification of lifestyle and diet. In addition, scientific evidence shows that some categories of vegetables are more important in the etiology of cancer than others (Terry *et al.*, 2001). These vegetables comprise those of the cruciferous family, also known as Cruciferae or Brassicaceae and include cabbage, collard greens, mustard greens, turnip greens and kale. Cruciferous vegetables have been studied extensively over the years because research has indicated an inverse relationship between the consumption of cruciferous vegetables and the incidence of cancer (Keck and Finley, 2004). Their secondary metabolites, glucosinolates and their hydrolysis

products play an important role in cancer prevention by offering protection against reactive oxygen species, altering detoxification pathways by inducing phase II and inhibiting phase I enzyme activities (Talalay and Fahey, 2001; Natanzi *et al.*, 2010).

Cruciferous vegetables such as turnip greens (*Brassica campestris* var. *rapifera*), cabbage (*Brassica oleracea* L. var. *capitata*), collard greens (*Brassica oleracea* var. *viridis*) are some of the most consumed vegetables in the United States. They are good sources of phytochemicals, vitamins and minerals that offer good health benefits (Ribaya-Mercado and Blumberg, 2004; West *et al.*, 2004; Miller-Cebert *et al.*, 2009; Rafatullah *et al.*, 2006) and are linked to the reduction of chemically induced Aberrant Crypt Foci (ACF), an important biomarker for cancer in animals (Lynn *et al.*, 2006). The canola plant also belongs to the family of cruciferous vegetables however there has been limited research on the utilization of canola's biomass as a green leafy vegetable. Previous research on canola has shown that canola leafy greens may also impart beneficial nutrients and phytochemicals similar to those of traditional green leafy vegetables (Miller-Cebert *et al.*, 2009; Clisby *et al.*, 2008).

The objective of this study was to examine the chemopreventive potential of selected cruciferous vegetables on azoxymethane (AOM)-induced Aberrant Crypt Foci (ACF). Since detoxification enzymes and antioxidant activities are used as biomarkers in chemoprevention studies, also examined the activity of Glutathione S Transferase (GST), Catalase (CAT) and Superoxide Dismutase (SOD) in the liver of cruciferous fed rats. The contents of total phenolics, total flavonoids, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Potential (FRAP) in extracts of cruciferous vegetables were also determined.

MATERIALS AND METHODS

Chemicals: All chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Company, St Louis, MO.

Cruciferous vegetables: Collard greens, cabbage and turnip greens were obtained from a local health-food store. Canola was obtained from the Winfred Thomas Agricultural Research Station (Alabama A and M University). All leafy vegetable samples were washed in deionized water, patted dry with paper towels and stored in a -80°C freezer. Frozen samples were later transferred from -80°C to a Consul 24 Virtis freeze-dryer (The Virtis Company, Gardiner, NY). The freeze-dried samples were milled using a Robot Coupe, Blixer RS1 BX3 Food Processor (Robot Coupe USA Inc. Ridgeland, MS).

Animal housing and diet: Fisher 344 male rats were obtained from Harlan, IN and housed in stainless steel cages. Following a one-week acclimatization period, the animals were randomly divided into 9 groups. The rats were fed AIN 93G control (American Institute of Nutrition) growth diet, canola greens, collard greens, cabbage and turnip greens at 5 and 10% for 13 weeks. Temperature and relative humidity were maintained at 21°C and 50%, respectively, with light and dark cycles at 12 h each. During the experimental period, biweekly body weights and feed intakes were recorded.

Carcinogen Injection: All animals received two subcutaneous injections of AOM (NCI Repository, Kansas, MO) at 7 and 8 weeks of age, at 16 mg kg⁻¹ body weight.

Sample preparation: Rats were killed by CO₂ asphyxiation at 17 weeks of age. Colons were removed and flushed with potassium buffer (0.1 M, pH 7.2). Livers were excised and rinsed in PBS, frozen and stored at -80°C for determination of enzyme activity.

Enumeration of aberrant crypt foci (ACF): Each colon was sectioned into 2 equal segments (proximal and distal) and further divided in 2 cm segment, stained with 0.2% methylene blue and examined using a light microscope. Total ACF were counted as previously described (Bird, 1987).

Glutathione S transferase (GST) assay: Hepatic GST activity was determined following Habig's protocol (Habig *et al.*, 1974). Briefly, rat livers were homogenized in ice-cold buffer at pH 7.4. The assay mixture containing 1 mL of potassium phosphate and 1-chloro-2, 4-dinitrobenzene was analyzed for enzyme activity of 340 nm.

Superoxide dismutase (SOD) assay: SOD activity was determined spectrophotometrically following established technique (Fridovich, 1989). Supernatant (1 mL) was diluted in 9 mL of distilled water. An aliquot of 2.0 mL was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2). The reaction was started with the addition of 0.3 mL freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. Buffer (2.5 mL), 0.3 mL of substrate (adrenaline) and 0.2 mL of water were added. A change in the absorbance at 480 nm was monitored every 30 sec for 150 sec. A single unit of enzyme is defined as the quantity of superoxide dismutase added to produce 50% inhibition of autoxidation.

Catalase (CAT) activity: CAT activity was determined by the reaction of formaldehyde produced from methanol with Purpald to produce a chromophore (Johansson and Borg, 1988). Quantitation was carried out by measuring the absorbance at 540 nm using a microplate reader (Biotek Synergy HT, Winooski, Vermont) and comparing the results with those obtained with formaldehyde calibrators.

Preparation of extracts: Vegetable extracts were prepared based on methods previously described (Kim *et al.*, 2003). Briefly, extraction was carried out using 5 g of powdered vegetable samples with 100 mL of 80% methanol, stirring for 2 h at room temperature. The samples were centrifuged at 4000 xg for 20 min and supernatant was removed. The process was repeated and the samples were pooled and then rotary evaporated at 40°C. The concentrate was made to a final volume of 10 mL using 80% methanol and stored at -80°C.

Determination of ferric reducing antioxidant power (FRAP): FRAP assay measures the change in absorbance at 593 nm owing to the formation of blue colored Fe(II)-tripyridyltriazine compound from colorless oxidized Fe(III) formed by the action of electron donating antioxidants. The FRAP assay was conducted using the protocol of Benzie and Strain (1996). Approximately 100 µL of diluted sample was combined with 3 mL of FRAP reagent consisting of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCL and 20 mM ferric chloride (FeCl₃·H₂O). The diluted mixture was subsequently incubated for 10 min at 37°C. Following incubation, the samples were analyzed at an absorbance of 593 nm. The change in absorbance was compared to a standard ferrous sulphate (FeSO₄·7H₂O) (0.1 mM-1.0 mM). The samples were analyzed in three replicates and the concentration of Fe³⁺ expressed as µmol of Fe²⁺ g⁻¹.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH): Approximately 0.1 mM solution of DPPH in 80% methanol was prepared and 40 μL of the solution was added to 200 μL of vegetable extracts at varied concentrations. The absorbance was measured at 517 nm, using a microplate reader (Synergy HT, Bio Tek Instrument, USA) after 30, 60 and 90 min. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Samples were analyzed in three replications; free radical scavenging activity was measured as the amount of extract required to decrease the initial absorbance (517 nm) of DPPH radical concentration by 50% (IC_{50}) as compared to the control according to the equation:

$$\% \text{DPPH} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

where, A_{control} is the absorbance of the control reaction (containing all the reagents except the test compound) and A_{sample} is the absorbance of the test compound.

Determination of total flavonoid content: Total flavonoid content was determined using a colorimetric assay (Kim *et al.*, 2003). Briefly, 25 μL of diluted sample was added to 40 μL of distilled H_2O after which 7.5 μL 5% NaCO_2 was added to the mixture. The mixture was allowed to stand for 5 min after which 15 μL of 10% AlCl_3 was added. The mixture was incubated for 5 min at room temperature (25°C) after which 50 μL of 1M NaOH were added. Absorbance of mixture was measured at 510 nm against a blank prepared with ddH_2O using a microplate reader (Synergy HT, Bio Tek Instruments, USA). Catechin was used as a standard and results expressed as mg g^{-1} dry weight.

Determination of total phenolics: Total phenolic contents of extracts were determined using the Folin-Ciocalteu colorimetric method (Singleton *et al.*, 1999). Briefly, approximately 12.5 μL of diluted samples was added 12.5 μL of Folin-Ciocalteu's phenol reagent and incubated for 5 min. Approximately 50 μL of distilled water and 125 μL of Na_2CO_3 were added to the mixture and incubated for 90 min at 25°C . Absorbance was read at 760 nm using a microplate reader (Synergy HT, Bio Tek Instruments, USA). A standard curve for total phenolics was constructed using a gallic acid standard solution. The results are expressed as mg g^{-1} dry weight.

Statistical analysis: Data were analyzed using the SAS system version 9.1 (SAS Institute, Cary, NC). Values are given as Means \pm SEM and separated using Duncan's Multiple Range Test. The significance was tested at $p < 0.05$ level.

RESULTS AND DISCUSSION

Effects of cruciferous vegetables on body weight, cecal weight and cecal pH: Table 1 shows the weight gain, cecal pH and cecal weight of rats fed control and treatment diets. Among the treatment groups, there were no significant differences in cecal weight, with the exception of the group fed CN at 5% (1.95 g). Similarly, significant difference in body weight was observed only in rats fed 5% CN (223.75 g/13 weeks) compared to the animals fed 5% CB (175.50 g/13 weeks).

Table 1: Weight gain, cecal weight and cecal pH in Fischer 344 male rats

Groups	Weight gain (g/13 week)	Cecal weight (g)	Cecal pH
Control	171.40±5.60 ^c	1.04±0.12 ^c	7.90±0.20 ^a
Cabbage 5%	175.50±13.10 ^c	1.20±0.21 ^b	7.70±0.10 ^a
Cabbage 10%	197.50±11.02 ^{abc}	1.15±0.10 ^b	7.68±0.07 ^a
Turnip 5%	213.25±9.10 ^{ab}	1.32±0.05 ^b	7.70±0.19 ^a
Turnip 10%	187.00±9.02 ^{bc}	1.22±0.07 ^b	7.60± 0.06 ^{ab}
Collard 5%	208.50±7.81 ^{ab}	1.12±0.21 ^b	7.55±0.06 ^{ab}
Collard 10%	207.50±8.67 ^{ab}	1.25±0.25 ^b	7.66±0.07 ^a
Canola 5%	223.75±5.40 ^a	1.95±0.12 ^a	7.31±0.06 ^b
Canola 10%	206.50±7.60 ^{abc}	1.42±0.16 ^b	7.51±0.09 ^{ab}

Values are Means±SEM, Means in a column with the same superscript do not significantly differ (p<0.05) using Duncan's multiple range test

Table 2: Incidence of aberrant crypt foci (ACF) in Fischer 344 male rats fed cabbage, turnip greens, collard greens and canola greens

Groups	Proximal	Distal	Total
Control (AIN 93 G)	35.10±2.49 ^a	116.12±6.87 ^a	151.10
Turnip green 5%	8.25±2.65 ^b	33.00±6.87 ^{abc}	41.25
Turnip green 10%	15.00±8.38 ^b	48.00±11.44 ^a	63.00
Collard green 5%	4.00±2.38 ^b	43.25±16.46 ^a	47.25
Collard green 10%	14.00±3.24 ^b	40.00±16.57 ^{ab}	54.00
Cabbage 5%	42.50±5.20 ^a	12.50±16.57 ^{bc}	55.00
Cabbage 10%	46.25±4.09 ^a	8.00±16.57 ^c	54.25
Canola 5%	13.00±1.47 ^b	46.50±8.30 ^a	59.50
Canola 10%	6.75±3.52 ^b	40.00±11.92 ^{ab}	46.75

Values are Means±SEM, Means in a column with the same superscript do not significantly differ (p<0.05) using Duncan's multiple range test

Cruciferous vegetables on the incidence of aberrant crypt foci (ACF) in rats: The incidence of aberrant crypt foci on cruciferous-fed rats is shown in Table 2. Present results indicate that total number of ACF in the rats fed the control diet was significantly (p<0.05) higher compared to those fed the treatment diets and ranged from a low of 46.75 (CN 10%) to a high of 151 (control). There was a 69 and 72% reduction in ACF in the rats fed CN 10% and TG 5% respectively, compared to the control fed group. The rats fed 5 and 10% CB had significantly (p<0.05) higher number of ACF in the proximal section of the colon (42.50 and 46.25, respectively) compared to the other treatment groups. With the exception of CN, the rats fed the 5% treatment diet had lower incidence of ACF in the proximal section of the colon compared to those fed the 10% diet. Among the 5% fed groups, ACF ranged from a low of 4.00 (CG) to a high of 42.50 (CB) in the proximal colon and a low of 12.50 (CB) to a high of 46.50 (CN) in the distal colon. However at the 10% level, ACF numbers ranged from a low of 6.75 (CN) to a high of 46.25 (CB) in the proximal colon, while ACFs in the distal colon ranged from a low of 8.00 (CB) to a high of 48.00 (TG). Of the treatment groups, the rats fed TG and CN had lower total number of ACF, 41.25 and 46.75, respectively. The incidence of ACF in the proximal colon of the rats fed the CB diet was higher than those seen in the distal colon. Previous studies indicated that ACF numbers increased in the distal portion of the colon compared to the proximal, which has prompted interest in understanding the mechanisms behind such occurrence (Roy *et al.*, 2004; Cheng and Lai, 2003; Guyton *et al.*, 2008; Miller *et al.*,

Table 3: Effect of cabbage, turnip green, collard green and canola greens on hepatic CAT, SOD and GST in Fischer 344 male rats

Groups	CAT ($\mu\text{mol mg}^{-1}$)	SOD ($\mu\text{mol mg}^{-1}$)	GST ($\mu\text{mol mg}^{-1}$)
Control	1.67±0.14 ^d	0.06±0.04 ^e	5.10±0.03 ^b
Cabbage 5%	51.29±3.11 ^c	0.21±0.01 ^a	54.22±10.84 ^a
Cabbage 10%	66.84±0.80 ^c	0.20±0.01 ^a	56.36±12.01 ^a
Turnip 5%	53.46±3.67 ^c	0.20±0.02 ^a	61.73±6.58 ^a
Turnip 10%	52.30±3.90 ^c	0.21±0.01 ^a	64.91±7.10 ^a
Collard 5%	52.30±3.90 ^c	0.20±0.01 ^a	42.45±3.28 ^a
Collard 10%	67.42±7.19 ^c	0.21±0.01 ^a	55.32±6.80 ^a
Canola 5%	184.81±8.26 ^a	0.10±0.01 ^b	0.22±0.01 ^b
Canola 10%	138.53±14.87 ^b	0.12±0.01 ^b	0.18±0.02 ^b

Values are Means±SEM, Means in a column with the same superscript do not significantly differ ($p < 0.05$) using Duncan's multiple range test

2010). Hong *et al.* (2001) suggested that differences in the proximal and distal colons as determined by DNA damage could be the response of diet and is an important contributor in tumor development in these regions of the colon. Lower tumor incidence in the proximal colon may be a result of the distal colon having the capability to overcome initial DNA damage compared to the distal (Hong *et al.*, 2001). However, the observed difference in the groups fed cabbage in this study warrants further investigation. Radical scavenging and total phenolic levels in vegetables may be affected by various factors such as cultivars, genetics, maturity and growing conditions (Wang, 2006). In addition, the antioxidant profile in soils in which plants are grown may also influence the plants antioxidant potential (Coria-Cayupan *et al.*, 2009; Rached *et al.*, 2010).

Effects of cruciferous vegetables on GST, SOD and CAT: Hepatic Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione S Transferase (GST) were significantly lower in the control fed rats compared to the treatment groups (Table 3). Of the treatment groups, CN had the lowest SOD (5%-0.10 $\mu\text{mol mg}^{-1}$, 10%-0.12 $\mu\text{mol mg}^{-1}$) and GST (5%-0.22 $\mu\text{mol mg}^{-1}$, 10%-0.18 $\mu\text{mol mg}^{-1}$) activities but were significantly ($p < 0.05$) higher in CAT activity (5%-184.81 $\mu\text{mol mg}^{-1}$, 10%-138.53 $\mu\text{mol mg}^{-1}$) compared to the other vegetables. GST activity was significantly ($p < 0.05$) higher in rats fed CB, TG and CG at both 5% and 10% compared to the control. A similar trend was observed in SOD levels, however, all the treatment groups had a hundred fold or greater increase compared to the control. Among the treatment groups, CAT activity ranged from a high of 184.81 $\mu\text{mol mg}^{-1}$ in CN (5%), to a low of 51.29 $\mu\text{mol mg}^{-1}$ in CB (5%). CN had significantly higher CAT activity (5%-184.81 $\mu\text{mol mg}^{-1}$ and 10%-138.53 $\mu\text{mol mg}^{-1}$) compared to the other treatment groups and the control (51.29 $\mu\text{mol mg}^{-1}$). Among the vegetables, TG fed rats had higher activity of GST (5%-61.73 $\mu\text{mol mg}^{-1}$ and 10%-64.91 $\mu\text{mol mg}^{-1}$), which is a phase II enzyme involved in the detoxification of xenobiotics. SOD and CAT are free radical scavenging enzymes that work together in the body's defense against DNA damage (Uma Devi and Chinnaswamy, 2008). Hydrogen peroxide, produced by SOD in catalyzing the dismutation of superoxides, is converted to water by CAT thus ridding the body of harmful carcinogens. Higher levels of these enzymes in cruciferous vegetable fed rats compared to the control may have resulted in the lower number of ACF seen in the treatment groups compared to the control.

Table 4: Scavenging activity of selected cruciferous vegetables on DPPH and FRAP

Assay	Cruciferous vegetables			
	Canola greens	Turnip greens	Collard greens	Cabbage
% DPPH	86.61 ^a	75.67 ^a	33.07 ^b	8.03 ^c
FRAP (mg g ⁻¹ DW)	12.31 ^a	8.59 ^{ab}	5.16 ^{bc}	3.51 ^c

Means with the same letter within row are not significantly different at (p<0.05) using Duncan's multiple range test

Antioxidant capabilities of cruciferous vegetables using FRAP and DPPH free radical scavenging activity: FRAP assay has been used to measure the antioxidant capacity from a wide range of biological samples. It is a quick and reliable method to perform and the reaction is reproducible and linearly related to the molar concentration of antioxidant present (Benzie and Strain, 1996). The presence of reductants such as antioxidants in samples causes the decrease of the Fe³⁺ complex to the ferrous form (Fe²⁺), noted by an intense blue color (Beyhan *et al.*, 2010). The change in color indicates the reducing ability of the samples (Benzie and Strain, 1996) and may serve as an indicator of its potential antioxidant activity (Beyhan *et al.*, 2010).

As shown in Table 4, there was no significant difference in antioxidant activity between CN (12.31 mg g⁻¹) and TG (8.59 mg g⁻¹). CB showed the lowest activity (3.51 mg g⁻¹) compared to all the leafy vegetables tested; however, was not significantly different from CG. The results, therefore, show that CN possesses a higher electron donating capability compared to the other vegetables tested and may be more effective in neutralizing free radicals by forming stable products. Free radical reactions are involved in many biological processes that cause damage to lipids, proteins, membranes and nucleic acids, thus giving rise to a variety of diseases (Huang *et al.*, 2002; Marjani *et al.*, 2007). The antioxidant activities of plant species are determined using different methods. As a result, a comparison of results from other scientists using similar plant species may yield varied results (Hodzic *et al.*, 2009).

DPPH is a stable free radical that accepts an electron or hydrogen donating antioxidant to form a stable molecule. An assay based on the use of DPPH, is the most accepted spectrophotometric method for determining antioxidant capacity of vegetable extracts (Hodzic *et al.*, 2009) and is based on the reduction of DPPH in methanol which causes the DPPH to decrease at 515 nm (Wong *et al.*, 2006). In this study, DPPH radical method was used to assess the potential radical scavenging activities of the green leafy vegetable extracts studied. At 517 nm the absorbance of DPPH was determined, resulting in a change in color from purple to yellow. Table 4 shows a significant decrease in the concentration of DPPH radical due to scavenging ability of cruciferous vegetable extracts in the order of CN>TG>CG>CB, which were 86.61, 75.67, 33.07 and 8.03%, respectively.

Phenolics and flavonoids: Green leafy vegetables provide great health benefits and are good sources of phenolics and flavonoids whose content varies based on plant species (Kuti and Konuru, 2004), soil conditions, geographical climatic differences and stress resulting from the application of herbicides and pesticides (Andarwulan *et al.*, 2010; Shafaei *et al.*, 2011). The highest phenolic content was observed in CN (55.59 mg GAE g⁻¹), followed by TG (53.39 mg GAE g⁻¹), CB (43.28 mg GAE g⁻¹) and CG (42.96 mg GAE g⁻¹). Of the vegetables tested, the highest flavonoid content was seen in canola greens (1.55 mg CE g⁻¹) and the lowest in CG (0.7 mg CE g⁻¹) (Fig. 1).

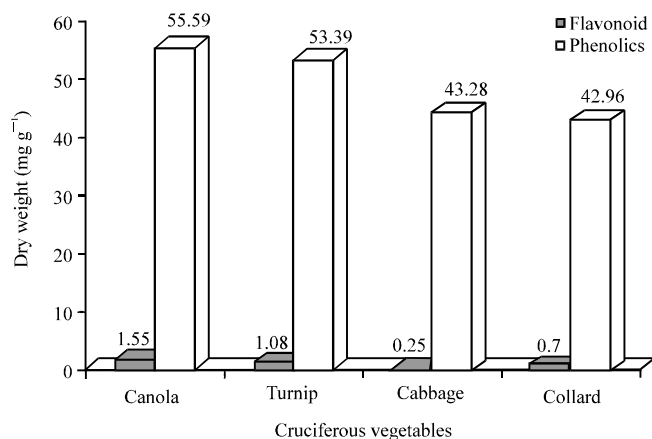


Fig. 1: Phenolic and flavonoid contents of selected cruciferous vegetables (canola greens, turnip, cabbage and collard greens)

CONCLUSIONS

This study provides important evidence that cruciferous vegetables are important components in the diet and may be linked to reduced incidence of ACFs in Fisher 344 male rats. A proposed mechanism for this reduction is the capability of these vegetables to help prevent further damage to DNA, which could result in increased incidence of cancer. Cruciferous vegetables have been studied over the years for their role as chemo-preventive agents. Compounds found in cruciferous vegetables are potent inducers of phase II enzymes while inhibiting phase I enzymes. The distal portion of the colon in rats fed cabbage in this study resulted in less ACFs compared to the other treatments. This result is not consistent with previous findings where distal ACFs are normally greater than those seen in the proximal portion of the colon and therefore, warrants further investigation.

The experimental data obtained also showed that canola leafy greens have comparable antioxidant activities to the traditional greens tested. The levels of antioxidant and scavenging activities were higher in canola leafy greens compared to the other vegetables assessed. This is an indication that canola leafy greens may offer greater protection in health and disease by aiding in the scavenging of free radicals which are known to play a key role in the pathology of diseases, such as cancer, atherosclerosis or inflammatory diseases.

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