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## Determination of Antioxidant Contents in Red Sorrel and its Anticarcinogenic Potential in Azoxymethane-Induced Colonic Aberrant Crypt Foci

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**Abstract:** The aim of the study was to determine the antioxidant properties in sorrel and to evaluate the effect of feeding red sorrel on azoxymethane-induced Aberrant Crypt Foci (ACF) in Fisher 344 rats. Total phenolics and flavonoids were spectrophotometrically determined using gallic acid and catechin as standards and antioxidant capacity was determined using 2, 2'-azinobis [(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)] radical scavenging assay, with vitamin C (ascorbic acid) as standard. Twenty-four Fisher 344 rats were divided into 3 groups and fed control-(C) diet (AIN-93G), 10 g/100 g red Sorrel Meal (SM) and Sorrel Juice (SJ). All rats received 2 subcutaneous injections of azoxymethane (AOM) at 7 and 8 week and were killed using CO<sub>2</sub> euthanasia at 17 week of age. Total phenolics (mg/100 g) as Gallic Acid Equivalents (GAE), flavonoids (mg/100 g) as Catechin Equivalents (CE) and VCAEC ranged from 256.8±1.0 to 433.7±1.4 for fresh and dry sorrel. Total number of ACF in rats fed C, SM and SJ were 154.4, 38.6 and 24.6, respectively. Sorrel is attracting the attention of food manufacturers and these results indicate that phytochemicals present in sorrel may have many possibilities for health improvement.

**Key words:** Aberrant crypt foci, azoxymethane, *Hibiscus sabdariffa*, VCEAC, total phenolics, total flavonoids

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## INTRODUCTION

Colon cancer is a significant cause of morbidity and mortality in Western industrialized countries and is the second most frequent cause of cancer deaths in the United States (Parker *et al.*, 1997). Human metabolic and laboratory animal model studies (Middleton *et al.*, 2000; Wang, 2007) indicate that the composition and physical properties of phytochemicals influence their beneficial effects in relation to cancer development. Based on current knowledge of the pathogenesis of colon cancer, it is reasonable to conclude that consumption of fruits and vegetables rich in phytochemicals are associated with a reduced risk of colon cancer. Phytochemicals impart color and are responsible for cranberries, strawberries and other fruits being good sources of antioxidants.

Sorrel (*Hibiscus sabdariffa*) is a potentially good source of antioxidants. Calyces of *Hibiscus* species contain polyphenolic acids, flavonoids and anthocyanins. Flavonoids are widespread in the plant kingdom and are especially common in leaves, flowering tissues and pollen (Beecher, 2003;

Liu, 2003). Known properties of flavonoids include free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (phospholipase A2, cyclooxygenases, lipoxygenase) and anti-inflammatory action (Frankel, 1995). Free radicals have been implicated in the inactivation of enzymes, degradation of DNA and cell membranes (Nijveldt *et al.*, 2001; Heijnen *et al.*, 2002; Chun *et al.*, 2003) all of which are linked to degenerative human diseases such as cancer, heart disease and cerebrovascular diseases.

Antioxidants such as vitamins and phytochemicals which are derived from foods have received growing attention, because they are known to function as chemopreventive agents against oxidative damage. Vitamin C is one of the most popular and least toxic antioxidant components in foods and is the most used dietary supplement to prevent oxidative stress-mediated diseases. However, the contribution of vitamin C to the total antioxidant activity of fruits is generally <15% (29). In addition to antioxidative properties, scientific studies have shown that phenolic phytochemicals are also associated with anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory activities (Cao and Prior, 1999). Phytochemical content in plants may be affected by level of maturity, cultivars, horticultural practices, geographic origin, growing season, post harvest storage conditions and processing procedures (Srivastava *et al.*, 2007; De Freitas and Glories, 1999; Kalt *et al.*, 1999; Donavon *et al.*, 1998). Therefore, evaluation of red sorrel as a potential source of phenolic phytochemicals is warranted.

Aberrant Crypt Foci (ACF) induced by azoxymethane (AOM) have been used extensively to investigate nutritional modulation of colon carcinogenesis in rats. AOM is a metabolite of the procarcinogen 1, 2-dimethylhydrazine and is one metabolic step closer to the proximate carcinogen capable of inducing colonic ACF (Bird, 1995). The glutathione S-transferases ( $\pi$ ,  $\mu$ ,  $\alpha$ ), a family of Phase II detoxification enzymes, play a critical role in protecting the colonic mucosa by catalyzing the conjugation of dietary carcinogens with glutathione (Boateng *et al.*, 2007; Kauser *et al.*, 2003). Overall, there is now new evidence that antioxidants in the human diet are beneficial for health and nutrition (Wang *et al.*, 1997). The objectives of the study were to determine total flavonoids, phenolics and Vitamin C Antioxidant Capacity (VCAC) in fresh and dry sorrel and to evaluate the effect of sorrel on azoxymethane-induced Aberrant Crypt Foci (ACF) in Fisher 344 male rats.

## MATERIALS AND METHODS

### Experiment 1

#### Plant Material

Dried red sorrel calyces were purchased from a local supermarket. Fresh red sorrel calyces were harvested in Summer 2006 from a greenhouse at Alabama A and M University (Normal, Alabama). Samples were ground and stored in amber jars at -20°C for further analysis.

#### Chemicals

Gallic acid, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), radical scavenging assay (ABTS) as diammonium salt, (+)-catechin and Folin and Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO.). 2'-azobis (2-amidinopropane) hydrochloride (AAPH), gallic acid and ascorbic acid were obtained from Fisher Scientific Co. (Suwannee, GA). All other chemicals were of analytical grade and purchased from Fisher Scientific Co. (Suwannee, GA).

#### Extraction of Phenolics

Phenolics in dried and fresh red sorrel calyces were extracted by an ultrasound-assisted method as described by Kim *et al.* (2003).

### **Determination of Total Phenolics**

Total phenolics were measured by the Folin-Ciocalteu method previously described by Singleton and Rossi (1995) with some modification (Kim *et al.*, 2003). Total phenolic content of sorrel was expressed as mg Gallic Acid Equivalents (GAE)/100 g fresh sample. All samples were analyzed five times.

### **Determination of Total Flavonoids**

A method described by Kim *et al.* (2003) was used to determine total flavonoids in red sorrel samples. Absorbance was determined at 510 nm versus prepared blank (distilled deionized water). Total flavonoid of sorrel was expressed on a fresh weight basis as mg/100 g Catechin Equivalents (CE). All samples were analyzed four times.

### **Vitamin C Equivalent Antioxidant Capacity (VCEAC) Assay Using 2,2'-Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) Radical**

Vitamin C Equivalent Antioxidant Capacity (VCEAC) assay was determined as described by Kim *et al.* (2003) with minor modifications. Briefly, 1 mM AAPH was mixed with 2.5 mM ABTS in phosphate-buffered saline (pH 7.4; 100 mM potassium phosphate buffer containing 150 mM NaCl). The solution was heated in a water bath at 68°C for 13 min and cooled to room temperature. The resulting blue-green ABTS solution was adjusted to an absorbance of 0.650±0.020 at 734 nm with phosphate-buffered saline. One hundred microliter of sample was added to 2.9 mL of the ABTS radical solution and incubated in a 37°C water bath under restricted light for 10 min, radical solution was prepared daily. A control consisting of 100 µL of 50 mg/100 mL methanol and 2.9 mL of ABTS radical solution was run with each series of samples. The decrease in absorbance at 734 nm was measured after endpoint of 10 min. Total antioxidant capacity of red sorrel, was expressed on a fresh weight basis as mg/100 g vitamin C equivalents. Experiment was done in four replications.

## **Experiment 2**

### **Animals, Housing and Diets**

Following a one-week period of acclimatization, 24 Fisher 344 male weanling rats (Harlan, IN) were randomly divided into 3 groups (8 rats each). Light and dark cycles were held at 12 h each and the temperature and relative humidity were held at 21°C and 50%, respectively. Rats were assigned to one of the following diets for 13 weeks: AIN 93G (Reeves *et al.*, 1993a, b) (Control -C), 10 g/100 g SM and 10 g/100 g SJ. Diets were formulated based on AIN 93G diet (Table 1). Ingredients were obtained from ICN (Costa Mesa, CA). Red sorrel calyxes were grounded to a fine powder using a food processor (Robot coupe, Blixer RSI, BS3). The ground sorrel was then mixed into the diet at 10 g/100 g level at the expense of cornstarch and fiber (Table 1). Feed was provided ad libitum. All diets were prepared weekly and refrigerated at -4°C, until fed. Biweekly weight gains and daily feed intakes were recorded. The Institutional Animal Care and Use Committee of Alabama A and M University approved all protocols involving rats.

### **Preparation of Red Sorrel Juice**

One hundred grams of dried red sorrel calyxes was added to 1 L of distilled deionized water and allowed to boil for 10 min. The sorrel infusion was allowed to cool to room temperature and then filtered with cheesecloth to remove spent calyxes. One hundred milliliter of the sorrel infusion, regarded as sorrel juice, was fed each day to the rats in place of water.

### **Carcinogen Injection**

Carcinogen injections were administered at 7 week and 8 week of age. All groups except saline group (n = 6) were subcutaneously injected with azoxymethane (Sigma Chemicals, St Louis, MO) in saline at 16 mg kg<sup>-1</sup> body weight.

Table 1: Composition of the diets<sup>1</sup>

Ingredients <sup>1</sup>	Control	Sorrel
	-----(g kg <sup>-1</sup> )-----	
Cornstarch	397.5	297.5
Sorrel	0.0	100.0
Common ingredients <sup>2</sup>	602.5	602.5

<sup>1</sup>: Formulation of diets based on AIN-93G (Reeves *et al.*, 1993 a, b), <sup>2</sup>: Common ingredients: casein (>85% protein), 200; dextrose, 132; sucrose, 100; soyabean oil, 70; fiber (Solk-Floc), 50; mineral mix (AIN-93), 35; AIN-93G, Vitamin mix, 10; L-cystein, 3; choline batartarate, 2.5

### Tissue Sample Collection and Counting Aberrant Crypt Foci

Rats were euthanized by CO<sub>2</sub> asphyxiation at 17 week of age and colons were collected for enumeration of Aberrant Crypt Foci (ACF). The colons were flushed with potassium phosphate buffer (0.1 M, pH 7.2), split open and ACF were enumerated as described by Bird (1987).

### Glutathione-S-Transferase (GST) Assay

Approximately 1 g of liver samples were homogenized in 10 volumes of potassium phosphate buffer (pH 7.0, 0.1M) and centrifuged at 10,000 x g for 30 min. Clear supernatant was collected and an aliquot was mixed 1, chloro 2, 4-dinitrobenzene, potassium phosphate buffer and reduced glutathione. GST activity was measured using a Cary1/3 UV/VIS dual beam spectrophotometer as outlined by Habig *et al.* (1974).

### Statistical Analysis

Data were analyzed using ANOVA (SAS, 2004). Means were separated using Turkey's studentized range test. Differences were considered significant at p<0.05 (28).

## RESULTS

### Experiment 1

#### Determination of Phenolics, Flavonoids and Vitamin C Antioxidant Capacity in Red Fresh and Dry Sorrel

Total phenolics expressed as Gallic Acid Equivalents (GAE) and flavonoid contents as Catechin Equivalents (CE) of fresh and dried red sorrel calyces were 433.7±1.4, 408.2±1.3, 279.5±1.5 and 271.3±1.3 mg/100 g, respectively. VCAEC in fresh and dry red sorrel were 293.4±2.2 and 256.8±1.0 mg/100 g, respectively. There were no significant differences (p<0.05) in total phenolics, flavonoids and vitamin C antioxidant capacity between fresh and dry sorrel (Table 2).

### Experiment 2

#### Feed Intake, Body Weight, Cecal pH and Cecal Weights

Body weight gains of rats were lower (p<0.05) in the control than in the sorrel fed rats. Rats fed 10% SJ had a significantly higher (p<0.05) weight gain. There were also significant (p<0.05) differences in feed intake in rats fed control (C) and SJ and SM. Cecal weights were higher in the control than in sorrel fed rats (Table 3). Cecal pH was significantly (p<0.05) lower in the rats fed sorrel meal and juice compared to the control. There were however, no significant (p<0.05) differences in cecal pH between rats fed SM and SJ.

#### Aberrant Crypt Foci

The rats administered saline (vehicle), showed no evidence of ACF formation in the colon (data not shown). In rats fed control, AOM induced an average of ≈150 ACF/colon. Totals ACF numbers in rats fed SJ and SM were 24.8 and 38.6, respectively (Table 4). Compared to the control

Table 2: Total phenolics, flavonoids and Vitamin C Antioxidant Capacity (VCAC) in fresh and dried sorrel

Variables (mg/100 g)	Fresh sorrel	Dry sorrel
Total phenolics	433.7±1.4 <sup>a</sup>	408.2±1.3 <sup>b</sup>
Total flavonoids	279.5±1.5 <sup>a</sup>	271.3±1.3 <sup>a</sup>
VCEAC	293.4±2.2 <sup>a</sup>	258.6±1.0 <sup>b</sup>

Values are Means±SEM. <sup>ab</sup>: Means in rows without a common letter(s) different significantly (p<0.05) using Tukey's studentized test

Table 3: Weight gain, feed intake and cecal weight and cecal pH of Fisher 344 male rats fed control, sorrel juice and sorrel meal

Variables	Control (C)	Sorrel juice	Sorrel meal
Weight gain (g/17 week)	159.25±10.41 <sup>a</sup>	230.25±8.43 <sup>b</sup>	213.75±16.59 <sup>c</sup>
Feed intake (g day <sup>-1</sup> )	14.11±0.20 <sup>a</sup>	16.11±0.31 <sup>b</sup>	16.30±0.33 <sup>b</sup>
Cecal weight (g)	1.31±0.12 <sup>a</sup>	0.85±0.12 <sup>b</sup>	1.04±0.14 <sup>a</sup>
Cecal pH	7.40±0.21 <sup>a</sup>	6.30±0.14 <sup>b</sup>	6.97±0.08 <sup>c</sup>

Values are means±SEM. <sup>abc</sup>: Means in rows without a common letter(s) different significantly (p<0.05) using Tukey's studentized test

Table 4: Effect of Sorrel juice and Sorrel meal on Aberrant Crypt Foci (ACF) and total crypts in colon of Fisher 344 male rats

Groups	Proximal colon	Distal colon	Total	Total crypts
Control-C	39.9±0.94 <sup>a</sup>	118.2±1.7 <sup>a</sup>	154.4±1.8 <sup>a</sup>	569.4±11.5 <sup>a</sup>
Sorrel juice	8.2±1.46 <sup>b</sup>	16.6±1.7 <sup>c</sup>	24.8±2.8 <sup>b</sup>	58.0±9.31 <sup>b</sup>
Sorrel meal	9.2±1.15 <sup>b</sup>	29.4±2.11 <sup>b</sup>	38.8±2.26 <sup>c</sup>	105.2±4.3 <sup>c</sup>

Values are Means±SEM. <sup>abc</sup>: Means in a column without common letter(s) different p<0.05 using Tukey's studentized test

Table 5: Glutathione S-Transferase (GST) activity in rats fed Sorrel as compared to the controls

Treatments	GST activity (μmol mg <sup>-1</sup> )
Control-C	9.2±0.4 <sup>a</sup>
Sorrel Meal	31.5±0.6 <sup>b</sup>
Sorrel Juice	49.4±0.4 <sup>a</sup>

Values are means±SEM. <sup>abc</sup>: Means in the column with a different letter(s) differ significantly (p<0.05) using Tukey's studentized test

there was 83.8 and 78% reduction in ACF in rats fed SJ and SM, respectively. Total number of aberrant crypts was significantly (p<0.05) higher in rats fed control compared to sorrel fed rats. The distal colon had higher numbers of ACF (p<0.05) than the proximal segment (Table 4). Foci with 4 and >5 crypts were lower (p<0.05) in rats fed SM and SJ than in the control (data not shown).

#### Glutathione-S-Transferase Activity (GST)

There was a significant (p<0.05) increase in liver GST (μmol mg<sup>-1</sup>) (a crucial detoxification enzyme) in rats fed SJ (81%) and SM (percent) compared to the control. GST activity (μmol mg<sup>-1</sup>) increased from 9.2 in the rats fed control diet to 31.5 and 49.4 in the rats fed sorrel meal and juice, respectively (Table 5).

## DISCUSSION

Recently, attention is being focused on the protective biochemical functions of naturally occurring antioxidants in biological systems and on their mechanisms of action. Results from this study indicate that total phenolics and flavonoid contents in red sorrel are greater than those in some traditional temperate fruits. The results are comparable with a study by Kim *et al.* (2003) on red plum cultivars where they reported values of 174 to 375 mg/100 g for phenolics, expressed as gallic acid equivalents and 118 to 237 mg/100 g for flavonoids, expressed as catechin equivalents. Total phenolic concentrations of various apple cultivars were reported to range from 50.9 to 140 mg GAE/100 g (Lee and Smith, 2000) while another study reported that total phenolics content of red plums was 144 mg/100 g, expressed as catechin equivalents (Karakaya *et al.*, 2001).

The flavonoid contents in red sorrel which accounts for its red color are also recognized as powerful antioxidants (Kandaswami and Middleton, 1994) with strong scavenging effects on superoxide radicals. This may be the most important function of flavonoids (Miller *et al.*, 1996). Many studies have considered fruits, vegetables and teas as the major sources of dietary antioxidative phenolics (Williams and Elliot, 1999; Kim *et al.*, 2003). Grapes and red wine have been recognized for their antioxidant activity and the improved health in populations that are large consumers of these products (Williams and Elliot, 1999). A study conducted on the antioxidant capacity of fresh plums and gala apples reported values ranging from 256 to 559 mg VCEAC/100 g and 205 to 210.6 mg VCEAC /100 g, respectively (Kim *et al.*, 2003). The antioxidant activity reported in this study for fresh and dried sorrel samples were 293.7 and 256.8 mg VCEAC/100 g, respectively.

Since sorrel is a prime source of phenolic antioxidants, we evaluated the potential inhibitory effects of sorrel on the formation of AOM-induced colonic ACF, which are putative preneoplastic lesions. The results showed that red sorrel when fed to fisher 344 rats significantly ( $p < 0.05$ ) reduced the number of ACF. Sorrel contains a composite of several antioxidants, which may have been involved in the reduction of ACF numbers. In their study, Chewonarin *et al.* (1999) observed a 22% reduction in the number of AOM induced ACF when rats were administered a gavage of sorrel extract once a day. According to the authors, one of the mechanisms associated with the decrease of ACF may be due to the inhibition of DNA methylation and cytochrome P450 1A2, although the exact mechanisms are yet to be determined.

Flavonoids have beneficial effects through their impact on the bioactivation of carcinogens. Most food-borne carcinogens require transformation by phase I metabolizing enzymes into a more reactive form to bind to DNA. If the resulting mutation is not repaired, it may initiate or promote the carcinogenesis process. The reactive chemical group introduced by phase I enzymes can be detoxified through conjugation by phase II metabolizing enzymes into a water-soluble compound which can be eliminated from the body (Khan *et al.*, 1992). Various flavonoids have also been shown to have anti-inflammatory activity by inhibiting cyclooxygenase-2 (COX 2) and inducible nitric oxide synthase (Raso *et al.*, 2001). Chronic inflammation is thought to play an important role in the etiology of a number of cancers and COX 2 inhibitors are being studied as chemopreventive agents against colon cancer (Mutoh *et al.*, 2000).

There was a significant ( $p < 0.05$ ) increase in hepatic Glutathione-S-transferase activity in rats fed SJ and SM compared to the control (Table 4). Animal and *in vitro* studies have shown that flavonoids including catechins and apigenin increase the activity of several detoxifying and antioxidant enzymes, such as glutathione reductase, glutathione peroxidase, catalase, quinone reductase and glutathione S-transferase (Valerio *et al.*, 2001). The glutathione S-transferases-crucial Phase II detoxification enzymes play a critical role in protecting the colon mucosa by catalyzing the conjugation of dietary carcinogens with glutathione, which renders them more water soluble and easily excreted. Hepatic GST activity may be used in colorectal cancer chemoprevention trials to monitor the responsiveness of colon tissue to regimens that modify Phase II detoxification enzymes (Mutoh *et al.*, 2000).

To present knowledge, this is the first known study to demonstrate that dietary administration of 10 ppm red sorrel significantly reduced ACF, suggesting that consumption of red sorrel may retard growth and/or development of neoplastic lesions in the colon. This suggests the usefulness of red sorrel as a chemopreventive agent for individuals at high risk for colon cancer development.

In conclusion, this study showed that phenolic phytochemicals such as flavonoids and phenolic acids are present in both fresh and dried sorrel calyces. These may function as effective natural antioxidants therefore an increased consumption of red sorrel may be beneficial. Red sorrel (fresh and/or dry) could possibly be utilized in commercial food processing to prevent lipid oxidation, which results in development of off-flavors and odors. Substituting or reducing the amount of synthetic antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) with red sorrel in food products may be beneficial.

The results of this study showed a significant decrease ( $p < 0.05$ ) in the total crypts in the sorrel juice and sorrel meal groups compared to the control group. The results justify the use of dietary phytochemicals as cancer preventive agents in population with high-risk for colon cancer in the US. Based on the results, red sorrel appears to hold promise as a potential cancer preventive agent in high-risk populations and should be further evaluated. End point tumor model studies may provide additional data suggestive of chemopreventive potential of red sorrel products.

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