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Inhibition of Chemically-Induced Colon Cancer by Dietary Treatment of *Hibiscus sabdariffa* L. Dried Calyx in Rats

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Abstract

This study examined the chemopreventive effect of feeding sorrel calyx as meal and juice against azoxymethane (AOM) induced colon cancer in Fisher 344 male rats. Rats were randomly assigned to five different groups and administered either sorrel meal (5% & 10%) or juice (2.5% & 5%) and control diet. Tumors were induced in rats with two subcutaneous injections (16 mg/kg body weight) of AOM at 6 & 7 weeks of age. Rats were killed at 45 weeks of age and samples (colon, liver) were collected. Tumor incidence, size and numbers were analyzed macroscopically. Activity of drug metabolizing (Cytochrome P2E1 (CYP2E1) & Glutathione S-Transferase (GST)) and antioxidative enzymes (Catalase and Superoxide dismutase) were determined in liver. Dietary feeding of sorrel calyx decreased ($P < 0.05$) tumor incidence and multiplicity in rats. Tumor size was reduced ($P < 0.05$) by 78% in rats fed with sorrel calyx meal at 10% compared to control. Rats administered with AOM alone increased liver CYP2E1 activity. Supplementation of sorrel increased ($P < 0.05$) activities of antioxidative enzymes. Highest reduction in GST activity was observed in rats that were treated with sorrel juice at 5%. Results of this study indicated the chemopreventive potential of sorrel meal and calyx agent chemically induced colon cancer in rats. This study also provided scientific evidence for using sorrel as a functional food in chemoprevention.

Keywords

Sorrel, Azoxymethane, Colon Cancer, Antioxidant

1. Introduction

Colon cancer is the third leading causes of cancer deaths in the United States. Colon cancer is on rise in developing countries and is gaining importance. Although mortality and morbidity rates of colon cancer decreased in

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US over the past decade due to improved awareness and screening, there will be an estimated 93,090 new cases of colon cancer in year 2015 [1]. Prevalence is far more for colon cancer as 5% of Americans would be diagnosed with colon cancer in life time. Genetics are main cause for colon cancer incidence. But, environmental factors and diet have an important effect on the colon cancer incidence and development [2] [3]. Much of the suffering and more than 30% of death rates from colon cancer could be prevented by changing life style factors that contribute to colon cancer. Efforts to reduce fat, improve diet and physical activity, include 5 servings of fruits and vegetables, and expand the use of established screening tests could play an important role in reducing colon cancer [4].

Carcinogenesis is a multistage multistep process. Animal models that mimic human carcinogenesis are important to determine dose and to test the efficacy, safety of chemopreventive agents. Chemical induction of colon cancer in rodents by azoxymethane was a widely studied model for testing efficacy of dietary chemopreventive agents [5] [6]. Azoxymethane is metabolized to methylazoxymethanol (MAM) by CYP2E1 to form DNA adducts, which is initial step in carcinogenesis process [7]. Preneoplastic lesions, aberrant crypt foci (ACF), are used as biomarkers to screen the potential of chemopreventive agents against colon cancer in short term [8] [9]. These ACF, if not all, could transform to adenomas and adenocarcinomas through promotion and progression [10]. Long term end point tumor studies represent the incidence of colon tumor and determine the potency of chemopreventive agent.

Oxidative stress, an imbalance between oxidant and anti-oxidant status, has an important role in every stage of carcinogenesis [11]. Accumulation of oxidative species, if not corrected by antioxidative systems, leads to damage of biological molecules and conversion of benign papillomas to carcinomas [12]. Endogenous antioxidants such as catalase and superoxide dismutase reduce oxidants produced by Phase 1 reactions and by metabolism to non-physiological levels. Modulation of xenobiotic enzymes is critical in the progression of cancer. Phase 1 enzymes are needed for metabolic activation of procarcinogen to ultimate carcinogen. Phase II enzymes, Glutathione-S-transferase and quinone reductase, detoxifies activated carcinogens [13] [14].

Cancer therapeutics are expensive, toxic and many are ineffective in treatment. Due to increased health care costs and consumer education, much focus is on natural or alternative agents that promote prevention of cancer. National cancer Institute (NCI) recommends consumption of diet with high fiber, more servings of fruit and vegetables, and less calories for prevention of cancer. However, natural agents lack scientific evidence for their safety, efficacy, bioavailability and mechanisms of action. Researchers in this decade have focused in testing the efficacy of these agents in chemoprevention. Epidemiological studies have suggested the reduced risk of cancer, diabetes, and other chronic diseases with plant polyphenols [15].

Sorrel (*Hibiscus sabdariffa* L.) was grown in tropical countries. Plant parts, leaves are generally used as green leafy vegetable and calyx is used to make hot and cold beverages. Calyx is also used to make jams and jellies in food processing industry. Sorrel calyx is rich in color and also used as natural color supplement. Many health benefits are reported for use of sorrel. Sorrel extract decreases blood pressure (BP) both in rats and human [16]. Daily consumption of 3 servings of *H. sabdariffa* (hibiscus) tea, an amount readily incorporated into the diet, effectively lowered BP in pre- and mildly hypertensive adults regardless of age, gender, or dietary supplement use [17]. Calyx of sorrel is rich in protocatechuic acid, pectin, anthocyanins, gossypectin and glucosides. Aqueous-methanolic extract of *H. sabdariffa* was found that it contained glycosides, flavonoids, saponins and alkaloids and exhibited antimicrobial activity and cytotoxicity [18]. Antitumor, immune modulating properties were found *in-vitro* by sorrel calyx extract [19].

Single compounds isolated from their native matrix of natural substances exhibited benefits of reducing cancer in controlled laboratory studies via single or multiple mechanisms. Using isolated compounds as chemopreventive agents was compromised with toxicity and safety issues. Dietary agents with complex mixtures and structures may provide multiple overlapping mechanisms with multi-targeted pattern impart anticancer activity [20] [21]. Therefore it is important to identify the whole foods that possess anticancer properties on long-term consumption. The present study is conducted to investigate the effect of feeding sorrel calyx as meal (SM) and juice (SJ) at nutritionally relevant quantities on azoxymethane induced colon cancer in Fisher 344 male rats.

2. Materials and Methods

2.1. Experimental Design and Animals

Three-week-old male Fisher 344 rats were purchased form Harlan laboratories (Harlan, IN, USA). Rats were

placed in stainless steel cages and acclimatized for a week. They were raised under standard laboratory conditions of temperature (24˚C ± 2˚C), humidity (55%) and 12 hrs light: dark cycles. At 5 weeks of age, rats were divided into 5 groups randomly and fed control (AIN 93 G or M) and experimental diets with sorrel (**Figure 1**). Sorrel meal (SM) was administered at 5% or 10% level and juice (SJ) was provided at 2.5% of 5% level to the rats in treatment groups. Rats were fed with purified chemical diet (M.P. Biomedicals, Costa Mesa, CA) of AIN 93 G for 20 weeks and shifted to AIN 93 M [22]. Sorrel was added to diet at the expense of cornstarch, sucrose and fiber to make isocaloric with control diet. Sorrel juice was provided instead of water for rats in juice treatment groups. Feed and water were provided *ad libitum*. Sorrel meal and juice were prepared as described earlier [23]. Weekly body weight gain and daily feed consumption were recorded. The experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of Alabama A&M University.

2.2. Induction of Tumor Development by Azoxymethane (AOM) and Sample Collection

At the age of 7 and 8 weeks, rats were injected subcutaneously with AOM (NCI Repository, Kansas City, MO) at 16 mg/kg body weight to induce tumor development (**Figure 1**). At 45 weeks of age rats were killed by CO_2 asphyxiation. Liver was removed, flash frozen with liquid nitrogen and stored at −70˚C for further analysis of enzyme activities. Cecum was removed, weighed and pH of cecal contents was noted. Colon was resected from the rat, opened longitudinally, washed with phosphate buffer (0.1 M, pH 7.2) and number of tumors were counted macroscopically. Location of the tumors, size of tumors and tumors/tumor bearing rat (T/TBR) were recorded.

2.3. Preparation of Liver Homogenate

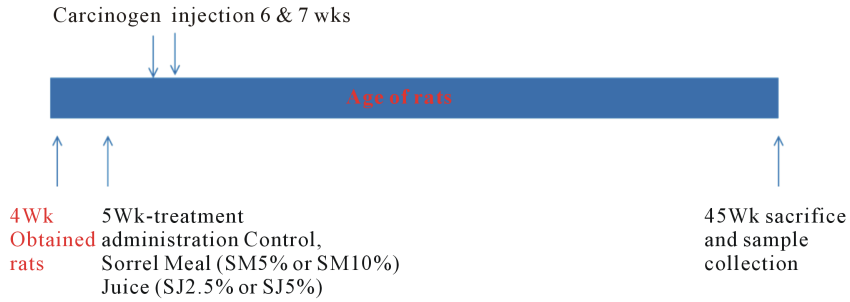
Liver was homogenized (1 g/10 ml) in ice-cold phosphate buffer (20 mM Hepes, 100 mM KCl, 1 mM MgCl2, 1 mM EGTA, 210 mM mannitol and 70 mM sucrose: pH 7.4) at 4˚C. The homogenate was centrifuged at 10,000 ×g for 30 min, supernatant was collected and a portion was used for analysis of antioxidative defense system enzymes catalase (CAT) and superoxide dismutase (SOD). The collected supernatant was further centrifuged at 100,000 × g for 60 min at 4˚C to yield the microsomal pellet for the assay of phase I CYP2E1 and Phase II Glutathione S-tranferase (GST). Equal volume of homogenization buffer was added to resuspend microsomal pellet, mixed and store at −70˚C for later analysis.

2.4. Biochemical Enzyme Analysis

Xenobiotic phase-1 metabolic enzyme, CYP2E1 was assayed by measuring the hydroxylation of p-nitrophenol at 600 nm spectromertrically [24]. Phase II, Glutathione-S-tranferase (GST) enzyme activity was analyzed by following the method of Habig *et al*. [25]. Catalase and SOD activities were measured by commercial kits (Cayman Chemical, Ann Arbor, MI) by following the manufacturers instruction.

2.5. Statistical Analysis

The results were expressed as mean ± SEM. Analysis of variance and Tukey's studentized test was used to



SM: Sorrel meal, SJ: Sorrel Juice

Figure 1. Experiential design.

compare the differences between the groups (SAS, 9.1, SAS institute, Cary, NC, USA). Differences were considered significant with $P < 0.05\%$.

3. Results

3.1. General Observations

Diet containing different levels of sorrel as meal (5% & 10%) and Juice (2.5% & 5%) did not produce any observable toxicity. All the rats remained healthy throughout the experimental period. Daily feed intake (g/day) did not differ ($P < 0.05$) among experimental groups and control (**Table 1**). The mean daily intake of feed was between 15.5 - 16.6 g/day/rat. There were no significant differences in the weight gain among the groups except rats fed with SM 10%. Rats fed with SM 10% weighed lower ($P < 0.05$) at end 45 wks compared to the rats fed with SJ 2.5%. Cecal weight and cecal pH were comparable and no differences were found between rats fed control diet and sorrel treatments.

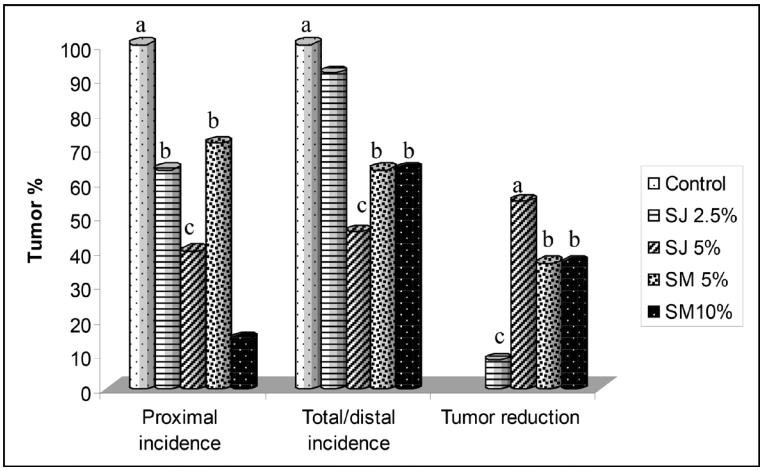
3.2. Incidence of Colon Tumors

Tumors were found in all groups of rats. However, tumor incidence in rats treated with sorrel was very low compared to the rats in control group (**Figure 2**). Macroscopic observations have revealed that most of the tumors were located in the distal section of rat colon. Proximal tumor incidence was higher in rats treated with SJ 2.5% (63.63%) and SM 5% (71.42%) compared to their counterparts (SJ 5% - 40.00%; SM 10% - 14.28%).

Table 1. Effect of sorrel calyx on weight gain, feed intake, cecal weight and cecal pH on AOM-induced Fisher 344 male rats.

	Feed Intake (g)	Weight Gain (g)	Cecal Weight (g)	Cecal pH
Control	16.63 ± 0.60	310.87 ± 8.61^{ab}	1.12 ± 0.05	7.81 ± 0.07
SJ 2.5%	16.39 ± 0.71	324.12 ± 3.70^{a}	1.13 ± 0.20	7.74 ± 0.02
SJ 5%	16.59 ± 1.09	323.87 ± 6.53^{a}	1.37 ± 0.05	7.71 ± 0.04
SM 5%	15.53 ± 1.10	306.37 ± 9.37^{ab}	1.03 ± 0.08	7.57 ± 0.12
SM 10%	16.51 ± 0.71	285.87 ± 8.47^{b}	0.93 ± 0.07	7.75 ± 0.04

Abbreviations are as follows: SJ: Sorrel Juice; SM: Sorrel Meal. Values are means ± SEM; $n = 6$. ^{a,b,c}vaules not sharing a common superscript in a column are significantly different ($P > 0.05$) with Tukey's studentized range test.



Abbreviations used are: SJ: Sorrel Juice; SM: Sorrel Meal. Values are means; $n = 6$. Bars not sharing a common superscript ^{a,b,c} are significantly different ($P < 0.05$) with Tukey's studentized range test.

Figure 2. Incidence of tumors in the colon of Fisher 344 male rats fed Sorrel Calyx.

Control fed rats had 100% incidence in both proximal and distal parts of the colon. Among the sorrel treatment groups, highest (91.66%) distal/total tumor incidence was observed in rats fed with SJ 2.5% followed by sorrel meal (SM 5% & 10% - 63.63%) and SJ 5% (45.45%). Highest reduction (54.55%) in tumor incidence was found in rats administered with SJ 5%. Sorrel meal treatments had no dose effect in reducing the incidence of tumor. However, rats fed with sorrel juice at high (5%) concentrations had low tumor incidence compared to its counterpart (8.34%).

3.3. Number of Tumors and Tumor Multiplicity

Effect of administration of sorrel as meal and juice on tumor number and tumor multiplicity was given in **Table 2**. Rats in control group had high number of rats with tumors, total number of tumors and also significantly higher tumors per tumor bearing rat (TBR) than the treatment groups. Although rats in both control and treatment groups had tumors, number of rats with tumors were low in sorrel fed groups. Number (7) of rats with tumors was similar with administration of sorrel as meal. Administration of SJ 5% had lowered the number of rats with tumors among all sorrel treated groups. Most of the rats treated with sorrel had lower number of tumors when observed macroscopically. Administration of SJ 5% and SM 10% reduced the number of tumors in rats by 5.9 fold; followed by SM 5% (2.95) and SJ 2.5% (2.10) fold. Tumor multiplicity (TBR) was lowest ($P < 0.05$) in rats fed with SM 10%. Though, no significant differences were observed in tumor multiplicity among the sorrel treated groups other than SM 10%, the level of tumor multiplicity was reduced ($P < 0.05$) 51% - 63% compared to the rats fed with control.

3.4. Tumor Size

Feeding sorrel as meal and administering as juice significantly reduced the size of tumors in rats compared to the control diet (**Figure 3**). Larger tumors were observed in distal colon of rats. Among the treatment groups, size of tumor in distal and total colon was significantly lower in rats fed with SM 10% and higher in SJ 2.5%. Long term feeding of sorrel reduced distal tumor size by 2.67 - 3.07 times in meal groups and 2 - 2.45 times in juice groups. A 52% - 78% reduction in tumor size was observed in rats with sorrel treatment compared to the control.

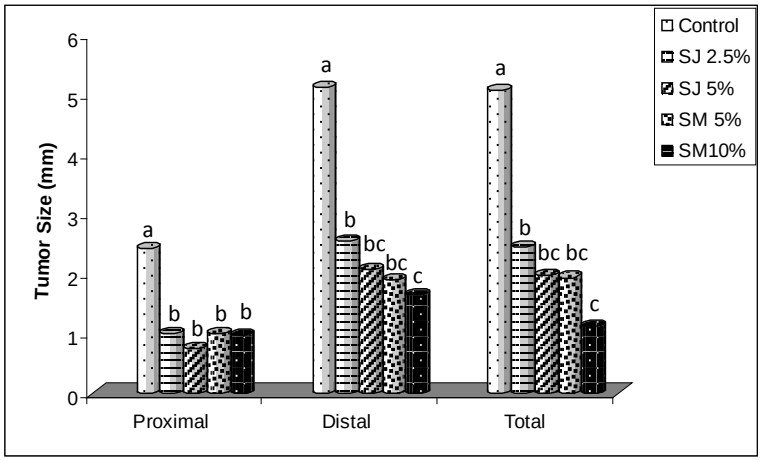
3.5. Activities of Hepatic Phase I and Phase II Enzymes

Effect of sorrel on xenobiotic metabolizing enzymes was shown in **Table 3**. Phase I (CYP2E1) (nmol/min/mg) activity was significantly higher in rats treated with AOM alone. Sorrel supplementation reduced ($P < 0.05$) the level of hepatic CYP2E1 in rats. Level of sorrel did not ($P < 0.05$) show any difference in reducing CYP2E1 among treatment groups. Hepatic Phase II enzyme, GST activity (mmol/min/mg) was lower ($P < 0.05$) in rats fed with control diet. Sorrel administration to rats as meal and juice increased GST activity significantly. Effect of sorrel in inducing GST activity was higher ($P < 0.05$) with sorrel supplementation at higher levels (SM 10% by 4.36 & SJ 5% times) compared to low level of sorrel (SJ 2.5% - 2.77% & SM 5% - 2.54% times) supply.

Table 2. Number of tumors and tumors/tumor bearing rat in the colon of Fisher 344 male rats fed sorrel calyx.

Treatments	Total Rats	Rats with Tumors	No of Tumors	TBR
Control	11	11	59	5.37 ± 0.49^{a}
SJ 2.5%	12	11	28	2.50 ± 0.18^{bc}
SJ 5%	11	5	10	2.00 ± 0.18^{bc}
SM 5%	11	7	20	2.62 ± 0.18^{b}
SM10%	11	7	10	1.37 ± 0.18^{c}

Abbreviations used are: SJ: Sorrel Juice; SM: Sorrel Meal. TBR: Tumors/tumor bearing rat. Values are means ± SEM; ^{a,b,c}Values not sharing a common superscript in a column are significantly different ($P < 0.05$) with Tukey's studentized range test.



Abbreviations used are: SJ: Sorrel Juice; SM: Sorrel Meal. Values are means; $n = 6$. Bars not sharing a common superscript ^{a,b,c} are significantly different ($P < 0.05$) with Tukey's studentized range test.

Figure 3. Size of tumor in different sections of the Fisher 344 rats colon fed with sorrel calyx.

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

Groups	CYP2E1	GST
	nmol/min/mg	mmol/min/mg
Control	0.81 ± 0.01^{a}	13.19 ± 0.35^{c}
SJ 2.5%	0.62 ± 0.01^{b}	36.63 ± 1.42^{b}
SJ 5%	0.54 ± 0.01^{b}	57.60 ± 1.36^{a}
SM 5%	0.58 ± 0.01^{b}	33.59 ± 1.60^{b}
SM 10%	0.61 ± 0.01^{b}	52.52 ± 1.25^{a}

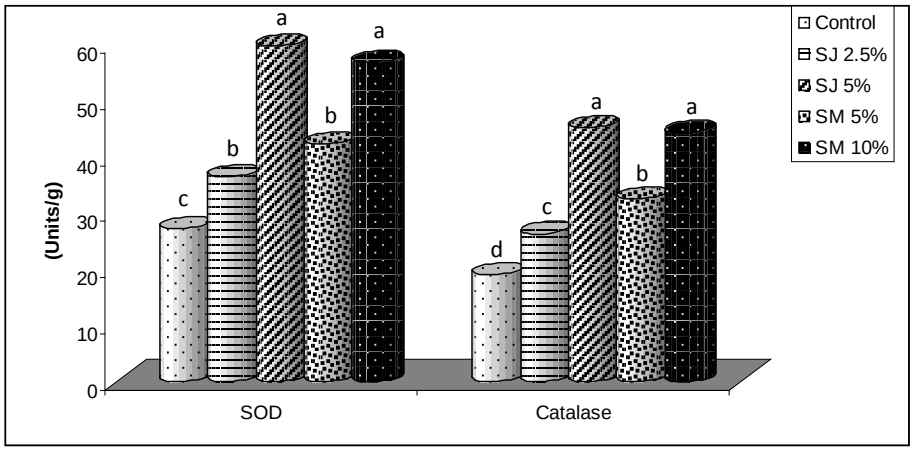
Abbreviations used are: SJ: Sorrel Juice; SM: Sorrel Meal. CYP: cytochrome; GST: Glutathione-S-Transferase. Values are mean ± SEM; $n = 6$. ^{a,b,c}Values not sharing a common superscript in a column are significantly different ($P < 0.05$) with Tukey's sudentized range test.

3.6. Antioxidative Enzyme Activity

Hepatic antioxidant enzymes (SOD & CAT) of rats were analyzed and given in **Figure 4**. Activities of these enzymes were found to be increased significantly in liver of sorrel treated rats compared with control rats. The activity of SOD in rats was increased ($p < 0.05$) by 2.19 folds in SJ 5%, 2.08 folds in SM 10%, 1.55 folds in SM 5%, 1.34 folds in SJ 2.5% compared to the control. Both antioxidative enzyme activities were significantly higher in SM10% and SJ 5% compared to their counterparts (SM 5% & SJ 2.5%). Dosing with sorrel elevated ($p < 0.05$) the level of CAT activity in rats by 1.34 - 2.19 fold compared to the rats with control diet.

4. Discussion

Plant based foods offer health benefits apart from providing nutrition due to presence of bioactive compounds. An inverse relation was found in epidemiological studies between high intake of fruits and vegetables and developing risk of colon cancer [26] [27]. Plant polyphenols and flavonoids, possess potent antioxidant, anti-atherosclerotic, anti-inflammatory, anti-mutagenic, antitumor, and antiviral activities *in-vitro* and *in-vivo* [28] [29]. Researchers have focused to identify the natural products with therapeutic potential against disease prevention and treatment. Many of the cancers developed due to lifestyle are preventable with diet modification. Previous studies indicated that plant foods and their constituent phytochemicals interfere at many stages and steps in the process of carcinogenesis [30]. Consumption of polyphenol-rich foods may induce beneficial changes in



Abbreviations used are: SJ: Sorrel Juice; SM: Sorrel Meal; CAT: Catalase; SOD: Superoxide Dismutase. Values are means; $n = 6$. Bars not sharing a common superscript [a,b,c] are significantly different ($P < 0.05$) with Tukey's studentized range Test.

Figure 4. Activity of hepatic antioxidative enzymes.

pathways related to cancer. Animal models are limited in understanding specific effect of *H. sabdariffa* in reducing colon cancer and its ability as anti-oxidative agent by consuming at nutritionally relevant quantities. The goal of the present study was to evaluate the chemopreventive potential of dried sorrel calyx extract and meal.

Feeding sorrel as in the form of juice or meal did not cause differences in daily food intake and weight gain of treatment groups compared to control diet fed rats. Therefore, sorrel administration did not cause any toxicity, effect on survival rate and could be considered as using functional food for obtaining health benefits. Lowest weight in rats fed with sorrel meal at high concentrations compared to juice groups might be due to the fiber in sorrel calyx. Cecal pH and weight were comparable in all groups.

Cancer is a multifactorial and multistage process. After forming DNA adducts by biological activation of carcinogen, the process of cancer may be further modified by a number of processes such as DNA repair mechanisms, cellular proliferative or transcriptional factors. There are opportunities for chemopreventive agents at every process to suppress process of carcinogenicity. AOM is a potent carcinogen and develops tumors in fisher 344 male rats by mimicking the development of tumors in middle and distal colon as of in human colon cancer [31]. Addition of sorrel as meal and Juice exhibited a decrease in tumor incidence, tumor size and tumor multiplicity compared to rats fed with control diet. Tumor number and tumor size are indicators of proliferation, differentiation and angiogenesis. Cell proliferation may lead to an increased risk of developing cancer whereas apoptosis is a protective innate mechanism for eliminating cells with DNA damage or genomic instability [32] [33]. Reduction in tumor growth found in rats fed with sorrel indicates anti-proliferative and anti-angiogenic and induced differentiation properties. Lower tumor incidence, smaller tumor size and TBR could be attributed to sorrel phytochemicals present which acts as anti-proliferative and anti-angiogenic factors.

Administration of sorrel at low doses (5% & 10% mg - meal and 2.5% & 5% - Juice) daily inhibited formation of ACF in AOM induced colon cancer in rats, suggested anti-carcinogenic properties of red sorrel [23]. Multiple genetic alteration in ACF would lead to formation of colon tumor [34] and some of them may not be developed into tumors because of DNA repair mechanisms and apoptosis. The present long term study further emphasized the ability of sorrel calyx in reducing tumor growth. A 30% - 50% reduction in tumor growth and tumor multiplicity further suggested the antitumor and anticancer properties of sorrel. Paired genetic changes and cell proliferation plays an important role in tumor progression [35]. Proanthocyanidins reduced growth of tumor cells and down regulated Proliferating cell nuclear antigen (PCNA) expression in colon [36].

Modulation of drug metabolizing enzymes is one of the characteristics of the phytochemicals, which makes them to be considered as chemopreventive agents against cancer. Many dietary components have been shown to modulate Phase I enzyme activities and alter the activity of carcinogen [37]. Inhibition of phase I activity and increased Phase II activity ($P < 0.05$) by 3 - 5 fold in liver of rats with sorrel treatment suggested the effect of sorrel and its components in altering initiation of carcinogenesis. These results also suggested the sorrel could act as blocking agent for AOM induced colon cancer.

Oxidative stress is an important factor in tumor development and affects all stages of cancer. Oxidation is a metabolic trigger for proliferation, inflammation and angiogenesis, which plays a major role in tumor progression [38]-[40]. Several studies suggested *in vitro* antioxidant activity of fruit juices or extracts from fruits [41]-[44]. Antioxidant potential of sorrel juice was evident in animal models with hepatotoxicity [45] [46]. In this study emphasis was given to identify antioxidative properties of sorrel in terms of inducing antioxidative enzymes in cancer induced rats and inhibiting the formation of free radical, which supported cell initiation and promotion. An increase ($P < 0.05$) in catalase and SOD activities were observed in sorrel administered groups compared rats fed with basal diet. The observed antioxidant activity was maybe due to presence of phenolic compounds and bioflavonoid protocatechuic acid, and glucosides in sorrel, which offered possible role in reducing the oxidative stress by inducing antioxidative enzymes. Flavonoids and phenolics are potent free radical scavengers and are known to modulate the activities of various enzyme systems [47] [48].

Our data suggested that sorrel acted as blocking agents by modulating biotransformation enzymes and as antitumor agents by inhibiting tumor proliferation as well as promoting progression. Therefore, sorrel could be used as a functional dietary chemopreventive agent against colon cancer development.

5. Conclusion

Dietary administration of sorrel as meal and juice for 45 weeks significantly reduced the development of colon tumors induced by AOM in Fisher 344 male rats via increased antioxidative enzyme activity and modulating phase I & II enzymes. Further animal models in varied cancer types and models with emphasis on molecular mechanisms are needed to confirm the chemopreventive potential of sorrel.

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