RESEARCH ARTICLE

Chemopreventive Effect of *Amorphophallus campanulatus* (Roxb.) Blume Tuber Against Aberrant Crypt Foci and Cell Proliferation in 1, 2-Dimethylhydrazine Induced Colon Carcinogenesis

Puthuparampil Nazarudeen Ansil, Santhibhavan Prabhakaran Prabha, Anand Nitha, Mukalel Sankunni Latha*

Abstract

Colorectal cancer is one of the leading causes of cancer death, both in men and women. This study investigated the effects of *Amorphophallus campanulatus* tuber methanolic extract (ACME) on aberrant crypt foci (ACF) formation, colonic cell proliferation, lipid peroxidative damage and the antioxidant status in a long term preclinical model of 1, 2-dimethylhydrazine (DMH) induced colon carcinogenesis in rats. Male Wistar rats were divided into six groups, viz., group I rats served as controls; group II rats treated as drug controls received 250 mg/kg body weight of ACME orally; group III rats received DMH (20 mg/kg body weight) subcutaneously once a week for the first 15 weeks; groups IV, V and VI rats received ACME along with DMH during the initiation, post- initiation stages and the entire period of the study, respectively. All the rats were sacrificed at the end of 30 weeks and the intestinal and colonic tissues from different groups were subjected to biochemical and histological studies. Administration of DMH resulted in significant ($p \leq 0.05$) reduction of intestinal and colonic lipid peroxidation (MDA) and antioxidants such as catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and reduced glutathione. Whereas the supplementation of ACME significantly ($p \leq 0.05$) improved the intestinal and colonic MDA and reduced glutathione levels and the activities of antioxidant enzymes in DMH intoxicated rats. ACME administration also significantly suppressed the formation and multiplicity of ACF. In addition, the DMH administered rats showed amplified expression of PCNA in the colon and decreased expression of this proliferative marker was clearly noted with initiation, post-initiation and entire period of ACME treatment regimens. These results indicate that ACME could exert a significant chemopreventive effect on colon carcinogenesis induced by DMH.

Keywords: *Amorphophallus campanulatus* - colon cancer - 1, 2-dimethylhydrazine - proliferating cell nuclear antigen

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer death worldwide and is the third most common form of malignancy in both men and women (Giftson et al., 2010; Hamiza et al., 2012). It is also one of the fastest emerging gastrointestinal cancers in the Asia Pacific region (Goh et al., 2005). Accumulating evidence from epidemiological and experimental studies suggest that diet is an important environmental factor in the aetiology of CRC. The typical Western diet contains high concentrations of protein including red or processed meat, animal fats, alcohol and refined carbohydrates, all of which are associated with elevated risks of CRC (Le Leu et al., 2007). Evidence from epidemiological studies also suggests that diets rich in fruits and vegetables are protective against a number of different cancers, including colon cancer (Yusof et al., 2012). Mortality due to non-hereditary colon cancer appears to be reduced with appropriate changes in diet and modifiable non-dietary factors, such as smoking (Vargas and Alberts, 1992).

The toxic environmental pollutant, 1, 2-dimethylhydrazine (DMH) is a known colon-specific carcinogen. Induction of colon tumors in rats by the administration of DMH or its metabolite azoxymethane (AOM) has been an excellent experimental model to study the pathogenesis of colon cancer in humans. DMH is metabolically activated in the liver by a series of reactions through intermediates AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite, highly reactive methyldiazonium ion. The activated carcinogen reaches the colon either via the blood or bile. Once methyldiazonium ion is formed it generates a carbonium ion, which is known to elicit oxidative stress, DNA alkylation and thereby DNA damage and mutations. (Perše and Cerar, 2011; Hamiza et al., 2012). The

*Biochemistry and Pharmacognosy Research Laboratory, School of Biosciences, Mahatma Gandhi University, P.D. Hills P.O., Kerala, India *For correspondence: mslathasbs@yahoo.com*

damaged cells undergoes apoptosis or acquired mutations that accumulate to cause cell proliferation leading to carcinogenesis (Sengupta et al., 2004).

Development of colon cancer is a multistep process involving a series of pathological alterations ranging from discrete microscopic mucosal lesions, like aberrant crypt foci (ACF), to malignant tumors (Takayama et al., 1998). ACF are putative preneoplastic lesions that occur in the colon of both animals and humans. ACF appear at an early period of colorectal carcinogenesis in tumor bearing rodents after treatment with chemical carcinogens and in patients with familial adenomatous polyposis (FAP) and other bowel tumors (Roncucci et al., 1991; Wargovich et al., 1995; Fernandes et al., 2011). ACF are now frequently used experimentally as effective surrogate biomarkers for the detection of cancer promoters or chemopreventive agents against colorectal cancers. ACF induced by carcinogens display hyperplastic or dysplastic changes and precede the development of adenomas and adenocarcinomas in the large bowel (Shpitz et al., 1996). The characterization of such early precursor lesions of colon cancer increase our understanding of oncogenesis and enable their use as intermediate endpoint biomarkers to screen potentially new chemopreventive agents (Jia and Han, 2000).

Chemopreventive agents are typically natural products or their synthetic analogues that inhibit the transformation of normal cells to premalignant cells or the progression of premalignant cells to malignant cells by modulating processes associated with xenobiotic biotransformation, along with the protection of cellular elements from oxidative damage (Sun et al., 2008). Several colon cancer chemopreventive agents are found in edible plants, including fruits and vegetables (Chung et al., 2013). *Amorphophallus campanulatus* (Roxb.) Blume is basically a tuber crop of south East Asian origin and is largely cultivated throughout the plains of India for using its corm (bulb) as food (Das et al., 2009). Further, the plant is valuable as medicine especially the corm has been used traditionally for the treatment of abdominal tumours, abdominal pain and liver diseases (Warrier et al., 1994). The present study was designed to investigate the effect of *Amorphophallus campanulatus* tuber methanolic extract (ACME) on ACF formation, colonic cell proliferation, lipid peroxidative damage and the antioxidant status in a long term preclinical model of DMH induced colon carcinogenesis.

Materials and Methods

Chemicals

The 1, 2-Dimethylhydrazine dihydrochloride (DMH), Monoclonal anti-proliferating cell nuclear antigen (PCNA), 3, 3'-Diaminobenzidine tetra hydrochloride (DAB), anti-mouse IgG (whole molecule)-peroxidase and streptavidin-HRP were purchased from Sigma-Aldrich, USA. All other chemicals and reagents used were of analytical grade.

Animals, diet and care

Animal studies were followed according to institute animal ethics committee regulations approved by the committee for the purpose of control and supervision of experiments on animals (Reg. No. B 2442009/6). Male Wistar rats weighing 162.6±13.3 gm (Mean±SD) were used in this study. Animals were housed in polypropylene cages with a wire mesh top and a hygienic bed of husk and were held in quarantine for one week. They were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12 h dark cycle. All the rats received modified pellet diet (commercial pellet diet containing 4.2% fat was powdered and mixed with 15.8% peanut oil making 20% total fat in the feed) to stimulate a high fat, western diet (Aranganathan et al., 2009). This modified pellet diet and drinking water were fed *ad libitum* throughout the experimental period of 30 weeks.

Collection and preparation of plant extracts

A campanulatus tubers were collected from the local market (Kottayam, Kerala, India) and authenticated. A voucher specimen (SBSBRL.02) is maintained in the institute. The powdered tubers were soxhlet extracted with methanol and were concentrated under reduced pressure using a rotary evaporator. The yield of methanolic extract was 9.3% (w/w). Extract was suspended in 5% Tween 80 to respective dosages and stored at -20°C.

Carcinogen administration

DMH was dissolved in distilled water containing 1 mM EDTA to ensure the stability of the chemical just prior to use and the pH was adjusted to 6.5 with 1 mM NaOH. Animals were given a weekly subcutaneous injection of DMH at a dose of 20 mg/kg body weight in the right thigh for the first 15 consecutive weeks (Aranganathan et al., 2009).

Treatment regimen

Rats were randomly divided into six experimental groups of 12 animals each (6 for ACF analysis and 6 for biochemical assays). Group I, received 5% Tween 80 and normal saline instead of ACME and DMH respectively, served as untreated control. Group II rats received ACME (250 mg/kg/day; p.o.) for 30 weeks (Control+ACME 250) served as drug control. Group III rats considered as the carcinogen control received DMH (20 mg/kg body weight) injections subcutaneously once a week for 15 consecutive weeks and then the animals were kept without any treatment till 30 weeks. Group IV [Initiation-(I)] animals received DMH (as in group III) and ACME (250 mg/kg/day; p.o.); the supplementation of ACME were started one week before the first DMH injection and continued until one week after the final exposure of carcinogen. Group V [Post-initiation-(PI)] rats received DMH as in group III and the treatment with ACME (250 mg/kg/day; p.o.) started after the cessation of DMH injections and continued till the end of the experimental period. Group VI [Entire period-(EP)] animals received DMH as in group III and ACME (250 mg/kg/day; p.o.) was supplemented from the day one of the experiment and continued till the end of the entire experimental period of 30 weeks. The schematic representation of the experimental protocol is given in Figure 1.

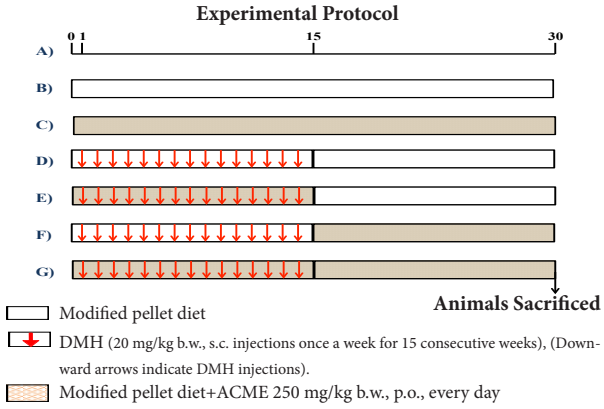

Figure 1. Experimental Design. A) Duration (in weeks); **B)** Group I (control); **C)** Group II (Drug control) (ACME 250 mg/kg); **D)** Group III (DMH control); **E)** Group IV (Initiation) (DMH+ACME 250 mg/kg); **F)** Group V (Post-initiation) (DMH+ACME 250 mg/kg); and **G)** Group VI (Entire period) (DMH+ACME 250 mg/kg)

Body weight changes

The body weight of control, DMH and ACME treated rats were measured at the beginning of the experiment, subsequently, once every week and finally before sacrifice. Growth rate was calculated as the difference between the final and the initial body weight divided by the total number of experimental days.

Identification of aberrant crypt foci (ACF)

Enumeration of ACF was performed as described by Bird (1987). At the end of treatment period, rat colons were removed and flushed with potassium phosphate buffered saline (0.1 M, pH 7.2). They were split open longitudinally from caecum to anus, divided into three segments viz., proximal, middle and distal and fixed flat between two pieces of filter paper in 10% buffered formalin for 24 hours. Microscopic slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. Each portion of the fixed colon was further cut into 2 cm long segments and stained with 0.2% methylene blue solution for 3 min. The segments were then transferred to the buffer to wash off excess stain. It was then placed mucosal side up, on a microscopic slide and observed under a light microscope at 40× magnification to score the total number of ACF as well as the number of crypts per focus. ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells. Since ACF size may relate more closely to the tumor end-point than ACF number, assessment of the aberrant crypt multiplicity was carried out as the number of crypts in each focus. Accordingly ACF was categorized as small (<3 crypts/focus), medium (3-6 crypts/focus) and large (>6 crypts/focus).

Homogenization of tissues

At the end of the experimental period, the animals were anesthetized with pentothal sodium followed by neck decapitation. Intestine, proximal colon and distal colon were immediately excised and washed with ice cold saline. The tissues were then cut into fragments and ten percent of homogenate was prepared in 0.1M Tris HCl buffer (pH-7.4). The homogenate was centrifuged at 3000 rpm for 20 min at 4°C and the supernatant was used for the estimation of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH) and lipid peroxidation (Thiobarbituric Acid Reactive Substances-TBARS).

Biochemical assays

Reduced GSH was determined based on the formation of a yellow colored complex with DTNB (Ellman, 1959). GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB (Habig et al., 1974). GR (EC 1.6.4.2) activity was assayed at 37°C and 340 nm by following the oxidation of NADPH by GSSG (Carlberg and Mannervik, 1985). GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3 (Rotruck et al., 1973). Tissue CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H_2O_2 (Beers and Sizer, 1952). Malondialdehyde (MDA), a product of lipid peroxidation was determined by thiobarbituric acid reaction as described by Niehius and Samuelsson (1968). Protein content in the tissue was determined using bovine serum albumin (BSA) as the standard (Lowry, 1951).

Histopathological examination

Small pieces of colon tissues fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with hematoxylin and eosin and examined for histopathological changes.

Immunohistochemistry

The 5 µm thick paraffin-embedded sections were heated for 60 min at 60°C, deparaffinized in xylene and rehydrated through graded alcohols at room temperature. The slides were incubated in citrate buffer (pH 6.0) for three cycles of 5 minutes each in a microwave oven for antigen retrieval. Phosphate buffered saline (PBS) (1×; pH 7.4) was used for washes between various steps. Sections were treated for 45 min at room temperature with 3% bovine serum albumin and then incubated overnight with monoclonal anti-proliferating cell nuclear antigen (PCNA) at 4°C. Horseradish peroxidase activity was visualized by treating the slides with 3, 3'-diaminobenzidine tetra hydrochloride hydrate (DAB, 3mg/5mL) in 0.024% H_2O_2 for 5 minutes. Finally, the sections were counterstained with Meyer's hematoxylin and examined microscopically. Quantitative analysis was made in a blinded manner under a light microscope. The labeling index was expressed as number of cells with positive staining per 100 counted cells in ten randomly selected fields at high magnification (Ramakrishnan et al., 2008).

Statistical analysis

Results are expressed as mean±SD and all statistical comparisons were made by means of one-way ANOVA test followed by Tukey's post hoc analysis and p-values less than or equal to 0.05 were considered significant.

Results

General observations

All the rats in the experimental groups tolerated subcutaneous injections of DMH as well as ACME feeding very well. Normal animal behavior, improved body weight gain and absence of mortality in ACME treated rats emphasize the safety of the drug. Effect of DMH and ACME on change in body weight and growth rate of control and experimental animals are shown in Table 1. Body weight of the animals in all the groups increased gradually during the 30 week experimental period. From week 0 to 30, variable changes were observed in the growth rate of the rats in the different groups. The growth rate of rats in DMH alone treated group (Group III) was significantly ($p \leq 0.05$) lower than control rats (Group I). There was a significant ($p \leq 0.05$) increase in the growth rate on ACME supplementation to DMH treated rats (Groups IV, V and VI) as compared to the DMH alone treated rats in group III. Figure 2 represents the morphological changes observed in longitudinally opened colon of one of the rats treated alone with DMH (Group III).

Effect of ACME on ACF formation

ACF analysis was carried out at the end of the experimental period. Effect of ACME and DMH on ACF incidence, total ACF, number of AC/ACF (crypt multiplicity) and percentage inhibition of ACF in experimental groups are shown in Table 2. The incidence of ACF was 100% in rats treated with DMH (Groups III-VI), whereas control rats and ACME alone treated rats (Group I and II) showed 0% ACF incidence. A statistically significant ($p \leq 0.05$) reduction in total ACF, number of AC and crypt multiplicity was observed in all the groups supplemented with ACME (Groups IV-VI). Percentage inhibitions of ACF in DMH treated rats fed with ACME were 49.3, 64.3 and 74.9% respectively in the initiation, post-initiation and entire period of study. Effects of ACME and DMH on the incidence of different category of ACF are shown in Table 3. When compared to the DMH alone treated group III animals, the number of small, medium and large crypts was significantly ($P \leq 0.05$) lowered in groups IV-VI rats supplemented with ACME. Topographical view of ACF from different groups are shown Figure 3. These results showed that ACME significantly inhibited DMH induced ACF formation and multiplicity and the effect was more pronounced in group VI (entire period) animals.

Effect of DMH and ACME on GSH and glutathione dependent enzymes

The effect of DMH and ACME on the activities of GSH and glutathione dependent enzymes of control and experimental groups of animals are depicted in Table 4. The level of GSH and activities of GSH dependent enzymes such as GST, GR and GPx were significantly decreased ($p \leq 0.05$) in intestine, proximal and distal colon tissues of DMH treated rats as compared to the control

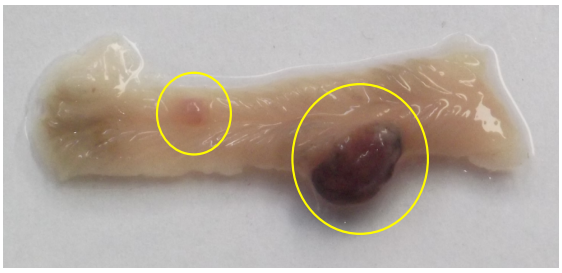

Figure 2. Morphological Changes Observed in the Longitudinally Opened Colon of one of the Rats Treated Alone with DMH

Table 1. Effect of DMH and ACME on Change in Body Weight and Growth Rate of Control and Experimental Animals

Groups	Initial weight (g)	Final weight (g)	Growth Rate (g)
Normal Control	150.3±8.9	284.5±13.5	0.65±0.04
Drug Control	153.7±6.3	288.2±15.4	0.64±0.06
DMH Control	177.8±10.3	218.8±19.5	0.19±0.04[a]
Initiation	160.7±12.7	233.3±15.3	0.34±0.01[b]
Post-initiation	165.5±13.4	270.2±16.5	0.49±0.02[b]
Entire Period	167.7±8.8	285.7±17.9	0.56±0.05[b]

*Values are expressed as mean±S.D; [a]p≤0.05 versus normal control. [b]p≤0.05 versus DMH control

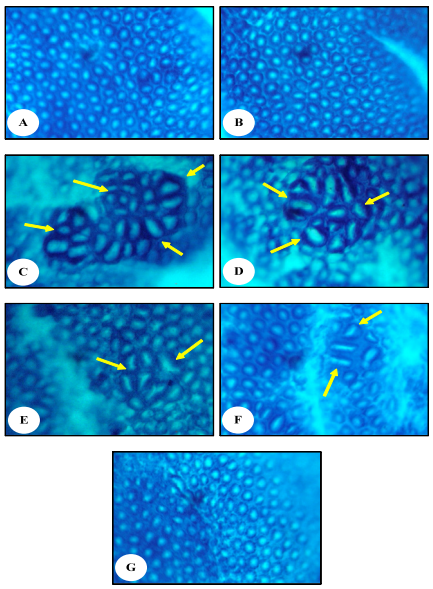

Figure 3. Effect of ACME on ACF Formation and Crypt Multiplicity (40×). A) and **B)** Topographical view of normal crypts in the colon of control rats and control+ACME treated rats; **C)** and **D)** ACF with >8 crypts in the colon of DMH alone treated rats; **E)** ACF with >5 crypts in the colon of DMH+ACME treated rats (Initiation); **F)** ACF with >3 crypts in the colon of DMH+ACME treated rats (Post-initiation); and **G)** Colon of DMH+ACME treated rats (Entire period). Arrows indicate the ACF

Table 2. Incidence of ACF in Control and Experimental Rats

Groups	ACF incidence (%)	ACF/ Colon	AC/ Colon	Crypt Multiplicity (AC/ACF)/Colon	Inhibition of ACF (%)
Normal Control	0/6 (0)	Nil	Nil	Nil	Nil
Drug Control	0/6 (0)	Nil	Nil	Nil	Nil
DMH Control	6/6 (100)	90.5±11.5	280.8±21.0	3.1±0.3	Nil
Initiation	6/6 (100)	45.8±8.3[a]	91.7±13.5[a]	2.0±0.1[a]	49.3
Post-initiation	6/6 (100)	32.3 ±11.3[a]	54.8±15.5[a]	1.7±0.2[a]	64.3
Entire Period	6/6 (100)	22.7±8.9[a]	30.5±9.7[a]	1.4±0.3[a]	74.9

*Values are expressed as mean±S.D; [a]p≤0.05 versus DMH control

group of animals. Administration of ACME (250 mg/kg body weight) to the experimental group of animals (initiation, post-initiation and entire period) markedly ($p \leq 0.05$) increased the reduced glutathione level as well as glutathione dependent enzymes activities, compared to rats treated alone with DMH. A more pronounced effect was observed in group VI animals, supplemented with ACME throughout the experimental period of 30 weeks.

Effect of DMH and ACME on catalase

The activity of CAT in intestine, proximal and distal colon tissues of control and experimental animals are shown in Table 5. DMH alone treated rats showed a significant ($p \leq 0.05$) reduction in CAT activity as compared to the normal control group. But on ACME supplementation (Group IV-VI) the CAT activity was significantly ($p \leq 0.05$) elevated as compared to the unsupplemented DMH treated group. The effect was more evident when ACME was supplemented throughout the experimental period (Entire period group).

Table 3. Effect of ACME and DMH on the Incidence of Different Category of ACF in Control and Experimental Animals

Groups	Number of ACF per colon		
	Small (<3 crypts/focus)	Medium (3-6 crypts/focus)	Large (>6 crypts/focus)
DMH Control	30.1±2.9	43.1±4.1	17.3±1.9
Initiation	19.2±1.8[a]	15.1±1.5[a]	11.5±1.2[a]
Post-initiation	13.8±1.3[a]	11.6±1.1[a]	6.9±1.6[a]
Entire Period	10.1±1.0[a]	8.8± 0.8[a]	3.8±0.6[a]

*Values are expressed as mean±S.D; [a]$p \leq 0.05$ versus DMH control

Effect of ACME on lipid peroxidation (MDA)

The extents of lipid peroxidation in intestine, proximal and distal colon tissues of control and experimental group of rats are depicted in Table 6. The intestinal and colonic MDA levels were significantly ($p \leq 0.05$) lower in DMH treated rats (Group III) as compared to that of untreated control animals (Group 1). ACME supplementation to the experimental group of animals (initiation, post-initiation and entire period) markedly ($p \leq 0.05$) increased the intestinal and colonic MDA level, compared to the group III animals treated alone with DMH. However, in group VI rats (entire period) the supplementation of ACME showed near normal levels of MDA in intestine, proximal and distal colon tissues.

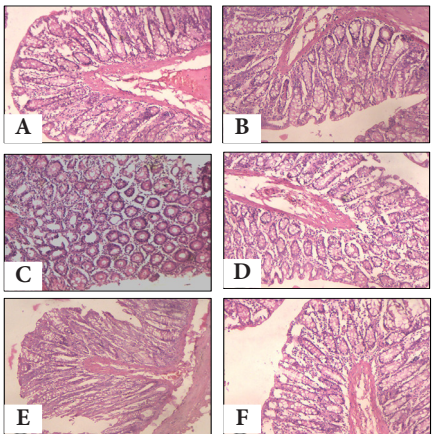

Figure 4. Histopathological Changes of the Colon in Control, DMH and ACME Treated Rats (100×). **A)** Normal Control; **B)** Drug Control; **C)** DMH Control; **D)** Initiation; **E)** Post-initiation; and **F)** Entire Period

Table 4. Effect of ACME on Glutathione and Glutathione Dependent Enzyme Activities in Intestine, Proximal and Distal Colon Tissues of Control and Experimental Animals

		Control	Control+ACME 250 mg/kg	DMH	DMH+ACME 250 mg/kg (I)	DMH+ACME 250 mg/kg (PI)	DMH+ACME 250 mg/kg (EP)
GSH (nmol/mg protein)	Intestine	20.0±0.5	20.0±0.9	13.2±0.5[a]	15.0±0.4[b]	16.3±0.8[b]	18.0±0.7[b]
	Proximal colon	28.3±0.8	27.0±0.4	14.9±0.6[a]	18.0±0.6[b]	21.1±0.8[b]	24.0±0.6[b]
	Distal colon	25.7±1.0	27.0±0.7	19.9±0.6[a]	22.1±0.9[b]	23.8±0.8[b]	25.5±1.0[b]
GST (μmol CDNB-GSH conjugate formed/min/mg protein)							
	Intestine	69.0±1.6	68.0±1.4	36.0±1.4[a]	47.9±2.0[b]	55.0±1.8[b]	61.4±1.7[b]
	Proximal colon	71.1±1.6	72.5±1.5	35.0±1.7[a]	49.9±1.8[b]	58.0±1.5[b]	65.8±1.7[b]
	Distal colon	70.0±2.0	69.2±1.9	36.9±1.5[a]	52.0±1.8[b]	61.0±1.9[b]	66.7±1.8[b]
GR (nmol of GSSG utilized/min/mg protein)							
	Intestine	17.9±1.0	17.5±0.9	8.0±0.7[a]	12.2±0.8[b]	14.0±0.4[b]	16.1±0.7[b]
	Proximal colon	22.1±0.5	23.0±0.5	15.2±0.8[a]	18.0±0.5[b]	19.1±0.8[b]	20.0±1.0[b]
	Distal colon	23.9±0.8	23.1±0.9	16.0±0.5[a]	18.9±0.7[b]	20.2±0.6[b]	21.3±0.9[b]
GPx (nmol of GSH oxidized/min/mg protein)							
	Intestine	279.3±2.9	283.3±3.7	154.7±3.1[a]	203.7±5.0[b]	220.0±4.2[b]	255.0±4.6[b]
	Proximal colon	285.8±4.4	279.0±5.1	142.2±3.5[a]	213.2±4.9[b]	236.0±5.5[b]	260.8±3.5[b]
	Distal colon	275.0±5.5	281.3±3.3	135.7±4.0[a]	209.7±4.6[b]	225.0±4.2[b]	251.3±5.0[b]

*Values are the mean±S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$; [a]DMH group differs significantly from control group; [b]DMH+ACME-250 mg/kg groups (Initiation, Post-initiation and Entire period) differs significantly from DMH alone treated group

Table 5. Effect of ACME on Catalase (CAT) Activity in Intestine, Proximal and Distal Colon Tissues of Control and Experimental Animals

		Control	Control+ACME 250 mg/kg	DMH	DMH+ACME 250 mg/kg (I)	DMH+ACME 250 mg/kg (PI)	DMH+ACME 250 mg/kg (EP)
CAT(U/mg protein)	Intestine	47.1±2.1	46.0±1.9	22.3±1.5[a]	30.1±2.2[b]	34.0±1.9[b]	41.0±1.7[b]
	Proximal colon	58.0±1.8	57.1±1.9	39.9±2.3[a]	44.3±1.7[b]	50.0±1.5[b]	54.1±2.3[b]
	Distal colon	59.2±1.7	60.0±1.9	45.0±1.9[a]	49.0±1.6[b]	51.9±2.0[b]	54.9±2.3[b]

*Values are the mean±S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. [a]DMH group differs significantly from control group. [b]DMH+ACME-250 mg/kg groups (Initiation, Post-initiation and Entire period) differs significantly from DMH alone treated group

Table 6. Effect of ACME on Malondialdehyde (MDA) Activity in Intestine, Proximal and Distal Colon Tissues of Control and Experimental Animals

		Control	Control+ACME 250 mg/kg	DMH	DMH+ACME 250 mg/kg (I)	DMH+ACME 250 mg/kg (PI)	DMH+ACME 250 mg/kg (EP)
MDA (nmol/g tissue)	Intestine	58.4±2.3	56.2±1.4	22.8±1.6[a]	33.8±1.9[b]	43.0±1.5[b]	50.1±1.7[b]
	Proximal colon	74.0±1.9	72.9±1.8	37.6±2.1[a]	43.0±1.5[b]	54.9±1.8[b]	65.9±1.6[b]
	Distal colon	78.9±1.6	81.0±1.6	43.7±1.7[a]	52.9±1.8[b]	59.0±1.9[b]	69.8±2.4[b]

*Values are the mean±S.D from 6 rats in each group. Statistical significance: p≤0.05. [a]DMH group differs significantly from control group. [b]DMH+ACME-250 mg/kg groups (Initiation, Post-initiation and Entire period) differs significantly from DMH alone treated group

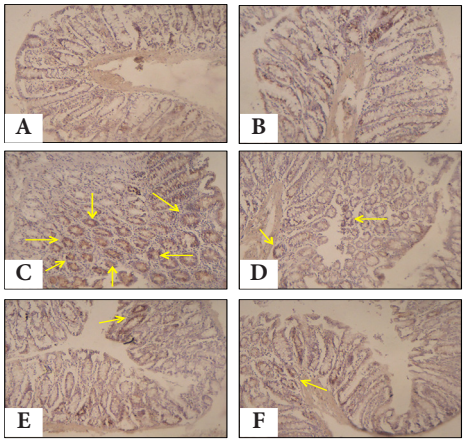

Figure 5. Immunohistochemical Staining of PCNA in the Colon of Control and Experimental Animals (100×). A) Normal Control; **B)** Drug Control; **C)** DMH Control; **D)** Initiation; **E)** Post-initiation; and **F)** Entire Period. Brown stained nuclei indicating the accumulation PCNA protein

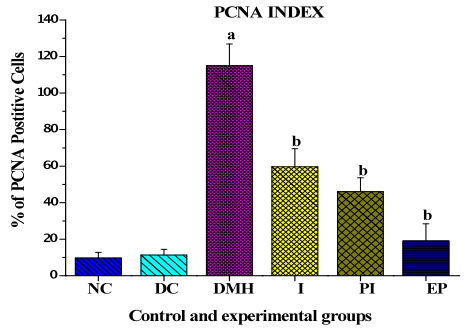

Figure 6. Bar Graph of the Percentage of PCNA-Positive Cells in Control and Experimental Groups. A) Normal Control; **B)** Drug Control; **C)** DMH Control; **D)** Initiation; **E)** Post-initiation; and **F)** Entire Period. [a]p≤0.05 versus normal control; [b]p≤0.05 versus DMH control; Values are expressed as mean±S.D.

Histopathological evaluation

Figure 4 represents the histopathological changes observed microscopically in the colon of control and experimental rats. Tissue sections of normal control and drug control rats (Group I and II) displayed normal crypts and colonic architecture with no signs of apparent abnormality (Figure 4A and 4B). Group III rats treated alone with DMH showed mucin filled carcinoma glands, enlarged nuclei and hyperchromatism with mitotic figures. Larger areas of thickened mucosal layer with densely packed inflammatory cell infiltration were also noted in group III animals (Figure 4C). In ACME treated rats (group IV-VI), the histology revealed no loss of nuclear polarity and also showed a reduction in mucin filled carcinoma gland formation. Few areas showed mucosal thickenings with scattered or no infiltration of the inflammatory cells in the mucosa (Figure 4D and 4F). The improvement of histopathological alterations of the colonic tissues in DMH treated rats by the supplementation of ACME in different treatment regimen were as follows: entire period>post-initiation>initiation.

Immunohistochemistry of PCNA

Figure 5 shows immunohistochemical staining of PCNA in the colon of control and experimental group of animals. The expression of PCNA which was observed as dense brown colour spots, significantly increased (p≤0.05) in group III DMH treated animals (Figure 5C) as compared to the group I normal control rats. Normal control (Figure 5A) and drug control (Figure 5B) rats, however, showed few positive expressions of PCNA. ACME supplementation to DMH treated rats [initiation (Figure 5D), post-initiation (Figure 5E) and entire period (Figure 5F)] showed decreased nuclear staining for PCNA. The effect of ACME in reducing cell proliferation was more pronounced in the post-initiation and entire period groups. Figure 6 depict the labeling index of PCNA in control and experimental group of animals.

Discussion

Dietary intervention is a convenient way for disease prevention or therapy, as it is usually not accompanied by severe side effects (Murakami et al., 1996). At least one third of all human cancers may be associated with diet and influenced by lifestyle and physical exercise. Nutritional or dietary factors have attracted a great deal of interest due to their perceived ability to act as highly effective chemopreventive agents. Thus dietary approaches are considered a rational strategy to prevent cancer (Venkatachalam et al., 2013). It is reported that the majority of large bowel cancers are attributable to environmental factors means that it is a potentially preventable disease (Kanna et al., 2003). The development of colon cancer involves three distinct stages, initiation that alters the molecular message of a normal cell, followed by promotion and progression that ultimately generates a phenotypically altered transformed malignant cell (Pandurangan, 2013). In the present study, we demonstrate that ACME supplementation during the different stages of carcinogenesis (initiation, post-initiation and entire period) has a potent inhibitory activity against DMH induced tumor formation in the rat colon.

DMH and its related compounds can induce neoplasms specifically in the rat colon. Primarily, DMH is an alkylating agent, alkylation of guanine at the O_6 position is

considered to be an important miscoding lesion, leading to G-A transition, with an important role in mutagenesis and carcinogenesis (Stephanou et al., 1996). In addition, DMH exposure can elicits substantial oxidative stress due to the formation of electrophilic diazonium ion. Weisburger, (1971) also reported that DMH produces free radicals in the blood, liver, and large bowel in experimental models.

In the present study all the animals received a diet containing 20% fat to induce the development of colonic ACF (Boateng et al., 2006). Reddy et al. (1974) reported that the number of DMH induced tumors increases with the percentage of dietary fat composition. Dietary fatty acid composition can influence the initiation, promotion and progression of experimental neoplasia in the rodent large bowel observed in studies where lipid intake had been manipulated both before and after treatment with DMH or AOM (Takahashi et al., 1993). The high incidence of colon tumors observed in our study in DMH administered rats fed with high fat diet may be due to the excretion of elevated amounts of bile acids, which act as colon tumor promoters (Kamaleeswari et al., 2006).

The significantly ($p \leq 0.05$) decreased growth rate observed in DMH exposed rats may be due to the occurrence of tumours in the colonic tract. However, the improved growth rate of ACME supplemented rats obviously shows its promising role as a chemopreventive agent. It is reported that colon cancer is often associated with an abdominal mass, weight loss, decreased appetite and blood in the stool (Malik and Kamath, 2011). Thus the body weight restoration upon ACME administration, observed in our study, emphasizes its chemopreventive potential against DMH induced colon cancer.

The earliest recognizable histopathological change that is the preneoplastic lesions of colorectal carcinoma are the ACF. These are considered to be the useful intermediate biomarkers to assess the chemopreventive potential of natural products against colon carcinogenesis (Khan et al., 2013). In this study, variable inhibitory effects of ACME on the occurrence of ACF were observed during different phases of colorectal carcinogenesis. Larger ACF (six or more aberrant crypts per focus) are considered more likely to progress into tumors (Bird and Good, 2000) and in our study, ACME treatment had a significant inverse influence on larger ACF formation in the colon. Significant reduction in the occurrence and multiplicity of ACF in DMH treated rats supplemented with ACME denotes that the extract have a remarkable potential in suppressing the occurrence of preneoplastic changes and the formation and progression of preneoplasia to malignant neoplasia.

Results of the present investigation correlate with previous studies that the level of lipid peroxidation in intestinal and colonic tissues of rats decreases on DMH exposure (Aranganathan et al., 2009). In addition, earlier reports suggest an inverse relationship between the concentrations of lipid peroxides and the rate of cell proliferation, i.e. the higher rate of lipid peroxidation in the cells with lower the rate of cell division (Das, 2002). In this study, ACME supplementation to DMH treated rats resulted in the increase of intestinal and colonic MDA levels. It clearly suggests that ACME can protect cells from loss of their oxidative capacity due to the administration of the procarcinogen DMH.

DMH treatment generates free radicals in colonic tissue and their level is controlled by antioxidants (Hamiza et al., 2012). Elimination of free radicals in biological systems is achieved through enzymatic (Catalase, GPx, GR etc.) and non enzymatic (GSH) antioxidants, which act as major defense systems against free radicals (Nandhakumar et al., 2012). CAT and GPx are considered to be the primary antioxidant enzymes because they are involved in the direct elimination of reactive oxygen species (Yu, 1994). Low level of CAT activity in the cancerous tissue promotes the growth of cancer and its infiltration into the surrounding tissues, which is important for invasion and metastasis (Janssen et al., 1999). Our study also demonstrated the decreased levels of intestinal and colonic CAT activity in rats treated alone with DMH. However the supplementation of ACME in different treatment regimens significantly ($p \leq 0.05$) elevated the CAT activity and which could be important in inhibiting the carcinogenic changes induced by DMH.

The glutathione antioxidant system includes GSH, GPx, GR and GST. GSH can act either as a non-enzymatic antioxidant by direct interaction of -SH group with reactive oxygen species (ROS) or it can be implicated in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme (Hamiza et al., 2012). GPx is an enzyme containing four selenium-cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydro peroxides. GST and GR are secondary antioxidant enzymes that help in the detoxification of reactive oxygen species by decreasing peroxide levels or by maintaining a steady supply of metabolic intermediates like GSH. Thus GSH and its dependent enzymes work in concert with other antioxidants and antioxidant enzymes to protect cells against reactive oxygen intermediates (Sreedharan et al., 2009). In this study, the significant increase of intestinal and colonic GST, GR, GPx and CAT activities with concomitant GSH replenishment on ACME treatment, could be important in inhibiting DMH induced colon carcinogenesis. In addition, a previous study conducted in our laboratory demonstrated that the methanolic extract of *A campanulatus* tuber has the potential to ameliorate DMH induced hepatotoxicity by markedly enhancing the antioxidant enzyme systems of the liver (Ansil et al., 2013). Therefore, the possible mechanism by which ACME mediates its anticancer activity could be through its ability to function as an antioxidant.

DMH enhances cellular proliferation in the colon but not in other organs of the experimental animals (Ohno et al., 2001). Cell proliferation is linked with the expression and synthesis of proliferative cell nuclear antigen (PCNA). It is a non-histone nuclear acidic protein (36 kDa) expressed in the nuclei of proliferating cells during G1 and S phase of cell cycle. PCNA act as a co-factor of DNA-polymerase delta (δ) and exert its function in DNA synthesis mainly at the S-phase of the cell cycle. Thus, PCNA has been widely used for evaluating cell proliferative activity in animal models used to test carcinogenesis or cancer prevention (Bravo et al., 1987; Salim et al., 2011). It is already reported that PCNA is an important biomarker in colorectal cancer (Kelman et al.,

1999; Nagendraprabhu and Sudhandiran, 2011). In the current study, DMH administered rats showed amplified expression of PCNA in the colon thereby indicating the hyper-proliferative activity of colon cells. Decreased expression of this important proliferative marker was clearly noted upon initiation, post-initiation and entire period of ACME treatment regimens, that in turn reflects a decrease in S phase cells and thus reduced proliferative activity.

We have previously reported the phytochemical constituents such as alkaloids, tannins, glycosides, phenols, flavonoids, saponins and carbohydrates in ACME (Ansil et al., 2011). Published report also establishes the presence of betulinic acid, lupeol, stigmasterol, β-sitosterol, glucose, galactose, rhamnose and xylose in the corm of *A campanulatus* (Khare, 2007). Thus, in the present study, the chemopreventive activity exhibited by the methanolic extract of *A campanulatus* tuber might be attributed to the presence of the reported phytochemical constituents in single or in combination.

To summarize, the histological findings, tissue lipid peroxidation and antioxidant profile of control and experimental group of rats together emphasize the chemopreventive effect of ACME against chemically induced colonic preneoplastic progression in rats. Though the supplementation of ACME during the initiation, post-initiation and entire period of the study significantly suppressed colonic neoplastic changes, the entire period treatment regimen was found to be the most effective method of treatment as compared to the other treatment regimens. Therefore *A campanulatus* tuber might have practical applications as a chemopreventive agent; however, further studies are required before ACME can be claimed as a therapeutic agent against colon cancer.

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