Chapter 4

CHEMOPREVENTIVE EFFECT OF AMORPHOPHALLUS CAMPANULATUS (ROXB.) BL. TUBER ON COLON CARCINOGENESIS
Chapter 4 (A)

Anticarcinogenic activity of *Amorphophallus campanulatus* (Roxb.) Bl. tuber on experimental colon cancer – A dose dependent study
4A.1. INTRODUCTION

Colon cancer, also called colorectal cancer or bowel cancer, is ranked as the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the Western world (American Cancer Society, 2011a). The incidence rates of colorectal cancer continue to increase in economically transitioning countries including most parts of Asia where the overall risk was formerly low. This increase may reflect the adoption of Western lifestyles and behaviours (Center et al., 2009). The risk factors of colon cancer include family history, mutation of genes, western style diet, inflammation, Crohn’s disease and inflammatory bowel disease. It is frequently a pathological consequence of persistent oxidative stress, leading to DNA damage; mutations in cancer related genes and epigenetic silencing of tumor suppressor genes (Kim and Milner, 2007).

Progression of the disease from benign colorectal adenoma to malignant carcinoma involves accumulation of molecular alterations, including chromosomal abnormalities, genetic mutations, and epigenetic changes over a period of time (Kaur et al., 2006). The long latency period for the development of full-blown disease provides a much needed opportunity for the intervention of colorectal cancer employing potential chemopreventive strategies. Chemoprevention refers to the use of natural or synthetic compounds to prevent, reverse, or delay the development of cancer. Because food-derived products exist universally and are expected to be safe, they are highly interesting for development as chemopreventive agents (Aranganathan et al., 2009; Winawer et al., 1993). In this context, identification and/or development of chemopreventive agents, which selectively target molecular
events linked to cancer progression, could be an effective approach (Arber and Levin, 2005).

Colorectal cancer is strongly related to diet, and thus dietary strategies may reduce the risk of this cancer. Epidemiologic studies have shown that consumption of fruits and vegetables based diet reduces the risk of cancer, especially cancers of digestive tracts (La Vecchia, 2004). *A. campanulatus* tuber, widely consumed as food, is traditionally used for the treatment of abdominal tumors (Warrier et al., 1994). Furthermore, the phytochemical constituents identified by the LC-MS analysis of ACME such as Ferulic acid, Retinol, Quercetin and Asiatic acid are well-known for its anti-colon cancer properties. In view of this, the present study was conducted to reveal the dose dependent chemopreventive nature of ACME on 1, 2-dimethylhydrazine (DMH) induced colon carcinogenesis in male Wistar rats.

4A.2. MATERIALS AND METHODS

4A.2.1. Chemicals

1, 2-Dimethylhydrazine dihydrochloride (DMH), Monoclonal anti-proliferating cell nuclear antigen (PCNA), 3, 3’-Diaminobenzidine tetrahydrochloride (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.

4A.2.2. Animals and diets

Male Wistar rats weighing 160 ± 5.3gm (Mean ± S.D, n = 30) were used in this study. The animals were housed in polypropylene cages and held in quarantine for one week and had access to drinking water *ad libitum*. The animals were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12
dark cycle. Animal studies were followed according to Institutional Animal Ethics Committee regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg. No. B 2442009/6) and conducted humanely. Commercial pellet diet containing 4.2% fat was powdered and mixed with 15.8% peanut oil making a total of 20% fat in the diet (Aranganathan et al., 2009). This modified powdered pellet diet was fed to rats in all groups throughout the experimental period of 15 weeks.

4A.2.3. Preparation of plant extracts

The powdered tubers of *A. campanulatus* were subjected to Soxhlet extraction with methanol and concentrated under reduced pressure using a rotary evaporator. The yield of methanolic extract was 9.3% (w/w). The concentrate was suspended in 5% Tween 80 for *in vivo* studies.

4A.2.4. Carcinogen administration

DMH was dissolved in 1mM EDTA just prior to use and the pH adjusted to 6.5 with 1mM NaOH and administered subcutaneously in the right thigh of rats at a dose of 20 mg/kg body weight once a week for the first 4 consecutive weeks (Aranganathan et al., 2009).

4A.2.5. Treatment schedule

A total of 30 rats were randomly divided into 5 groups. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of drug and DMH respectively (Control). Group II rats received ACME (250 mg/kg/day; p.o.) for 15 weeks (Control + ACME 250). Group III (DMH) rats received DMH (20 mg/kg body weight) injections subcutaneously once a week for 4 consecutive weeks and then rats were kept without any treatment till 15 weeks. Groups IV - V
(DMH+ACME 125; DMH+ACME 250) animals received subcutaneous injections of DMH as in group III along with ACME at dose of 125 and 250 mg/kg body weight/day; peroral respectively for the entire experimental period of 15 weeks. The schematic representation of the experimental protocol is given in Figure 4.1.

![Experimental protocol diagram]

### Figure 4.1. Experimental protocol

#### 4A.2.6. Serum enzyme analysis

The blood collected from each animal was allowed to clot for 45 min at room temperature and the serum was separated by centrifugation at 2000 rpm at 4°C for 15 min. The hepatic oxidative damage was assessed by quantifying the serum levels of AST (EC 2.6.1.1), ALT (EC 2.6.1.2) and ALP (EC 3.1.3.1) by kinetic method.
using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes were measured using semi autoAnalyzer (RMS, India).

Percent protection was calculated using the formula,

\[
\text{Percentage protection} = \left( \frac{\text{Toxic control} - \text{Extract treated}}{\text{Toxic control} - \text{Normal control}} \right) \times 100
\]

**4A.2.7. Homogenization of tissues**

At the end of the experimental period, the animals were anesthetized with pentothal sodium followed by neck decapitation. Liver, 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).

**4A.2.8. Biochemical assays**

GSH levels in tissues were 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 H₂O₂ and NaN₃ (Rotruck et al., 1973). Tissue CAT (EC 1.11.1.6) activity was determined from
the rate of decomposition of $\text{H}_2\text{O}_2$ (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 contents of the tissues were determined using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

(Detailed protocols are given under chapter 2, section 2.2.13. Procedures for in vivo antioxidant assays)

4A.2.9. Histopathological examination

Small pieces of colon and liver tissues fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 5–6 $\mu$m were cut and stained with hematoxylin and eosin and examined for histopathological changes.

4A.2.10. Immunohistochemistry

Immunohistochemistry of PCNA was performed as described by Ramakrishnan et al. (2008). (Protocol is described in detail under chapter 2, section 2.2.9. Immunohistochemistry)

4A.2.11. Statistical analysis

Results are expressed as mean ± standard deviation (SD). 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.

4A.3. RESULTS

4A.3.1. Effect of DMH and ACME on serum marker enzymes

The results are graphically represented in figure 4.2 (A, B and C). Rats treated alone with DMH (group III) showed significantly ($p \leq 0.05$) elevated levels
of liver specific serum marker enzymes such as AST, ALT and ALP, when compared to the control group of animals. However the supplementation of ACME along with DMH significantly ($p \leq 0.05$) and dose dependently lowered the increased levels of serum enzymes. Treatment with 125 and 250 mg/kg of ACME exhibited a protection of 47.0 and 83.0% in AST levels, 62.2 and 79.1% in ALT levels and 61.5 and 83.8% in ALP levels respectively. In other words, these DMH dependent increases in serum AST, ALT and ALP levels were clearly inhibited by ACME (125 and 250 mg/kg) treatments, suggesting protective properties of ACME against DMH induced hepatic damage.

4A.3.2. 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 4.1. The level of GSH and activities of GSH dependent enzymes such as GST, GR and GPx were significantly decreased ($p < 0.05$) in liver, intestine, proximal and distal colon tissues of DMH treated rats as compared to the control group of animals. Administration of ACME at two different doses (125 mg/kg and 250 mg/kg) markedly ($p < 0.05$) 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 V animals, supplemented with ACME at 250 mg/kg body weight.
Figure 4.2. Effect of ACME on serum enzyme levels of rats intoxicated with DMH (A). Aspartate aminotransferase (B). Alanine aminotransferase

N - Normal control; DC - Drug control (ACME - 250 mg/kg); T - Toxic control (DMH); D1 - DMH +ACME - 125 mg/kg; D2 - DMH +ACME - 250 mg/kg.

Values are expressed as mean ± S.D. (n = 6). Error bar indicating the standard deviation. Statistical significance: $p < 0.05$. $^{a}$ DMH group differs significantly from control group. $^{b}$ DMH + ACME – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group $^{c}$ DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.
Figure 4.2. (Cont.) Effect of ACME on serum enzyme levels of rats intoxicated with DMH (C). Alkaline phosphatase

N - Normal control; DC - Drug control (ACME - 250 mg/kg); T - Toxic control (DMH); D1 - DMH + ACME - 125 mg/kg; D2 - DMH + ACME - 250 mg/kg.

Values are expressed as mean ± S.D. (n = 6). Error bar indicating the standard deviation. Statistical significance: *p* <0.05.  

- DMH group differs significantly from control group.
- DMH + ACME – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group.
- DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.
Table 4.1. Effect of ACME on glutathione and glutathione dependent enzyme activities in liver, intestine, proximal and distal colon tissues of control and experimental animals

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 125 mg/kg</th>
<th>DMH + ACME 250 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DMH</td>
<td>DMH + ACME 125 mg/kg</td>
<td>DMH + ACME 250 mg/kg</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>25.2 ± 0.5</td>
<td>26.1 ± 0.7</td>
<td>19.0 ± 0.3</td>
<td>22.1 ± 0.4</td>
<td>23.5 ± 0.5</td>
</tr>
<tr>
<td>Intestine</td>
<td>19.0 ± 0.4</td>
<td>20.0 ± 0.5</td>
<td>12.1 ± 0.6</td>
<td>15.1 ± 0.5</td>
<td>18.2 ± 0.6</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>27.1 ± 0.5</td>
<td>26.3 ± 0.5</td>
<td>16.8 ± 0.7</td>
<td>20.2 ± 0.6</td>
<td>23.1 ± 0.7</td>
</tr>
<tr>
<td>Distal colon</td>
<td>26.3 ± 0.6</td>
<td>25.3 ± 0.5</td>
<td>20.5 ± 0.8</td>
<td>22.3 ± 0.5</td>
<td>24.0 ± 0.4</td>
</tr>
<tr>
<td>GST (µmol CDNB-GSH conjugate formed/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>73.0 ± 0.4</td>
<td>74.1 ± 0.3</td>
<td>42.2 ± 0.5</td>
<td>56.1 ± 0.5</td>
<td>68.1 ± 0.3</td>
</tr>
<tr>
<td>Intestine</td>
<td>68.2 ± 0.5</td>
<td>69.0 ± 0.5</td>
<td>38.1 ± 0.5</td>
<td>49.0 ± 0.7</td>
<td>59.9 ± 0.2</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>70.1 ± 0.4</td>
<td>71.1 ± 0.6</td>
<td>39.1 ± 0.5</td>
<td>57.0 ± 0.4</td>
<td>64.9 ± 0.6</td>
</tr>
<tr>
<td>Distal colon</td>
<td>70.8 ± 0.8</td>
<td>70.0 ± 0.4</td>
<td>40.5 ± 1.0</td>
<td>54.0 ± 0.3</td>
<td>67.1 ± 0.4</td>
</tr>
<tr>
<td>GR (nmol of GSSG utilized/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>24.4 ± 0.7</td>
<td>22.3 ± 1.2</td>
<td>16.0 ± 1.0</td>
<td>19.0 ± 0.6</td>
<td>20.1 ± 0.8</td>
</tr>
<tr>
<td>Intestine</td>
<td>19.0 ± 0.9</td>
<td>19.6 ± 0.9</td>
<td>9.9 ± 1.3</td>
<td>14.2 ± 0.9</td>
<td>16.1 ± 0.6</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>23.3 ± 0.9</td>
<td>23.8 ± 0.9</td>
<td>18.0 ± 1.0</td>
<td>20.2 ± 0.7</td>
<td>21.9 ± 1.0</td>
</tr>
<tr>
<td>Distal colon</td>
<td>25.3 ± 0.8</td>
<td>24.5 ± 1.1</td>
<td>17.2 ± 0.9</td>
<td>21.2 ± 0.7</td>
<td>23.0 ± 0.7</td>
</tr>
<tr>
<td>GPx (nmol of GSH oxidized/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>296.2 ± 3.1</td>
<td>298.2 ± 2.6</td>
<td>151.4 ± 2.1</td>
<td>240.5 ± 1.8</td>
<td>260.5 ± 2.5</td>
</tr>
<tr>
<td>Intestine</td>
<td>286.2 ± 1.2</td>
<td>289.7 ± 1.3</td>
<td>160.9 ± 2.4</td>
<td>214.9 ± 2.7</td>
<td>243.6 ± 3.9</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>289.8 ± 3.7</td>
<td>294.1 ± 2.8</td>
<td>158.7 ± 2.5</td>
<td>229.5 ± 4.5</td>
<td>265.2 ± 2.1</td>
</tr>
<tr>
<td>Distal colon</td>
<td>274.7 ± 2.8</td>
<td>279.2 ± 2.8</td>
<td>140.4 ± 4.3</td>
<td>238.1 ± 3.6</td>
<td>250.1 ± 1.8</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D from 6 rats in each group. Statistical significance: *p* ≤ 0.05. "DMH group differs significantly from control group. "DMH + ACME – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group. "DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg."
4A.3.3. Effect of DMH and ACME on catalase

The activity of CAT in liver, intestine, proximal and distal colon tissues of control and experimental animals are shown in Table 4.2. DMH alone treated rats showed a significant \((p < 0.05)\) reduction in CAT activity as compared to the control group. However, treatment with ACME at a dose of 125 mg/kg and 250 mg/kg significantly \((p < 0.05)\) increased the activity of this enzyme. The effect was more evident when ACME supplemented at a dose of 250 mg/kg body weight.

**Table 4.2. Effect of ACME on catalase (CAT) activity in liver, intestine, proximal and distal colon tissues of control and experimental animals**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 125 mg/kg</th>
<th>DMH + ACME 250 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>51.9 ± 0.9</td>
<td>51.3 ± 0.7</td>
<td>40.9 ± 1.0 (^a)</td>
<td>46.1 ± 1.1 (^b)</td>
<td>49.0 ± 0.8 (^bc)</td>
</tr>
<tr>
<td>Intestine</td>
<td>46.0 ± 1.1</td>
<td>46.4 ± 0.9</td>
<td>24.6 ± 1.4 (^a)</td>
<td>38.3 ± 1.0 (^b)</td>
<td>44.9 ± 1.0 (^bc)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>58.4 ± 0.9</td>
<td>60.4 ± 0.7</td>
<td>43.9 ± 0.7 (^a)</td>
<td>50.2 ± 1.1 (^b)</td>
<td>56.2 ± 0.9 (^bc)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>61.0 ± 1.0</td>
<td>60.3 ± 0.9</td>
<td>52.3 ± 1.0 (^a)</td>
<td>56.3 ± 1.1 (^b)</td>
<td>58.6 ± 0.9 (^bc)</td>
</tr>
</tbody>
</table>

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 – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group \(^c\) DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.

4A.3.4. Effect of ACME on lipid peroxidation (MDA)

Table 4.3 shows the extent of lipid peroxidation in liver, intestine, proximal and distal colon tissues of control and experimental group of rats. When compared to the control group of animals (group I), a significant increase \((p \leq 0.05)\) in hepatic
MDA level was observed in DMH treated rats (group III). Administration of ACME to the rats injected with DMH (group IV and V) significantly \((p \leq 0.05)\) prevented the elevation of hepatic MDA level. But the intestinal and colonic MDA level was significantly decreased \((p < 0.05)\) in DMH treated rats at the end of 15 weeks as compared to the untreated control animals (group I). However, the supplementation of ACME to DMH treated rats showed near normal levels of MDA in intestine, proximal and distal colon tissues.

**Table 4.3. Effect of ACME on malondialdehyde (MDA) in liver, intestine, proximal and distal colon tissues of control and experimental animals**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 125 mg/kg</th>
<th>DMH + ACME 250 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>45.3 ± 0.3</td>
<td>46.2 ± 0.3</td>
<td>80.4 ± 0.3(^a)</td>
<td>72.5 ± 0.4(^b)</td>
<td>54.4 ± 0.3(^bc)</td>
</tr>
<tr>
<td>Intestine</td>
<td>54.2 ± 0.3</td>
<td>56.2 ± 0.4</td>
<td>25.0 ± 0.3(^a)</td>
<td>34.2 ± 0.5(^b)</td>
<td>51.4 ± 0.6(^bc)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>75.9 ± 0.5</td>
<td>77.8 ± 0.5</td>
<td>41.1 ± 0.6(^a)</td>
<td>55.1 ± 0.8(^b)</td>
<td>66.1 ± 0.5(^bc)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>80.9 ± 0.5</td>
<td>83.1 ± 0.4</td>
<td>47.0 ± 0.4(^a)</td>
<td>59.0 ± 0.4(^b)</td>
<td>72.1 ± 0.6(^bc)</td>
</tr>
</tbody>
</table>

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 – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group. 
\(^c\) DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.

**4A.3.5. Histopathological analysis**

Histopathological examination of the liver revealed that the normal architecture of hepatic tissue (Figure 4.3.A) was completely lost in rats treated alone with DMH (group III) (Figure 4.3.C) with the appearance of cell necrosis, haemorrhage, fatty infiltration and pleomorphic nuclei. But the administration of
ACME (125 and 250 mg/kg) to DMH treated animals (Figure 4.3.D and 4.3.E) seems to prevent such hepatic changes as evident from hepatic architectural pattern with mild to moderate hepatitis.

Figure 4.4 represents the histopathological changes observed microscopically in the colon of control and experimental rats. Tissue sections of control rats (group I) displayed normal crypts and colonic architecture with no signs of apparent abnormality (Figure 4.4.A). In group II rats (supplemented alone with 250 mg/kg of ACME) also the colonic mucosal epithelium appeared normal on histological examination (Figure 4.4.B). Mucin filled carcinoma glands, enlarged nuclei and hyperchromatism with mitotic figures were observed in rats treated alone with DMH (group III). Larger areas of thickened mucosal layer with densely packed inflammatory cell infiltration were also noted in group III animals (Figure 4.4.C). In ACME treated rats (group IV and group V), the histology revealed no loss of nuclear polarity and also showed a dose dependent reduction in mucin filled carcinoma gland formation. Few areas showed mucosal thickenings with scattered or no infiltration of the inflammatory cells in the mucosa (Fig. 4.4.D and 4.4.E).
Figure 4.3. Effect of ACME on DMH induced histopathological changes in liver tissue. (Hematoxylin and eosin, 100×)

(A) Normal Control. (B) Drug control (ACME - 250 mg/kg). (C) DMH control (20 mg/kg; s.c. once a week for the first 4 consecutive weeks) (D) DMH + ACME (125 mg/kg) and (E) DMH + ACME (250 mg/kg).
Figure 4.4. Histopathological changes in the colon tissues of control and experimental rats. (Hematoxylin and eosin, 100×)

(A) Colon of control rat with normal mucosal and submucosal layers. (B) Colon of ACME alone (250 mg/kg) treated rat with normal mucosal and submucosal layers. (C) Colon of DMH alone treated rat showing carcinoma glands filled with mucin, enlarged nuclei, hyperchromatism, mitotic figures and densely packed inflammatory cell infiltration. (D) and (E) Colon of ACME treated rats (125 mg/kg and 250 mg/kg respectively) showing normal mucosal folds and scattered inflammatory cell infiltration.
4A.3.6. Immunohistochemistry of PCNA

Immunohistochemical staining for PCNA in the colon of control and experimental group of animals are depicted in Figure 4.5. The expression of PCNA which was observed as dense brown colour spots, significantly increased (p<0.05) in group II animals treated with DMH alone as compared with group I normal control animals (Figure 4.5.C). Control (Figure 4.5.A) and ACME treated (Figure 4.5.B) rats, however, showed few positive expressions of PCNA. In case of ACME administration (125 mg/kg and 250 mg/kg in group IV and group V respectively) to DMH treated rats, it extensively abridged (p < 0.05) the expression of PCNA when compared with rats treated alone with DMH (Figure 4.5.D and Figure 4.5.E). The quantification of PCNA is presented in Figure 4.5.F
Figure 4.5. Immunohistochemical staining of PCNA in control and experimental animals (100x)

(A) Control. (B) Drug control (ACME – 250 mg/kg). (C) PCNA staining in rats treated with DMH alone, showing brown stain nuclei, indicating PCNA protein accumulation. (D) DMH + ACME – 125 mg/kg. (E) DMH + ACME – 250 mg/kg.

(F) Graph of percentage of PCNA positive cells in control and experimental groups. Arrow indicating the expression of PCNA.

Values are expressed as mean ± S.D. (n = 6). Statistical significance: $p <0.05$. $^a$ DMH group differs significantly from control group. $^b$ DMH + ACME – 125 mg/kg and DMH + ACME – 250 mg/kg groups differs significantly from DMH alone treated group $^c$ DMH + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.
4A.4. DISCUSSION

Our previous study established that *A. campanulatus* tuber particularly its methanolic extract is a potential source of natural antioxidant. Johnson et al. (1994) reported that many anti-oxidant substances have anti-cancer or anti-carcinogenic properties. Hence in the present study, we evaluated the tuber for its chemopreventive potential on DMH induced experimental colon carcinogenesis.

The organotropic colon carcinogen DMH is an alkylating agent widely used to induce benign and malignant neoplasms in the colon of rodents. The experimental colon cancer induced by DMH in rats mimic human colon cancer and is therefore, an ideal model for chemoprevention studies (Cooper et al., 1978). DMH is a procarcinogen, which requires metabolic activation within the host to become an active carcinogen. The cells at the subcutaneous site do not possess the enzymes capable of reacting with DMH. Hence, subcutaneously administered DMH is released slowly into the circulation and reaches the liver. DMH is metabolized in the liver into methylazoxymethanol (MAM), which is catalyzed by the enzyme cytochrome P450 IIE1 (Sohn et al., 1991). Metabolic activation of MAM to highly reactive carcinogenic electrophiles (methylidiazonium ion and carbonium ion) occurs in the liver and colon, which is known to elicit oxidative stress (Fiala et al., 1987). Oxidative stress occurs when the production of free radicals increases, scavenging of free radicals or repair of oxidatively damaged macromolecules decreases, or both (Fiala, 1977; Van Rossen et al., 2000).

Cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes. PCNA is an auxiliary protein of the DNA polymerase delta, reaching an expression peak during the S-phase of the cell cycle and playing
an important role in cellular proliferation. PCNA has been used as an intermediate biomarker in the chemoprevention studies of colorectal cancer (Cohen, 1998; Hall et al., 1990). In the current study the over expression of PCNA observed in rats treated alone with DMH reflects increased cell proliferation. DMH administered animals treated with ACME showed a decline in the number of PCNA positive cells that in turn reflects a decrease in S phase cells and thus reduced proliferative activity. It is already reported that most potential chemopreventive agents against chemical-induced colon carcinogenesis suppress the proliferation of colonic cells and it can be quantitatively expressed through PCNA labeling index (Sangeetha et al., 2010).

Histopathological examination of liver tissue revealed the hepatic damage and appearance of pre-neoplastic lesions in rats treated alone with DMH (Fig. 4.3.C). Liver sections of ACME supplemented rats showed the signs of recovery from DMH induced hepatotoxicity and carcinogenicity in a dose dependent manner. Results from histopathological studies of colon tissues showed a great degree of variation in the different experimental groups. Carcinoma glands filled with mucin were observed in DMH treated rats, resulted after 4 weeks of carcinogen administration. Supplementation of ACME throughout the experimental period to DMH treated rats showed the reduced malignant glands and it emphasize the anticarcinogenic effect of the extract.

In the present study, the subcutaneous injection of DMH (20 mg/kg body weight) once a week for 4 consecutive weeks led to hepatic damage, which has been proven by the significant difference in biochemical markers between the DMH control and normal control rats. The increase in the activities of AST, ALT and ALP in serum of toxic control rats might be due to the increased permeability of plasma
membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Bumrela and Naik, 2012). Treatment with ACME at a dose of 250 mg/kg produced a better restoration of serum enzyme levels than 125 mg/kg, shows the dose response action of the extract against DMH induced increase of serum enzymes. This may be related to the activity of ACME in stabilization of the plasma membranes as well as the repair of hepatic tissue damage due to DMH.

Studies have shown that the generation of reactive oxygen species may be involved in various carcinogenic processes. Cancer cells can be subjected to increased and persistent oxidative stress due to elevated levels of intracellular ROS generation. Reducing oxidative stress can therefore suppress the proliferation of tumor cells and enhance apoptosis (Sun et al., 2004). One of the plausible ways to prevent reactive oxygen species mediated cellular injury is to augment or fortify endogenous defense capacity against oxidative stress through dietary or pharmacological intake of antioxidants (Fang et al., 2002).

The intra cellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants like reduced glutathione (GSH) and other thiols. GSH, GST, GR, GPx and CAT are the priority reactive species scavengers of the antioxidant system in defense for oxidative stress occurring in many pathological conditions including cancer. Its deficiency may leads to the accumulation of reactive oxygen metabolites and this may cause the initiation of carcinogenesis (Cobanoglu et al., 2010). The observed decrease in hepatic GSH level and GST, GR, GPx and CAT activities of DMH treated animals in the present study indicates that the liver is susceptible to oxidative damage during colon carcinogenesis. ACME supplementation significantly ($p \leq$
0.05) aided to maintain their activities near to normal level in hepatic tissue. Thus, it is evidently clear that one of the ways by which ACME exerts its chemopreventive effect is by modulating the activities of antioxidant enzymes in hepatic tissues. Furthermore, the biochemical evidences from our study demonstrated that the GSH level and GST, GR, GPx and CAT activities of the intestine, proximal and distal colon tissues were lower in rats treated with DMH (group III), as compared to the control group. Low levels of antioxidant activity in the cancerous tissue promote the growth of cancer and its infiltration into the surrounding tissues, which is important for invasion and metastasis (Janssen et al., 1999). However, ACME supplementation (group IV and V) significantly (P<0.05) elevated the level of antioxidants in the intestines, proximal and distal colon tissues. This may be due to the reduced oxidative stress and possibly enhanced repair mechanisms in the colonic mucosa and both could be important in inhibiting the DMH induced carcinogenesis.

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Devasagayam et al., 2003). In the present study, the elevation of hepatic lipid peroxidation as evidenced by the increased levels of MDA in DMH alone exposed group (group III) of animals could be due to the reactive oxygen metabolites released during the metabolism of DMH in the liver. ACME supplementation potentially reduced MDA level, suggesting that ACME might have antioxidant principles to produce such response. However, the cancer cells are known to have highly evolved protective mechanisms to prevent lipid peroxidation so that rapid cell proliferation can occur (Conklin, 2000). Similar to our present study, several studies have demonstrated that lipid peroxidation is decreased significantly in tumor cells
and tissues as compared to that in corresponding normal cells and tissues (Bartoli and Galeotti, 1979). We observed that ACME supplementation to DMH treated rats at a dose of 250 mg/kg (group V) resulted in near normal levels of MDA in intestinal and colonic tissues. Previous reports also suggest an inverse relationship between the concentrations of lipid peroxides and the rate of cell proliferation, i.e. higher the rates of lipid peroxidation in the cell, the lower will be the rate of cell division (Sreedharan et al., 2009).

The chemopreventive activity of ACME, observed in the present study, against colon carcinogenesis might be attributed to the combined activity of anti-colon cancer phytochemicals viz., Ferulic acid, Retinol, Quercetin and Asiatic acid, which are already identified by the LC-MS analysis of ACME. It is reported that ferulic acid, a phenolic compound, possess antiproliferative effect against colon cancer cell lines (Janicke et al., 2011). Delage et al. (2005) reported that retinol also known as vitamin A, exhibited a protective role against disturbances associated with hyperlipidic diet exposure in DMH induced colon carcinogenesis. Besides, quercetin - the ubiquitous bioactive flavonoid, has also been shown to induce growth inhibition in colon cancer cells. Kim et al. (2010) reported that quercetin induces apoptosis via AMP-activated protein kinase (AMPK) activation and p53-dependent apoptotic cell death in HT-29 colon cancer cells. Asiatic acid, a triterpene, is known to be cytotoxic to several tumor cell lines and can induce apoptosis in colon cancer RKO cells (Cho et al., 2006).

To summarize, the results of the present study evidently indicates that the administration of colon specific procarcinogen DMH brings about profound alterations in tissue lipid peroxidation and antioxidant status of hepatic, intestinal
and colonic tissues. Whereas the supplementation of ACME significantly protected the animals from DMH induced carcinogenic changes. In addition, the results revealed that in DMH induced rat colon cancer, ACME exert its protective effect in a dose dependent manner. ACME at a dose of 250 mg/kg was able to exert a more pronounced effect, as shown histologically by a significant reduction in the extent and severity of lesions in colon tissue, abridged expression of PCNA and also biochemically by the modulation of hepatic, intestinal and colonic antioxidant status and lipid peroxidation. The present dose dependent preliminary investigation also depicts that 250 mg/kg b.w. of ACME would be an effective dose for long-term studies. Further, it is obvious that one of the mechanisms by which ACME exerted its chemopreventive potential on DMH induced colon tumorigenesis is by the enhancement of antioxidant enzyme systems in the liver and thereby the metabolic disposal of carcinogenic DMH metabolites. The role of ACME as a free radical quencher and its role in conserving the pro-oxidant–antioxidant balance suggested that ACME is a promising agent for hepatoprotection as well as a good candidate for chemoprevention.
Chapter 4 (B)

Chemopreventive efficacy of *Amorphophallus campanulatus* (Roxb.) Bl. tuber against 1, 2-dimethylhydrazine induced colon carcinogenesis - a long term preclinical model
4B.1. 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).

Induction of colon tumors in rats by the administration of 1, 2-dimethylhydrazine (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 methylidiazonium ion. The activated carcinogen reaches the colon either via the blood or bile. Once methylidiazonium 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.,
The damaged DNA 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 (Fernandes et al., 2011; Roncucci et al., 1991). 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). Our previous studies revealed the antioxidant and anticancer properties of the methanolic extract of *Amorphophallus campanulatus* tuber (ACME), as discussed in the preceding chapters. More precisely, the ACME at a dose of 250 mg/kg was found to possess promising chemopreventive activity against 1, 2-dimethylhydrazine (DMH) induced colon carcinogenesis in rats. Thus the present study was designed to investigate the effect of 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.

4B.2. MATERIALS AND METHODS

4B.2.1. Chemicals

1, 2-Dimethylhydrazine dihydrochloride (DMH), Monoclonal anti-proliferating cell nuclear antigen (PCNA), 3, 3’-Diaminobenzidine tetrahydrochloride (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.

4B.2.2. Animals, diets and care

Animal studies were followed according to Institutional animal ethics committee regulations approved by committee for the purpose of control and supervision of experiments on animals (Reg. No. B 2442009/6). Male Wistar rats weighing 162.6 ± 13.3g (Mean ± S.D) 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.

4B.2.3. Collection and preparation of plant extract

The shade-dried tubers of *A. campanulatus* were powdered and subjected to Soxhlet extraction with methanol (50 g in 400 mL) and 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.

4B.2.4. Dose selection

The previous dose dependent study established that ACME at a dose of 250 mg/kg b.w. is most effective against DMH induced colon carcinogenesis in rats. Accordingly, in the present long term pre-clinical model experiment the dose of ACME was fixed to 250 mg/kg b.w.

4B.2.5. 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).

4B.2.6. 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 4.6.
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.

4B.2.8. Serum enzyme analysis

The blood collected from each animal was allowed to clot for 45 min at room temperature and the serum was separated by centrifugation at 2000 rpm at 4°C for 15 min. The carcinogen (DMH) induced hepatic damage was assessed by
quantifying the serum levels of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), ALP (EC 3.1.3.1) and LDH (EC 1.1.1.27) by kinetic method using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes were measured using semi autoanalyzer (RMS, India).

Percent protection was calculated using the formula,

\[
\text{Percentage protection} = \frac{\text{Toxic control} - \text{Extract treated}}{\text{Toxic control} - \text{Normal control}} \times 100
\]

4B.2.9. Identification of aberrant crypt foci (ACF)

Enumeration of ACF was performed as described by Bird (1987). Detailed protocol of ACF counting is given in chapter 2, section 2.2.10. Identification of aberrant crypt foci.

4B.2.10. Homogenization of tissues

At the end of the experimental period, the animals were anesthetized with pentothal sodium followed by neck decapitation. Liver, 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).

4B.2.11. Biochemical assays

GSH levels in tissues were 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$_2$O$_2$ and NaN$_3$ (Rotruck et al., 1973). Tissue CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H$_2$O$_2$ (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 contents of the tissues were determined using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

(Detailed protocols are given in chapter 2, section 2.2.13. Procedures for in vivo antioxidant assays)

**4B.2.12. Histopathological examination**

Small pieces of liver and 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.

**4B.2.13. Immunohistochemistry**

Immunohistochemistry of PCNA was performed as described by Ramakrishnan et al. (2008). (Protocol is described in detail under chapter 2, section 2.2.9. Immunohistochemistry)
4B.2.14. Statistical analysis

Results are expressed as mean ± standard deviation (SD). 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.

4B.3. RESULTS

4B.3.1. 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 4.4. 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 was significantly (\( P < 0.05 \)) 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 4.7 represents the morphological changes observed in the longitudinally opened colon of rats treated alone with DMH (Group III).
Table 4.4. Effect of DMH and ACME on change in body weight and growth rate of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Growth Rate (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>150.3 ± 8.9</td>
<td>284.5 ± 13.5</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Drug Control</td>
<td>153.7 ± 6.3</td>
<td>288.2 ± 15.4</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>DMH Control</td>
<td>177.8 ± 10.3</td>
<td>218.8 ± 19.5</td>
<td>0.19 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Initiation</td>
<td>160.7 ± 12.7</td>
<td>233.3 ± 15.3</td>
<td>0.34 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Post-initiation</td>
<td>165.5 ± 13.4</td>
<td>270.2 ± 16.5</td>
<td>0.49 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Entire Period</td>
<td>167.7 ± 8.8</td>
<td>285.7 ± 17.9</td>
<td>0.56 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup><i>p</i> ≤ 0.05 versus normal control. <sup>b</sup><i>p</i> ≤ 0.05 versus DMH control. Values are expressed as mean ± S.D.

Figure 4.7. Morphological changes observed in the longitudinally opened colon of one of the rats treated alone with DMH

4B.3.2. 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 4.5. 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 ≤ 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 4.6. When compared to the DMH alone treated group III animals, the number of small, medium and large crypts was significantly (P ≤ 0.05) lowered in groups IV-VI rats supplemented with ACME. Topographical view of ACF from different groups are shown figure 4.8. 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.

Table 4.5. Incidence of ACF in control and experimental rats

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

aP ≤ 0.05 versus DMH control. Values are expressed as mean ± S.D.
Table 4.6. Effect of ACME and DMH on the incidence of different category of ACF in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of ACF per colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small (&lt;3 crypts/focus)</td>
</tr>
<tr>
<td>DMH Control</td>
<td>30.1±2.9</td>
</tr>
<tr>
<td>Initiation</td>
<td>19.2±1.8\textsuperscript{a}</td>
</tr>
<tr>
<td>Post-initiation</td>
<td>13.8±1.3\textsuperscript{a}</td>
</tr>
<tr>
<td>Entire Period</td>
<td>10.1±1.0\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}p ≤ 0.05 versus DMH control. Values are expressed as mean ± S.D

4B.3.3. Effect of DMH and ACME on serum marker enzymes

The results are graphically depicted in figure 4.9 (A, B, C and D). Rats treated alone with DMH (group III) showed significantly (p ≤ 0.05) elevated levels of liver specific serum marker enzymes such as AST, ALT, ALP and LDH, when compared to the control group of animals (group I). However the supplementation of ACME along with DMH significantly (p ≤ 0.05) lowered the increased levels of serum enzymes in all the treatment groups. In initiation, post-initiation and entire period groups treatment with 250 mg/kg of ACME exhibited a protection of 73.4, 83.4 and 97.8% in AST levels, 72.3, 86.5 and 92% in ALT levels, 43.8, 69.2 and 86% in ALP levels and 70.7, 88.9 and 100% in LDH levels respectively.
Figure 4.8. 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); G - Colon of DMH + ACME treated rats (Entire period). Arrows indicate the ACF.
4B.3.4. Effect of DMH and ACME on GSH and glutathione dependent enzymes

Table 4.7 shows the effect of DMH and ACME on the activities of GSH and glutathione dependent enzymes of control and experimental groups of animals. The level of GSH and activities of GSH dependent enzymes such as GST, GR and GPx were significantly decreased ($p < 0.05$) in liver, intestine, proximal and distal colon tissues of DMH treated rats as compared to the 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 ($p < 0.05$) 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.

![Figure 4.9. Effect of ACME in serum enzyme levels of rats intoxicated with DMH (A). Aspartate aminotransferase](image)

(Control and experimental groups: NC - Normal Control; DC - Drug Control; C - DMH Control; I – Initiation; PI - Post-initiation; EP - Entire Period.

* $p \leq 0.05$ versus normal control. $p \leq 0.05$ versus DMH control. Values are expressed as mean ± S.D.)
Figure 4.9. (Cont.) Effect of ACME in serum enzyme levels of rats intoxicated with DMH (B). Alanine aminotransferase (C). Alkaline phosphatase

NC - Normal Control; DC - Drug Control; C - DMH Control; I – Initiation; PI - Post-initiation; EP - Entire Period.

\( ^a p \leq 0.05 \) versus normal control. \( ^b p \leq 0.05 \) versus DMH control. Values are expressed as mean ± S.D.
Figure 4.9. (Cont.) Effect of ACME in serum enzyme levels of rats intoxicated with DMH (D). Lactate dehydrogenase.

NC - Normal Control; DC - Drug Control; C - DMH Control; I – Initiation; PI - Post-initiation; EP - Entire Period.

\[ a^p \leq 0.05 \] versus normal control. \[ b^p \leq 0.05 \] versus DMH control. Values are expressed as mean ± S.D.
Table 4.7. Effect of ACME on glutathione and glutathione dependent enzyme activities in liver, intestine, proximal and distal colon tissues of control and experimental animals

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 250 mg/kg (I)</th>
<th>DMH + ACME 250 mg/kg (PI)</th>
<th>DMH + ACME 250 mg/kg (EP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>23.2 ± 0.7</td>
<td>24.1 ± 0.4</td>
<td>16.0 ± 1.1 a</td>
<td>18.9 ± 0.6 b</td>
<td>20.0 ± 0.3 b</td>
<td>21.4 ± 0.9 b</td>
</tr>
<tr>
<td>Intestine</td>
<td>20.0 ± 0.5</td>
<td>20.0 ± 0.9</td>
<td>13.2 ± 0.5 a</td>
<td>15.0 ± 0.4 b</td>
<td>16.3 ± 0.8 b</td>
<td>18.0 ± 0.7 b</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>28.3 ± 0.8</td>
<td>27.0 ± 0.4</td>
<td>14.9 ± 0.6 a</td>
<td>18.0 ± 0.6 b</td>
<td>21.1 ± 0.8 b</td>
<td>24.0 ± 0.6 b</td>
</tr>
<tr>
<td>Distal colon</td>
<td>25.7 ± 1.0</td>
<td>27.0 ± 0.7</td>
<td>19.9 ± 0.6 a</td>
<td>22.1 ± 0.9 b</td>
<td>23.8 ± 0.8 b</td>
<td>25.5 ± 1.0 b</td>
</tr>
<tr>
<td>GST (µmol CDNB-GSH conjugate formed/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>73.0 ± 1.4</td>
<td>74.5 ± 1.6</td>
<td>40.0 ± 1.5 a</td>
<td>53.9 ± 1.8 b</td>
<td>59.0 ± 2.0 b</td>
<td>67.0 ± 1.9 b</td>
</tr>
<tr>
<td>Intestine</td>
<td>69.0 ± 1.6</td>
<td>68.0 ± 1.4</td>
<td>36.0 ± 1.4 a</td>
<td>47.9 ± 2.0 b</td>
<td>55.0 ± 1.8 b</td>
<td>61.4 ± 1.7 b</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>71.1 ± 1.6</td>
<td>72.5 ± 1.5</td>
<td>35.0 ± 1.7 a</td>
<td>49.9 ± 1.8 b</td>
<td>58.0 ± 1.5 b</td>
<td>65.8 ± 1.7 b</td>
</tr>
<tr>
<td>Distal colon</td>
<td>70.0 ± 2.0</td>
<td>69.2 ± 1.9</td>
<td>36.9 ± 1.5 a</td>
<td>52.0 ± 1.8 b</td>
<td>61.0 ± 1.9 b</td>
<td>66.7 ± 1.8 b</td>
</tr>
<tr>
<td>GR (nmol GSSG utilized/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>23.1 ± 0.9</td>
<td>22.0 ± 0.5</td>
<td>12.9 ± 0.8 a</td>
<td>16.0 ± 0.8 b</td>
<td>17.9 ± 1.0 b</td>
<td>20.0 ± 0.5 b</td>
</tr>
<tr>
<td>Intestine</td>
<td>17.9 ± 1.0</td>
<td>17.5 ± 0.9</td>
<td>8.0 ± 0.7 a</td>
<td>12.2 ± 0.8 b</td>
<td>14.0 ± 0.4 b</td>
<td>16.1 ± 0.7 b</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>22.1 ± 0.5</td>
<td>23.0 ± 0.5</td>
<td>15.2 ± 0.8 a</td>
<td>18.0 ± 0.5 b</td>
<td>19.1 ± 0.8 b</td>
<td>20.0 ± 1.0 b</td>
</tr>
<tr>
<td>Distal colon</td>
<td>23.9 ± 0.8</td>
<td>23.1 ± 0.9</td>
<td>16.0 ± 0.5 a</td>
<td>18.9 ± 0.7 b</td>
<td>20.2 ± 0.6 b</td>
<td>21.3 ± 0.9 b</td>
</tr>
<tr>
<td>GPx (nmol of GSH oxidized/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>297.0±4.6</td>
<td>296.0±4.0</td>
<td>144.7±4.9 a</td>
<td>200.3±5.0 b</td>
<td>229.7±5.0 b</td>
<td>265.3±3.3 b</td>
</tr>
<tr>
<td>Intestine</td>
<td>279.3±2.9</td>
<td>283.3±3.7</td>
<td>154.7±3.1 a</td>
<td>203.7±5.0 b</td>
<td>220.0±4.2 b</td>
<td>255.0±4.6 b</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>285.8±4.4</td>
<td>279.0±5.1</td>
<td>142.2±3.5 a</td>
<td>213.2±4.9 b</td>
<td>236.0±5.5 b</td>
<td>260.8±3.5 b</td>
</tr>
<tr>
<td>Distal colon</td>
<td>275.0±5.5</td>
<td>281.3±3.3</td>
<td>135.7±4.0 a</td>
<td>209.7±4.6 b</td>
<td>225.0±4.2 b</td>
<td>251.3±5.0 b</td>
</tr>
</tbody>
</table>

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.
4B.3.5. Effect of DMH and ACME on catalase

Table 4.8 shows the activity of CAT in liver, intestine, proximal and distal colon tissues of control and experimental animals. DMH alone treated rats showed a significant \( p < 0.05 \) reduction in CAT activity as compared to the normal control group. But on ACME supplementation (Group IV-VI) the CAT activity was significantly \( p < 0.05 \) 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 4.8. Effect of ACME on catalase (CAT) activity in liver, intestine, proximal and distal colon tissues of control and experimental animals

<table>
<thead>
<tr>
<th>CAT (U/mg protein)</th>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 250 mg/kg (I)</th>
<th>DMH + ACME 250 mg/kg (PI)</th>
<th>DMH + ACME 250 mg/kg (EP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>50.0 ± 1.7</td>
<td>52.0 ± 1.6</td>
<td>35.9 ± 2.0(^a)</td>
<td>41.0 ± 1.9(^b)</td>
<td>43.9 ± 1.5(^b)</td>
<td>46.2 ± 2.0(^b)</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>47.1 ± 2.1</td>
<td>46.0 ± 1.9</td>
<td>22.3 ± 1.5(^a)</td>
<td>30.1 ± 2.2(^b)</td>
<td>34.0 ± 1.9(^b)</td>
<td>41.0 ± 1.7(^b)</td>
</tr>
<tr>
<td></td>
<td>Proximal colon</td>
<td>58.0 ± 1.8</td>
<td>57.1 ± 1.9</td>
<td>39.9 ± 2.3(^a)</td>
<td>44.3 ± 1.7(^b)</td>
<td>50.0 ± 1.5(^b)</td>
<td>54.1 ± 2.3(^b)</td>
</tr>
<tr>
<td></td>
<td>Distal colon</td>
<td>59.2 ± 1.7</td>
<td>60.0 ± 1.9</td>
<td>45.0 ± 1.9(^a)</td>
<td>49.0 ± 1.6(^b)</td>
<td>51.9 ± 2.0(^b)</td>
<td>54.9 ± 2.3(^b)</td>
</tr>
</tbody>
</table>

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.

4B.3.6. Effect of ACME on lipid peroxidation (MDA)

The extents of lipid peroxidation in liver, intestine, proximal and distal colon tissues of control and experimental group of rats are depicted in table 4.9. When compared to the control group of animals (group I), a significant increase \( p \leq 0.05 \) in
hepatic MDA level was observed in rats treated alone with DMH (Group III). Supplementation of ACME to the rats injected with DMH (Group IV - VI) significantly ($p \leq 0.05$) prevented the elevation of hepatic MDA level. But the intestinal and colonic MDA levels were significantly ($p < 0.05$) 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 ($p < 0.05$) 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.

**Table 4.9. Effect of ACME on malondialdehyde (MDA) in liver, intestine, proximal and distal colon tissues of control and experimental animals**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Control + ACME 250 mg/kg</th>
<th>DMH</th>
<th>DMH + ACME 250 mg/kg (I)</th>
<th>DMH + ACME 250 mg/kg (PI)</th>
<th>DMH + ACME 250 mg/kg (EP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>45.8 ± 1.6</td>
<td>47.1 ± 1.3</td>
<td>87.9 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.4 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.9 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53.0 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intestine</td>
<td>58.4 ± 2.3</td>
<td>56.2 ± 1.4</td>
<td>22.8 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.8 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.0 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.1 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>74.0 ± 1.9</td>
<td>72.9 ± 1.8</td>
<td>37.6 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.0 ± 1.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.9 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.9 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distal colon</td>
<td>78.9 ± 1.6</td>
<td>81.0 ± 1.6</td>
<td>43.7 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.9 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.0 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.8 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$.<sup>a</sup> DMH group differs significantly from control group. <sup>b</sup> DMH + ACME – 250 mg/kg groups (Initiation, Post-initiation and Entire period) differs significantly from DMH alone treated group.
4B.3.7. Morphological evaluation

Morphological changes observed in the liver of control and experimental group of rats are represented in figure 4.10. Morphological evaluation revealed that the administration of DMH for the first 15 weeks of the experiment to group III rats resulted in the formation of nodules on the surface of the liver. The altered morphology of the livers of rats treated alone with DMH was also evident from its pale colour. However the supplementation of ACME, predominantly during the post-initiation and entire period of the study, completely protected the hepatic tissues from morphological changes induced by DMH.

4B.3.8. Histopathological evaluation

Figure 4.11 depicts the histopathology of the hepatic tissues of control and experimental group of animals. Histopathological examination of the liver revealed that the normal architecture of hepatic tissue (Figure 4.11.A) was completely lost in rats treated alone with DMH (group III) (Figure 4.11.C) with the appearance of cell necrosis, haemorrhage, fatty infiltration and pleomorphic nuclei. But the supplementation of ACME to DMH treated animals (Figure 4.11.D - 4.11.F) seems to prevent such hepatic changes as evident from hepatic architectural pattern with mild to moderate hepatitis.

Figure 4.12 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 4.12.A and 4.12.B). 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 4.12.C). 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 4.12.D - 4.12.F). 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.

4B.3.9. Immunohistochemistry of PCNA

Figure 4.13 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 4.13.C) as compared to the group I normal control rats. Normal control (Figure 4.13.A) and drug control (Figure 4.13.B) rats, however, showed few positive expressions of PCNA. ACME supplementation to DMH treated rats [initiation (Figure 4.13.D), post-initiation (Figure 4.13.E) and entire period (Figure 4.13.F)] 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 4.14 depict the labeling index of PCNA in control and experimental group of animals.
Figure 4.10. Morphological changes of the liver in control, DMH and ACME treated rats

A - Normal Control; B - Drug Control; C - DMH Control; D - Initiation; E - Post-initiation; F - Entire Period.
Figure 4.11. Histopathological changes of the liver in control, DMH and ACME treated rats (Hematoxylin and eosin, 100×)

A - Normal Control; B - Drug Control; C - DMH Control; D – Initiation; E - Post-initiation; F - Entire Period.
Figure 4.12. Histopathological changes of the colon in control, DMH and ACME treated rats (Hematoxylin and eosin, 100×)

A - Normal Control; B - Drug Control; C - DMH Control; D – Initiation; E - Post-initiation; F - Entire Period.
Figure 4.13. Immunohistochemical staining of PCNA in the colon of control and experimental animals (100x)

A - Normal Control; B - Drug Control; C - DMH Control; D - Initiation; E - Post-initiation; F - Entire Period.
Brown stained nuclei indicating the accumulation PCNA protein.
Figure 4.14. Bar graph of the percentage of PCNA-positive cells in control and experimental groups

NC - Normal Control; DC - Drug Control; DMH - DMH Control; I – Initiation; PI - Post-initiation; EP - Entire Period. \(^a p \leq 0.05\) versus normal control. \(^b p \leq 0.05\) versus DMH control. Values are expressed as mean ± S.D.

4B.4. 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 O⁶ 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≤0.05) decreased growth rate observed in DMH exposed rats may be due to the occurrence of tumors 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.

In the present study, the hepatic damage caused by the administration of DMH was evident by the significant difference in biochemical markers between DMH control and normal control rats. The increase in the activities of AST, ALT ALP and LDH in serum of carcinogen treated rats might be due to the increased
permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Bumrela and Naik, 2012). In different treatment regimens viz. initiation, post-initiation and entire period, supplementation of ACME restored the DMH induced increase of serum enzyme levels. This may be related to the activity of ACME in stabilization of the plasma membranes as well as the repair of hepatic tissue damage due to DMH.

The DMH induced changes in hepatic lipid peroxidation and enzymic and non-enzymic antioxidant status were brought near to normal by the supplementation of ACME highlights that the extract possess antioxidant property in the liver in order to meet the oxidative challenge caused by the carcinogen. Giftson et al, (2010) also reported an increased lipid peroxidation and decreased activities of antioxidant enzymes in the hepatic tissues of rats treated with DMH.

Unlike liver lipid peroxidation, intestinal and colonic lipid peroxidation was not increased on treatment with DMH. Our results also 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<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. Therefore, the possible mechanism by which ACME mediates its anticancer activity could be through its ability to function as an antioxidant.

Histopathological observations of the hepatic tissues of drug control (treated alone with ACME) rats showed a well preserved normal architecture with no evidence of necrosis that reflects the non toxicity of ACME as compared to DMH alone treated rat liver which showed necrosis and haemorrhage. ACME supplementation to DMH treated rats markedly restored normalcy in the hepatic tissues confirms the efficacy of the drug to challenge the oxidative stress induced by DMH and thereby the detoxification of the carcinogen. The histopathological observations of colonic tissues amply imply that supplementation of ACME to DMH exposed rats can efficiently prevent the carcinogen induced pathological changes. The ability of ACME to restore the histological changes induced by DMH indicates the antiproliferative potential of the extract.

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.

In conclusion, 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.