Chapter 5

CURATIVE EFFECT OF AMORPHOPHALLUS CAMPANULATUS (ROXB.) BL. TUBER ON N - NITROSODIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOMA IN RATS
5.1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the world’s deadliest cancers, ranking third among all cancer related mortalities (El-Serag and Rudolph, 2007). In most cases, HCC develops within an established background of chronic liver disease (70–90% of all patients) (Sherman, 2010). The major known risk factors for HCC are viral (chronic hepatitis B and hepatitis C), toxic (alcohol and aflatoxins), metabolic (diabetes and non alcoholic fatty liver disease, hereditary haemochromatosis), immune related (primary biliary cirrhosis and autoimmune hepatitis), food additives, environmental and industrial toxic chemicals and air and water pollutants (Farazi and DePinho, 2006).

\(N\) nitrosodiethylamine (NDEA) is one of the most important environmental carcinogen belongs to the family of carcinogenic \(N\)-nitroso compounds. Evidence from previous investigations suggests that the dialkynitrosoamines are hazardous to human health and cause a wide range of tumors in most of the animal species. Exposure of man to preformed \(N\)-nitrosamines occurs through the diet, in certain occupational setting and also due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals (Loeppky, 1994; Hecht, 1997; Thirunavukkarasu and Sakthisekaran, 2001). Administration of NDEA to animals causes cancer in liver and at low incidence in other organs also. Hence NDEA is used as a potent hepatocarcinogen to produce reproducible hepatocellular carcinoma after repeated administration in experimental animals (Singh et al., 2009).

Although there are many strategies for the treatment of HCC, its therapeutic outcome remains very poor. Therefore, preventive strategies are of paramount importance and need to be actively explored in order to reduce the incidence of this
disease. The reduced cancer risk and lack of toxicity associated with high intake of natural products suggest that specific concentrations of phytochemicals from plant sources may produce cancer chemopreventive effects. Furthermore, natural products are believed to suppress the inflammatory process that lead to neoplastic transformation, hyperproliferation, promotion and progression of carcinogenic process and angiogenesis (Blum, 2005; Ramasamy and Agarwal, 2008).

Many plant compounds have been shown to inhibit tumorigenesis in a variety of animal models of carcinogenesis, involving organ sites such as the skin, lungs, oral cavity, esophagus, stomach, liver, pancreas, small intestine, colon, and prostate (Lambert et al., 2005). Likewise, several herbal drugs have also been evaluated for its potential as liver protectant against N-nitrosodietylamine induced hepatotoxicity in rats (Shahjahan et al., 2005; Sultana et al., 2005).

*A. campanulatus* tubers are traditionally used for liver diseases and tumors (Nair, 1993), and it was evident from our initial study that the methanolic extract of *A. campanulatus* tuber (ACME) possesses hepatoprotective activity in experimental animals. In addition, the phytochemical constituents identified by the LC-MS analysis of ACME such as Cinnamaldehyde, Ferulic acid, Retinol, Quercetin and Asiatic acid are known for its cytotoxic and/or anticancer effects, particularly, cinnamaldehyde is reported as an apoptotic inducer that acts on the mitochondrial death pathway in human hepatoma PLC/PRF/5 cells (Lin et al., 2013). In view of these the present study was undertaken to evaluate the curative effect of *A. campanulatus* tuber on NDEA induced hepatocellular carcinoma in experimental rats.
5.2. MATERIALS AND METHODS

5.2.1. Chemicals

N-nitrosodiethylamine (NDEA) was purchased from Sigma Chemical Co., USA. Alpha feto-protein (AFP) assay kit was purchased from Yuvraj biobiz, Chennai, India. Assay kits for serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT) and total bilirubin (TBL) were purchased from Agappe Diagnostics, India. All other chemicals were of analytical grade.

5.2.2. Collection of plant material and preparation of drugs

*A. campanulatus* tubers were powdered and 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 extract was suspended in 5% Tween 80 to respective dosages and stored at -20°C. Silymarin - a well known hepatoprotectant, at an oral dose of 100 mg/kg body weight was used as standard drug control in the experiment (Shyamal et al., 2010).

5.2.3. Animals and diets

Male Wistar rats weighing 165.8 ± 8.4g (Mean ± S.D) were used in this study. The animals were housed in polypropylene cages and had free access to standard pellet diet (Sai Durga Feeds, Bangalore, India) and drinking water. The animals were maintained at a controlled condition of temperature of 26–28°C with a 12 h light: 12 h 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).
5.2.4. Induction of hepatocellular carcinoma

Hepatocellular carcinoma was induced by oral administration of 2 ml 0.02% NDEA (5 days/week) for 20 weeks (Wills et al., 2006).

5.2.5. Experimental design

Thirty rats were randomly divided into five groups and that were treated as follows:

- Group I : Normal control
- Group II : NDEA control (2 ml 0.02% NDEA, 5 days/week, p.o.)
- Group III : NDEA + Silymarin (100 mg/kg, b.w)
- Group IV : NDEA + ACME (125 mg/kg, b.w)
- Group V : NDEA + ACME (250 mg/kg, b.w)

Group I rats served as normal control in the experiment. All the rats except group I received 0.02% NDEA (2 ml, 5 days/week) for the first 20 weeks of the experiment. After 20 weeks of NDEA intoxication, group III rats received silymarin at a dose of 100 mg/kg for the last 28 days. Whereas group IV and group V animals were supplemented with 125 mg/kg and 250 mg/kg of ACME respectively, for the last 28 days, after 20 weeks of NDEA challenge. Animals were sacrificed 48 h after the last dose of drug administration. The schematic representation of the experimental protocol is given in figure 5.1.
5.2.6. Analysis of relative liver weight

The body weight of control and experimental groups of rats were measured at the beginning of the experiment and finally before sacrifice. After the experimental period, the rats were anesthetized with pentothal sodium followed by neck decapitation. Liver tissue was immediately excised and washed thoroughly in ice-cold saline to remove the blood. Then the tissues were blotted dry and liver weights were measured. The relative liver weight was calculated using the following formula and expressed as liver weight in grams/100 g body weight (Ramakrishnan et al., 2006).

\[
\text{Relative liver weight} = \frac{\text{Liver weight (g)}}{\text{Final body weight (g)}} \times 100
\]
5.2.7. Serum analysis

The blood was collected from each animals and were 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. NDEA 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) LDH (EC 1.1.1.27) and GGT (EC 2.3.2.2) by kinetic method. The serum levels of AFP and TBL were also determined.

5.2.8. Tissue analysis

Dissected livers were initially evaluated for its morphometry and then the tissues were cut into separate portions for biochemical assays, histopathological examination and immunohistochemical analysis.

5.2.8.1. Morphometric evaluation

Hepatic tissues of control and experimental groups of rats were examined morphologically for visible macroscopic lesions (neoplastic nodules). Nodules were easily recognized and distinguished from the surrounding non- nodular reddish brown liver parenchyma. The nodules were spherical in shape. The percentage of nodule incidence, total number of nodules and the nodule multiplicity (Average number of nodules/Nodule bearing liver) were calculated.

5.2.8.2. Biochemical assays

Dissected livers were 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 reduced glutathione (GSH), glutathione-S-transferase (GST), glutathione reductase
(GR), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (Thiobarbituric Acid Reactive Substances – TBARS).

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 H₂O₂ (Beers and Sizer, 1952). Malondialdehyde (MDA), a product of lipid peroxidation was determined by thiobarbituric acid reaction as described by Niehuis 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)

5.2.8.3. Histopathological examination

Small pieces of liver 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 under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam 1000 camera at original magnification of 100x.
5.2.8.4. Immunohistochemistry

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

5.2.9. 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.

5.3. RESULTS

5.3.1. Effect of NDEA and ACME on body weight, liver weight and relative liver weight

The results are summarized in Table 5.1. The relative liver weight was significantly (P < 0.05) increased in group II animals treated alone with 0.02% NDEA for the first 20 weeks of the experiment, when compared with group I normal control rats. However, administration of ACME at a dose of 125 and 250 mg/kg for the last 28 days of the experiment significantly (P < 0.05) reduced the relative liver weight, compared to the group II NDEA control animals. There was also a significant decrease in the relative liver weight of silymarin treated group III rats when compared with group II animals treated alone with NDEA. Whereas ACME treatment in group V rats exhibited a more pronounced result than the standard silymarin treated group III rats at the concentration used.
Table 5.1. Effect of ACME on body weight, liver weight and relative liver weight of control and experimental groups of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Relative liver weight (g liver/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>163.0 ± 8.0</td>
<td>270.0 ± 8.9</td>
<td>7.5 ± 1.1</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>NDEA Control (0.02%)</td>
<td>169.0 ± 8.9</td>
<td>201.8 ± 7.4</td>
<td>19.3 ± 2.3</td>
<td>9.5 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NDEA + Silymarin (100 mg/kg)</td>
<td>167.0 ± 7.2</td>
<td>240.3 ± 9.4</td>
<td>9.4 ± 1.0</td>
<td>3.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NDEA +ACME (125 mg/kg)</td>
<td>165.3 ± 9.0</td>
<td>223.0 ± 7.2</td>
<td>11.4 ± 1.6</td>
<td>5.1 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NDEA +ACME (250 mg/kg)</td>
<td>164.6 ± 10.3</td>
<td>255.5 ± 9.1</td>
<td>8.5 ± 0.8</td>
<td>3.3 ± 0.2&lt;sup&gt;b,c&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> NDEA control group differs significantly from Normal control group. <sup>b</sup> NDEA + Silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group. <sup>c</sup> NDEA + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.

5.3.2. Effect of NDEA and ACME on serum marker enzymes

Figure 5.2 (A, B, C, D and E) represents the results. Rats treated alone with NDEA (group II) showed significantly \( p \leq 0.05 \) elevated levels of liver specific serum marker enzymes such as AST, ALT, ALP, LDH and GGT, when compared to the normal control group of animals. However, ACME post-treatment significantly \( p \leq 0.05 \) and dose dependently lowered the increased levels of serum marker enzymes. Group V animals treated with ACME at a dose of 250 mg/kg, b.w. exhibited better results than group III rats treated with the standard drug, silymarin.
Figure 5.2. Effect of NDEA and ACME on serum marker enzymes (A). Aspartate aminotransferase (B). Alanine aminotransferase

(N) Normal control, (T) Toxic - NDEA control, (S) Silymarin – 100 mg/kg, (D1) ACME – 125 mg/kg, (D2) ACME – 250 mg/kg.

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

- Group \( a \): NDEA group differs significantly from normal control group.
- Group \( b \): NDEA + silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group.
- Group \( c \): NDEA + ACME – 250 mg/kg group differs significantly from NDEA + ACME – 125 mg/kg.
Figure 5.2. (Cont.) Effect of NDEA and ACME on serum marker enzymes (C). Alkaline phosphatase (D). Lactate dehydrogenase

(N) Normal control, (T) Toxic - NDEA control, (S) Silymarin – 100 mg/kg, (D1) ACME – 125 mg/kg, (D2) 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 NDEA group differs significantly from normal control group. b NDEA + silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group. c NDEA + ACME – 250 mg/kg group differs significantly from NDEA + ACME – 125 mg/kg.
5.2. Effect of NDEA and ACME on serum marker enzymes

(E). Gamma glutamyl transferase.

(N) Normal control, (T) Toxic - NDEA control, (S) Silymarin – 100 mg/kg, (D1) ACME – 125 mg/kg, (D2) 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 NDEA group differs significantly from normal control group. b NDEA + silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group c NDEA + ACME – 250 mg/kg group differs significantly from NDEA + ACME – 125 mg/kg.

5.3.3. Effect of NDEA and ACME on serum levels Alpha fetoprotein and Total Bilirubin

Figure 5.3A and 5.3B depicts the effect of NDEA and ACME on serum levels of AFP and TBL respectively. When compared to the normal control animals (group I), the serum levels AFP and TBL were significantly ($p < 0.05$) elevated in group II animals treated alone with NDEA. Whereas supplementation of ACME dose dependently lowered the increased levels of AFP and TBL, when compared with group II tumor bearing animals. Silymarin treated group III rats also significantly restored the serum levels of AFP and TBL, when compared to the rats treated alone with 0.02% NDEA (group II).
Figure 5.3. Effect of NDEA and ACME on serum levels Alpha fetoprotein and Bilirubin (A). Alpha fetoprotein and (B). Bilirubin.

(N) Normal control, (T) Toxic - NDEA control, (S) Silymarin – 100 mg/kg, (D1) ACME – 125 mg/kg, (D2) ACME – 250 mg/kg.

Values are expressed as mean ± S.D. (n = 6), error bar indicating the standard deviation. Statistical significance: p <0.05. \(^{a}\) NDEA group differs significantly from normal control group. \(^{b}\) NDEA + silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group \(^{c}\) NDEA + ACME – 250 mg/kg group differs significantly from NDEA + ACME – 125 mg/kg.
5.3.4. Morphological and Morphometric evaluation

Morphological changes observed in the liver of control and experimental group of rats are represented in figure 5.4. Livers dissected out from the control group of rats (group I) were normal in its appearance, without any morphological changes. Administration of 0.02% NDEA for the first 20 weeks of the experiment to group II rats resulted in the enlargement of the liver and formation of nodules on its surface. The nodule incidence was 100% in group II NDEA control rats, in other words, nodules were formed in the liver of all the rats in group II administered with NDEA. However, the incidence of nodules decreased to 33.3% in group III and group IV rats treated with silymarin (100 mg/kg, b.w.) and ACME (125 mg/kg, b.w.) respectively. Treatment with ACME at a dose of 250 mg/kg b.w. (group V) for the last 28 days of the experiment produced a more pronounced result with zero percentage nodule incidence and nodule multiplicity (Table 5.2). The liver morphology of group V rats was also similar to that of group I rats served as normal control in the experiment.

5.3.5. Effect of NDEA and ACME on tissue antioxidants

The effect of NDEA and ACME on the level of tissue GSH and activities of antioxidant enzymes such as GST, GR, GPx and CAT are shown in table 5.3. The reduced glutathione level and activities of glutathione dependent enzymes and catalase were significantly decreased ($p < 0.05$) in the hepatic tissues of group II animals treated with NDEA, as compared to the control group of animals (group I). After 20 weeks of NDEA challenge, the administration of silymarin and ACME (125 and 250 mg/kg body weight) to the experimental group of animals markedly ($p < 0.05$) increased the reduced glutathione level as well as glutathione dependent
enzymes and catalase activities, compared to rats treated alone with 0.02% NDEA (group II). Among the drug treated group of animals, the results were more prominent in group V animals treated with ACME 250 mg/kg body weight.

Figure 5.4. The gross appearance of liver in control and experimental rats

A - Normal Control; B - NDEA Control; C - NDEA + Silymarin (100 mg/kg); D - NDEA + ACME (125 mg/kg); E - NDEA + ACME (250 mg/kg).
Table 5.2. Effect of ACME on NDEA induced hepatic nodules development in experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats with nodule/ Total no. of rats</th>
<th>Nodule incidence (%)</th>
<th>Total no. of nodules</th>
<th>Nodule multiplicity (Average no. of nodules/ Nodule bearing liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NDEA Control (0.02%)</td>
<td>6/6</td>
<td>100</td>
<td>131 ± 29</td>
<td>21.8</td>
</tr>
<tr>
<td>NDEA + Silymarin (100 mg/kg)</td>
<td>2/6</td>
<td>33.3</td>
<td>8 ± 3</td>
<td>4.0</td>
</tr>
<tr>
<td>NDEA +ACME (125 mg/kg)</td>
<td>2/6</td>
<td>33.3</td>
<td>7 ± 4</td>
<td>3.5</td>
</tr>
<tr>
<td>NDEA +ACME (250 mg/kg)</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.3. Curative effects of ACME against NDEA induced changes of liver antioxidants

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>GSH(^1)</th>
<th>GST(^2)</th>
<th>GR(^3)</th>
<th>GPx(^4)</th>
<th>CAT(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>24.3 ± 1.0</td>
<td>71.3 ± 1.4</td>
<td>22.7 ± 1.4</td>
<td>291.3 ± 11.7</td>
<td>51.0 ± 1.8</td>
</tr>
<tr>
<td>NDEA Control (0.02%)</td>
<td>14.0 ± 0.9(^a)</td>
<td>36.0 ± 1.8(^a)</td>
<td>9.5 ± 0.8(^a)</td>
<td>145.0 ± 8.9(^a)</td>
<td>35.3 ± 1.4(^a)</td>
</tr>
<tr>
<td>NDEA + Silymarin (100 mg/kg)</td>
<td>20.3 ± 1.3(^b)</td>
<td>61.7 ± 1.9(^b)</td>
<td>18.7 ± 1.2(^b)</td>
<td>261.3 ± 13.7(^b)</td>
<td>44.5 ± 1.5(^b)</td>
</tr>
<tr>
<td>NDEA +ACME (125 mg/kg)</td>
<td>17.3 ± 1.4(^b)</td>
<td>56.0 ± 0.9(^b)</td>
<td>15.3 ± 1.0(^b)</td>
<td>224.0 ± 12.1(^b)</td>
<td>39.3 ± 1.4(^b)</td>
</tr>
<tr>
<td>NDEA +ACME (250 mg/kg)</td>
<td>21.7 ± 1.6(^b,c)</td>
<td>65.3 ± 1.3(^b,c)</td>
<td>20.0 ± 0.9(^b,c)</td>
<td>275.7 ± 11.5(^b,c)</td>
<td>47.7 ± 1.9(^b,c)</td>
</tr>
</tbody>
</table>

\(^1\)(nmol/mg protein); \(^2\)(µmol CDNB-GSH conjugate formed/min/mg protein); \(^3\)(nmol of GSSG utilized/min/mg protein); \(^4\)(nmol of GSH oxidized/min/mg protein); \(^5\)(U/mg protein).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: \(p \leq 0.05\). \(^a\) NDEA control group differs significantly from Normal control group. \(^b\) NDEA + Silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group \(^c\) NDEA + ACME – 250 mg/kg group differs significantly from DMH + ACME – 125 mg/kg.
5.3.6. Histopathological examination

Figure 5.5A represents the normal architecture of the rat liver; characteristically the cells are uniformly arranged with granulated cytoplasm, oval hepatocytes and small uniform nuclei. The normal architecture of hepatic tissue was completely lost in rats treated alone with NDEA (group II). Enlarged nuclei, hyperchromatism, proliferating hepatocytes and mild congestion of sinusoids with central vein dilation were detected in most areas of the tissue sections of NDEA intoxicated rats. Microscopic examination also revealed the trabecular structure of liver tissue, which is a typical feature of hepatocellular carcinoma (Figure 5.5B). Tissue sections of silymarin (100mg/kg b.w) treated rats showed necrotic tissues in certain areas (Figure 5.5C). However, a few degenerating hepatic cells were detected in group IV, ACME 125 mg/kg b.w. treated rats (Figure 5.5D). In ACME 250 mg/kg b.w. treated group (group V), the liver tissue showed almost normal architecture with normal hepatocytes and uniform sinusoids (Figure 5.5E).

5.3.7. Immunohistochemistry of PCNA

Figure 5.6 shows the photomicrographs of immunohistochemical staining of PCNA in the liver tissues 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 II NDEA treated rats (Figure 5.6B), as compared to the group I normal control animals. Normal control rats, however, showed few positive expressions of PCNA (Figure 5.6A). Treatment with silymarin and ACME for the last 28 days of the experiment significantly (p<0.05) reduced the expression of PCNA (Figure 5.6C-5.6E). The effect of ACME in reducing cell proliferation was
more pronounced at a dose of 250 mg/kg than the effect rendered by silymarin at a concentration of 100 mg/kg. The PCNA labeling index is shown in figure 5.7.

Figure 5.5. Histopathological changes of the liver in control and experimental animals (Hematoxylin and eosin, 100×).

A - Normal Control; B - NDEA Control; C - NDEA + Silymarin (100 mg/kg); D – NDEA + ACME (125 mg/kg); E - NDEA + ACME (250 mg/kg).
Figure 5.6. Immunohistochemical staining of PCNA in control and experimental animals

A - Normal Control; B - NDEA Control; C - NDEA + Silymarin (100 mg/kg); D – NDEA + ACME (125 mg/kg); E - NDEA + ACME (250 mg/kg).

Brown stained nuclei indicating the accumulation PCNA protein.
Figure 5.7. Bar graph of the percentage of PCNA-positive cells in control and experimental groups

(N) Normal control, (T) Toxic - NDEA control, (S) Silymarin – 100 mg/kg, (D1) ACME – 125 mg/kg, (D2) ACME – 250 mg/kg.

Values are expressed as mean ± S.D. (n = 6), error bar indicating the standard deviation. Statistical significance: \( p < 0.05 \). \(^{a}\) NDEA group differs significantly from normal control group. \(^{b}\) NDEA + silymarin – 100 mg/kg, NDEA + ACME – 125 mg/kg and NDEA + ACME – 250 mg/kg groups differs significantly from NDEA alone treated group.

5.4. DISCUSSION

\( N\)-nitrosodiethylamine is used as a carcinogen to induce liver cancer in animal models (Loeppky, 1994). NDEA is mainly metabolized in the liver by the action of cytochrome p450 enzymes and the reactive metabolites are primarily responsible for its hepatotoxic effects. These reactive oxygen species (ROS) induce oxidative stress and cytotoxicity by damaging biomolecules such as DNA, lipids and proteins. NDEA is bioactivated to ethyldiazonium ion which alkylates DNA bases to
form promutagenic adducts such as $O^6$-ethyldeoxyguanosine and $O^4$ and $O^6$-ethyldeoxythymidine (Verna et al., 1996). The ROS, continuously generated as a result of NDEA administration causes oxidative stress that wreak havoc in biological system by damaging tissues, altering biochemical compounds, causing chromosomal instability, corroding cell membranes and inducing mutations, which play an important role in the development of cancer (Harman, 1980). It is also reported that NDEA cause perturbations in the nuclear enzymes involved in DNA repair/replication and induce pericentral foci of small dysplastic hepatocytes and acts by ethylating nucleophilic sites in DNA, causing cirrhosis and multifocal HCC within 18 weeks. (Bhosale, et al., 2002; Newell et al., 2008).

Results of the present investigation indicated that administration of NDEA leads to the induction of HCC and augmentation of oxidative stress in NDEA treated rats. The present study also demonstrated that NDEA intoxication to the rats for a period of 20 weeks leads to a marked elevation in the levels of total bilirubin and serum enzymes such as AST, ALT, ALP, LDH and GGT. The increase in the activities of serum marker enzymes may potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation after liver cell damage (Shaarawy et al., 2009). Serum transaminases, ALP and GGT are representative of liver function and their increased levels are indicators of liver damage. The increase in ALT activity is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST (Azri et al., 1992). Similarly, increase in ALP reflects pathological alteration in biliary flow and discharge of TBL reflects a non-specific alteration in the plasma membrane integrity and/or permeability. GGT is embedded in the hepatocyte plasma membrane, mainly in the canalicular domain and its
liberation into serum indicates damage of the cells and thus injury to liver (Jeena et al., 1999; Sivaramakrishnan et al., 2008). In addition, Shaarawy et al. (2009) reported that the increase of serum GGT activity is a biomarker of preneoplastic lesion. Experimental studies have also shown that GGT was strikingly activated during the course of hepatocarcinogenesis induced by several hepatocarcinogens in animals (Fiala and Fiala, 1973). Likewise, LDH is found to be increased in various types of tumors and is a fairly sensitive marker of solid neoplasm (Lipport et al., 1981; Engan and Hannisdal, 1990; Thangaraju et al., 1998). Proliferating malignant cells exhibit very high rates of glycolysis, which subsequently lead to elevated LDH activity (Dao, 1980). ACME treatment in the present study showed a significant inhibitory effect on the increase of serum marker enzyme activities initiated by NDEA, which indicated that ACME could suppress NDEA induced liver damage. Thus it is suggested that ACME aids in hepatic parenchymal cell regeneration and thereby protecting membrane integrity by decreased enzyme leakage.

Elevation of serum AFP level has been reported in several diseases; however, it is most extensively used in the diagnosis of HCC (Abeleb, 1986; Banker, 2003). It has also been recognized that exposure of rats to certain carcinogens like NDEA increases the circulating AFP levels (Sivaramakrishnan et al., 2008). In our study also there was an increase in the level of AFP in carcinogen administered animals, confirming the development of HCC in group II rats. Whereas supplementation of ACME resulted in the significant reduction of AFP levels indicates that the extract possess chemopreventive activity against NDEA induced experimental liver cancer.
NDEA intoxication results in an uncompromised free radical generation in the liver that overwhelms the antioxidant status and ultimately proceeds to oxidative stress paving way to carcinogenesis (Gey, 1993). Boitier et al. (1995) also reported that in hepatocellular carcinoma there is a disequilibrium between oxidant and antioxidant balance which is tilted towards oxidant side. Antioxidants possess a variety of biological activities, including the inhibition of carcinogen induced mutagenesis and scavenging of free radicals (Hirose et al., 1994). Antioxidants may protect the cell from ROS toxicity by prevention of ROS formation, by the interruption of ROS attack, by scavenging the reactive metabolites and converting them to less reactive molecules and/or by enhancing the resistance to sensitive biological targets to ROS attack, by facilitating the repair caused by ROS and by providing cofactors for the effective functioning of other antioxidants (Sen, 1995).

For the purpose of preventing cellular damage induced by ROS, the organism has a lot of antioxidative defense system, including the non-enzymatic (mainly GSH) and enzymatic antioxidant defenses (e.g. GR, GST, GPx, CAT etc.). GSH plays an important role in maintaining the normal reduced state of cells and counteracting the harmful effects of oxidative stress. GSH can effectively scavenge free radicals and other oxygen species through non-enzymatic and enzymatic process by conjugation with GPx and GST. GPx ubiquitously exists both in cytosol and mitochondria of the hepatocytes. GST locates in cytosol and plays an important role in detoxification and excretion of xenobiotics. GST catalyzes the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics, leading to elimination or conversion of xenobiotic-GSH conjugate. In such reaction, the GSH is oxidized into GSSG, which can be reduced to GSH by GR with the consumption
of NADPH. In addition to GSH and GSH-related antioxidant enzymes, other antioxidant enzymes such as CAT also take important role in the antioxidant defense system. CAT act as supporting antioxidant enzyme by transforming H₂O₂ to H₂O, thereby providing protection against ROS (Saydam et al., 1997; Zhang et al., 2012).

In the present study, the depletion in GSH level and GSH dependent enzymes (GST, GPx and GR) in NDEA treated rats might be attributed to the reduction in their biosynthesis during hepatocellular damage or their excessive utilization in scavenging the free radicals formed during the metabolism of NDEA. Furthermore, the decreased levels of cellular GSH might have caused a reduction in the activities of GSH dependent enzymes, as GSH is a vital co-factor for GST, GPx and GR (Seven et al., 2004; Pradeep et al., 2007). Natural antioxidants are capable of inhibiting the ROS production and thereby reducing the associated intracellular oxidative stress (Feng et al., 2001). Supplementation of the extract under investigation (ACME) to NDEA treated animals effectively modulated the deterioration in the antioxidant indices such as GSH, GR, GPx, GST and CAT implied the beneficial antioxidant abilities of the extract. Moreover, the increase in GSH level may reduce the DNA-carcinogen interaction by providing a large nucleophilic pool for electrophilic carcinogen (e.g. NDEA). GSH neutralizes the electrophilic site by providing SH group and renders the metabolite more water soluble (Habig et al., 1974).

The biochemical findings of our study were supported by the histopathological examinations of the liver tissues of control and experimental rats. The histopathological analysis of the livers of animals treated alone with NDEA revealed disorganization of hepatic lobular architecture and obvious cellular damage
with megalocytosis, foamy cytoplasm and hyperchromatic nuclei. The observed changes in the histopathology of NDEA treated rats corroborate with the study of Gupta et al. (2010), reported the vacuolization, loss of normal hepatocellular architecture and the presence of pycnotic nuclei in the liver tissues of rats treated with NDEA. This may be attributed to that NDEA is primarily metabolized in the liver and reactive metabolites generated thereby are known to damage hepatocytes (El Mesallamy et al., 2011). Histopathology of the liver tissue of rats post-treated with ACME revealed an improvement in the hepatocytes exhibiting less disarrangement and degeneration of hepatocytes compared to the rats treated alone with NDEA. This improvement may be credited to the activity of ACME, which could competently reduce the intracellular ROS levels of hepatocytes and thus prevented the oxidative stress induced cellular damage. In addition, the results of histopathological examinations are in correlation with morphological and morphometric evaluations. Post - treatment of ACME also recovered the NDEA induced increase in liver weight and nodule multiplicity towards normalcy. This evidently shows the curative effect of the extract in NDEA induced HCC.

Abnormal proliferation of cells is the main feature of carcinogenesis, and therefore exploration of drugs that can affect malignant proliferation of liver cells is of primary importance in chemical prevention of liver cancer. PCNA is a non-histone nuclear acidic protein expressed in the nuclei of proliferating cells during G1 and S phase of cell cycle. It has been found that the positive expression of PCNA were a common index for proliferation of hepatocytes. The positive expression of PCNA was mainly found in the precancerous proliferation focus and cancerous liver tissues during NDEA induced hepatocarcinogenesis (Bravo et al., 1987; Chodon et
In the present study, NDEA administered rats showed amplified expression of PCNA in the liver tissues and thereby indicating the hyper-proliferation of hepatocytes and its malignant transformation. The results also indicated that the administration of ACME could remarkably decreases the expression of PCNA in the hepatic tissues of NDEA intoxicated rats, suggesting that ACME had the potential to suppress malignant proliferation of hepatocytes in experimental liver cancer and inhibited tumor growth through the inhibition of DNA synthesis.

In summary, the results of the present study evidently indicate that the administration of NDEA brings about profound alterations in the activities of serum marker enzymes, serum levels of bilirubin and alpha fetoprotein and hepatic antioxidant status. In addition, the results revealed that in NDEA induced hepatocellular carcinoma, ACME exerts its protective effect in a dose dependent manner. ACME at a dose of 250 mg/kg was able to exert a marked effect, as shown histologically by a significant reduction in the extent and severity of lesions in hepatic tissue, abridged expression of PCNA and also biochemically by the modulation of hepatic antioxidant status. Silymarin, a known tumor suppressive agent against hepatocellular carcinoma, was used as the positive drug control in the experiment (Shaarawy et al., 2009). But interestingly, treatment with ACME at a dose of 250 mg/kg showed better results than the silymarin (100 mg/kg) treatment. The present investigation further demonstrates that the significant defense exhibited by ACME against NDEA induced hepatocarcinogenesis might be mediated through the antioxidant and/or free radical scavenging activities and the inhibition of hepatic cell proliferation. Furthermore, the chemopreventive activity exhibited by ACME
against HCC might be attributed to the combined activity of the previously identified cytotoxic/anticancer phytoconstituents of ACME viz., Cinnamaldehyde, Ferulic acid, Retinol, Quercetin and Asiatic acid. Therefore ACME may prevent the tumor promoting property of NDEA through antioxidant and anti-proliferative properties and might be useful clinically as chemopreventive agent for liver cancer after further molecular chemopreventive studies.