CHAPTER – 3

3.1. *IN VIVO* ANTICANCER PROPERTIES OF 1, 3 BPMU IN CONTROL AND EXPERIMENTAL RATS.

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most frequently occurring forms of a solid tumor and has limited therapeutic options. Hence, a thorough understanding of the biological bases of this malignancy might suggest new strategies for effective treatment (Alessandro *et al.*, 2007). Hepatocarcinogenesis induced animal by DEN is an ideal model to investigate liver tumor formation because, it proceeds in stages similar to that of human liver cancer i.e., formation of preneoplastic foci, neoplastic nodules and HCC nodules (Peto *et al.*, 1991). Various macro and micro elements are necessary to maintain the cell development and processing. If these macro element or micro elements leads to abnormal condition either by excess level or low level than the normal, cause disease. Albumin is made mainly in the liver. It helps to keep the blood from leaking out of blood vessels, carries some substances through the blood and is important for tissue growth and healing. If liver is injured by risk factors, the serum albumin will not be their normal levels.

Globulin is a generic term used to describe a set of sixty proteins including the antibodies or gamma globulins and protein-carbohydrate compounds known as glycoprotein. These are used to help transport proteins through the lipoproteins and assisting the blood in clotting. They also act as plasma cells which indicate whether there is an antibody deficiency in the blood stream. The liver produces much of the alpha and beta globulins used for this purpose.

Approximately 85% of the total bilirubin is derived from the heme moiety of hemoglobin, while the remaining 15% is produced from the red blood cell precursors destroyed in the bone marrow and from the catabolism of other heme-containing proteins. After production in peripheral tissues, bilirubin is rapidly taken up by hepatocytes where it is conjugated with glucuronic acid to produce mono- and diglucuronide, which are excreted in the bile.
Aminotransferases in general AST (serum glutamic oxaloacetic transaminase (SGOT) and ALT associated with Hepatocellular damage and not associated with cholestasis. ALT is more specific for liver damage than AST (Fredericks. et al., 1990). Aspartate aminotransferase (AST) catalyses conversion of nitrogenous portion of amino acid. It is essential for energy production in Krebs cycle. AST is released into serum in proportion to cellular damage and most elevated in acute phase of cellular necrosis. Found in decreasing levels in liver, cardiac, skeletal muscle, kidney, brain, pancreas, red blood cells and used in the detection and differential diagnosis of hepatic disease. Monitor patients with cardiac and hepatic disease – levels are dependent on the stage of disease (Sivaramakrishnan et al., 2008). Alkaline Phosphatase has up to 60 different isoenzymes, collectively measured as ALP. ALP influences the bone calcification, lipid and metabolite transport. It is produced by Bile cannalicular membrane of hepatocytes, bone, placenta and small intestine. Elevated ALP is often associated with biliary obstruction with cholestasis and usually before a rise in bilirubin (Plaa et al., 1989). Liver plays a vital role in the production and catabolism of plasma lipoproteins such as LDL, HDL and VLDL. Plasma lipid profiles could be changed in HCC. Analysis of serum levels of lipids, lipoproteins (LDL, HDL and VLDL) in the patients suffered from HCC may reflect the condition of hepatic cellular impairment and may also be used as an indicator to evaluate patient's prognosis. It is suggested that variations in the levels of plasma lipids and lipoproteins may assist in describing the nature of HCC with or without cirrhosis. Levels of lipoproteins may be considered as the index of liver impairments under chronic disease or HCC. In addition, HDL and LDL had been used as a carrier for delivering chemotherapeutic drugs in HCC and other cancers. Lipids are transported in the circulation packaged in to lipoproteins. The clinical relevance of blood lipid levels is that abnormal levels of lipids in certain lipoproteins are linked to an increase risk of atherosclerosis.

VLDL delivers TAG to the cells in the body. Two types of lipoproteins are triglyceride-rich chylomicrons and VLDL. Chylomicrons are synthesized by enterocytes from lipidsand absorbed in the small intestine. VLDL is synthesized in the liver. The function of these lipoproteins is to deliver energy-rich triacylglycerol (TAG) to cells in the body (pink pathway). TAG is stripped from chylomicrons and VLDL through the action of lipoprotein lipase, an enzyme that is found on the surface of endothelial cells. This enzyme digests the TAG to fatty acids and monoglycerides, w
into the cell to be oxidized. In the case of an adipose cell it is to be re-synthesized into TAG and stored in the cell. LDL delivers cholesterol to cells in the body. As VLDL particles are stripped of triacylglycerol, they become dense. These particles are remodeled at the liver and transformed into LDL. The function of LDL is to deliver cholesterol to cells, where it is used in membranes, or for the synthesis of steroid hormones (blue pathway). Cells take up cholesterol by receptor-mediated endocytosis. LDL binds to a specific LDL receptor and is internalized in an endocytic vesicle. Receptors are recycled to the cell surface, while hydrolysis for endolysosome releases the cholesterol for use of cell.

HDL is generally involved in reverse cholesterol transport. Excess cholesterol is eliminated from the body via the liver, which secretes cholesterol in bile or converts it to bile salts. The liver removes LDL and other lipoproteins from the circulation by receptor-mediated endocytosis. Additionally, excess cholesterol from cells is brought back to the liver by HDL in a process known as reverse cholesterol transport (green pathway). HDL (or really, the HDL precursor) is synthesized and secreted by the liver and small intestine. It travels in the circulation where it gathers cholesterol to form mature HDL, which then returns the cholesterol to the liver via various pathways.

Fig. 3.1. Lipid functions in liver.

Gamma Glutamyl Transferase (γGT) is associated with transfer of amino acids across cell membranes. It is produced in the renal tubules, liver, biliary tract, pancreas, lymphocytes, brain and testes. GGT is most useful when lookin
damage. GGT is more sensitive than ALP and AST but its specificity is less. Particularly sensitive to effects of alcohol on liver, increased production of GGT as ductal enzymosis, with increased enzymes produced in response to hepatocellular damage. Lactate Dehydrogenase (LDH) catalyses the reversible conversion of lactic acid to pyruvic acid. The final step in Embden-Meyerhoff pathway provides bridge to Krebs cycle and thus cellular energy. If the liver is in abnormal condition the formation of pyruvic acid will be blocked.

The kidneys regulate the amount of water and salts that we have in our body. They do this by filtering the blood through millions of structures called nephrons. The kidneys also pass out certain waste products from the body. Urine is made up of the excess water, salts and waste products passed out by the kidney. The usual blood test which checks that the kidneys are working properly measures the level of urea, creatinine, and certain dissolved salts. Urea is a waste product formed from the breakdown of proteins. Urea is usually passed out in the urine. An abnormal blood level of urea (uraemia) indicates that the kidneys may not be working properly or that you are dehydrated (have a low body water content). Creatinine is a waste product made by the muscles. Creatinine passes into the bloodstream, and is usually passed out in urine. A high blood level of creatinine indicates that the kidneys may not be working properly. Creatinine is usually a more accurate marker of kidney function than urea down to the bladder.

3.1.1. Materials and Methods
3.1.1.1. Animals

Male albino rats of Wistar strain approximately weighing 150-165g were used in this study. The healthy animals purchased from the Indian Institute of Science, Bangalore. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature 27 ± 2° C and 12 hour light/dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet and water ad libitum. They were acclimatized to the environment for one week prior to experimental use. All the animal experimentations were permitted and executed in compliance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional animal Ethical Committee Guidelines (743/03/abc/CPCSEA dt3.3.03).
3.1.1.2-Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dintiro benzene (CDNB), 5,5’-dithio-bis (2-nitrobenzoic acid), glutathione (reduced), glutathione (oxidized), Diethylnitrosamine (DEN) and L-ascorbic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from Glaxo Laboratories, Mumbai, India and Sisco Research Laboratories, Mumbai, India.

3.1.1.3-Fixation of optimum dosage for 1, 3 Bpmu

Formerly, 1, 3 BPMU at different concentrations ranging from (20, 30, 40, 50 and 60mg/kg body weight) was administered to DEN induced group of rats to determine its minimal effective optimum dosage. After the experimental periods the rats were sacrificed under the anesthetic condition and blood sample were collected for analysis of liver marker enzymes. Hence, the minimal dose of 50 mg/kg b.wt was fixed as the optimum dosage for subsequent studies.

3.1.1.4-Experimental Design

The rats were divided into five groups; each group consists of six animals analyzed for a total experimental period of 16 weeks as follows:

Group 1: Control rats fed with standard diet with ad-libitum of water.
Group 2: 1, 3 BPMU was orally given to rats (50 mg / kg bwt.) by aqueous form for 16 weeks once in a day.
Group 3: Rats were induced HCC with DEN by ip injection (0.02%) after two weeks cancer promoted by the administration of Phenobarbital (oral/250mg/kg bwt.) for 6 weeks. (Kyle et al., 1996)
Group 4: Rats were induced HCC with DEN by ip injection (0.02%) after two weeks cancer promoted by the administration of Phenobarbital (oral/250mg/kg bwt.) for 6 weeks then rats were treated with 1,3BPMU oral 50mg / kg/bwt.) for the next 8 Weeks.
Group 5: A well known hepato protective compound silymarin (100 mg/kg) administered from the period of 16 weeks.
After 16 weeks, experimental rats (n=6 per group) were anesthetized with sodium pentothal after overnight fasting. Blood were collected from tail vein, immediately all the groups were sacrificed by cervical dislocation. Liver samples were collected for histological studies and biochemical estimation. The liver was excised immediately and rinsed in ice cold saline. A portion of the liver was homogenized in 0.1M Tris buffer, pH7.4 and used for the assays.

3.1.1.5-Preparation of Liver Tissue Homogenate

The liver tissues were excised and rinsed in ice-cold saline. Known amount of the tissue were homogenized in 0.1 M Tris–HCl buffer, pH 7.4 at 4°C, in a Potter– Elvehjem homogenizer with a Teflon pestle at 600 rpm for 3 minutes. The homogenate was centrifuged at 3000 μg for 10 minutes. The supernatant was collected as tissue homogenate, which was used to assay various parameters.

3.1.1.6-Analysis of liver marker enzymes and cancer marker proteins in control and experimental rats.

3.1.1.6.1-Activity of ALT (Reitman and Frankel, 1957).

0.2ml of Sample and 1.0ml of the buffered substrate were incubated for 30 min at 37 °C. To the control tubes, enzyme was added after arresting the reaction with 1.0ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The color developed was read at 520 nm. The enzyme activity in serum was expressed as IU / L.

3.1.1.6.2-Activity of AST (Reitman and Frankel, 1957).

0.2 ml of sample and 1.0 ml of the buffered substrate was incubated for 60 min at 37 °C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The color developed was read at 520nm. The enzyme activity in serum was expressed as U / L.
3.1.1.6.3-Determination of Alkaline Phosphatase activity (ALP) (King and king, 1954).

0.5ml of buffered substrate was diluted with 1.5ml distilled water and incubated at 37°C for few minutes. Then 0.1 ml of serum was added into tests and incubated for 15 minutes at 37°C. After 1 ml of chromogen reagent was added to all tubes and colour developed was read at 510nm. Values were expressed as IU/L.

3.1.1.6.4-Determination of Lactate Dehydrogenase (LDH) Activity (King, 1965).

0.02ml of serum was mixed with 1.0ml of buffered substrate and placed in a water bath at 37°C, 0.2ml of NAD solution was added only in test, exactly after 15 minutes 1.0ml of 2, 4 DNPH was mixed with test and control and left in the water bath for 15 minutes. Then 10ml of 0.4N NAOH was added and absorbance was read at 440nm. Values are expressed as IU/L.

3.1.1.6.5-Determination of Acid phosphatase (ACP) (Annon, 1963).

The p-nitrophenol (400mg in 0.5ml) was mixed with an equal volume of 0.1ml citrate buffer (pH 4.8). The post-lysosomal supernatant was added and incubated at room temperature for 30min. 4ml of 0.1N NaOH was added to stop the reaction. The absorbance of the solution was measured at 410nm. The amount of p-nitrophenol liberated by the enzyme per hour per mg protein gives the specific activity. The acid phosphatase activity was expressed as IU/L.

3.1.1.6.6-Determination of Gamma Glutamyl Transferase (γ GT) (Rosalki and Rau 1972)

The activity of γ - glutamyl transferase (γ-GT) was estimated in serum.1 ml of Tris-HCl, 2.2 ml of glycyl glycine, 0.2 ml of homogenate was added to the incubation mixture containing 0.5 ml of the substrate and the total volume was made upto 4 ml with water and incubated for 30 min at 37 °C. Then the samples were heated at 100 °C for 5 min and centrifuged at 3000 rpm. The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of γ -glutamyl transferase was expressed as IU/L.
3.1.1.6.7-Assay of 5’-Nucleotidase activity (Rieder and Otero, 1968).

The reaction mixture contains 1.0 ml of Tris-HCl buffer and 0.1 ml each of MgSO₄, KCl, EDTA, substrate and water. The reaction was initiated by the addition of 0.2 ml plasma or tissue homogenate and incubated at 37 °C for 15 min. The reaction was arrested by the addition of 2.0 ml of 10% TCA and centrifuged at 4,000 rpm. The phosphorous liberated in the suspension was estimated. The enzyme activity was expressed as U/L.

3.1.1.6.8-Determination of α2-Macroglobulin (α2M) and carcinoembryonic antigen (CEA) (Prłmus et al., 1988).

Serum homocysteine levels were estimated by ELISA method. Serum α2M was determined using radial-immuno diffusion kit (Biocientífica SA, Buenos Aires-Argentina). CEA was measured in serum by chemiluminescent immunoassay (Fully Automated ADVIA Centaur, Bayer, and USA). Values were expressed as mg/dl for α2M and ng/ml for CEA.

3.1.1.6.9-Assay of alpha-fetoprotein. (Sell and Becker, 1978)

20 ml of standards, specimens and controls were added to the appropriate wells. A hundred microliters of buffer was added to each well and mixed for 10sec. Wells were covered and incubated at room temperature for 30 min. After aspirating the contents, the wells were washed five times with distilled water. The remaining fluid was removed by tapping the strips on a tissue paper. Enzyme conjugate (150 ml) was added to each well and mixed. Wells were incubated for 30 min and the strips were washed. TMB (3, 30, 5, 50-tetramethylbenzidine) substrate solution (200 ml) was added to all the wells and the plate was incubated in the dark at room temperature. Enzyme reaction was stopped by the addition of 50ml of 2M H₂SO₄ per well and the absorbance was measured at 450 nm. The absorbance was measured for each set of reference standards, specimens and controls. A standard curve was constructed by plotting the absorbance obtained from each reference standard against its concentration (ng/ml). The amount of AFP (ng/ml) in each specimen was quantitated against the standard curve obtained by plotting the absorbance of the reference standards against its concentration. Values were expressed as ng/ml.
3.1.1.7-Analysis of lipid profiles in control and experimental rats

3.1.1.7.1-Determination of Total Cholesterol (Allian et al., 1974)

To 0.1 ml of serum, 4.9 ml of ferric chloride, precipitating reagent was added and centrifuged. To 2.5 ml of supernatant 2.5 ml of ferric chloride diluting reagent was mixed. 4.0 ml of concentrated sulphuric acid was added. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0 ml of concentrated sulphuric acid. A set of standards (0.5 - 2.5 ml) were taken and made up to 5.0 ml with FeCl₂ diluting reagent. Then add 4.0 ml of con. H₂SO₄. After 30 min. the intensity of the color developed was read at 540 nm against reagent blank. The amount of cholesterol in the serum was expressed as mg/dl.

3.1.1.7.2- Estimation of Triglycerides (TG) (Rice, 1970)

0.1 ml of the serum was taken and made up to 4.0 ml with isopropanol. The content was mixed well and 400 mg of silicic acid was added, placed in a mechanical shaker and centrifuged. To 2.0 ml of the supernatant 0.6 ml of saponification reagent was added and incubated at 60-70 °C for 15 min. After cooling 1.0 ml of sodium metaperiodate was added and mixed well. Then 0.5 ml of acetyl acetone reagent was added and mixed again, incubate the tubes at 50 °C for 30 min. After cooling the absorbance was read at 405 nm. Triglycerides were expressed as mg/ml in serum.

3.1.1.7.3-Determination of LDL Cholesterol

LDL cholesterol was calculated by the following formulae:

\[
LDL\text{ cholesterol (mg/dl)} = \frac{TCL}{5} - HDL\text{ cholesterol}
\]

3.1.1.7.4-Determination of VLDL Cholesterol

VLDL cholesterol was calculated by the following formulae:

\[
VLDL\text{ cholesterol (mg/dl)} = \frac{TCL}{5}
\]
3.1.1.7.5—Determination of HDL Cholesterol

To 200 µl of serum sample mixed with 300 µl of HDL precipitate reagent was added and mixed well, kept at room temperature for 10 mins then centrifuged at 3000rpm for 10 mins. The pellet was discarded and 1 ml of enzyme reagent was added to 100 µl if supernatant, incubated for 5 mins at 37 °C and the absorbance was read at 505nm. The activity was calculated by HDL Cholesterol by conc.mg/dl: Abs of standard x Conc of Std (50)

3.1.1.8- Analysis of blood glucose, Protein, Urea and Creatinine profiles in control and experimental rats

3.1.1.8.1- Estimation of blood glucose (Sasaki and Matsui, 1972).

To 0.1 ml of blood, 1.9 ml of 10% TCA solution was added to precipitate proteins and then centrifuged. One ml of the supernatant was mixed with 4.0 ml of O-toluidine reagent and was kept in a boiling water bath for 15 minutes. The green color developed was read at 600 nm by spectrophotometer. A series of standard glucose solutions (1mg/ml) were also treated similarly. The values were expressed as mg of glucose/dl of whole blood.

3.1.1.8.2—Estimation of total bilirubin (Autozyme Kit)

To 50 µl of serum 1000 µl of total bilirubin reagent and direct bilirubin reagent was added and 20 µl of respective activator reagent was added. The reaction mixture were mixed well and incubated for 10 minutes at 37°C. At the same time, blank and standard solution was prepared. The absorbance of sample against reagent blank was read at 546 nm. Values are expressed in mg/dl.

3.1.1.8.3—Estimation of Total Albumin (Autozyme Kit)

To 0.01 ml of serum, 1.0 ml of working solution was added and the assay mixture was incubated for 1 minute at 37 °C. After completion of incubation period, the absorbance was measured at 600 nm. Values are expressed in gm/dl.

3.1.1.8.4—Estimation of total protein (Lowry et al., 1951).

To 0.1 ml of homogenate/serum, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 min. To this 0.5 ml of Folin’s reagent was added and the blue color developed was read after 20 min at 640 nm. Protein level was expressed as mg/ml for serum.
3.1.1.8.5-Estimation of Urea (Natelson, 1956).

To 0.1ml of plasma, 3.3ml of distilled water, 0.3ml of 10% sodium tungstate and 0.3ml of 2/3N sulphuric acid were added, mixed well and centrifuged. 1ml of supernatant fluid was added to 1ml of water, 0.4ml of diacetyl monoxime and 1.6ml of the sulphuric acid-phosphoric acid mixture and placed in a boiling water bath for thirty minutes, cooled and absorbance was read at 480nm. The urea was expressed as mg/dl plasma.

3.1.1.8.6-Estimation of creatinine (Bones and Taussky, 1954).

1ml of plasma was diluted with 3ml of distilled water and precipitated by adding 2ml of sodium tungstate and 2ml of 2/3 N H2SO4 which was added drop by drop with constant shaking and allowed to stand for 2 minutes and filtered. 3ml of protein free filtrate was pipptted out and 1ml of picric acid wasvadded followed by 1 ml of NaOH. The colour intensity was read at 470nm after 15 minutes. The values were expressed as mg/dl.

3.1.2. Results

1, 3 BPMU was dissolved in water, at different doses (20, 30, 40, 50 and 60 mg/kg b.wt) to determine the optimum dosage. It was observed that the effective dose of 50 mg (kg bwt) of 1,3 BPMU treatment has significantly (P<0.05) altered the levels of liver markers such as AST, ALT, ALP and γ GT in serum near normal values in DEN induced rats after 28 days of experimental study. Hence, the dose of 50 mg/kg bwt was chosen for further study. Values of liver marker enzyme levels are given in the Table 3.1.

<table>
<thead>
<tr>
<th>Concentration of 1,3 BPMU(mg)</th>
<th>AST(U/L)</th>
<th>ALT(IU/L)</th>
<th>ALP(IU/L)</th>
<th>γGT(IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.12±0.83</td>
<td>18.36±1.37</td>
<td>32.14±2.62</td>
<td>5.76±1.73</td>
</tr>
<tr>
<td>20</td>
<td>8.28±0.76</td>
<td>36.74±1.33</td>
<td>58.82±3.91</td>
<td>9.86±2.98</td>
</tr>
<tr>
<td>30</td>
<td>7.12±0.71</td>
<td>30.68±0.98</td>
<td>50.74±3.01</td>
<td>7.73±2.90</td>
</tr>
<tr>
<td>40</td>
<td>5.01±1.01</td>
<td>28.92±1.21</td>
<td>48.97±2.91</td>
<td>6.98±1.88</td>
</tr>
<tr>
<td>50</td>
<td>4.37±1.23</td>
<td>20.93±1.46</td>
<td>34.84±2.80</td>
<td>6.01±1.17</td>
</tr>
<tr>
<td>60</td>
<td>4.46±0.85</td>
<td>21.38±1.97</td>
<td>33.17±3.63</td>
<td>6.38±1.36</td>
</tr>
</tbody>
</table>
Table 3.2 shows the body weight of control and experimental group of rats. The final body weight of DEN induced group of rats significantly decreased after treatment with DEN. 1, 3BPMU treatment significantly increased the final body weight as compared to DEN induced rats. But, no significant changes could be observed in the final body weight of 1, 3BPMU and Sylimarin treated rats when compared with normal control rats. Oral administration of 1, 3BPMU significantly recouped the body weight and organ weights as compared with group 3 rats.

Table: 3. 2-Effect of 1, 3 BPMU on Body and liver weight in control and experimental rats.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Wt in gm)</td>
<td>156± 10.21</td>
<td>162 ±12.8</td>
<td>157 ± 2.9</td>
<td>159 ± 12.5</td>
<td>159±12.58</td>
</tr>
<tr>
<td></td>
<td>Initial bodyweight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Final body weight</td>
<td>252± 19.52</td>
<td>251 ±20</td>
<td>196± 5.8a</td>
<td>249 ± 19.84b</td>
<td>237±18.65</td>
</tr>
<tr>
<td></td>
<td>Liver weight</td>
<td>7.45 ± 0.59</td>
<td>8.20± 0.65</td>
<td>13.29±1.06a</td>
<td>8.32± 0.67b</td>
<td>9.18± 0.97</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SD for six rats in each group. aAs compared with group I, bAs compared with group III.p<0.001.

Figure 3.2 shows the activity of AST, ALT, ALP GGT, 5’NT in the serum of control and experimental animals. It is observed that administration of DEN (Group-III) to rats produced a significant increase in the activities of all the marker enzymes, the increase being two-fold for AST, ALT, and ALP, when compared to control rats. An increased activity of these enzymes was observed in serum of rats after administration of DEN (Group-IV) when compared to control rats. Treatment with 1, 3 BPMU administration (Group-IV) significantly decreased the activities of these enzymes to normal and resemble with hepato protective sylimarin treated rats (GroupV). 1, 3 BPMU treatment markedly decreased the activity of these enzymes in rats towards normal when compared to DEN treated rats.
Each value is expressed as mean ± SD for six rats in each group (p<0.001).

**Fig.3.2.-Effects of 1, 3BPMU on plasma marker enzymes of control and experimental group of rats.**

Table- 3.3 indicates the plasma concentration of tumor marker proteins such as AFP and CEA in control and experimental group of rats. The plasma concentrations of AFP and CEA marker proteins were elevated in DEN-induced rats as compared with control rats \((P<0.05)\). However, cancer bearing rats treated with 1,3BPMU significantly reduced tumor marker levels as compared with DEN-induced rats \((P<0.05)\). There were no variations observed in rats treated with only 1,3 BPMU as compared with control rats \((P<0.05)\). The observed significant increase of serum α2-macroglobulin in DEN-induced rats is in harmony.

**Table: 3.3-Determination of α2-Macroglobulin (α2M), carcinoembryonic antigen (CA) and Assay of alpha-fetoprotein (AFP).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>0.04±0.003</td>
<td>0.05±0.004</td>
<td>0.46±0.15 (^a)</td>
<td>0.18±0.09 (^b)</td>
<td>0.08±0.002</td>
</tr>
<tr>
<td>AFP</td>
<td>0.06±0.04</td>
<td>0.07±0.03</td>
<td>0.58±0.07 (^a)</td>
<td>0.17±0.05 (^b)</td>
<td>0.05±0.06</td>
</tr>
<tr>
<td>α2M</td>
<td>135.8±4.2</td>
<td>139.7±3.6</td>
<td>228.8±5.4 (^a)</td>
<td>134.17±3.99 (^b)</td>
<td>140.5±5.1</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SD for six rats in each group. \(^a\)As compared with group I, \(^b\)As compared with group III\((p<0.001)\). AFP & CEA- ng/ml, α2M- mg/dl.
The effect of 1, 3 BPMU on serum cholesterol, triglyceride, low density lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL) is presented in Tables 3.4. Rats induced with DEN exhibited higher serum cholesterol, triglyceride, LDL, VLDL and decreased HDL level in serum compared to that of control rats. However, administration of 1,3 BPMU to DEN induced rats showed significant reduction in serum cholesterol, triglyceride, very low density lipoprotein and low density lipoprotein, the HDL levels significantly increased compared to those group induced by DEN. The levels of lipid profile in DEN induced group, 1, 3 BPMU alone treated, DEN induced then 1,3 BPMU treated compared to each other. In Sylimarin (Positive drug) there is no significant changes observed throughout the experiment (Table 3.4).

Table: 3.4-Effect of 1,3 BPMU on lipid profile in DEN induced hepatocellular carcinoma rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>VLDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>97.78±6.84</td>
<td>86.48±6.05</td>
<td>32.96±2.30</td>
<td>19.55±1.36</td>
<td>33.97±2.37</td>
</tr>
<tr>
<td>Group II</td>
<td>94.44±6.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84.61±5.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.61±2.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.88±1.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.12±2.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>153.33±10.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>132.70±9.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.74±1.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.66±2.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.33±5.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>93.33±6.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92.48±6.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.78±2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.66±1.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.04±2.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>96.62±6.78</td>
<td>91.62±6.52</td>
<td>33.89±2.52</td>
<td>19.91±1.47</td>
<td>31.84±2.91</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SD for six rats in each group.  
<sup>a</sup>As compared with Group I (p<0.01),  
<sup>b</sup>As compared with Group III (p<0.01).

The effect of 1, 3 BPMU on the contents of serum glucose, total bilirubin, total protein, urea and creatinine in control and experimental rats is presented in Table 3.5. The level of serum, glucose, total bilirubin, total protein, urea and creatinine were found to be elevated in DEN induced rats. In the 1, 3 BPMU administered rats, the levels of glucose, total bilirubin, total protein, urea and creatinine were reduced when compared with DEN induced rats. There were no significant changes in the levels of above biochemical parameters of 1,3BPMU and sylimarin treated rats when compared to control rats.
Table: 3.5-Determination of glucose, total bilirubin, total protein, urea and creatinine in the serum of control and experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose (mg/dl)</th>
<th>Bilirubin (mg/dl)</th>
<th>Albumin (gm/dl)</th>
<th>Total protein (gm/dl)</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>92.60±1.21</td>
<td>1.63±0.31</td>
<td>1.21±0.32</td>
<td>8.45 ± 0.27</td>
<td>30.42±2.13</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>Group II</td>
<td>91.14±1.15</td>
<td>1.65±0.30</td>
<td>1.1±0.2</td>
<td>7.65 ± 0.35</td>
<td>33.28±2.33</td>
<td>0.68±0.03</td>
</tr>
<tr>
<td>Group III</td>
<td>150.03±1.79 a</td>
<td>3.71±0.75 a</td>
<td>0.64±0.1 a</td>
<td>5.37±0.28 a</td>
<td>44.57±3.30 a</td>
<td>0.98±0.06 a</td>
</tr>
<tr>
<td>Group IV</td>
<td>98.32±1.42 b</td>
<td>1.86±0.42 b</td>
<td>1.02±1.3 b</td>
<td>6.61±0.14 b</td>
<td>34.71±2.42 b</td>
<td>0.73±0.05 b</td>
</tr>
<tr>
<td>Group V</td>
<td>93.42±.78</td>
<td>2.10±0.86</td>
<td>1.13±0.1</td>
<td>6.58±0.26</td>
<td>32.54±2.31</td>
<td>0.69±0.04</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at p <0.05. Activity is expressed as mg/dl. aComparisons are made with group1 (control). bComparisons are made with group3 (DEN-induced).

3.1.3. Discussion

Administration of 1, 3 BPMU improved the metabolic function of liver by decreasing the plasma enzyme levels in cancer treated rats. The level of AST and ALT were increased the incidence of hepatic diseases and abnormal metabolic function. AST is an enzyme found primarily in the cells of the liver, heart, skeletal muscles, kidneys, and to a lesser extent, in the pancreas. Its plasma concentration is in proportion to the amount of cellular leakage or damage. Its increased levels are usually associated with cardiac arrest or liver disease. ALT, an enzyme found primarily in the liver, It enhanced to release into the blood stream when the liver is abnormal, therefore serves as a specific indicator of liver status and its elevated levels in plasma indicate abnormal metabolic function of liver. The 1, 3 BPMU decrease the AST and ALT levels, which is an indication of the therapeutic effect on liver from carcinoma.

Liver damage due to the DEN, it reflects the liver instability and abnormal liver metabolism which leads to the drastic changes in serum enzymes and its activities. Transaminases are very important enzymes in liver function, which are LDH, ALT, ALP, ACP and γ GT. Role of these enzymes in liver is to maintain cellular integrity and
maintenence of cell membrane from the toxic effect. If these enzymes increased in serum level, it denotes that liver damage occurs by DEN. An elevated level of ALT in serum represents the hepatocellular damage which normally accompanied with AST. A highest level of ALP in serum indicates and reflects alteration of pathophysiologicals in biliary flow. Due to the carcinogenic effect of DEN, an increased range of AST and ALT found in serum. The main role is transport function by hepatocytes in liver damaged by DEN and leads to leakage of plasma membrane and finally increased level of transaminase enzymes. However treatment with 1,3 BPMU to DEN induced animals, the enzyme activity is gradually decreased and stability of cell as well as plasma membrane integrity repair mechanism carried out in hepatic tissue damaged by DEN. The serum trasnaminases levels were normalized in 1, 3 BPMU treated animals by normal cell regeneration and cellular formation also normalized in hepatocytes of liver. Discharged level of LDH in serum reflects the alteration of permeability of plasma membrane and integrity of plasma membrane in hepatic tissues. 1, 3 BPMU treated animal shows the effect of repair mechanism of mannich base compound by normalized level of LDH in serum.

$\gamma$ GT is another important enzyme for parenchymal cell maintenance in liver. It protects the cell by maintaining in normal condition and reducing the enzyme leakages in diseased condition. In our study, the abnormal level of $\gamma$ GT in DEN induced serum of animals indicates that loss of membrane integrity and stability in liver hepatocytes. The same $\gamma$ GT leakages blocked by maintaining membrane integrity is stimulated by 1, 3 BPMU. AFP is an oncofetal protein. Generally, these proteins absent in the normal, healthy adult. By the carcinogenic effect of DEN, an irregular, highest level of oncofetal protein in serum denotes the abnormal/ diseased condition of liver and it act as a marker protein for liver cancer (Ying et al., 2012). A drastic reduction of AFP observed in 1, 3 BPMU treated group of rats and it shows it has the anti cancer effect.

Carcinoembryonic antigen (CEA) is a glycoprotein and it comes under immunoglobulin family. It clinically used to diagnose many types of tumors especially HCC, it is a tumor marker protein (Zimmer and Thomas, 2001). The main function of CEA is an adhesion molecule, forming homotypic and heterotypic aggregation between the cells. CEA is cleared from the circulation by the liver with significant traces taken up by the spleen and lungs, Present study revealed that an increased $\kappa$
DEN induced animals associated with production of rates of tumor. But reduction level of CEA in 1, 3 BPMU treated animals were presumably due to the decreased production rate of tumor. The α2M might be tightly linked to the rat hepatocarcinogenesis from the initial stage to tumour progression even in conditions, which are undetectable, by established cytochemical markers such as placental glutathione-S-transferase (GST-P) and γ-GT-positive lesions (Sukata et al., 2004), also confirmed that the observed increases in serum α2M concentrations during hepatocarcinogenesis and in animals, bearing hepatic tumours was not a result of secretion by the host liver of α2M as an acute-phase reactant in response to inflammatory injury. Mainly, α2M functions as a carrier protein and re regulator for various growth factors and cytokines such as transforming growth factor-β (known to be involved in the onset of hepatocyte apoptosis). Furthermore, α2M partially counteracts the inhibitory effects of transforming growth factor-β on proliferation of neoplastic hepatocytes, suggesting that under some conditions, α2M can promote hepatocarcinogenesis by perturbing transforming growth factor-β-induced apoptosis. In the present study observed that the increased content of AFP, CEA and α2M concentrations in cancer bearing animals. Supplementation of compound to cancer bearing animals restored the content of AFP, CEA and α2M concentrations. Our findings are in concordance with (Nermin et al., 2008). Our study, which have been reported that supplementations of 1,3 BPMU decrease the content of AFP and α2M concentrations on DEN induced liver cancer.

Lipoproteins are particles that contain triacylglycerol, phospholipids, cholesterol and amphipathic proteins called apolipoproteins. Lipoproteins can be differentiated on the basis of their density, but also by the types of apolipoproteins they contain. The degree of lipid in a lipoprotein affects its density when lower the density of a lipoprotein, the more lipid it contains relative to protein. The four major types of lipoproteins are chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The liver is central to the regulation of cholesterol levels in the body. Not only does it synthesize cholesterol for export to other cells, but it also removes cholesterol from the body by converting it to bile salts and putting it into the bile where it can be eliminated in the feces. Furthermore, the liver synthesizes the various lipoproteins involved in transporting cholesterol and lipids throughout the body. Cholesterol synthesis in hepatocytes is under negative feedback regulation. Lipids are transported in the circulation system packaged in the form of lipoproteins. The clinic
lipid levels is that abnormal levels of lipids in certain lipoproteins are linked to an increase risk of atherosclerosis. Atherosclerosis is a cardiovascular disease in which lipids and inflammatory cells accumulate in plaques within the walls of blood vessels. As a result, vessel walls are narrowed and clots may form, impeding blood flow and oxygen delivery and causing tissue injury. Heart disease occurs because of the coronary arteries supply to the heart are a major site where atherosclerotic plaques form.

Abnormal lipid synthesis or defective degradation of lipids are implicated in the pathological condition like cancer. Peroxidation of lipids in biomembranes and tissues causes the leakage of these lipids into circulation and consequently leads to hyperlipidemia. Hyperlipidemia has been shown to increase the risk of metastasis in several cancers (Abel et al., 2009). Hepatoma is usually associated with hyperlipidemia as well as a notable decrease in the high-density lipoprotein (HDL) fraction and an enormous increase in the VLDL and LDL fractions. The major metabolic vital organ, liver, plays a key role in cholesterol metabolism in mammals. Several reports reveal the hypercholesterolemia in rats with hepatomas. The LDL cholesterol is more susceptible to oxidation in various pathologic conditions resulting in higher lipid peroxidation (LPO) during oxidative stress (Park et al., 2010). The high density lipoproteins on the other hand, have a great potential to counter the oxidative damage of LDL cholesterol in cell membrane, thereby preventing LPO.

VLDL converted to LDL in blood stream and it transported the endogenous products. The excess of cholesterol from cells reach to liver and converted in to VLDL, then from the hepatocytes chylomicrons carried by VLDL and converted into TAG, it carries energy to cells where required throughout the body. Due to this circulation VLDL level will be high when the liver is damaged. In the present study DEN induction showed higher level of VLDL and it indicates more circulation of VLDL in blood stream. It is gradually decreased by administration of 1,3 BPMU. HDL involves the reverse cholesterol transport process via liver (Skill et al., 2011). The excess of cholesterol eliminated from the body in bile. Liver removes LDL and other lipoproteins from the circulation by receptor mediated endocytosis and excess cholesterol from the cells of body bring back to liver is called as reverse cholesterol transport. HDL travel in the circulation and return the cholesterol to liver by various pathways. Due to an increased level of VLDL the circulation in blood, the HDL is
condition. The HDL circulation and reverse cholesterol transport process normalized by 1,3 BPMU.

Glucose is an important nutrient in human body and it act as a major energy source for many cells. Liver bilirubin is an orange-yellow pigment and it is a waste product of the normal breakdown of red blood cells. Bilirubin passes through the liver and eventually out of the body, mostly in feces, a small amount in urine. Higher than normal levels of direct or indirect bilirubin may indicate different types of liver problems. Occasionally, higher bilirubin levels may indicate an increased rate of destruction of red blood cells. The liver also makes albumin, an essential protein that circulates in blood. Albumin levels are low in people with severe chronic liver disease, because the liver does not make normal amounts of albumin. However, albumin levels may fall in a variety of medical conditions. A low albumin level is often temporary, so it is not a reliable way to diagnose liver disease. Glucose is an essential nutrient for the human body. It is the major energy source for many cells, which depend on the bloodstream for a steady supply therefore blood glucose levels are carefully to be maintained. The liver plays a central role in this process by balancing the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis. The several substrate cycles in the major metabolic pathways of the liver play key roles in the regulation of glucose production. The abnormal levels of glucose, albumin and bilirubin in DEN induced groups indicate that highest cell proliferation which caused by carcinogen. Similarly the 1,3 BPMU treated group showed the reduction of abnormal condition of urea and creatinin levels.

Urea is a waste product formed from the breakdown of proteins and metabololic byproduct of muscle metabolism. These are usually passed out by the urine. A high blood level of urea and creatinine indicates that the kidneys may not be working properly. Creatinine is usually a more accurate marker of kidney function than urea. In our study the serum contains an enormous various proteins such as marker protein liver protein enzymatic proteins in DEN induced group of animals, these all proteins breakdown and converted into urea in circulation path. But these highest level of urea formation was reduced by protein normalization due to the activity of 1, 3 BPMU.
3.2. Investigation of enzymatic and non enzymatic antioxidants and lipid

Peroxide in control and experimental rats

Introduction

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell 1995). These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body. Other lighter antioxidants are found in the diet. Although there is several enzymes system within the body that scavenges free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene. The body cannot manufacture these micronutrients, so they must be supplied in the diet (Shi et al., 1999).

The body creates free radicals and reactive oxygen species relentlessly and continuously. Antioxidants are compounds which provide our body with protection against the harmful effects of damaging free radicals and other reactive oxygen species. By definition, an antioxidant is a compound that is able to react with free radicals, forming harmless unreactive molecules and protecting other biological molecules from damage. Antioxidants are either reactive chemicals such as vitamin E or specialised enzymes like catalase. The body produces enzymatic antioxidants but it cannot make antioxidant chemicals such as vitamin E, C and flavanoids. These antioxidant chemicals protect the sites in the body which the enzymatic systems cannot reach. We obtain these antioxidant chemicals from our diet but they are rapidly turned over in the body and need to be constantly replenished. Cells are protected against oxidative stress by an interacting network of antioxidant enzymes (Sies 1997). Here, the superoxide released by the processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide (Magnenat et al., 1998).
Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Zelko et al., 2002). SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Johnson and Giulivi 2005). There are three major families of superoxide dismutase (SOD1, SOD2, SOD3), depending on the metal cofactor (Cu/Zn (which binds both copper and zinc) the activity of SOD may differ. Reactive oxygen species are produced in both unstressed and stressed cells. Plants have a well developed defence systems against ROS and involving both limiting the formation of ROS as well as its removal. Super oxide dismutase (SODs) constitute the first line of defence against ROS. The main function of SOD may due to the combination of the more enzymes and commonly of elements in the upstream sequence of Fe, Mn and Cu/Zn SODs suggest a relatively recent origin for those regulatory regions. The finding that the upstream sequence of Mn and peroxisomal Cu/Zn SODs has three common elements suggests a common regulatory pathway. Fe and Mn types of SOD present in mitochondria of higher plants. SOD1 is located in the cytoplasm, SOD2 in mitochondria, SOD3 in extra cellular matrix.(Corpas et al., 2006, Cao et al., 2008). Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Banniste et al., 1987, Chelikani et al., 2004).

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms. Glutathione peroxidase is an enzyme containing four selenium cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes et al., 2005). Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesized by humans and must be obtain
(Smirnoff, 2001). Most other than animals are able to produce this compound in their bodies and not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins. Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide (Padayattay et al., 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants. Lipid peroxidation is probably the most extensively investigated free radical induced process. One of the earliest descriptions of the different stages of lipid peroxidation was given in the late 1820s by de Saussure, who used a simple mercury manometer to study the uptake of oxygen by a layer of walnut oil on water are particularly susceptible to peroxidation and once the process is initiated, it proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination. An increased concentration of end products of lipid peroxidation is the evidence most frequently quoted for the involvement of free radicals in human disease. However, it is likely that increased oxidative damage occurs in most, if not all, human diseases and plays a significant pathological role in only some of them. For example, peroxidation appears to be important in atherosclerosis and in worsening the initial tissue injury caused by ischemic or traumatic brain damage. Oxidative stress can damage many biological molecules; indeed, proteins and DNA are often more significant targets of injury than are lipids, and lipid peroxidation often occurs late in the injury process (Halliwell and Chirico, 1993).

3.2.1-Materials and methods.
3.2.1.1. Assay of Manganese Superoxide dismutase (Mn SOD)( Kakkar et al.,1984)

0.5ml of serum sample was diluted to 1ml with water. Then 2.5ml of ethanol and 1.5ml chloroform (all reagents chilled) were added. This mixture was shaken for one minute at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.01ml of phenazine methosulphate, 0.3ml of nitroblue tetrazolium, 0.2ml of NADH, appropriately diluted enzyme preparation; 0.01ml of KCN and water in a total volume of 3ml. Reaction was initiated by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1ml glacial acetic acid. The
stirred vigorously and shaken with 4ml of n-butanol. The intensity of the chromogen in the n-butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control. Enzyme activity was expressed as U/mg protein in tissues.

### 3.2.1.2 Assay of Catalase (Beers and Sizer, 1952).

1.9 ml of distilled water and 1ml of the hydrogen peroxide reagent, as substrate was added and incubated for 4-5 minutes. 0.1 ml of serum sample was added and the decrease in absorbance for 2-3 minutes was recorded at 240nm. Enzyme activity was expressed as U/mg protein in tissues.

### 3.2.1.3 Assay of Glutathione Peroxidase (Rotruck et al., 1973).

The reaction mixture consists of 0.2ml of EDTA, sodium azide, H₂O₂, 0.4ml of phosphate buffer; 0.1ml of serum sample and it was incubated at 37°C. The reaction was arrested by the addition of 0.5ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5ml of supernatant, 4ml of disodium hydrogen phosphate and 0.5ml DTNB were added and the colour developed was read at 420nm immediately. The activity of GPₓ was expressed as U/mg protein in tissues.

### 3.2.1.4 Assay of Glutathione reductase (Staal et al., 1969).

The reaction mixture containing 1ml of phosphate buffer, 0.5ml of EDTA, 0.5ml of oxidized glutathione and 0.2ml of NADPH was made up to 3ml with distilled water. After the addition of 0.1ml of mitochondrial fraction, the change in optical density at 340nm was monitored for 2min at 30sec intervals. The activity of GR was expressed as nmole of NADPH oxidized/min/mg protein in tissues.

### 3.2.1.5 Assay of Glutathione-S-Transferase (Habig et al., 1974).

The reaction mixture contains 1.0ml of phosphate buffer, 0.01ml of CDNB, 0.1ml of sample and 0.7ml of distilled water. This mixture was pre incubated at 37°C for 5 minutes and then the reaction was started by the addition of 0.1ml of GSH. The change in the absorbance was read at 340nm for 5 minutes, the reaction mixture without the enzyme was used as the blank. The enzyme activity was expressed as μmole of glutathione utilized/min/mg protein in tissues.
3.2.1.6. Estimation of Ascorbic acid (Vitamin C) (Omaye et al., 1979, Baker., et.al., (1980).

To 0.5ml of serum sample, 0.5ml of water and 1ml of TCA were added, mixed thoroughly and centrifuged. To 1ml of the supernatant, 0.2ml of DTC reagent was added and incubated at 37° C for 3hours. Then 1.5ml of sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30min. The colour developed was read at 520nm. The level of ascorbic acid was expressed as µg/mg protein in tissues.

3.2.1.7. Estimation of α-tocopherol (Vitamin E) (Omaye et al., 1979).

To 0.5ml of serum sample, 1.5ml of ethanol was added, mixed and centrifuged. The supernatant was dried at 80° C, to this 0.2ml of 2,2'-dipyridyl and 0.2ml of ferric chloride solution were added, mixed well and 4.0ml of butanol was added. The developed red colour was read at 520nm. The level of α-tocopherol was expressed as µg/mg protein in tissues.


The serum sample was combined with 2.0ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15minutes in a boiling water bath. The flocculants were centrifuged at 1000rpm for 10 min. The absorbance of the sample was read at 535nm against blank without sample. Values were expressed as n mole of MDA formed/mg protein in tissues.

3.2.2. Results

Table 3.6 shows the effect of 1, 3 BPMU on serum antioxidant enzymes activities. In the present study, changes in the activities of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione-S-transferase (GST) and reduced glutathione (GSH) levels were investigated. The activities of the antioxidant enzymes did not show variation between the control and 1, 3 BPMU alone and sylimarin treated rats. When DEN alone was administrated to the group III rats, there was a reduction in the activities of all antioxidant enzymes when compared to control rats. The GPx level was found to be drastically increased in the DEN induced rats after administration of 1, 3 BPMU.
Table 3.6. Investigation of enzymatic antioxidants in control and experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD(U/mg)</th>
<th>CAT(U/mg)</th>
<th>GPx(U/mg)</th>
<th>Gr(U/mg)</th>
<th>GST(U/mg)</th>
<th>GSH(U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>3.99 ± 0.19</td>
<td>4.82 ± 0.23</td>
<td>6.21 ± 0.43</td>
<td>5.28 ± 0.26</td>
<td>0.89 ± 0.02</td>
<td>9.84 ± 0.49</td>
</tr>
<tr>
<td>Group II</td>
<td>4.35 ± 0.21</td>
<td>4.61 ± 0.22</td>
<td>6.72 ± 0.33</td>
<td>5.63 ± 0.28</td>
<td>0.96 ± 0.02</td>
<td>10.56 ± 0.52</td>
</tr>
<tr>
<td>Group III</td>
<td>2.56 ± 0.27 a</td>
<td>3.88 ± 0.28 a</td>
<td>4.63 ± 0.38 a</td>
<td>3.82 ± 0.19 a</td>
<td>0.59 ± 0.01 a</td>
<td>6.03 ± 0.60 a</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.87 ± 0.24 b</td>
<td>4.72 ± 0.24 b</td>
<td>6.73 ± 0.33 b</td>
<td>4.83 ± 0.24 b</td>
<td>0.71 ± 0.02 b</td>
<td>10.11 ± 0.50 b</td>
</tr>
<tr>
<td>Group V</td>
<td>3.89 ± 0.31</td>
<td>4.81 ± 0.23</td>
<td>6.67 ± 0.29</td>
<td>5.01 ± 0.21</td>
<td>0.87 ± 0.11</td>
<td>9.97 ± 0.50</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SD for six rats in each group. a As compared with group I (p<0.01), b As compared with group III (p<0.01).

The levels of serum and liver non-enzymatic antioxidants namely total glutathione, vitamin C and vitamin E levels are presented in Fig 3.3. In the present study, changes in the levels of GSH, vitamin C and vitamin E were investigated. The levels of the non-enzymatic antioxidants did not show much variation between the control, 1, 3 BPMU and silymarin alone treated rats. When DEN alone was administrated to the rats (Group III), there was a significant reduction in the levels of all non-enzymatic antioxidants when compared to control. However, rats treated with 1, 3 BPMU (Group IV) showed significant increase in the non enzymatic antioxidant status.

Fig. 3.3. Investigation of non enzymatic antioxidants in control and experimental rats

The levels of serum and liver lipid peroxides in control and experimental rats are given in Fig.3.4. The DEN induced rats exhibited higher level of MDA in serum as compared to that of control rats. However, 1, 3 BPMU treatments to group IV rats showed reduced MDA level when compared to DEN induced rats. Similar results obtained in liver MDA levels. No significant variation was observed in the rats treated with 1, 3 BPMU.
3.2.3. Discussion

Free radicals produced by carcinogens and it causes oxidative stress which in turn leads to the damage of nucleic acid, protein and lipid resulting in development of cancer by chromosomal instability, mutations, loss of organells deformation and function, membrane damage and its mechanism. Unreactive super oxide anion free radical is converted into H$_2$O$_2$ in the presence of the enzyme super oxide dismutase (SOD).

Antioxidants are substances that either directly or indirectly protects cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions (Ramakrishnan et al., 2006). SOD is the primary step of defense mechanism in the antioxidant system against the oxidative stress. It demolish the superoxide radical by converting it to peroxide and molecular oxygen in turn, that can be counteracted by catalase or GPx reactions, thereby reducing the level of cellular damage. During the reaction of H$_2$O$_2$ scavenging, GSH is oxidized to GSSG by the enzyme GPx (Sundaresan and Subramanian, 2008) and GR reduces GSSG to GSH (Sreepriya and Bali, 2005).

Deleterious effects of oxidants progression to neoplastic condition are affronted by primary antioxidants such as SOD, CAT and GPx. In a variety of malignancies, the elevated LPO is associated with reduced activity of antioxidants. Deficiency of SOD and CAT results in decreased detoxification of oxygen radicals, which leads to attack of ROS on protein and nucleic acids. Hence, the activities of these enzymes are decreased in cancerous condition (Van der Oost et al., 2003). Such studies substantiate with the findings of the present study as there was significant depletion in the activities of enzymatic antioxidants in both serum of animals treated with DEN when compared to
normal animals. The findings of this study signify that 1, 3 BPMU treatments to DEN induced rats were able to reverse the reduced activities of antioxidant status.

Vitamin C, Vitamin E and reduced glutathione are well known non-enzymic antioxidant defense system of cells. These are interrelated with each other by recycling process. Vitamin C is water soluble antioxidant and can react with radicals to regenerate vitamin E (Sehrawat and Sultana, 2006). Vitamin E is chain breaking antioxidant present in the cell membrane. It provides protection against superoxides as well as H$_2$O$_2$ (Gupta et al., 2010).

GSH is the major cytosolic thiol compound and is required to maintain the normal reduced state of the cells and to counteract ROS, thereby reducing the oxidative stress (Sivalokanathan et al., 2006). GSH also preserves the cellular levels of active forms of vitamin C and vitamin E (Pradeep et al., 2007). The levels of these non-enzymic antioxidants were decreased in hepatoma bearing animals (Kim et al., 2008). The results of present study also correlate with such findings. It might be due to over utilization of these antioxidants to scavenge free radicals. On other hand, the simultaneous administration of 1, 3 BPMU reversed the changes induced by DEN exposure to near normal, and supporting the hypothesis that 1, 3 BPMU is an effective chemotherapeutic agent.1, 3 BPMU treatment has been able to enhance the non-enzymic antioxidants to near normal when compared to tumor bearing rats. Hence 1, 3BPMU exhibits its chemotherapeutic effects against DEN-induced HCC by enhancing the antioxidant status probably through its free radical scavenging and having potential of protecting endogenous non-enzymic antioxidants

3.3. Evaluation of mitochondrial enzymes and membrane bounded enzymes in control and experimental rats

Introduction

Isocitrate dehydrogenases (IDHs) comprise a family of enzymes that catalyze oxidative decarboxylation of isocitrate to alpha-ketoglutarate (2-oxoglutarate). Eukaryotic cells express two distinct classes of IDHs that utilize either NAD or NADP as the electron acceptor and serve diverse biological functions:
- NAD-dependent IDH, IDH3, is located at the mitochondrial matrix and is well known for its central role for energy production in the Krebs cycle.

- The two NADP dependent forms are primarily located either in mitochondria (IDH2) or cytoplasm (IDH1). In addition to their potential catabolic role in the Krebs cycle, both mitochondrial and cytosolic IDHs are shown to play an important role in cellular defense against oxidative damage as a source of NADPH.

The three IDH isoenzymes are important players in the exchange of metabolites between the mitochondria and the cytosol. IDH3 is part of the TCA cycle where it generates NADH as a fuel for energy production IDH1 and IDH2 are important for shuttling electrons between the mitochondria and the cytosol. Moreover, all eukaryotic cytosolic IDHs contain a type 1 peroxisomal targeting sequence at their C terminus that is sufficient to direct proteins into peroxisomes. Indeed cytosolic IDHs have been found in peroxisomes of yeast, human and rat liver cells and are shown to be required for the beta-oxidation of unsaturated fatty acids as a provider of NADPH inside peroxisomes. Metabolites entering the mitochondria can be processed for energy generation usually through the production of NADH in the TCA cycle whereas metabolites exported back to the cytosol take part in anabolic processes. The transport of metabolites is also coupled to electron exchange between mitochondrial and cytosolic NADH and NADPH both of which cannot move across the mitochondrial inner membrane.

Biological membranes are the first fence that has to be overcome by toxic compounds targeting the cell. The minimum functional unit of Na\(^+\), K\(^+\), and ATPase is an oligomer composed of stoichiometric amounts of two major polypeptides, the so-called α and β subunits. The different kinetic parameters for activating cations (Na\(^+\) and K\(^+\)), the substrate ATP for each Na\(^+\),K\(^+\) ATPase isoyme imply that each isoform has distinct properties The expression of Na\(^+\), K\(^+\), ATPase isoforms can be altered by pathological conditions. For instance, in several cardiac diseases, the Na\(^+\), K\(^+\) ATPase isoform composition of the heart is modified (Muller et al., 2002).

Numerous studies have reported changes in Na\(^+\),K\(^+\) ATPase subunit expression and activity in the course of malignant transformation, including gliomas, with evidence that these occur at the very early stages of tumorigenesis (Mijatovic et al., 2007). Moreover, it was previously shown that both non-small cell lung
overexpress Na⁺,K⁺ ATPase compared with healthy tissues (Mijatovic et al., 2008). Histopathological studies using Light microscope showed that in the rats induced with DEN, there was a loss of liver architecture and the lobules of neoplastic hepatocytes exhibited focal area of fatty change (Luly et al., 1972). Neoplastic cells have vesicular to hyper chromatic nuclei with a typical mitotic change. It also showed sinusoidal dilatation with cords of neoplastic hepatocytes (Janani et al., 2010).

3.3.1. Materials and Methods.

3.3.1.1. Analysis of mitochondrial enzymes in control and experimental rats.

3.3.1.1.1. Isolation of mitochondria (Johnson and Lardy, 1967).

The liver tissues were homogenized in ice-cold 50 mM of Tris-HCl (pH 7.4) containing 0.25M of sucrose. According to Ignarro (1971) this buffer helps to maintain the integrity of membrane during homogenization. The homogenate of tissues were centrifuged at 600 ×g for 20 min and then the supernatant obtained was centrifuged at 12,000rpm for 15 min to obtain mitochondria pellets. The mitochondrial pellets were washed with 10 mM of Tris-HCl (pH 7.8) containing 0.25 mole of sucrose and finally resuspended in the same buffer. The suspended mitochondrial pellets were used for various biochemical analyses.

3.3.1.1.2. Determination of Lactate Dehydrogenase (LDH) Activity (King, 1965).

0.02ml of serum was mixed with 1.0ml of buffered substrate and placed in a water bath at 37°C. 0.2ml of NAD solution was added. Only in test exactly after 15 minutes, 1.0ml of 2,4 DNPH was added, mixed and left in the water bath for 15 minutes. Then 10ml of 0.4N NAOH added and read at 440nm. Values are expressed as IU / L.

3.3.1.1.3. Assay of Succinate dehydrogenase (SDH) (Slater and Bonner, 1952).

The reaction mixture containing 1ml of phosphate buffer, 0.1ml of EDTA, 0.1ml of BSA, 0.3ml of sodium succinate and 0.2ml of potassium ferricyanide were made up to 2.8ml with water. The reaction was started by the addition of 0.2ml of mitochondrial suspension. The change in OD was recorded at 15 seconds interval for 5 min at 420 nm. The succinate dehydrogenase activity was expressed as μmoles of succinate oxidized/min/mg protein in tissues.
3.3.1.4. Assay of Isocitrate dehydrogenase (ICDH) (King, 1965).
To 0.1ml of Tris-HCl, 0.2ml of trisodium isocitrate, 0.3ml of manganese chloride and
0.2ml of mitochondrial suspension and 0.2ml of NADP⁺ (0.2ml of water for controls)
were added. After 60 min of incubation, 1 ml of DNPH was added followed by 0.5ml of
EDTA and kept at room temperature for 20 min. Then 10 ml of NaOH was added and the
colour developed was read at 390 nm. A standard containing α-ketoglutarate was run
simultaneously. The isocitrate dehydrogenase activity was expressed as nmoles of α-
ketoglutarate liberated/min/mg protein.

3.3.1.5. Assay of Malate dehydrogenase (MDH). (Mehler et al., 1948).
To 0.3ml of buffer, 0.1ml of NADH and 0.1ml of oxaloacetate were added and the
total volume was made upto 2.9ml with water. The reaction was started by adding 0.1ml
of mitochondrial suspension. The change in optical density was measured at 350 nm in an
interval of 15 seconds for 5 min in a spectrophotometer. The enzyme activity was
expressed as nmoles of NADH oxidized/min/mg protein.

3.3.1.2 – Analysis of membrane bound enzymes in control and experimental rats.
3.3.1.2.1. Assay of Na⁺, K⁺ ATPase (Bonting, 1970).
1 ml of Tris-HCl buffer, 0.2 ml of MgSO₄, NaCl, EDTA and the homogenate were
added to form the incubation mixture with a final volume of 2 ml. Equilibration of the
mixture was carried out for 10 min at 37 °C, the reaction was started by the addition of
ATP, 2.2 ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the
supernatant was estimated according to the method of Fiske and Subbarow (1925). The
enzyme activity is expressed as µmoles of phosphorus liberated/min/mg of protein.

3.5.1.2.2. Assay of Mg²⁺ ATPase (Ohnishi et al., 1982).
0.1 ml of Tris-HCl buffer, MgCl₂, ATP, homogenate were added to form the
reaction mixture and was incubated at 37 °C for 15 min. The reaction was arrested by the
addition of 1.0 ml TCA and the liberated phosphorus was estimated according to the
method of Fiske and Subbarow (1925). The enzyme activity is expressed as µmoles of
phosphorus liberated/min/mg of protein under incubation conditions.
3.3.1.2.3. Assay of Ca\textsuperscript{2+} ATPase (Ohnishi et al., 1982).

0.1 ml of Tris-HCl buffer, CaCl\textsubscript{2}, ATP and homogenate were added to form the reaction mixture and then incubated at 37 °C for 15 min. The reaction was arrested by the addition of 1.0 ml of TCA. The test mixture was centrifuged and the supernatant was estimated for the phosphorous content. The amount of phosphorus liberated was quantified (Fiske and Subbarow, 1925). The enzyme activity was expressed as µmoles of phosphorus liberated/min/mg of protein under incubation conditions.

3.3.1.2.4.-Estimation of nucleic acids (Schneider, 1957).

Known amount of tissues were homogenized in 5.0ml of ice-cold distilled water using homogeniser with a Teflon pestle. 5.0ml of 5% TCA was added to the homogenate and this was kept in ice for 30 minutes to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. Then it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 90°C for 15 minutes with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.

3.3.1.2.5. Deoxy ribonucleic acid (DNA) (Burton, 1956).

A known volume of the nucleic acid extract was made up to 3.0ml with 1N perchloric acid. This was mixed with 2.0ml of diphenylamine reagent. A reagent blank and standards were also carried out concurrently. This was kept in a boiling water bath for 10 minutes and the blue colour developed was read at 640nm in a spectrophotometer. The DNA level was expressed as mg/g wet tissue.

3.3.1.2.6.-Ribonucleic acid (RNA) (Rawal et al., 1977).

Aliquots of nucleic acid extracts were made up to 2.0ml with 5%TCA. To this 3.0ml of orcinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 minutes. Reagent blank and standards were also treated in the same way. The tubes were cooled and the colour developed was measured at 640nm using a spectrophotometer. The RNA level was expressed as mg/g wet tissue.
3.3.2. Results

The restorative effect of 1, 3 BPMU on mitochondrial enzymes in control and experimental animals is presented in Table 3.1. The activity of isocitrate dehydrogenase (IDH) reduced in the DEN induced rats. However, treatment with 1, 3BPMU significantly increased its activity when compared to DEN induced rats. No significant difference could be observed between the control rats and rats treated with 1, 3BPMU and sylimarin. The finding indicates that the 1,3BPMU has the potentiality to protect the mitochondrial damage in the DEN induced HCC animals. The activity of succinate dehydrogenase (SDH) decreased in the DEN induced rats compared with control rats. However, 3BPMU administration restored the altered activity of succinate dehydrogenase when compared with DEN induced rats. The activity of malate dehydrogenase (MDH) significantly reduced in DEN induced rats. But after 1,3BPMU activity increased when compared with DEN induced rats. There was no marked difference in the activities of the TCA cycle enzyme between the rats treated with sylimarin and 1, 3BPMU alone and control rats.

Table 3.7. Analysis of mitochondrial enzymes in control and experimental rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>ICDH(mg)</th>
<th>SDH(mg)</th>
<th>MDH(mg)</th>
<th>LDH(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>820+22.1</td>
<td>28.54+3.8</td>
<td>345+13.25</td>
<td>79.27 ± 4.36</td>
</tr>
<tr>
<td>Group II</td>
<td>834+20.65</td>
<td>26.98+3.21</td>
<td>356+14.21</td>
<td>79.34 ± 4.35</td>
</tr>
<tr>
<td>Group III</td>
<td>540+15.24 a</td>
<td>18.56+1.97 a</td>
<td>230+10.32 a</td>
<td>120.34 ± 6.30 a</td>
</tr>
<tr>
<td>Group IV</td>
<td>790+19.32 b</td>
<td>23.76+2.95 b</td>
<td>305+12.45 b</td>
<td>81.53 ± 7.24 b</td>
</tr>
<tr>
<td>Group V</td>
<td>809+21.52</td>
<td>25.31+2.11</td>
<td>349+13.54</td>
<td>80.39+6.72</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ± SD for six rats in each group. aAs compared with group I (p<0.01), bAs compared with group III (p<0.01).

Fig.3.6 represents the quantification of DNA and RNA in control and experimental groups of rats. The content of DNA and RNA increased in DEN-induced rats as compared with control group of rats, whereas cancer bearing rats treated with 1, 3 BPMU decreased the content of DNA and RNA as compared with DEN-induced rats (P<0.05). Abnormal content of DNA is related with malignancy development. It indicates cell proliferation in tumor condition. It is thus indicated that 1, 3BPMU significantly controls tumour development. No significant changes were observed in rats treated with only 1, 3 BPMU as compared with control rats (P<0.05).
Table 3.8 represents the activities of liver mitochondrial membrane bound ATPases such as sodium-potassium (Na⁺-K⁺) ATPase, calcium (Ca²⁺) ATPase and magnesium (Mg²⁺) ATPase in control and experimental animals. The activity of Na⁺-K⁺ ATPase decreased significantly in the liver of DEN induced rats compared with control rats. However, treatment with 1, 3 BPMU to DEN induced rats increased the activity of Na⁺-K⁺ ATPase in the liver compared to DEN induced rats. The activity of Ca²⁺ ATPase was found to be increased in DEN induced rats compared to control group.

![Graph showing nucleic acid levels in control and experimental group of rats](image)

**Fig. 3.6-Nucleic acid levels in control and experimental group of rats**

There was a decrease in the activity of Ca²⁺ ATPases in the experimental rats treated with 1, 3 BPMU compared with DEN induced rats. The activity of Mg²⁺ ATPase increased in DEN induced groups compared to control group. There was a decrease in the activity of Mg²⁺ ATPases in experimental rats treated with 1, 3 BPMU compared with DEN induced rats. No significant changes were distinguished in rats treated with 1, 3 BPMU alone compared to that of control group.
Table 3.8. Effect of 1, 3 BPMU on membrane bound enzymes in control and experimental group of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>$\text{Na}^+\text{K}^-\text{ATPase}$</th>
<th>$\text{Ca}^+\text{ATPase}$</th>
<th>$\text{Mg}^{2+}\text{ATPase}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>2.16±0.96</td>
<td>0.79±0.12</td>
<td>0.73±0.16</td>
</tr>
<tr>
<td>Group II</td>
<td>1.98±0.83</td>
<td>0.81±0.18</td>
<td>0.74±0.21</td>
</tr>
<tr>
<td>Group III</td>
<td>0.32±0.46 a</td>
<td>1.63±0.34 a</td>
<td>2.78±0.57 a</td>
</tr>
<tr>
<td>Group IV</td>
<td>1.58±0.73 b</td>
<td>0.89±0.21 b</td>
<td>0.81±0.18 b</td>
</tr>
<tr>
<td>Group V</td>
<td>1.98±0.82</td>
<td>0.85±0.09</td>
<td>0.78±0.21</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at p <0.05. Activity is expressed as, μmoles of phosphorus liberated/min/mg of protein. aComparisons are made with group1 (control). bComparisons are made with group3 (DEN-induced).

3.3.3. Discussion

The liver plays a pivotal role in regulation of physiological processes. Oxidative stress is associated with damage to a wide range of macromolecular species such as lipids, proteins and nucleic acids thereby producing major interrelated de arrangements of cellular metabolism. Free radicals and non-radicals oxidizing species were produced in animals treated with carcinogens and also in human tissues (Ellis et al., 1991). DNA acts as a genetic determinant and functional aspect of tumor formation. It indicated the tumor condition with more proliferation. The tumor cell size increased due to the presence of excessive DNA content. In the present study the increased level of DNA synthesis in cancer bearing animal group (DEN-induced) were identified by the expression of enzymes for cell differentiation and function. The increased level of RNA in cancer bearing animals may be because of excess DNA content which leads to increased level of transcription in cancerous conditions. Simultaneously, 1, 3BPMU treated animals controlled the nucleic acid excessive biosynthesis bringing about the anticancer effect. Abnormalities in DNA content are associated with malignancy. It is an indicator of proliferating activity in tumor conditions. Hence, it is important to determine DNA content in cancer state. The effect of 1, 3BPMU on the levels of nucleic acids (DNA and RNA) in control and experimental group of rats are shown in figure 3.6. In DEN induced (group III) animals, the levels of nucleic acids were found to be si
increased when compared to control group of rats. Conversely, these elevated levels were significantly (p<0.05) decreased in 1, 3BPMU treated group 4 animals when compared to group III animals.

In malignancy, the cell membrane plays a crucial role in stimulation and control of cell adhesiveness, mortality and proliferation in a much damaged condition (Berson et al., 2006). The protection of membranes is of potential importance in the treatment of disease processes. ATPases are membrane-bound enzymatic proteins that maintain ionic gradients between aqueous intra-and extracellular phases. Membrane-bound enzymes such as Na⁺/K⁺ ATPase, Ca²⁺ ATPase and Mg²⁺ ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP by hydrolysis (Torres et al., 2009).

In the present study, the decrease in the activities of Na⁺/ K⁺ ATPase, in liver cancer-bearing animals may be due to increased production of free radicals leading to cell injury (Kantihaldar et al., 2011). Free radicals are clearly involved in the pathogenesis of various diseases such as atherosclerosis, inflammatory diseases and cancer. Free radicals have been suggested to exert their cytotoxic effects by causing peroxidation of membrane phospholipids. Damage of plasma membrane occurs directly through interaction with the membrane components such as the ion-dependent ATPases and ion channels and indirectly as a consequence of overt cytosolic damage. Inhibiting function of ion-dependent ATPases leads to disturbances in ion homeostasis resulting in impaired signal transduction, altered cellular metabolism, and changes in cell membrane permeability, integrity and disturbances of vital function (Dhanarasu et al., 2010).

Ca²⁺-ATPase is also located in the plasma membrane pumping Ca²⁺ out of the cell and thereby helping to maintain the concentration gradient of Ca²⁺ between the cytosol and the extracellular fluid (ECF). Many ATPases, including Ca²⁺ ATPases contain essential free sulfhydryl groups. Impairment of this enzyme may be due to peroxidative stress, which may act on the sulphhydryl groups present in the active site of Ca²⁺ ATPase. Thiol modification (I.E loss of protein sulfhydryl groups) has been recognized as a critical event in cytotoxicity (Chan et al., 2004) damage to these thiol moieties may result in inhibition of Ca²⁺ ATPases function and increase in the 90 intracellular Ca²⁺ concentration (Wei xu et al., 2010). Elevated Ca²⁺ is thought to be
invasion and calcium ATPase alterations are associated with tumorigenesis (Evtodienko et al., 1998). The decreased activities of Na\(^+\)/K\(^+\) ATPase and increase in the activities of Ca\(^{2+}\) ATPase and Mg\(^{2+}\) ATPase were found in cancer-bearing animals. 1, 3 BPMU treatment significantly restored the tissue ATPase activities to near normal values. The results suggest that 1, 3 BPMU protects ion pump ATPases, presumably by limiting the degree of oxidation and levels of oxidation by products due to an increase in the reduced glutathione (GSH) content in rats treated with 1, 3 BPMU. The results of the present study demonstrate that 1, 3 BPMU can modulate ATPase and reduce free radical formation in DEN-induced liver cancer in albino rats. Thus inhibitory effect of 1, 3 BPMU may play an important role in preserving membrane asymmetry by suppressing free radicals, implying efficacy of this agent against tumorigenesis.

The activity of isocitrate dehydrogenase (IDH) reduced in the DEN induced rats. However, treatment with 1, 3 BPMU significantly increased its activity when compared to DEN induced rats. No significant difference could be observed between the control rats and rats treated with 1,3 BPMU alone. The finding indicates that the 1, 3 BPMU has the potentiality to protect the mitochondrial damage in the DEN induced HCC animals. The activity of succinate dehydrogenase (SDH) decreased in the DEN induced rats compared with control rats. However, 1, 3 BPMU administration restored the altered activity of succinate dehydrogenase when compared with DEN induced rats. The activity of malate dehydrogenase (MDH) significantly reduced in DEN induced rats. But after 1, 3 BPMU treatment, their activity increased when compared with DEN induced rats. There was no marked difference in the activities of the TCA cycle enzyme between the rats treated with 1, 3 BPMU alone and control rats.

In the present study, significant decrease in these citric acid cycle enzymes were observed in DEN administered rats (Group III). DEN significantly reduced MDH, SDH and IDH in the mitochondria. This decline in the activities of these enzymes might be due to the alterations in cancer cell morphology, ultrastructural changes and the ability of mitochondria to undergo metabolic changes. However, treatment of 1, 3 BPMU had shown a significant increase in the levels of IDH, SDH and MDH in the mitochondria. 1, 3 BPMU treatment increased the activities of these mitochondrial enzymes suggesting its chemotherapeutic nature.
Nucleic acids play an important role during neoplastic transformation and the determination of DNA content was more meaningful with regard to biological and functional aspects of the tumor, because it is an index of proliferative activity in tumor conditions. Additionally DNA content is found to be an independent indicator of prognosis, since the size of the tumor often correlates well with the DNA content of tumor (Chakraborty et al., 2007). In the present study, increased level of DNA synthesis in cancer bearing animals may be due to the increased expression of the enzymes necessary for differentiated cell function. Increased RNA level in cancer bearing animals may be due to the increased DNA content, this lead to an increased transcription and there by elevated RNA content in cancerous condition. Contrarily, 1, 3 BPMU administration controlled the nucleic acid biosynthesis and exerts anti cancer effect.