

## HEPATOPROTECTIVE ACTIVITY OF METHANOLIC EXTRACT OF CROTON SPARCIFLORUS ON DEN INDUCED HEPATOTOXICITY IN WISTAR ALBINO RATS

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### ABSTRACT

The methanolic extract of *Croton sparciflorus* was studied for the hepatoprotective activity using DEN induced liver damage in wistar albino rats. Methanolic extract of this plant showed significant ( $p < 0.05$ ) hepatoprotective effect by lowering the serum levels of various biochemical parameters like AST, ALT and ALP in the selected model. These biochemical observations were confirmed by histopathological examinations of liver sections and are comparable with the standard hepatoprotective drug Silymarin which served as a positive control. The results suggest that the biologically active phytoconstituents such as flavonoids, glycosides, alkaloids present in the methanolic extract of plant which is confirmed from the qualitative analysis may be responsible for the significant hepatoprotective activity. The results justify the use of the plant as a hepatoprotective agent in folklore practice against liver disorder.

**Keywords:** *Croton sparciflorus*, Hepato toxicity, AST, ALT, DEN.

### INTRODUCTION

Cancer is a multi-step disease incorporating environmental, chemical, physical, metabolic and genetic factors which play a direct and/or indirect role in the induction and deterioration of cancers<sup>1</sup>. Among the different varieties of cancer, the Hepato Cellular Carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related mortality in human beings worldwide<sup>2,3</sup>. Several risk factors, including alcohol consumption, obesity, iron overload, environmental pollution and dietary carcinogens, such as aflatoxins and nitrosamines have been reported to induce HCC<sup>4,5</sup>. High mortality rate in patients with HCC can be attributed to the lack of effective treatment and high recurrence rates after surgery. In the absence of a proven effective therapy, there is an urgent need for novel chemo preventive strategies to lower the high death rates of HCC.

Natural Products have long been a fertile source of cure for cancer, which is projected to become the major causes of death in this century<sup>6</sup>. However, there is a continuing need for development of new anticancer drugs, drug combinations and chemotherapy strategies, by methodical and scientific exploration of enormous pool of synthetic, biological and natural products<sup>7</sup>. There are at least 2,50,000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties. *Croton sparsiflorus* Morong (synonym = *Croton bonplandianum* Bail.) belongs to the family Euphorbiaceae is a small annual herb, growing up to 1-2 feet tall found in southern parts of Indian subcontinent. The latex of this plant is being used in folk medicines by tribal people of Tamil Nadu to treat wasp sting<sup>8</sup>. The powdered leaves are useful in controlling high blood pressure and for the treatment of skin diseases,

cuts and wounds<sup>9-11</sup>. Exhaustive literature survey shows that, in this particular plant research is not carried out in this particular area. So this will be the first attempt carried out to investigate the hepatoprotective activity of the plant as far as we are concerned.

## MATERIALS AND METHODS

### Experimental animals

Male Wistar albino rats (n = 30; 8 week old), weighing 180–200 g, were obtained from the Biomedical Research Unit and Laboratory Animal centre (BRULAC) of Saveetha University, Chennai, India and were housed in solid bottom polycarbonate cages under controlled environmental conditions (22 ± 2°C with 55 ± 5% humidity, and a 12/12 hour light/dark cycle). The rats were acclimatized to the environment for 1 week prior to the initiation of the experiment. The rats were offered standard diet supplied by Hindustan Lever Ltd, Mumbai and water *ad libitum*. The protocol of this study was approved by the Animal Ethics Committee of the Institute [SU/BRULAC/RD/008/2014]. All procedures were in strict compliance with relevant laws, the Animal Welfare Act, Public Health Services Policy, and guidelines established by the Institutional Animal Care and Use Committee of the University.

### Plant collection

*Croton sparciflorus* is an herb that grows throughout south India in fields and forest. The plant was collected in the month of August from Auxilium College campus, Vellore, Tamil Nadu, India and was taxonomically identified by the Department of Botany, Auxilium College, Vellore, Tamil Nadu, India and a voucher specimen [CS-ACK-CHEM-001] is retained as an herbarium in the department for future reference.

### Extraction

The freshly cut plants were dried in the shade with active ventilation at ambient temperature and pulverized to a coarse powder using mechanical grinder and sieved with the help of 40 mesh size. The powder was percolated in methanol for about 20 days. The miscella was then concentrated using a vacuum rotary evaporator under reduced pressure. The dark brownish semisolid extract was preserved in tightly closed container and used for the analysis.

### Experimental Design

The animals were divided into 5 groups, each consisting of 6 animals.

**Group I:** the control group, serve as negative control received a control diet and a single intra peritoneal (i.p.) injection of normal saline (2.5ml/kg).

**Group II:** the DEN induced group, serve as a positive control was given a single i.p. injection of DEN, 200 mg/kg of bodyweight and a control diet. Hepatocarcinogenesis was provoked by a single i.p. injection of DEN (Sigma-NO 258, USA) at a dose of 200 mg/kg body weight (mixed with olive oil). Following a two week recovery period, the promoter phenobarbital (Sigma-Aldrich, St. Louis, MO) was incorporated into the drinking water at a concentration of 0.05% for 24 successive weeks. The dose of DEN and phenobarbital and the time required to study initiation stage of hepatic carcinogenesis in rats were adopted from Bishayee et al.<sup>12</sup>

**Group III:** the DEN induced group, after the development of cancer, was treated with the methanolic extract of *Croton sparciflorus*, 100 mg/bw.

**Group IV:** the DEN induced group, after the development of cancer, was treated with the methanolic extract of *Croton sparciflorus*, 200 mg/bw.

**Group V:** the DEN induced group, after the development of cancer, was treated with the standard drug Silymarin [Sigma- S0292, USA] 100 mg/kg orally.

The duration of the treatment was 20 and 40 days. At the end of the treatment, blood was collected from all the groups by puncturing the retro-orbital plexus and was allowed to clot at room temperature and serum was separated by centrifugation at 2500rpm for 10 min. The animals were sacrificed and the abdomen was cut open to remove the liver for histopathological studies.

### Acute toxicity studies

The dose limits were selected on the basis of oral acute toxicity studies in rats, in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines<sup>13</sup>. The acute toxicity test was carried out in 5 rats by giving doses of 100, 200, 300, 400, 500 mg/kg body weight. All groups of test drug showed neither any toxic effect nor any lethal effect in the dose range of 100 to 500 mg/kg body weight. So we had taken a minimum dose of 100mg/kg and 200 mg/kg of body weight for further studies.

### Assessment of liver function

The serum collected was used for estimation of biochemical parameters to determine the functional state of the liver. Aspartate Transaminase (AST) or Serum Glutamate

Oxaloacetate Transaminase (SGOT) is increased in conditions like hepatitis, hepatic congestion, obstructive jaundice and kidney diseases. It is estimated in blood by UV kinetic method<sup>14,15</sup>. Alanine Transaminase (ALT) or Serum Glutamate Pyruvate Transaminase (SGPT) which is predominantly present in liver tissues is helpful in evaluation of liver functions and conditions like hepatitis, hepatic congestion, metastatic carcinoma, obstructive jaundice and kidney diseases. It is estimated by a UV kinetic method<sup>14,15</sup>. Alkaline phosphatase (ALP) is an isoenzyme, present in all tissues of the body, particularly high levels in liver is a helpful diagnostic tool in conditions such as Hepatobiliary disease and Bone disease associated with osteoblastic activity. ALP was estimated by method described by Comb and Bowers<sup>16</sup> involving hydrolysis of p-nitrophenol which gives strong yellow colour in alkaline solution. The increase in absorbance due to its formation is directly proportional to ALP activity. Total cholesterol was determined by CHOD-PAP method adapted by Richmond<sup>17</sup>, in which the free cholesterol is hydrolyzed by cholesterol oxidase to cholestenone-4-en-3-one and hydrogen peroxide. Hydrogen peroxide by the action of peroxidase liberates oxygen which reacts with 4-amino antipyrine and phenol to form red coloured compound which is measured at 500nm.

Total triglycerides (TGL), High density lipoproteins (HDL) and Low density lipoproteins (LDL) were determined by Polyethylene glycol -CHOD-PAP method adapted by Richmond<sup>17</sup>. Lipid peroxidation (LPO) was determined by the method of Okawa<sup>18</sup> in which the malondialdehyde released served as the index of LPO. Total protein was estimated by Biuret method<sup>19</sup>, where the proteins produce a violet colour complex with copper ions in an alkaline solution. The absorbance of the colour complex formed was directly proportional to the protein content in the sample. Blood glucose level was estimated by Orthotoluidine method.

#### Histological Studies

After sacrificing the rats by cervical dislocation, liver was collected, washed in normal saline and was perfused with 10% formalin and stored in the same for histopathological studies. It was fixed by using 40% formaldehyde as fixative for 24 hours and dehydrated with alcohol. All tissues were cleaned and embedded by using xylene and molten paraffin wax (melting point 58-60<sup>o</sup> C). Sections were cut at 5  $\mu$ m thickness and were stained by double staining. To differentiate the nucleus and cytoplasm, the

basic dye haematoxylin and the acid dye eosin were used<sup>20,21</sup>. Electron micrographs were performed using transmission electron microscope and photographed by photomicrography. The sections were then viewed under the Nikon microscope, ECLIPSE E400, model 115, Japan.

#### Statistical Analysis

The data were collected, calculated and tabulated as Mean  $\pm$  SD and testing significance between the groups were analyzed by students 't' test using SPSS software version 20 and the 'p' values were used to judge the significance level.

#### RESULTS AND DISCUSSION

In Indian system of medicine, certain herbs were claimed to provide relief against liver disorders which has to be verified in a scientific manner. In the present study one such herb *Croton sparciflorus* was taken for the study. The methanolic extract of *Croton sparciflorus* possesses significant ( $P < 0.05$ ) hepatoprotective effect in the DEN induced rats. DEN is commonly used as a hepatocarcinogen that influences cancer initiation by inducing DNA carcinogen adducts and DNA-strand breaks formation, and in turn causing hepatocarcinogenesis without cirrhosis through the development of putative preneoplastic focal lesions<sup>22</sup>. DEN can react covalently with several biomolecules such as protein, nucleic acid and lipid, resulting in cellular membrane degeneration, increased permeability, and leakage of cytoplasmic enzymes such as ALT, AST and ALP. The release of these biochemical indicators in the blood stream could be a event following DEN-induced peroxidation of hepatocytomembrane lipids. It has been reported that the analysis of liver enzymes in serum reflects cellular damage and serve as hepatotoxicity indexes<sup>23</sup>. Liver functional enzymes such as ALT, AST and ALP and other biochemical parameters such as total protein, Lipid profile and glucose were generally used in human beings and animals as indicators of liver injury as well as liver response to medicines<sup>24</sup>.

#### Acute toxicity test

The oral acute toxicity study of methanolic extract of *Croton sparciflorus* showed no mortality up to 500 mg/kg body weight in rats and did not produce any toxic symptoms. Hence the extract was considered to be safe and non-toxic for further pharmacological screening. The maximum non lethal dose was found to be 4000mg/Kg body weight, orally was also reported in the previous studies<sup>25</sup>. This may be

due to the broad non-toxic range of the plant, where the plant extract showed a high safety index.

#### Assessment of Liver marker enzymes

The results of hepatoprotective activities of methanolic extract of *Croton sparciflorus* against DEN induced hepatotoxicity were shown in Table-1. Serum marker enzymes AST, ALT and ALP levels were higher in the DEN groups than in normal rats, which most likely reflected the hepatic injury induced by DEN. However, treatments of rats with the methanolic extract of *Croton sparciflorus* at a concentration of 100 and 200 mg/ Kg bw and silymarin showed a reduction ( $p < 0.05$ ) in the levels of AST, ALT and ALP, which comes down to the normal range, in both short and long term treatment periods (20 and 40 days).

The levels of AST was found to be  $78.5 \pm 0.56$  in Group I (normal rats) and the levels increased to  $115.2 \pm 1.12$  and  $120.2 \pm 0.56$  when the rats are treated with DEN. Then the rats on treatment for 20 and 40 days continuously with the plant extract it showed a decrease in the values. The results correspondingly reduced at 10.4%; 25.9% and showed the value of  $103.4 \pm 0.56$ ,  $89 \pm 0.33$  in group III. Further reduced at 25.5%; 35.1% to  $85.7 \pm 1.15$ ;  $78.0 \pm 0.33$  in Group IV. The results were comparable with the Silymarin treated group whose values were  $55.02 \pm 0.45$  and  $48.06 \pm 0.78$  in Group V. The levels of ALT was found to increase in Group II ( $134.21 \pm 1.44$  and  $154.18 \pm 1.05$ ) because of the hepatocellular activity of DEN, which was brought down to the normal levels in the treatment group III at a percentage of 44.5% and 57.8% and showed a result of  $74.42 \pm 0.56$  and  $65.00 \pm 0.79$ . Again on treatment for 40 days it reduced at 48.7%; 68.3% and the values became  $68.89 \pm 1.22$  and  $51.89 \pm 0.56$  which is comparable with the values of silymarin treated group whose ALT are found to be  $35.66 \pm 1.09$  and  $22.45 \pm 0.78$  for 20 and 40 days respectively. The levels of ALP was found to be  $78.48 \pm 0.56$  in Group I normal rats which increased to  $169.06 \pm 0.51$  and  $180.55 \pm 0.46$  when the rats are treated with DEN. Then the rats on treatment for 20 and 40 days continuously with the plant extract it showed a decrease in the values, which was found to be  $111.67 \pm 0.48$ ,  $100.44 \pm 1.05$  and  $98.09 \pm 0.56$ ,  $86.07 \pm 0.26$  respectively which is comparable with the Silymarin treated group whose values are  $99.78 \pm 0.47$  and  $84.00 \pm 0.55$ . The prevention of the leakage of ALT, AST, and ALP from the liver in Group III and Group IV suggested a potential protective effect of the plant extract against DEN-induced liver damage. The effect

could be due to the stabilization of hepatocyte membrane by the plant extract with a consequent decrease in the leakage of these marker proteins. Estimation of the marker enzymes in the serum was a useful quantitative marker of the extent and type of hepatocellular damage<sup>26</sup>. The tendency of these enzymes to return to near normal in extract administered group was a clear manifestation of hepatoprotective activity of the extract.

#### Estimation of lipid levels

The results of the levels of lipid profile for 20 and 40 days treatment in wister albino were given in Table-2. The significant acute hepatocellular damage was indicated by the elevated levels of total cholesterol, Triglycerides (TG) and low density lipoproteins (LDL) in Group II. In the treated groups III and IV, the levels were reduced nearest to the normal values, because of the action of plant extract. The results were significantly comparable with the standard group V. The same type of result was also seen in the treatment conducted for 40 days. The levels of HDL reduced considerably in DEN induced group, reflecting the reduction of Good cholesterol. But in the treated groups III and IV the HDL levels increased significantly.

#### Lipid peroxidation

Lipid peroxidation was regarded as one of the basic mechanisms of tissue damage caused by free radicals<sup>27</sup>. Administration of DEN has been reported to generate LPO products in general<sup>28</sup> and Phenobarbital enhanced the formation of the activated oxygen species in the preneoplastic nodules<sup>29</sup> in rat liver. Here the administration of DEN and Phenobarbital have shown to increase the levels of liver tissue LPO during hepatocarcinogenesis and this vigorous action may be lead by the uncompromised production of free radicals. It has been extensively reported that free radicals participated in DEN induced hepatocarcinogenesis<sup>30,31</sup>. The levels of LPO in liver tissues of control and experimental animals were depicted in Table-3 and found that there was an increase in LPO in group II ( $p < 0.05$ ) rats when compared to control animals. These significant effects were reversed in treated groups III and IV ( $p < 0.05$ ) on dose dependent manner and also on group V.

#### Estimation of protein level

The levels of total protein was reduced due to the hepatotoxin intoxication (Recknagal, 1967). The reduction was attributed to the damage produced and localised in the endoplasmic reticulum which results in the loss of P450

leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides. The results of the protein content in wister albino rats were given in Table-3 for the short and long term treatment periods. The levels of total protein content reduced significantly ( $p < 0.05$ ) for both the treatment periods, in the DEN treated groups  $3.62 \pm 0.56$ ,  $2.87 \pm 0.22$  when compared with normal rats  $5.91 \pm 0.33$ . The levels of the total protein increased after the treatment period 20 and 40 days significantly ( $p < 0.05$ ) in Group III  $5.03 \pm 0.77$ ,  $5.68 \pm 0.77$  and in group IV  $5.78 \pm 0.25$ ,  $5.95 \pm 0.22$  which was comparable with the Standard Silymarin  $5.95 \pm 0.45$ ,  $5.95 \pm 0.45$ . This results suggested the stabilization of endoplasmic reticulum leading to protein synthesis.

#### Estimation of Glucose level

Glucose is a reducing monosaccharide that serves as the principal fuel of all the tissues which enters the cell through the influence of insulin and undergoes a series of chemical reactions to produce energy. Lack of insulin or resistance to its action at the cellular level causes the blood glucose levels to raise in the blood stream. The levels of Glucose increased to  $140.0 \pm 0.78$  and  $150.5 \pm 0.87$  in DEN induced group compared with the normal group  $68.4 \pm 0.22$ , which again came to the normal range on treatment with the plant extract.

#### Histopathological studies

Results of the histopathological studies for the treatment periods 20 days and for treatment period 40 days are presented in Figure. 1(a-e) and Figure. 2 (a-e). Histopathological liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein. Disarrangement and Degeneration of normal hepatic cells with centrilobular necrosis and

vacuolization are observed in DEN intoxicated liver cells. The liver sections of the rat treated with 100 and 200mg/kg bw of methanolic extract of *Croton sparciflorus* followed by DEN intoxication showed less vacuole formation and absence of necrosis observed were comparable with standard Silymarin, supplementing the protective effect of the test drug and the standard hepatoprotective drug.

The phytochemical analysis carried out in conventional methods<sup>32-35</sup> showed the presence of phytoconstituents such as Alkaloids, Flavonoids, Phenols, Tannins, Saponin and Terpenoids in methanolic extract of *Croton sparciflorus* may be responsible for the significant hepatoprotective activity. The presence of these phytoconstituents were analyzed by various qualitative tests<sup>36-40</sup>.

#### CONCLUSION

The Hepatoprotective activity of methanolic extract of *Croton sparciflorus* exhibited good results against DEN induced hepatotoxicity. Efforts were in progress to isolate and characterize the active principle, which were responsible for the hepatoprotective efficacy of this valuable medicinal plant. The search for new pharmacologically-active compounds for drug development is an important issue, but not the only one, as the trend toward using standardized plant extracts of high quality, safety and efficacy will continue. Therefore, all efforts have to be targeted to reveal the chemical-pharmacological profiles of extracts and fixed combinations and to rationalize their therapeutic application.

#### ACKNOWLEDGEMENT

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**Table 1: Effect of Croton sparciflorus in Marker enzymes on DEN induced toxicity in rats**

Marker enzymes	AST (IU/L)		ALT (IU/L)		ALP (IU/L)	
	20	40	20	40	20	40
Group I	78.50±0.56	78.50±0.56	25.78±0.89	25.78±0.89	78.48±0.56	78.48±0.56
Group II	115.00±1.12	120.20±0.56	134.21±1.44	154.18±1.05	169.06±0.51	180.55±0.46
Group III	103.40±0.56	89.00±0.33	74.42±0.56	65.00±0.79	111.67±0.48	98.09±0.56
Group IV	85.70±1.15	78.00±0.33	68.89±1.22	51.89±0.56	100.44±1.05	86.07±0.26
Group V	55.02±0.45	48.06±0.78	35.66±1.09	22.45±0.78	99.78±0.47	84.00±0.55

Values are mean±SD; Significant reduction in Group III, IV and V compared to Group II ( $P < 0.05$ ).

**Table 2: Effect of Croton sparciflorus in Lipid profile on DEN induced toxicity in rats**

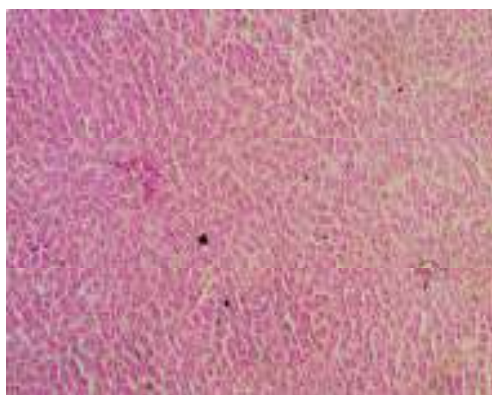
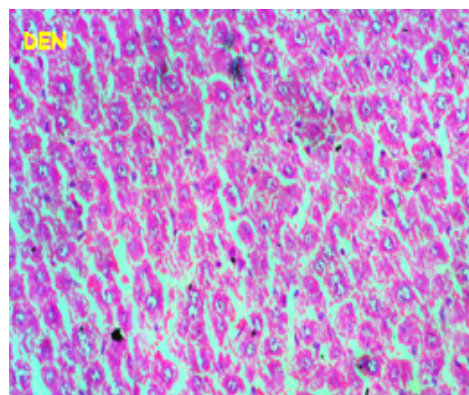
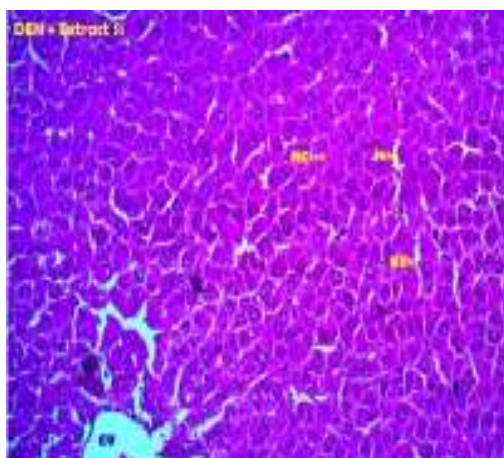
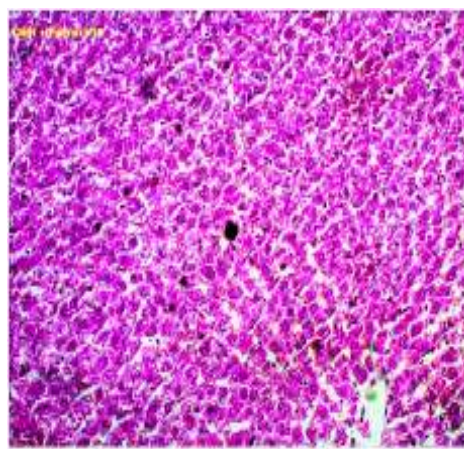
Lipid Profile	Cholesterol (mg/dl)		TGL (mg/dl)		HDL (mg/dl)		LDL (mg/dl)	
	20	40	20	40	20	40	20	40
Group I	120.0±0.56	120.0±0.56	125.4±0.78	125.4±0.78	40.5±0.52	40.5±0.52	105.7±0.45	105.7±0.45
Group II	201.0±0.33	225.0±0.33	194.2±0.45	200.0±0.66	21.6±0.45	15.3±0.11	217.8±0.12	237.3±0.11
Group III	186.4±0.85	140.4±0.48	178.7±1.04	140.4±0.95	35.8±0.12	49.5±0.11	183.6±1.00	156.6±0.45
Group IV	159.7±1.03	110.0±0.79	135.6±0.78	128.3±0.46	45.4±0.45	55.4±0.22	145.3±0.12	123.5±0.33
Group V	154.8±1.02	100.0±1.05	130.5±0.44	115.8±0.45	43.3±0.56	48.0±0.22	123.3±0.12	104.9±0.55

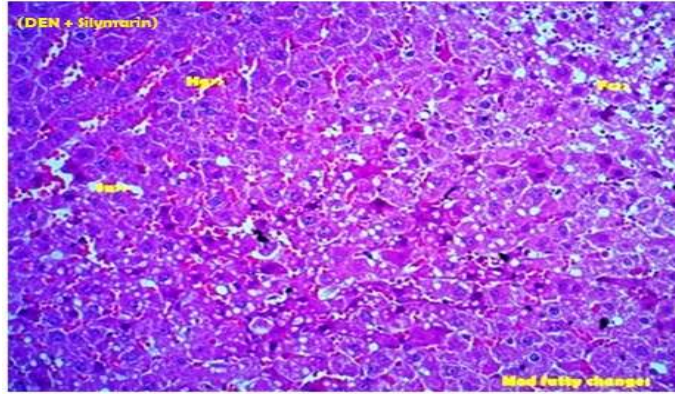
Values are mean±SD; Significant reduction in Group III, IV and V compared to Group II ( $P < 0.05$ ).

**Table 3: Effect of Croton sparciflorus in LPO, Protein and Glucose on DEN induced toxicity in rats**

Parameters	LPO (nmol/ml)		Protein (mg/dl)		Glucose (mg/dl)	
	20	40	20	40	20	40
Group I	1.2±0.21	1.2±0.21	5.91±0.33	5.91±0.33	68.4±0.22	68.4±0.22
Group II	2.8±0.22	3.6±0.55	3.62±0.56	2.87±0.22	140.0±0.78	150.5±0.87
Group III	2.21±1.02	1.98±0.21	5.03±0.77	5.68±0.77	125.6±0.85	119.3±0.45
Group IV	1.86±1.33	1.45±0.45	5.78±0.25	5.95±0.22	115.0±0.44	95.0±0.58
Group V	1.21±0.55	1.08±0.44	5.95±0.45	5.95±0.45	100.1±0.45	85.4±0.78

Values are mean±SD; Significant reduction in Group III, IV and V compared to Group II ( $P < 0.05$ ).

**(a) Normal liver section****(b) Den induced liver section****(c) Treatment with 100mg/bw of extract****(d) Treatment with 200mg/bw of extract**

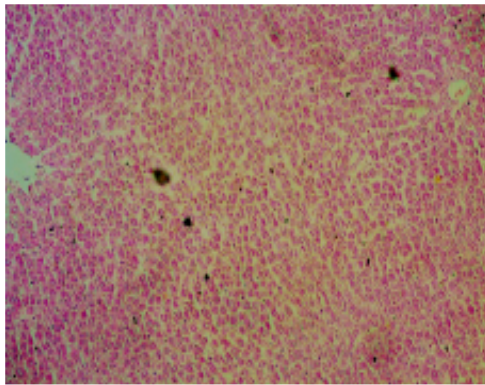


(e) Treatment with Silymarin drug

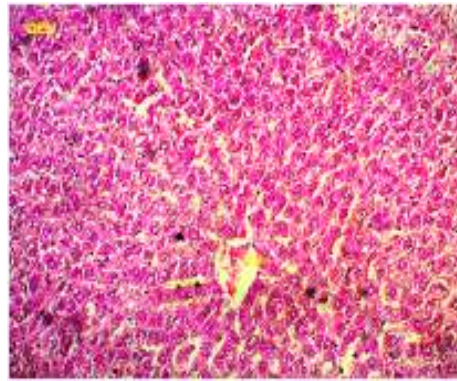
Magnification: 400×, Stain: Haematoxylin-Eosin.

cv: Central vein; hc: Hepatocyte; ss: Sinusoidal space; vc: Vacuole.

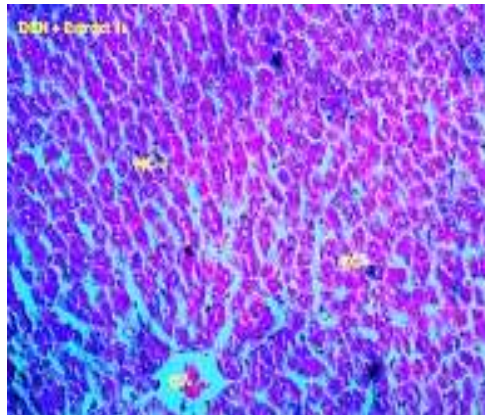
Fig. 1: Histopathological section of liver after a treatment period of 20 days



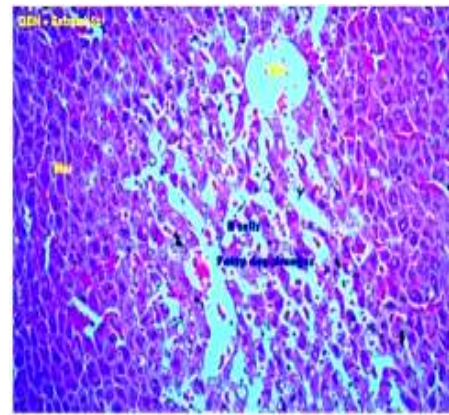
(a) Normal liver section



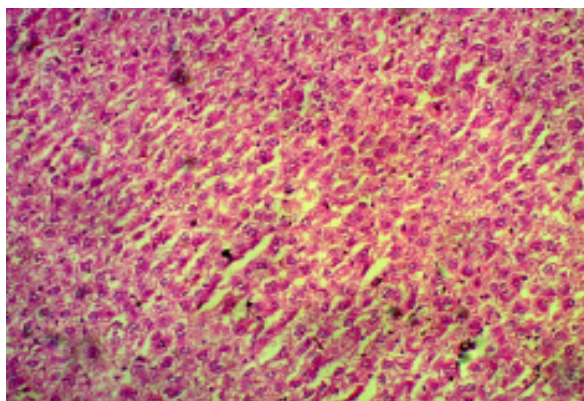
(b) Den induced liver section



(c) Treatment with 100mg/bw of extract



(d) Treatment with 200mg/bw of extract



(e) Treatment with Silymarin drug

Magnification: 400×, Stain: Haematoxylin-Eosin.

cv: Central vein; hc: Hepatocyte; ss: Sinusoidal space; vc: Vacuole.

Fig. 2: Histopathological section of liver after a treatment period of 40 days

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