Efficacy of propriety herbal formulation (PHF) against carbon tetrachloride (CCl₄) induced liver damage was investigated in adult rats. Administration of CCl₄ (0.2 ml/kg; ip) twice a week for 12 weeks resulted in significant elevation in serum transaminases activity. Level of reduced glutathione was significantly decreased. On the contrary, significant elevation was found in the hepatic lipid peroxidation level. Proliferation of fibroblast replaced the hepatic parenchyma cells in focal areas. Cell organelles like mitochondria, endoplasmic reticulum and nucleus showed severe degeneration after CCl₄ exposure. PHF was effective in restoring the CCl₄ induced biochemical and histological ultrastructural changes.

Healthy male rats were divided into 4 groups (Gr 1-4) of 5 animals in each group. Gr 1 was given normal saline (4 ml/kg) and served as normal control. The animals of Gr 2 received CCl₄ (0.2 ml/kg; ip) twice a week for 12 weeks. In the Gr 3, extract (250 mg/kg; orally) was administered daily for 12 weeks and in the Gr 4 the animals were administered with CCl₄ (as in Gr 2) and plant extract (as in Gr 3). The doses were selected as described earlier.

Blood samples of rats were withdrawn by puncturing the retro-orbital venous sinus and serum samples were used for estimation of aspartate amino transferase (AST) and alanine amino transferase (ALT). Liver of each rat was promptly removed to determine the tissue level of hepatic lipid peroxidation and reduced glutathione. Quantitative measurement of lipid peroxidation was done by measuring the concentration of TBARS in liver. Amount of malondialdehyde (MDA) formed was quantitated by reaction with thiobarbituric acid (TBA) and used as an index of lipid peroxidation. Reduced glutathione was estimated in the liver homogenate using DTNB.

For histopathological studies, small pieces of liver and kidney were fixed in aqueous Bouin’s fixative and embedded in paraffin wax. Haematoxylin and eosin stained sections were observed under light microscope. For ultrastructural study, the tissues were primarily fixed in phosphate buffer followed by osmium tetroxide. Ultra thin sections were cut with Reichert OME3 ultra microtome and observations were made on Philips EM-300 transmission electron microscope. The data obtained were analyzed by one way analysis of variance (ANOVA).

Materials and Methods

Adult healthy male rats of Sprague dawley strain (130±10 g) were kept under 14 hr light and at 26°C. They were fed on standard pelleted diet (Lipton's India Ltd., Kolkata, India; metal contents of diet in ppm dry weight: Cu, 10; Mn, 33; Zn, 45; Co, 5) and drinking water ad libitum.

Plant parts used in the present study were collected from the plants growing locally and were identified by the Botany Department of Jiwaji University, Gwalior. These plant parts were dried, chopped and prepared crude extract by mixing the plants as per the composition shown in Table 1.
Results

Biochemical changes—Administration of CCl₄ caused significant increase in the activity of serum AST and ALT (Table 2). Statistical analysis indicated recovery with extract therapy. A significant increase was observed in MDA concentration of liver tissue after CCl₄ administration. On the contrary, a marked fall was observed in the reduced glutathione level. Conjoint treatment with aqueous extract when given orally, prevented progression of CCl₄ induced chronic liver injury. A remarkable recovery was seen in the lipid peroxidation and the level of reduced glutathione was also recouped (Table 3).

Light microscopical changes—Liver of rats exposed to CCl₄ at a dose of 0.2 ml/kg for 3 months exhibited lesions. Vacuolation of hepatocytes was common in the peripheral cells. Portal fibrosis, bile duct hyperplasia and fatty degeneration were observed. Leucocytic infiltration was commonly observed (Fig. 1). The central sinus showed congestion (Fig. 2). Administration of PHF showed well-maintained histoarchitecture. Sinusoids were normal with well-formed nuclei. There was no granulation and perinuclear vacuolation in the hepatocytes, however, at some places normal kupffer cells were observed. The biliary ductules were normal (Figs 3, 4).

Administration of CCl₄ caused deformity in the cellular organization of kidney. Epithelial cells of proximal tubules showed hypertrophy. Debris was seen in the lumen of tubules. Severe vacuolation was observed in the epithelial cells. Nuclei assumed irregular shape and apical position in the collecting tubules. Enlarged glomeruli occupied whole space of Bowman’s capsule (Fig. 5). Monocellular infiltration was prominent (Fig. 6). Conjoint treatment with the extract showed significant recovery (Figs 7,8). Bowman’s capsule also showed better organization. Proximal and medullary tubules were well organized.

Ultrastructural changes—Chronic CCl₄ exposure caused changes in the liver. Proliferation of fibroblasts replaced the hepatic parenchyma cells in focal areas. Large vacuolar spaces could be seen in hepatocytes, and nuclei were retained in such cells. Focal dilated blood vessel and proliferation of few bile ducts were also observed. Extensive fatty changes and centrilobular necrosis were also observed. Swelling in mitochondria was observed along with damaged outer and inner membranes. Degenerated crests were also seen. Severe degeneration in the structure of nucleus along with accumulation of chromatin material was seen. Nuclear membrane was also irregular (Fig. 9). Conjoint administration of PHF showed regenerative changes. Mitochondria and endoplasmic reticulum were well formed. Nuclear membrane was clear with distinct nuclear pores. Glycogen bodies were evenly distributed. Uniform distribution of fat droplets were clearly visible. The cytoplasm showed rich distribution of endoplasmic reticulum, closely packed around the mitochondria. There were a few cytoplasmic vacuoles, however, the nucleoplasm also showed presence of some vacuoles (Fig. 10).

Table 2—Effect of PHF against CCl₄ treated rats on the activity of transaminases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST IU/l</th>
<th>ALT IU/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55± 2.77</td>
<td>35± 1.7</td>
</tr>
<tr>
<td>PHF</td>
<td>54± 2.61</td>
<td>35± 1.7</td>
</tr>
<tr>
<td>CCl₄</td>
<td>170± 7.57</td>
<td>180± 7.4</td>
</tr>
<tr>
<td>CCl₄+PHF</td>
<td>80± 4.80</td>
<td>52± 3.4</td>
</tr>
</tbody>
</table>

F-variance days at 5% level: 0.44<sup>ns</sup> 0.46<sup>ns</sup>

Analysis of variance*F=P<0.05, ns=not significant.

Table 3—Effectiveness of PHF in liver against CCl₄ treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO nmole of MDA/mg protein</th>
<th>GSH μmole/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.42± 0.04</td>
<td>8.3+ 0.4</td>
</tr>
<tr>
<td>PHF</td>
<td>0.39± 0.02</td>
<td>8.0+ 0.3</td>
</tr>
<tr>
<td>CCl₄</td>
<td>0.74± 0.04</td>
<td>5.9+ 0.6</td>
</tr>
<tr>
<td>CCl₄+PHF</td>
<td>0.43± 0.04</td>
<td>8.1+ 0.3</td>
</tr>
<tr>
<td>F-variance days at 5% level</td>
<td>3.45&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.33&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Analysis of variance*F=P<0.05, ns=not significant.

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Table 1—Composition of plant parts used for preparation of crude extract

<table>
<thead>
<tr>
<th>Name of Plant</th>
<th>Source</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographis paniculata</td>
<td>Roots &amp; leaves</td>
<td>20</td>
</tr>
<tr>
<td>Boerhaavia diffusa</td>
<td>Root</td>
<td>30</td>
</tr>
<tr>
<td>Cassia occidentalis</td>
<td>leaves</td>
<td>05</td>
</tr>
<tr>
<td>Eclipta alba</td>
<td>Whole plant</td>
<td>15</td>
</tr>
<tr>
<td>Fumaria indica</td>
<td>Entire plant except root</td>
<td>10</td>
</tr>
<tr>
<td>Plumbago zeylanica</td>
<td>Root</td>
<td>05</td>
</tr>
<tr>
<td>Teocmella undulata</td>
<td>Bark</td>
<td>05</td>
</tr>
<tr>
<td>Terminalia arjuna</td>
<td>Bark</td>
<td>05</td>
</tr>
<tr>
<td>Solanum nigrum</td>
<td>Entire plant except root</td>
<td>05</td>
</tr>
</tbody>
</table>
Ultrastructural studies of kidney revealed degenerated proximal convoluted tubules. Plasma membrane of these tubules exhibited basal infoldings into the cells. These infoldings contained degenerated mitochondria in which outer and inner membrane was not clear. Microvilli were scanty and short. At some places phagocytes could be identified in the lumen of capillaries with prominent chromatin condensation in patches near the plasma membrane. Around such capillaries, abundant microvilli were seen (Fig. 11). Conjoint administration of extract showed significant recoupment in the glomeruli. Thin layer of fenestrated

Fig. 1-6—(1) Fatty degeneration in the hepatocytes after 12 weeks of CCl₄ administration. [X120]; (2) Sinus showing congestion after 12 weeks of CCl₄ administration [X400]; (3) Normal hepatocytes with maintained chord arrangement after treatment with extract [X120]; (4) Same as Fig. 3 under high magnification showing normal nuclei after conjoint treatment with extract. [X400]; (5) Note necrotic changes in proximal convoluted tubules after 12 weeks of CCl₄ administration. [X400] and (6) Note intense monocellular infiltration after 12 weeks of CCl₄ administration. [X400].
endothelium lined the capillaries. Outer and inner mitochondrial membranes were intact with well-developed cristae and these were closely packed. Excretory cells showed close approximation to capillaries, which could be identified with the presence of RBC and phagocytes. Apical margin of these cells showed numerous infoldings extending at variable into the cytoplasm. Nuclear wall was continuous with well-defined nucleolus and chromatin material (Fig. 12). Proximal tubules were well formed clearly attached to...
the capillary wall. Medullary region had defined columnar epithelium with the absence of brush border.

Discussion

Significant hepatoprotective efficacy has been reported earlier in various plants. The combination of nine medicinal plants that are used in the present investigation have also been reported to have medicinal value. Liver cells participate in a variety of metabolic activities and thus contain a host of enzymes. In severe acute liver damage, serum transaminases level parallel to those of the organs indicating that both cellular and mitochondrial membranes have been damaged. It is reported that large doses of CCl₄ result in cell lysis and cytoplasmic hepatic enzymes are released into blood circulation. Many fold increase of enzyme leakage as demonstrated by an increased level of serum enzymes ALT and AST has been noted, indicating liver cell damage by CCl₄. It has also been supported by other workers. Since, membrane integrity is linked with intracellular metabolic states, disturbance in latter results in membrane lesion with concomitant increase in enzyme leakage giving rise to hypoxia and membrane hyperpermeability.

Results demonstrated significant increase in lipid peroxidation and decreased level of reduced glutathione with CCl₄ exposure. It could be hypothesized that one of the causes of CCl₄ induced liver injury was lipid peroxidation. CCl₄ is metabolically activated by cytochrome P450-dependent mixed oxidases in endoplasmic reticulum to form a trichloromethyl free radical (CCl₃•) which combines with cellular lipid and proteins in the presence of oxygen to induce lipid peroxidation by hydrogen abstraction. This results in changes of structures of endoplasmic reticulum and other membranes, and loss of metabolic enzyme activation leading to liver damage. In states of oxidative stress, GSH is converted to GSSG and depleted leading to lipid peroxidation. Therefore, the role of GSH as a reasonable marker for evaluation of oxidative stress is important as it acts as an antioxidant both extracellularly and intracellularly, and is produced in the liver. PHF inhibited lipid peroxidation significantly and recovered the decreased hepatic GSH level induced by CCl₄ towards normal. It may be suggested that PHF preserves the activity of glutathione reductase which maintains the level of GSH and inhibits lipid peroxidation by reducing the formation of free radicals thereby accelerating the repair mechanism and showing significant protective effect. CCl₄ administration increased lipid peroxidation in serum and in liver tissue that was recouped significantly by the treatment of Emblica officinalis and chywanprash. These findings are also supported by various authors where the treatment of Emblica officinalis, turkish folk remedies, Cichorium intybus, Ventilago leiocarpa, and Ginkgo biloba also showed recoulement after CCl₄ intoxication.

Histopathological studies demonstrated that CCl₄ induces degeneration in hepatic cords and hepatocytes, infiltration of lymphocytes, and kupffer cell proliferation. These findings are further supported by earlier reports. Significant recoulement in histoarchitecture was seen with PHF therapy.

In the present study, liver of rats treated with CCl₄ exhibited extensive degenerative lesions in all the cell organelles of liver. Electron microscopic studies revealed marked dilatation, degranulation and loss of organization of the RER. CCl₄ induced nephrotoxic changes have been recorded suggesting the separation of lysosomes and endoplasmic reticulum in kidney. Severe degeneration of smooth and rough endoplasmic reticulum and significant reduction in microsomal enzymes has been reported after administration of single dose of CCl₄ (ref. 25).

It can be concluded that the significant antihepatoxic activity shown by PHF may possibly be due to its inhibitory effect on microsomal enzymes or on lipid peroxidation. The plant extract may interfere with cytochrome P-450 and ultimately hinder the formation of hepatotoxic CCl₄ free radical and exert its hepatoprotective action. The extract may also have antioxidant property, which inhibited the deleterious effect of free radicals generated by CCl₄, influencing the membrane rigidity by prevention or inhibition of membrane peroxidation. Thus the present study demonstrated the efficacy of PHF as an effective hepatoprotective agent.

Acknowledgement

The authors are indebted to Professor R Mathur, for his kind suggestions and valuable support during the work. The financial assistance from UGC, New Delhi is also acknowledged.

References