RESULTS & DISCUSSION
Oxygen derived free radicals have been implicated in the pathogenesis of many diseases such as atherosclerosis, diabetes mellitus, cancer, epilepsy and inflammatory diseases. These reactive oxygen species are the by products of metabolism and cells have well balanced mechanisms to deal with these species. It so happens that often these systems become overwhelmed. This brings in the need for supplementation with dietary antioxidants. A variety of medicinal plants are being used in different types of traditional medicine. Though medicinal plants are rarely used as antioxidants in traditional medicine, their claimed therapeutic properties could be partly because of their capacity to scavenge free radicals. Therefore, this study evaluated the antioxidant potential of *Crataegus oxyacantha*, commonly called as Hawthorn, which is used as a cardiotonic in herbo-homeopathic medicines. This plant extract is part of European Pharmacopoeia.

3.1 EVALUATION OF THE ANTIOXIDANT POTENTIAL OF *CRATAEGUS* EXTRACT, *IN VITRO*, IN RAT LIVER HOMOGENATE USING FeSO₄

Initially, the antioxidant potential of the extract was tested *in vitro* in rat liver homogenate. Its capacity to reduce lipid peroxidation, induced by FeSO₄, was studied. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl perferryl complex or through OH⁻ radical by Fenton reaction. The extract was added at different concentrations (10μl/ml, 20μl/ml, 30μl/ml, 40μl/ml and 50μl/ml of liver homogenate) At a concentration of 30μl/ml of the liver homogenate the extract showed maximum prevention of lipid peroxidation (Table 3.1). It was
Table 3.1 Effect of *Crataegus* extract on Ferrous sulphate (15mM) induced lipid peroxidation and glutathione content in rat liver homogenate, *in vitro*. Values are mean ± SD of 6 replicates

<table>
<thead>
<tr>
<th>Concentration of drug µl/ml</th>
<th>10 µl/ml</th>
<th>20µl/ml</th>
<th>30µl/ml</th>
<th>40µl/ml</th>
<th>50µl/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage inhibition of lipid peroxidation</td>
<td>52.47 ± 0.51</td>
<td>48.43 ± 1.27</td>
<td>61.87 ± 0.31</td>
<td>55.98 ± 1.26</td>
<td>55.23 ± 1.23</td>
</tr>
<tr>
<td>Percentage reduction in GSH content</td>
<td>80.85 ± 01</td>
<td>70.15 ±1.11</td>
<td>45.96 ± 0.71</td>
<td>51.17 ± 0.73</td>
<td>51.23 ± 0.69</td>
</tr>
</tbody>
</table>
also able to maintain the reduced glutathione levels at 30µl/ml of drug concentration (Table 3.1).

3.2 TOXICOLOGICAL STUDIES

Once the antioxidant potential was established, the extract was given to rats for a month at a dose of 400µl/kg body weight and its toxicological effects were evaluated. The dosage was chosen based on toxicological evaluation by Ammon and Haendal, 1981\textsuperscript{74}. Dosage fixation was not done in this study in strict compliance with CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines. There was no change in body weight (Fig. 3.1). There was also no change in liver and kidney weight (Table 3.2). The results also showed no disturbances whatsoever in the haematological parameters such as the RBC count, total WBC count, platelet count, haemoglobin levels and erythrocyte sedimentation rate (Table 3.3). No change in the levels of serum protein, urea and creatinine levels indicate that the protein metabolism was unaffected by the drug (Table 3.2). Similarly, there was no change in the blood glucose content (Table 3.2). This ensured that the carbohydrate metabolism was unaffected.

3.3 EVALUATION OF THE ANTIOXIDANT CAPACITY OF CRATAEGUS OXYACANTHA EXTRACT IN RAT USING CCL\textsubscript{4} INDUCED OXIDATIVE STRESS

Carbon tetrachloride is the best known example of a chemical whose toxicity is presumably the consequence of the formation of free radicals. Carbon tetrachloride is microsomally reduced by mixed function oxidases to the reactive
Fig. 3.1 Body weight changes in control (Group I) and Crataegus extract treated (Group II) rat
Table 3.2 The liver weight, kidney weight and biochemical parameters of control and *Crataegus* treated rat (400μl/kg body weight /day) for 30 days. Values are mean ± SD for six rats in each group.

<table>
<thead>
<tr>
<th></th>
<th>Group I - Control</th>
<th>Group II - <em>Crataegus</em> treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>5.74 ± 0.16</td>
<td>6.08 ± 0.17&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.52 ± 0.01</td>
<td>1.59 ± 0.01&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>8.4 ± 0.062</td>
<td>8.4 ± 0.063&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>122.17 ± 1.47</td>
<td>123.5 ± 1.39&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood urea (mg/dl)</td>
<td>59.78 ± 0.46</td>
<td>60.01 ± 0.69&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.30 ± 0.01</td>
<td>1.30 ± 0.02&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NS- Not significant
Table 3.3  The haematological parameters of control and *Crataegus* treated rat (400µl/kg body weight /day) for 30 days. Values are mean ± SD for six rats in each group.

<table>
<thead>
<tr>
<th></th>
<th>Group I - Control</th>
<th>Group II - <em>Crataegus</em> treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC count (cells x 10^6/mm³)</td>
<td>3.7 ± 0.18</td>
<td>3.7 ± 0.16&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total WBC count (cells/mm³)</td>
<td>7191.66 ± 66.45</td>
<td>7220 ± 113.1&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelet count (lakhs/mm³)</td>
<td>3.71 ± 0.15</td>
<td>3.73 ± 0.08&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>10.83 ± 0.08</td>
<td>10.9 ± 0.14&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>9.08 ± 0.08</td>
<td>9.03 ± 0.10&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NS- Not significant
trichloromethyl radical (CCl$_3$). At low oxygen tension, the CCl$_3$ radical forms the trichloromethyl peroxy radical (CCl$_3$OO$^\cdot$). The trichloromethyl peroxy radical causes lipid peroxidation that initially affects the cellular endoplasmic reticulum, followed by mitochondrial and diffuse cellular damage. This results in cellular swelling. CCl$_4$ produces significant hepatic damage, including fatty infiltration, centrlobular necrosis and cirrhosis$^{110}$. Though the effects of carbon tetrachloride are primarily seen in the liver, kidney tissue and erythrocytes were also studied along with the liver.

3.3.1a Hepatoprotective effects of *Crataegus oxyacantha*

From a morphologic standpoint, the liver is an inherently simple organ with a limited repertoire of responses to injurious events. Any significant insult to the liver may cause hepatocyte necrosis. A single dose of CCl$_4$ (1.5ml-2.5ml/kg body weight) administered to rat produces centrlobular necrosis$^{111}$. Haematoxylin and eosin stained sections of the liver revealed centrlobular necrosis and the cells in the centrlobular region showed vacuolated cytoplasm (Plate 3.1c). Cellular swelling was also observed as mentioned before$^{112,113,114,115}$. There was no damage in the control and drug control groups (Plates 3.1a and b). The *Crataegus* extract pretreated group showed milder damage after CCl$_4$ administration (Plate 3.2d).

Periodic Acid Schiff (PAS) staining of the liver showed decreased glycogen levels in the carbon tetrachloride administered rats. A 1000x magnification showed considerable glycogen depletion in the centrlobular zones (Plate 3.2c). Decreased levels of glycogen and altered glycogenesis has been observed with CCl$_4$ poisoning before$^{116}$. In contrast, vehicle and drug treated liver sections showed that most cells
Plate 3.1 Sections of haematoxylin and eosin stained liver in control and experimental animals. (x 200)

a) Liver of control control group showing normal architecture.

b) Liver of Crataegus treated animals also showing normal architecture.

c) Carbon tetrachloride treated animals showing extensive centrilobular necrosis. Cells also show vacuolated cytoplasm.

d) Crataegus pretreated animals showing a lesser degree of carbon tetrachloride induced damage.
Plate 3.2  Sections of PAS stained liver in control and experimental animals
(x 1000)

a) Liver of control group showing the presence of heavy amounts of glycogen
in every cell. Each cell has an intact nucleus with an unperturbed
cytoplasmic boundary.

b) *Crataegus* pretreated liver shows no pathologic features and the
hepatocellular features mimic the control group.

c) Carbon tetrachloride treated liver section shows severely glycogen depleted
cells. An apoptotic hepatocyte showing a nucleus with condensed,
fragmented and well margined chromatin is seen (arrow).

d) *Crataegus* pretreated animals show only slight depletion of glycogen No
apoptotic cells are seen.
Plate 3.2 Sections of PAS stained liver tissue in normal and experimental rats (x1000)
were uniformly stained and deep-pink colouration signified that nearly every cell was glycogen-loaded with intact cytoplasm (Plate 3.2a and b). Apoptotic cells were characterized as described by Ray, et al, 1999\textsuperscript{17}. Cellular glycogen stores serve as endogenous sensors of cellular crisis. The glycogen depleted hepatocytes showed signs of apoptosis. In routine sections, the best cytological marker of apoptosis is karyorhexis\textsuperscript{18}. Here, the nucleus breaks up into fragments\textsuperscript{19}. The nucleus showed condensed, fragmented and well-marginated chromatin in the CCL\textsubscript{4} treated liver (Plate 3.2c). No such pathologic features were seen in the control groups. Glycogen depletion was lesser in the Crataegus pretreated liver sections (Plate 3.2d).

Enzymes are retained within their cells of origin by the plasma membrane surrounding the cell. The plasma membrane is a metabolically active part of the cell, and its integrity depends on the cell's production of ATP. The earliest sign of impaired energy metabolism is the efflux of potassium and influx of sodium. This causes water to accumulate within the cell, causing it to swell. The next, and the most serious is the entry of Ca\textsuperscript{2+}, which stimulates intracellular enzymes, leading both to cell damage and disruption of the cell membrane. Finally, free radicals formed during lipid peroxidation may cause further damage. The membrane becomes leaky, and if cellular injury becomes irreversible, the cell will die although substantial enzyme loss may occur without irreversible injury occurring. The biochemical features of CCL\textsubscript{4} toxicity are similar to other instances of cytotoxicity, with markedly elevated serum levels of Aspartate transaminase (AST) and Alanine transaminase (ALT). Elevation of serum ALT is a direct reflection of free radical mediated lipid peroxidation of liver cell membranes\textsuperscript{120}. Both these enzymes were elevated significantly in the CCL\textsubscript{4} treated group (Fig. 3.2 & Fig. 3.3). This
Fig 3.2  Activity of serum aspartate amino transferase (AST) in the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS  Group I vs Group III (CCl₄) p < 0.05  
Group III vs Group IV (drug + CCl₄) p < 0.05

![Graph showing AST activity across different groups.]

Fig 3.3  Activity of serum alanine amino transferase (ALT) in the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS  Group I vs Group III (CCl₄) p < 0.05  
Group III vs Group IV (drug + CCl₄) p < 0.05

![Graph showing ALT activity across different groups.]

significant increase in the enzyme levels correlated with the histological changes seen in the liver tissue. Elevated AST and ALT levels associated with CCl₄ induced cellular injury has been reported by many groups which have worked on silymarin, methamphetamine, rutin, tender coconut water and *Coscinium fenestratum* extract. The protective effect of silymarin is related to decreased metabolic activation and to a chain-breaking antioxidant effect. Protective effect of methamphetamine is related to interactions between parenchymal and Kupffer cells. Rutin, a well known flavonoid prevents cytological damage in CCl₄ induced toxicity. The possible mechanisms underlying the hepatoprotective properties of tender coconut water include prevention of GSH depletion and destruction of free radicals. The methanolic extract of *Coscinium fenestratum* is supposed to provide protection to the microsomal membrane.

Similarly, there was a significant elevation in alkaline phosphatase levels (ALP) in the CCl₄ treated group (Fig. 3.5). On the other hand, the control and drug control groups exhibited no significant difference in the ALP levels. Elevated ALP levels generally indicate cholestatic lesions, particularly damage to the bile duct. ALP levels were also maintained at near normal levels after pretreatment with *Crataegus* extract. Other Indian medicinal supplements such as Haridra ghrita have been shown to do the same. Its hepatoprotective activity has been suggested to be because it acts as a scavenger of free radicals like hydroxyl radicals and superoxides.

There was an alarming increase of LDH in the CCl₄ treated group (Fig. 3.4) whereas the drug control groups had normal levels. This was partially reversed in
Fig 3.4  Activity of serum lactate dehydrogenase (LDH) in the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL) p < 0.05
Group III vs Group IV (drug + CCL) p < 0.05

Fig 3.5  Activity of serum alkaline phosphatase (ALP) in the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL) p < 0.05
Group III vs Group IV (drug + CCL) p < 0.05
the *Crataegus* treated group. Lactate dehydrogenase (LDH) activity is present in all cells of the body and is invariably found only in the cytoplasm of the cell. Enzyme levels in tissues are much higher than those normally seen in the serum. Leakage of this enzyme from even a small mass of damaged tissue can increase the observed serum level of LDH to a significant extent. Elevations of LDH activity are high in liver disease such as toxic hepatitis, viral hepatitis, liver anoxia and cirrhosis. LDH decrease would show the cytoprotective nature of the drug as decrease in the serum LDH levels would indicate lesser cytological damage. Such a decrease in LDH levels after administration of drug has been documented in the protective effect of *Terminalia chebula* extract against experimental myocardial infarction induced by isoproterenol where the extract had significant cardioprotective effect and maintained membrane integrity.

During hepatic damage, levels of bilirubin in blood plasma are increased. Here also bilirubin levels were increased significantly (Fig. 3.6) The fact that the drug pretreated group showed decreased levels reveals the hepatoprotective nature of the drug. Such a rise in bilirubin levels as a result of CCl₄ toxicity has been shown to be decreased by Haradradi ghrita.

There was a significant increase in the lipid peroxide levels in the liver (Fig. 3.7) in the CCl₄ treated group. But the drug control group did not show any increase in lipid peroxide levels. The prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Consequent peroxidation results in a loss in membrane fluidity and receptor alignment. This initiates lipid peroxidation of the liver membranes. These lipid peroxides decompose under physiologic conditions in
Fig 3.6 Serum Bilirubin levels in the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

- Group I (control) vs Group II (drug control): p NS
- Group I vs Group III (CCL): p < 0.05
- Group III vs Group IV (drug + CCL): p < 0.05

Fig 3.7 Lipid peroxide levels in the liver tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

- Group I (control) vs Group II (drug control): p NS
- Group I vs Group III (CCL): p < 0.05
- Group III vs Group IV (drug + CCL): p < 0.05
the presence of iron or copper ions to generate highly toxic aldehydes\textsuperscript{131}. Of such aldehydes, malondialdehyde can be estimated and considered as an index of lipid peroxidation\textsuperscript{132}. The lipid peroxide levels in the liver more than doubled the normal levels. This is clearly a result of hepatic oxidative stress induced by CCl\textsubscript{4} toxicity\textsuperscript{133}. These effects were partially reversed, though significantly in the drug pretreated group. Amelioration of such CCl\textsubscript{4}-induced hepatotoxicity has been shown by other groups using different protective agents such as astibilin and thymol. The protective effect of astibilin has been associated to its flavonoid nature and because of the effect it has on liver arachidonate metabolism\textsuperscript{134} The protection rendered by thymol has been related to its capacity to inhibit lipid peroxidation\textsuperscript{135}.

Evaluation of plasma lipids showed a slightly significant rise in the levels of total cholesterol, and triglycerides (Fig. 3.8 and Fig. 3.9), whereas the drug control group did not exhibit any increase in lipid levels. It has been suggested that CCl\textsubscript{4} administration induces an increased synthesis of fatty acids as well as a decreased release of lipoproteins\textsuperscript{136,137,138,139}. But here, there was a significant increase in both high density and low density lipoproteins in the plasma (Fig. 3.10, Fig. 3.11 and Fig. 3.12). This increase was slightly reversed in the Crataegus pretreated rats. It has clearly been shown that Crataegus extract could prevent the rise of plasma lipids in rats which were fed with a hyperlipidemic diet\textsuperscript{140}. CCl\textsubscript{4}-induced lipoprotein alteration has also been shown to be alleviated by other herbal supplements such as Spirulina maxima extract which lowered the accumulation of fatty acids in rat livers and lowered lipid peroxide levels\textsuperscript{140}.

The liver glutathione levels in the CCl\textsubscript{4} group was significantly decreased (Fig. 3.13). It was normal in the control and drug control groups. Glutathione plays a
Fig 3.8 Total cholesterol levels in plasma of the different groups of rats. 
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS 
Group I vs Group III (CCl₄) p < 0.05 
Group III vs Group IV (drug + CCl₄) p < 0.05

Fig 3.9 Triglyceride levels in plasma of the different groups of rats. 
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS 
Group I vs Group III (CCl₄) p < 0.05 
Group III vs Group IV (drug + CCl₄) p < 0.05
Fig 3.10 HDL levels in plasma of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug control) p NS. Group I Vs Group III (CCL-4) p< 0.05
Group III Vs Group IV (drug +CCL-4) p< 0.05

Fig 3.11 LDL levels in plasma of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug control) p NS. Group I Vs Group III (CCL-4) p< 0.05
Group III Vs Group IV (drug +CCL-4) p<0.05
Fig 3.12 VLDL levels in plasma of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL) p<0.05, Group III vs Group IV (drug +CCL) p<0.05

Fig 3.13 Reduced glutathione levels in the liver tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL) p<0.05, Group III vs Group IV (drug +CCL) p<0.05
key role in the liver in several detoxification reactions and in the reduction of lipid peroxides. It also plays a key role in a variety of cell functions, including amino acid transport and storage of thiol moieties\textsuperscript{141}. Irreversible cell damage, mediated by lipid peroxidation, could be due to a severe GSH depletion\textsuperscript{142}. Moreover, 4-hydroxyalkenals resulting from \textit{CCL}_4 metabolism can readily react with low molecular weight thiols such as GSH, thereby further contributing towards its depletion\textsuperscript{143}. The overall effect of depletion of glutathione with a concomitant increase in the lipid peroxide levels provides strong evidence about the vital role played by glutathione during oxidative stress\textsuperscript{144,145}. Glutathione levels significantly improved in the drug pretreated group.

The liver also contains antioxidant enzymes which are involved in scavenging free radicals. The superoxide dismutase enzyme is involved in the quenching of superoxide free radical\textsuperscript{146}. SOD's biological importance has been clearly demonstrated in simple organisms, such as bacteria and yeast mutants devoid of this enzyme. These mutants are more susceptible to oxidative stress\textsuperscript{147}. SOD activity was significantly decreased in the liver homogenate of \textit{CCL}_4 treated rats (Fig. 3.14). This decrease was significantly reversed in the drug pretreated group.

Catalase is a major primary antioxidant defense component that primarily works to catalyse the decomposition of H\textsubscript{2}O\textsubscript{2} to water. In hepatocytes, the peroxisomes exhibit high levels of catalase activity, although activity is also found in the microsomes and in the cytosol\textsuperscript{148}. Catalase activity was also decreased in the \textit{CCL}_4 treated group (Fig. 3.15) which was partially reversed in the drug treated group.
Fig 3.14 Activity of superoxide dismutase in the liver tissue of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p< NS, Group I vs Group III (CCL4) p< 0.05,
Group III vs Group IV (drug +CCL4) p<0.05

Fig 3.15 Activity of catalase in the liver tissue of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p< NS, Group I vs Group III (CCL4) p< 0.05,
Group III vs Group IV (drug +CCL4) p< 0.05
Glutathione peroxidase (GPx) can reduce organic hydroperoxides in addition to hydrogen peroxide. Both selenium-dependent and independent GPx have been shown to reduce the above substrates. It has also been reported to be involved in inhibition of lipid peroxidation along with GSH. A significant decrease in its activity was observed in CCl₄ group (Fig. 3.16). This decrease was significantly reversed in the drug pretreated group.

Similarly, glutathione-s-transferase which conjugates GSH to toxic metabolites, was also decreased in the CCl₄ group (Fig. 3.17). This enzyme can be a potential GSH depleter. *Crataegus* extract could improve the levels of this enzyme significantly. It is possible that the reversal effects of CCl₄ in *Crataegus* pretreated group is because of its ability to induce drug detoxifying enzymes, as seen in the case of kolaviron where the basal levels of glutathione-s-transferase were raised.

3.3.1b Effect of *Crataegus* extract on the liver mitochondria

Mitochondria play a key role in energy metabolism. The final steps in breakdown of carbohydrates and fatty acids are located in mitochondria where energy which is released during oxidation of NADH and FADH₂ is transduced into chemical energy of ATP. The liver is highly dependent on aerobic metabolism processes for its various functions. Mammalian hepatocytes contain between 800 and 2000 mitochondria.

Although the relationship between cirrhosis and impaired liver mitochondria function has not been clearly established, there is a growing bulk of evidence
Fig 3.16 Activity of glutathione peroxidase in the liver tissue of the different
groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS
Group I vs Group III (+CCL4) p<0.05
Group III vs Group IV (drug +CCL4) p<0.05

Fig 3.17 Activity of glutathione-s-transferase in the liver tissue of the different
groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS
Group I vs Group III (+CCL4) p<0.05
Group III vs Group IV (drug +CCL4) p<0.05
suggesting functional and morphological alterations of mitochondria in liver disease. It has been shown that mitochondria isolated from animals poisoned 20 hours previously with CCl₄ had a disruption of oxidative phosphorylation¹⁵²,¹⁵³. Gross ultrastructural changes have been documented in the mitochondria of isolated hepatocytes treated with CCl₄. The electron density and the degree of swelling of mitochondria have been shown to change. Upon extended exposure, some mitochondria develop vacuoles and swirls of lamellar membranes within the matrices⁵⁴.

The results obtained in the liver mitochondria mirrored the changes obtained in the cytosolic fraction of liver. As seen before, there was an increase in the lipid peroxide levels in the CCl₄ group (Fig. 3.18). This increase was reduced significantly by Crataegus extract pretreatment. It is possible that this drug has a protective action on the membrane and that it stabilizes the mitochondrial membranes. This idea has been supported by another group working on the effect of adenosine administration on the function and membrane composition of liver mitochondria in carbon tetrachloride induced cirrhosis¹⁵⁴. The increase in LPO levels showed a concomitant decrease in GSH levels (Fig. 3.19). Similarly, there was a decrease in activity of antioxidant enzymes, SOD and GPx (Fig. 3.20 and Fig. 3.21). It is likely that such injury induced by CCL₄ is because of the action of CCl₃ produced by microsomal cytochrome P-450. It has been shown that mitochondria and microsomes themselves are able to activate CCl₄ to a free radical species¹⁵⁵,¹⁵⁶. Levels of antioxidant enzymes improved upon administration of the drug extract.
Fig 3.18 Lipid peroxide levels in the liver mitochondria of the different groups of rats.

Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS
Group I vs Group III (CCL₄) p < 0.05
Group III vs Group IV (drug + CCL₄) p < 0.05

Fig 3.19 Reduced glutathione levels in the liver mitochondria of the different groups of rats.

Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS
Group I vs Group III (CCL₄) p < 0.05
Group III vs Group IV (drug + CCL₄) p < 0.05
Fig 3.20 Activity of superoxide dismutase in the liver mitochondria of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) Vs Group II (drug control) p NS, Group I Vs Group III (CCL4) p<0.05,
Group III Vs Group IV (drug+CCL4) p<0.05

Fig 3.21 Activity of glutathione peroxidase in the liver mitochondria of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) Vs Group II (drug control) p NS, Group I Vs Group III (CCL4) p<0.05
Group III Vs Group IV (drug+CCL4) p<0.05
In the present study, the enzymes of the tricarboxylic acid cycle; isocitrate dehydrogenase, malate dehydrogenase and succinate dehydrogenase, decreased upon CCl₄ administration (Fig. 3.22, Fig. 3.23 and Fig. 3.24). The drug control group exhibited no change in these enzyme levels. Mild oxidative stress has been known to induce mitochondrial enzymes such as malate dehydrogenase. Altered mitochondrial function has been documented in paracetamol hepatotoxicity also. CCl₄ administration causes an early disturbance in hepatocellular Ca²⁺ homeostasis. It has been suggested that this altered intracellular Ca²⁺ homeostasis may be responsible for impairment in mitochondrial functions. Similarly, NADH dehydrogenase levels were also reduced significantly (Fig. 3.25). The transfer of electrons from NADH in the mitochondrial matrix to ubiquinone in the membrane core is catalyzed by this highly organized enzyme complex which is located in the inner mitochondrial membrane. Cytochrome-c-oxidase, which catalyses the transfer of electrons from cytochrome c to oxygen, also exhibited decreased activity upon CCl₄ administration (Fig. 3.26).

*Crataegus* pretreatment reversed the effects of CCl₄ observed in all the enzymes assayed in mitochondria, significantly. This is in accordance with other results obtained in the study of hepatoprotective action of schisandrin B.

### 3.3.2 Effect of *Crataegus* extract on the Kidney of CCl₄ treated rats

The kidney is a site of significant aerobic metabolism. As in other organs, the kidney produces reactive oxygen species as a consequence of normal cellular metabolism. The function of intrinsic antioxidant enzymes in the kidney are continuously being defined. Superoxide dismutase, both manganese and copper-
Fig 3.22  Activity of isocitrate dehydrogenase in the liver mitochondria of the
different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL4) p<0.05,
Group III vs Group IV (drug +CCL4) p<0.05

Fig 3.23  Activity of malate dehydrogenase in the liver mitochondria of the
different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL4) p<0.05, Group III vs Group IV
(drug +CCL4) p<0.05.
Fig 3.24  Activity of succinate dehydrogenase in the liver mitochondria of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL4) p<0.05
Group III vs Group IV (drug +CCL4) p<0.05

Fig 3.25  Activity of NADH dehydrogenase in the liver mitochondria of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) vs Group II (drug control) p NS, Group I vs Group III (CCL4) p<0.05
Group III vs Group IV (drug +CCL4) p<0.05
Fig 3.26 Activity of cytochrome-c-oxidase in the liver mitochondria of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) Vs Group II (drug control) p < 0.01
Group I Vs Group III (CCL4) p < 0.05
Group III Vs Group IV (drug +CCL4) p < 0.05

![Graph showing activity of cytochrome-c-oxidase in different groups of rats.]

Fig 3.27 Lipid peroxide levels in the kidney tissue of the different groups of rats.
Values are expressed as mean ± SD for six animals in each group.
Group I (control) Vs Group II (drug control) p NS
Group I Vs Group III (CCL4) p < 0.05
Group III Vs Group IV (drug +CCL4) p < 0.05

![Graph showing lipid peroxide levels in different groups of rats.]

zinc dependent forms, catalase, glutathione peroxidase and haemoxxygenase have all been identified within the kidney\textsuperscript{164}. As in other tissues, kidneys are susceptible to oxidant damage when antioxidant defenses are overwhelmed. The kidney is susceptible to chemical induced injury and halogenated alkanes and alkenes are known to cause renal damage. This damage may occur to lipoprotein membranes, extracellular matrix, DNA, proteins and aminoacids\textsuperscript{165}. Such oxidant damage has been shown to play an essential role in the mechanisms of experimental models of several renal diseases like ischemic acute renal failure, renal graft rejection, acute glomerulonephritis and toxic renal diseases\textsuperscript{166}.

Histological investigations of the kidney revealed normal architecture in the control and drug control groups (Plate 3.3a and b). Intact tubules and normal glomerulus are seen in the control group (Plate 3.3a). The CCl\textsubscript{4} treated animals showed exudation in the Bowman’s capsule, tubular regeneration and hyperaemia (Plate 3.3c). In kidney, the proximal tubule is particularly vulnerable to ischemic damage and toxins are frequently reabsorbed by the proximal tubule rendering it susceptible to chemical injury\textsuperscript{167,168}. Preadministration of the \textit{Crataegus} extract showed a lesser degree of injury to kidney tissues (Plate 3.3d).

Lipid peroxidation was increased in the kidney significantly (Fig. 3.27) with the drug control group showing no increase. Tubular degeneration seen in the kidney sections could be explained by the increased LPO levels. There was a reduction in the renal GSH levels in the CCl\textsubscript{4} treated group (Fig. 3.28) with the drug control group showing no decrease. Again, the increased LPO levels could have caused the resultant decrease in GSH levels. There was a significant reduction in the
Plate 3.3 Sections of H&E stained kidney in control and experimental animals (x200)

a) Section of control kidney showing normal architecture with an intact Bowman’s capsule

b) *Crataegus* extract treated animals showing normal architecture.

c) Carbon tetrachloride induced animals showing tubular degeneration, hyperaemia and exudation of the Bowman’s capsule

d) *Crataegus* extract pretreated animals showing lesser degree of damage.
PLATE 3.3 SECTIONS OF H&E STAINED KIDNEY TISSUE IN CONTROL AND EXPERIMENTAL RATS (X200)
Fig 3.28 Reduced glutathione levels in the kidney tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug control) p NS
Group I Vs Group III (+CCL) p< 0.05
Group III Vs Group IV (drug +CCL) p< 0.05

Groups

Fig 3.29 Activity of superoxide dismutase in the kidney tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug, control) p NS
Group I Vs Group III (+CCL) p< 0.05
Group III Vs Group IV (drug, +CCL) p< 0.05

Groups
LPO levels in the drug pretreated group. Similarly, there was a significant increase upon administration of drug extract.

Activities of all the antioxidant enzymes showed a significant decrease in the CCl₄ administrated rat kidney (Fig. 3.29, Fig. 3.30, Fig. 3.31 and Fig. 3.32). Such a decrease in enzyme activity has also been documented in certain pathological states of the kidney. CCl₄ administration has also shown decrease in lysosomal enzymes in the kidney tissue of rat.

The decreased levels of enzymes in the CCl₄ group showed an increase in the group which was pretreated with *Crataegus* extract. It is obvious that cells must have ways to amplify antioxidant enzyme activity to counter sudden increases in oxygen metabolites. The genetic expression of antioxidant enzymes are controlled in bacteria and higher organisms. For example, the *oxyR* regulon in bacteria controls the expression of catalase, glutathione reductase and alkyl hydroperoxidase. It is possible that the drug could be a direct free radical scavenger or could be an inducer of antioxidant enzymes.

### 3.3.3 Effect of *Crataegus* extract on the erythrocytes of CCl₄ treated rats

Erythrocytes are more commonly employed in the evaluation of oxidative stress as they are prone to oxidative reactions because of relatively high oxygen tension and the presence of polyunsaturated lipid rich plasma membrane.

The lipid peroxide levels of both the erythrocyte membrane and haemolysate exhibited an increase (Fig. 3.33 and Fig. 3.34). The drug control groups showed no increase in the lipid peroxide levels. This is because the polyunsaturated fatty acid
Fig 3.30 Activity of catalase in the kidney tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug, control) p NS  Group I vs Group III (C C L) p < 0.05  Group III vs Group IV (drug, + C C L) p < 0.05

Fig 3.31 Activity of glutathione peroxidase in the kidney tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug, control) p NS  Group I vs Group III (C C L) p < 0.05  Group III vs Group IV (drug, + C C L) p < 0.05
Fig 3.32 Activity of glutathione -s-transferase in the kidney tissue of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (+CCL) p < 0.05, Group III vs Group IV (drug +CCL) p < 0.05

Fig 3.33 Lipid peroxide levels in the erythrocyte membrane of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS, Group I vs Group III (+CCL) p < 0.05, Group III vs Group IV (drug +CCL) p < 0.05
side chains of the membrane lipids are also highly susceptible to attack by oxidizing radicals with the formation of lipid hydroperoxides\textsuperscript{174}.

As observed in the liver and kidney, there was a decrease in the GSH levels of haemolysate of the CCl\textsubscript{4} treated group (Fig. 3.35). The drug control groups exhibited no decrease in its GSH levels. Erythrocytes contain large amounts of GSH and one role of this GSH is to protect haemoglobin and other thiol group-containing proteins from denaturation under oxidative stress. When erythrocytes are exposed to oxidative stress, the reduced form of glutathione gets oxidized. This may occur through interaction of the oxidative compound with haemoglobin to form peroxides, with the subsequent oxidation of GSH through the mediation of glutathione peroxidase\textsuperscript{175,176}. \textit{Crataegus} extract was able to reduce LPO levels and increase GSH levels significantly.

Similarly, the drug extract improved the reduced erythrocyte antioxidant enzyme status which was reduced in the CCl\textsubscript{4} treated group (Fig. 3.36, Fig. 3.37 and Fig. 3.38). Generally, high levels of free radicals enhance the activities of enzymes such as glutathione peroxidase\textsuperscript{177}. Since CCl\textsubscript{4} is an overwhelming intoxicant, the decrease in the levels of antioxidant enzymes can be explained.

The different ATPases were assayed in the RBC membrane. All the three: Na'K'-ATPase, Ca\textsuperscript{2+}-ATPase, and Mg\textsuperscript{2+}-APTase levels decreased in the CCl\textsubscript{4} treated group (Fig. 3.39, Fig. 3.40 and Fig. 3.41). The drug control group showed no decrease in its enzyme levels.
Fig 3.34  Lipid peroxide levels in the erythrocyte haemolysate of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug control) p NS  Group I vs Group III (CCL4) p< 0.05  
Group III vs Group IV (drug +CCL4) p< 0.05

Fig 3.35 Reduced glutathione levels in the erythrocyte haemolysate of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (controls) vs Group II (drug controls) p NS  Group I vs Group III (CCL4) p< 0.05  
Group III vs Group IV (drug +CCL4) p< 0.05
Fig 3.36 Activity of superoxide dismutase in the erythrocyte haemolysate of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug, control) p NS. Group I vs Group III (C.C.L) p < 0.05
Group III vs Group IV (drug, +C.C.L) p < 0.05

Fig 3.37 Activity of catalase in the erythrocyte haemolysate of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) vs Group II (drug, control) p < 0.001. Group I vs Group III (C.C.L) p < 0.05
Group III vs Group IV (drug, +C.C.L) p < 0.05
Fig 3.38 Activity of glutathione peroxidase in the erythrocyte haemolysate of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug, control) p NS  Group I Vs Group III (+ CCL4) p< 0.05  Group III Vs Group IV (drug + CCL4) p< 0.05

Fig 3.39 Activity of Na⁺K⁺-ATPase in the erythrocyte membrane of the different groups of rats. Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug, control) p NS  Group I Vs Group III (+ CCL4) p< 0.05  Group III Vs Group IV (drug + CCL4) p< 0.05
ATPases are enzymes that are closely associated with the plasma membrane, which participate in energy metabolism. They are lipid and thiol-dependent, membrane bound enzymes which also maintain cellular electrolyte concentration and transmembrane electrochemical gradients. In active transport, the high energy phosphate bond of ATP is utilized to transport compounds against a concentration gradient. For example, Na⁺K⁺-ATPase uses ATP energy to pump Na⁺ out of the cell\textsuperscript{178}. Erythrocyte membrane lipid peroxidation would have contributed to the decrease in ATPase activities since membrane damage by free radical chain reaction has been proposed as the major cause of injury induced by CCl\textsubscript{4}\textsuperscript{179} The administration of the drug improved the levels of the ATPases significantly. This has established the cytoprotective nature of the drug.

3.4 \textit{IN VITRO STUDIES OF ANTIOXIDANT EFFECT OF CRATAEGUS OXYACANTHA}

Due to ethical constraints on performing toxicity studies in humans, relevant safety assessment is usually studied in laboratory animals. But extrapolation of assessment from animals to humans is very challenging\textsuperscript{180}. Therefore usually, risk assessment and drug metabolism studies are performed, \textit{in vitro}, in cell lines. But the genetic make up of cell lines are completely different from their tissues of origin. This model also does not completely mimic the human model.

Therefore, for this study, normal human blood tissue was used for evaluation of the antioxidant capacity of the drug extract. Here, hydrogen peroxide was used to induce oxidative stress. Both the peripheral lymphocytes and erythrocytes were used for assessment.
Fig 3.40 Activity of Ca$^{2+}$-ATPase in the erythrocyte membrane of the different groups of rats.

Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug control) p NS, Group I Vs Group III (CCL) p < 0.05
Group III Vs Group IV (drug +CCL) p > 0.05

Fig 3.41 Activity of Mg$^{2+}$-ATPase in the erythrocyte membrane of the different groups of rats.

Values are expressed as mean ± SD for six animals in each group.

Group I (control) Vs Group II (drug control) p NS, Group I Vs Group III (CCL) p < 0.05
Group III Vs Group IV (drug +CCL) p > 0.05
3.4.1 Antioxidant capacity of *Crataegus* extract in human erythrocytes

The erythrocyte is the most readily available source of pure membrane in the human body. This cell contains large amounts of haemoglobin and its membrane is not only rich in polyunsaturated fatty acids, but also has an anion channel through which superoxide can enter. The superoxide radical is continuously generated in human erythrocytes\(^\text{181}\). This exposes the cell to a constant mild oxidative stress. In spite of this, the normal erythrocyte is highly resistant to oxidative damage because of its efficient protective mechanisms. The cell interior is rich in catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione\(^\text{182}\).

Hydrogen peroxide was used to induce toxicity here because it crosses the cell membrane easily and can cause hydroxyl radical to be formed. This hydroxyl radical initiates lipid peroxidation\(^\text{183}\). Here also, there was increased lipid peroxidation in the H\(_2\)O\(_2\) treated erythrocytes (Table 3.4). This was followed by a decrease in the glutathione levels (Table 3.4). There was a decrease in the superoxide dismutase activity (Table 3.5). The toxin induced deficiency of glutathione may have aggravated oxidative stress. This would explain the decrease in superoxide dismutase activity. The enzymes specific for the removal of hydrogen peroxide, namely catalase and glutathione peroxidase, also exhibited a decrease in activity (Table 3.5).

Generally, the superoxide dismutases of Man are more active than that of rat\(^\text{184}\). Superoxide dismutase levels have actually showed increased levels in certain malignant conditions\(^\text{185}\). Since these experiments were conducted *in vitro*, the
Table 3.4  Levels of lipid peroxides and reduced glutathione in the haemolysate of human RBC’s treated in vitro with *Crataegus* extract (5μl, 10μl and 20μl/ml packed RBC’s) and hydrogen peroxide (n=10)

<table>
<thead>
<tr>
<th></th>
<th>Group I - Control</th>
<th>Group II - H₂O₂ treated</th>
<th>Group III - <em>Crataegus</em> treated (5 μl/ml) + H₂O₂</th>
<th>Group IV - <em>Crataegus</em> treated (10μl/ml) + H₂O₂</th>
<th>Group V - <em>Crataegus</em> treated (20μl/ml) + H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxides</td>
<td>4.48 ± 0.13</td>
<td>8.295 ± 0.25</td>
<td>5.62 ± 0.28</td>
<td>5.58 ± 0.20</td>
<td>6.21 ± 0.02</td>
</tr>
<tr>
<td>(nmoles MDA/mg Hb)</td>
<td></td>
<td>a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>100.19 ± 1.3</td>
<td>43.0 ± 3.7</td>
<td>95.8 ± 2.7</td>
<td>98.7 ± 2.1</td>
<td>81.8 ± 2.3</td>
</tr>
<tr>
<td>(nmoles/mg Hb)</td>
<td></td>
<td>a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a- Group I compared with group II, P<0.05  
b- Group III compared with Group II, P<0.05  
c- Group IV compared with Group II, P<0.05  
d- Group V compared with Group II, P<0.05
<table>
<thead>
<tr>
<th>Group IV-</th>
<th>Group V-</th>
<th>Group II- H₂O₂ treated (20 μl/ml) + H₂O₂</th>
<th>Group III- Crataegus treated (5 μl/ml) + H₂O₂</th>
<th>Group I- Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (U/mg Hb)</td>
<td>2.24 ± 0.1</td>
<td>1.27 ± 0.13(^a)</td>
<td>20.14 ± 0.14</td>
<td>6.42 ± 0.16</td>
</tr>
<tr>
<td>Catalase (μmoles of H₂O₂ utilized/min/mg Hb)</td>
<td>12.28 ± 0.13(^a)</td>
<td>16.24 ± 0.16</td>
<td>3.96 ± 0.11(^a)</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (μmoles of GSH utilized/min/mg Hb)</td>
<td>1.42 ± 0.11(^c)</td>
<td>15.23 ± 0.21(^d)</td>
<td>5.02 ± 0.15(^d)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Group I compared with Group II, P < 0.05
\(^b\) Group III compared with Group II, P < 0.05
\(^c\) Group IV compared with Group II, P < 0.05
\(^d\) Group V compared with Group II, P < 0.05
\(^e\) Group V compared with Group II, P < 0.05
decrease in superoxide dismutase activity can be explained as superoxide radical is formed during autoxidation of oxyhaemoglobin.

Lipid peroxides, hydrogen peroxide, superoxide and other oxidants contribute to the instability of the erythrocyte cell membrane. Catalase and glutathione peroxidase probably play complementary roles in protecting the erythrocyte from haemolysis due to hydrogen peroxide. This explains the quenching of the activities of these enzymes. The enzyme activities improved after pretreatment with the extract. The effect was more at a concentration of 10μl/ml of packed RBC's. Similarly, lipid peroxide levels decreased with preincubation with drug.

The activities of the different ATPases in the RBC membrane decreased significantly after treatment with H₂O₂(Table 3.6). The levels of ATPases increased significantly in the drug pre-incubated erythrocytes. There was maximum increase at a concentration of 10μl/ml of packed RBC's. The erythrocyte membrane is known to regulate the movement of certain ionic substances like Na⁺ and K⁺ ions into and out of the cell. Improper functioning of Na⁺K⁺-ATPase may disturb the membrane Na⁺-K⁺ pump which may increase cellular excitability through prolonged depolarization of cells. Inhibition of this enzyme increases intracellular Ca²⁺ and decreases intracellular Mg²⁺ concentration. Ca²⁺ is an important intracellular messenger that regulates cellular processes as varied as muscle contraction and carbohydrate metabolism. Ca²⁺ is actively transported into cells across the plasma membrane by a Ca²⁺-APTase which is structurally and mechanistically similar to Na⁺K⁺-ATPase. Similarly, decreased activity of Mg²⁺-ATPase can also lead to
Table 3.6 Activities of membrane bound, Na⁺K⁺-ATPase, Ca²⁺-ATPase and Mg²⁺-ATPase in the RBC membrane of human RBC’s treated *in vitro* with *Crataegus* extract (5μl, 10μl and 20μl/ml packed RBC’s) and hydrogen peroxide (n=10)

<table>
<thead>
<tr>
<th></th>
<th>Group I-Control</th>
<th>Group II- H₂O₂ treated</th>
<th>Group III- <em>Crataegus</em> treated (5 μl/ml) + H₂O₂</th>
<th>Group IV- <em>Crataegus</em> treated (10μl/ml) + H₂O₂</th>
<th>Group V- <em>Crataegus</em> treated (20μl/ml) +H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺K⁺-ATPase (μmole Pi liberated/min/mg protein)</td>
<td>7.18 ± 0.11</td>
<td>5.08 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.05 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.92 ± 0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.77 ± 0.11&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ca²⁺-ATPase(μmole Pi liberated/min/mg protein)</td>
<td>4.05 ± 0.42</td>
<td>1.66 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.07 ± 0.13&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mg²⁺-ATPase(μmole Pi liberated/min/mg protein)</td>
<td>2.34 ±0.14</td>
<td>1.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60 ±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.76 ±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.52 ±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a- Group I compared with group II, P<0.05  
b- Group III compared with Group II, P<0.05  
c- Group IV compared with Group II, P<0.05  
d- Group V compared with Group II, P<0.05  
e- Group V compared with Group II, P non significant
alteration in cellular magnesium homeostasis. The eventual impact of elevated intracellular Ca\(^{2+}\) and intracellular Mg\(^{2+}\) is the escalation of reactive oxygen species generation and oxidative injury to cells\(^{188}\). The loss of ATPase activity is believed to result from the oxidation of thiol groups\(^{189,190}\). Since the levels of glutathione improved in the drug treated group, the levels of ATPases also improved.

3.4.2 Antioxidant capacity of *Crataegus* extract in human lymphocytes

Peripheral lymphocytes from normal donors provide an excellent model for studying the cytoprotective nature of drugs even though proliferating lymphocytes are used to test the cytotoxic nature of drugs\(^{191}\).

Lymphocytes also showed an increase in the lipid peroxide levels (Table 3.7). Lipid peroxide levels are also altered in certain diseased states\(^{192,193}\). Glutathione levels were also decreased significantly (Table 3.7). This can be explained by the increased LPO levels. Activities of SOD, CAT and GPx exhibited a decrease (Table 3.8). This sort of a decrease is seen in some diseased and physiologic conditions\(^{194,195,196}\). Pre-incubation with the drug effectively reversed the effects. Reversal of effects was maximum at a concentration of 10µl/10,000 cells.

Fluorescent staining with ethidium bromide and acridine orange showed that 100% of the untreated normal lymphocytes exhibiting a fluorescent green colour showing they were viable (Plate 3.4). The hydrogen peroxide treated cells showed extensive membrane blebbing and these cells showed a deep orange colour as ethidium bromide had entered into the cells which were not viable and therefore had a leaky membrane. Ethidium bromide enters into dead cells and intercalates between
Table 3.7 Levels of lipid peroxides and reduced glutathione in the human peripheral blood lymphocytes treated *in vitro* with *Crataegus* extract (5μl, 10μl and 20μl/10,000 cells) and hydrogen peroxide (n=10)

<table>
<thead>
<tr>
<th></th>
<th>Group I- Control</th>
<th>Group II- H₂O₂ treated</th>
<th>Group III- <em>Crataegus</em> treated (5 μl/10,000 cells) + H₂O₂</th>
<th>Group IV- <em>Crataegus</em> treated (10μl/10,000 cells) +H₂O₂</th>
<th>Group V- <em>Crataegus</em> treated (20μl/10,000 cells) +H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxides</td>
<td>295.07 ± 0.91</td>
<td>1273.83 ± 1.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>535.61 ± 5.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>481.83 ± 1.61&lt;sup&gt;c&lt;/sup&gt;</td>
<td>608.38 ± 5.32&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(nmoles MDA/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>96.93 ± 1.38</td>
<td>32.35 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.75 ± 2.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.80 ± 0.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.23 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(nmoles/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Group I compared with group II, P<0.05
<sup>b</sup> Group III compared with group II, P<0.05
<sup>c</sup> Group IV compared with group II, P<0.05
<sup>d</sup> Group V compared with group II, P<0.05
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Superoxide Dismutase (U/mg Hb)</th>
<th>Catalase (μmoles of H₂O₂ utilized/min/mg Hb)</th>
<th>Glutathione Peroxidase (μmoles of GSH utilized/mm/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>62.15 ± 1.28</td>
<td>34.72 ± 3.07</td>
<td>7.00 ± 0.24</td>
</tr>
<tr>
<td>II</td>
<td>H₂O₂ treated</td>
<td>31.78 ± 1.34</td>
<td>9.08 ± 0.20</td>
<td>2.51 ± 0.25</td>
</tr>
<tr>
<td>III</td>
<td>20 μl/10,000 cells + H₂O₂</td>
<td>42.85 ± 1.99\textsuperscript{a}</td>
<td>20.26 ± 0.95\textsuperscript{b}</td>
<td>4.20 ± 0.21\textsuperscript{b}</td>
</tr>
<tr>
<td>IV</td>
<td>Citrus treated</td>
<td>44.60 ± 1.87\textsuperscript{c}</td>
<td>25.83 ± 1.06\textsuperscript{c}</td>
<td>5.62 ± 0.14\textsuperscript{c}</td>
</tr>
<tr>
<td>V</td>
<td>Citrus treated</td>
<td>38.51 ± 0.87\textsuperscript{d}</td>
<td>18.32 ± 0.24\textsuperscript{d}</td>
<td>3.55 ± 0.21\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Group I compared with group II, \textit{P}<0.05
\textsuperscript{b} Group III compared with Group II, \textit{P}<0.05
\textsuperscript{c} Group IV compared with Group II, \textit{P}<0.05
\textsuperscript{d} Group V compared with Group II, \textit{P}<0.05

Table 3.8 Levels of primary antioxidant enzymes in the human peripheral blood lymphocytes treated in vitro with Citrus extract (5 μl, 10 μl, and 20 μl/10,000 cells) and hydrogen peroxide (n=10)
the DNA strands\textsuperscript{197}. Here, almost all the cells were non viable showing extensive membrane blebbing. Membrane blebbing is a distinctive morphological feature of apoptosis\textsuperscript{198}. There were lesser number of non viable cells in the drug preteated cells (Plate 3.4c and Plate 3.4d). The degree of membrane blebbing was reduced in the drug pretreated cells especially at the concentration of 10\(\mu\)l/10,000 cells. At this concentration, nearly 75\% of the lymphocytes were viable in nature. At a concentration of 5\(\mu\)l/10,000 cells, about 60\% of the lymphocytes were viable in nature.

Cellular DNA is prone to oxidative damage\textsuperscript{199,200}. Hydroxyl radicals attack DNA and produce several types of damage. DNA strand breaks are formed when cells are exposed to \(\text{H}_2\text{O}_2\). Hydrogen peroxide can damage DNA in a manner that is mediated by intracellular iron. This has been proved, as lipophilic Fe(II) chelators, such as 1,10-phenanthroline and dipyridyl can inhibit DNA strand break formation\textsuperscript{201,202}. This damage can induce apoptosis in cells as evidenced by the morphological features seen in the lymphocytes\textsuperscript{203}. DNA degradation seen in most cells undergoing apoptosis is an useful biochemical marker to characterize apoptosis. Hydrogen peroxide induced DNA degradation could be effectively reduced by the drug (Plate 3.5) at a concentration of (10\(\mu\)l/10,000 cells). Other herbal extracts and compounds of herbal origin such as epigallocatechin gallate, lignin, Chaga mushroom extract, tannic acid and \textit{Inonotus obliquus} extract have been shown to reduce damage to DNA resulting from hydrogen peroxide\textsuperscript{204,205,206,207,208,209}.
Plate 3.4  Fluorescent staining of lymphocytes with ethidium bromide and acridine orange (x400)

a)  Control group showing 100% viable cells exhibiting bright green fluorescence.
b)  Hydrogen peroxide treated cells with bright orange fluorescence. Extensive membrane blebbing is seen.
c)  *Crataegus* pretreated cells (10 µM/10,000 cells) showing 75% viable cells.
d)  *Crataegus* pretreated cells (5 µM/10,000 cells) showing 60% viable cells.
PLATE 3.4 FLUORESCENT STAINING OF LYMPHOCYTES WITH ETHIDIUM BROMIDE AND ACRIDINE ORANGE (X400)
Therefore, it is evident that *Crataegus* is a potent cytoprotective agent. That it can attenuate the accumulation of lipid peroxidation products indicate that it can act as an *in vivo* antioxidant and protect biomembranes from damage. It could also scavenge free radicals via conjugation with glutathione with enhanced activity of GST. The mechanism by which it promotes antioxidant effect could be by up regulation of antiapoptotic genes\(^{210}\). It could possibly up regulate anti apoptotic genes such as *Bcl-2* or it could down regulate apoptosis promoting genes such as *p53* and *Bax*\(^{211}\). It could also be a Ca\(^{2+}\) antagonist, thereby, making calcium ions unavailable for programmed cell death\(^{212,213}\). But work is needed in this area to confirm the mechanism of action of drug.

The cytoprotective nature of the drug could be an explanation for its use in United States and European countries where berries and the flowers of the common hawthorn have been traditionally used as a popular cardiac tonic.
Plate 3.5  Reversal of DNA degradation induced by hydrogen peroxide by *Crataegus* extract

Lane1 :  φX174 DNA digest. Bands seen are 1353bp, 1078bp, 872bp, 603bp and 310bp

Lane2 :  Control lymphocyte DNA

Lane3 :  DNA of H$_2$O$_2$ treated lymphocytes

Lane4 :  DNA of lymphocytes incubated with 5µl drug and then treated with H$_2$O$_2$.

Lane5 :  DNA of lymphocytes incubated with 10µl drug and then treated with H$_2$O$_2$.

Lane6 :  DNA of lymphocytes incubated with 20µl drug and then treated with H$_2$O$_2$. 
Plate 3.5 Effect of drug extract on DNA degradation induced by Hydrogen Peroxide on normal human Lymphocytes