6.1 INTRODUCTION:

Liver is one of the largest organs in human body and the chief site for metabolism and excretion. So it has an astonishing role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (Azri et al., 1992). The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. But it is continuously and variedly exposed to environmental toxins, abused by poor drug habits, alcoholism, prescribed and over-the-counter drug which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease. Thus, liver disorders are some of the fatal disease in the world today. They pose a serious challenge to international public health. Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for their treatment of liver disorders. But there are not much drug available for the treatment of liver disorders.

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver diseases. Carbon tetrachloride (CCl\textsubscript{4}) is a xenobiotic producing hepatotoxicity in human beings and animals (Braltin et al., 1985 and Azer et al., 1997). In fact, it has been shown that the
trichloromethyl radical (CCl₃O·) formed in the metabolism of CCl₄ via the liver microsomal cytochrome P450 system reacts rapidly with molecular oxygen to produce trichloromethyl peroxy radicals (CCl₃O₂). These radicals react with unsaturated fatty acids of phospholipids present in cell membranes, initiating lipid peroxidation in liver cells (Recknagel et al., 1984). Hydrogen atoms are removed from unsaturated fatty acids by such radical created carbon centred lipid radicals (Cay et al., 1989). These lipid radicals quickly add molecular oxygen to form lipid peroxy radicals, which in turn abstract hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation (Charless Nelson, 1979). Antioxidants play a crucial role in hepatoprotective ability and hence the search for crude drugs of natural origin with this property has become a central focus of study of hepatoprotection today (Pradeep et al., 2010).

Thus present study, a best effort is made to evaluate the hepatoprotective effect of ethanolic extract of seed coat and cotyledon of C. cajan, against CCl₄ induced hepatotoxic mice.

6.2 REVIEW OF EARLIER WORKS:

There are several studies made on hepatoprotective aspects of leguminous plants by different researchers (Handa et al., 1989; Hikino et al., 1988; Abbiw, 1990; Evans et al., 1996; Sharma et al., 1991; Iweala Emeka and Eleka, 2001; Wu et al., 2001; Joseph Ashidi et al., 2004; Mankani et al., 2005; Ksturi Sarkar et al., 2006; Vipul Gujrati et al., 2007; Avijeet et al., 2008; Rekha Rajendran et al., 2009; Rajib Ashan et al., 2009; Sunetra Ptwardhan et al., 2009;
Qureshi Mohammad et al., 2010; Ighodaro and Omole, 2010; Suman Pattanayak et al., 2011; Sidharath Singh et al., 2011; Oluseyi and Moshood, 2011; Anjana Male et al., 2012; Mohd Ali et al., 2013; Rhitajit et al., 2013). We shall give a following brief review of works related to present studies.

Wu et al. (2001) have evaluated the hepatoprotective activity on foods and folk medicines of legumes in Taiwan such as mung bean, adzuki bean, black bean and rice bean. The results revealed that aqueous extract of mung bean showed the best hepatoprotective effect on APAP-induced hepatotoxicity. So, mung bean acted as a potential hepatoprotective agent in dietary supply. Further, Mohd Ali et al. (2013) have reported the antioxidant and hepatoprotective effect of aqueous extract of germinated and fermented mung bean on ethanol-mediated liver damage. Tested results suggested that freeze-dried, germinated, and fermented mung bean enriched with amino acids and GABA possessed better hepatoprotective effect as compared to normal mung bean.

Iweala Emeka and Elekwa (2001) have observed the liver function status and lipid peroxidation level in rats, supplemented with ‘bambara groundnut’ (Voanzela subterranea) and ‘pigeon pea’ (Cajanus cajan). They observed the significant decrease (P>0.05) in serum aspartate amino transferase (ALT) and alkaline phosphates activities. There was a marginal reduction in lipid peroxidation levels.

Kasturi Sarkar et al. (2006) have purified and characterized a 43 kD hepatoprotective protein from the herb Cajanus indicus. They suggested the
protein also possesses preventive role against a number of toxin induced hepatic damages.

Ighodaro and Omole (2010) have studied the effect of *Cajanus cajan* aqueous leaf extract (at 0.5 g/kg and 1 g/kg b.w) on serum amino transferase, alkaline phosphatise and electrolytes concentrations of normal wistar rats for 14 days. With the exception of ALP, the extract at a dose of 0.5 g/kg produced no significant (p < 0.05) changes in ALT and AST activities and in the concentrations of the electrolytes in rats. Though the levels of serum electrolytes were not significantly raised, 1.0 g/kg of the extract markedly (p < 0.05) increased the activities of serum enzymes. These results revealed that aqueous leaf extract of *Cajanus cajan* at a dose of 0.5 g/kg did not cause obvious damage to the liver and kidneys of rats. However, 1.0 g/kg b.w. of the plant extract showed clear signs of hepatocellular derangement in rats. Hence, *Cajanu s cajan* leaves may be hepatotoxic when consumed at concentrations about or above 0.5 g/kg of body weight.

Sidharth Singh et al. (2011) have studied the hepatotrotective activity of hydroalchoholic extract of leaf of *C. cajan* (100, 200 and 400 mg/kg) against CCl$_4$ induced liver damage. The extract was effective in protecting the liver against the injury as there was significant reduction in serum enzyme aspartate aminotransferase (AST), alanine aminotransferase (ALT) and increase in total protein.
Suman Pattanayak et al. (2011) have investigated the hepatoprotective activity of crude flavonoids extract (50 mg/kg) of Cajanus scarabaeoides (L) in paracetamol intoxicated albino rats. Treatment of crude flavonoids has reduced the AST, ALT, ALP and total bilirubin level and which is compared to with standard silymarin (100 mg/kg). Histopathological studies also provided supportive evidence for biochemical analysis. They suggested that crude flavonoids extract of C. scarabaeoides possessed hepatoprotective activity.

Further, Oluseyi and Moshood (2011) have investigated the hepatoprotective effect of Cajanus cajan ethanolic extract on hepatic antioxidant status in D-galactosamine-induced hepatitis in rats. Results showed that a significant (p<0.05) increase in the activities of liver marker enzymes (ASP, ALT) levels and decreased in antiperoxidative enzymes (Catalase, Superoxide dismutase, Glutathione peroxidise, Glutathione-S-transferase) activities were observed in D-galactosamine-induced hepatitis rats. Whereas, C. cajan extract (100 mg/kg) reversed the D-galactosamine-induced increase in the level of ASP and ALP with corresponding increase in antiperoxidative enzymes activities. This study revealed that induction of the antioxidant enzymes is considered to be reliable marker for evaluating antiperoxidative efficacy of medicinal plants in hepatic system.

Similarly, Anjana Male et al. (2012) have studied the hepatoprotective activity of ethanol and water extracts leaves of Cajanus cajan L. against CCl4 induced hepatic damage. The results revealed that administration of ethanol water extract (600 mg/kg) significantly reduced the serum marker enzymes
ASP, ALT, ALP and bilirubin levels and enhance the protein contents showed the significant liver protection.

Rhitajit et al. (2013) have studied antioxidative protection against iron overload-induced liver damage in mice by methanolic extract of C. cajan leaf. Post oral administration of methanolic extract of leaf to iron overloaded mice, the levels of antioxidant and serum enzymes, hepatic iron, serum ferritin, lipid peroxidation and protein carboxyl and hydroxyproline contents were measured, in comparison to deferasirox treated mice. Oral treatment of the plant extract effectively lowered the elevated levels of hepatic antioxidants. The histopathological observations also substantiated the ameliorative function of the plant extract. The results suggested that C. cajan leaf can be a useful herbal remedy to suppress oxidative damage caused by iron overload.

6.3 MATERIALS AND METHODS:

C. cajan seeds have been used in the traditional system of medicine for the treatment of number of disease complications including hepatic diseases (Abbiw, 1990). The ethanolic extract of seed coat and cotyledon are chosen for the present investigation because ethanol extract have more bioactive compounds and scavenging activity compared to aqueous, pet ether and chloroform extract showed in chapter - IV. Therefore, in present investigation the ethanol extract of seed coat and cotyledon of C. cajan were screened for the determination of acute toxicity and hepatoprotective activities on Albino mice.
6.3.1 Determination of Acute toxicity:

Acute toxicity of *C. cajan* ethanol extract of seed coat and cotyledon was carried out on albino mice (procured from M/s Sri. Venkateshwar Enterprises, Hyderabad, Andhra Pradesh), either sex weighing between 25-35 g was selected and divided into seven groups of four animals each. These animals used after acclimatization for a period of 5 days to the laboratory conditions. These animals were housed in standard metal cages and were provided food and water *ad libitum*. The animals under study were kept on fasting for period of 18 h prior to the experiment with water *ad libitum*. The animals of Group - I were given 0.5% gum acacia (w/v) suspension to serve as negative control. Whereas Group-II to Group-IV animals received orally (p.o.) the ethanol extract of seed coat at the doses of 50, 500 and 1000 mg/kg body weight (b.w.) respectively. The remaining Group V, VI and VII animals received the cotyledon extracts at the doses of 50, 500 and 1000 mg/kg b.w. respectively. These animals were observed for the toxicity of extracts of *C. cajan* in terms of their survival and/or mortality at different intervals i.e., ½, 1, 2, 4, 8, 24, 48, 72 h for a period of seven days.

6.3.2 Experimental design of Hepatoprotective activity:

Liver injuries induced by CCl₄ are commonly used hepatotoxine in experimental models for the screening of hepatoprotective drugs (Rajesh et al. 2007). The ethanolic extract of seed coat and cotyledon of *C. cajan* was studied by their ability to inhibit CCl₄ induced liver damage in albino mice.
Procedure:

A total at 42 albino mice of either sex weighing between 25-35 g were equally divided into 7 groups (n=6 in each group). The treatment period was for 6 days. Group-I was maintained as normal control which was given distilled water only and Group-II received CCl₄ (2 ml/kg) diluted with liquid paraffin (1:1) given orally on third and sixth day. Group III received CCl₄ and standard drug Liv 52 (100 mg/kg p.o.). Group IV and V received CCl₄ and seed coat extract 100 and 500 mg/kg p.o. Similarly, Group VI and VII received CCl₄ and cotyledon extract 100 and 500 mg/kg p.o. respectively, once simultaneously for 6 days.

**Group I** - Normal control (10 ml/kg normal saline, p.o.)

**Group II** - Toxicant CCl₄ (2 ml/kg p.o. diluted with liquid paraffin (1:1) on third and sixth day).

**Group III** - Standard Liv-52 (100 mg/kg p.o.) and CCl₄ (2 ml/kg p.o.)

**Group IV** - Ethanol extract of seed coat (100 mg/kg p.o.) and CCl₄ (2 ml/kg p.o.)

**Group V** - Ethanol extract of seed coat (500 mg/kg p.o.) and CCl₄ (2 ml/kg p.o.)

**Group VI** - Ethanol extract of cotyledon (100 mg/kg p.o.) and CCl₄ (2ml/kg p.o.)

**Group VII** - Ethanol extract of cotyledon (500 mg/kg p.o.) and CCl₄ (2 ml/kg p.o.).

Food was withdrawn 12 h before CCl₄ administration on the sixth day to enhance the acute liver damage in all the groups except Group-I animals. Mice were sacrificed on seventh day, 24 h after administration of the last
dose. The animals were then anesthetized using anaesthetic ether and blood collected by retro orbital puncture and used for biochemical tests. The animals were sacrificed by overdose of ether and autopsied. Livers from all animals were removed, washed with ice-cold saline small piece of liver tissue collected and preserved in 10% formalin solution for histopathological studies and another small piece of liver tissue (200 mg) was homogenized with ice-chilled 10% KCl solution and centrifuged at 1200 rpm for 15 min at 4°C. The supernatant was used for the assay of antioxidant enzymes.

6.3.2.1 Biochemical Analysis of Blood Serum of Mice:

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 2500 rpm for 10 min. The serum was used for estimation of biochemical parameters to determine the functional state of the liver.

6.3.2.1.1 Estimation of Proteins:

The level of total protein was estimated in serum of experimental animals by Biuret method (Peters, 1968), standard kit was obtained from Span diagnostics.

6.3.2.1.2 Estimation of Bilirubins:

The total and direct bilirubin was estimated in serum of experimental animals by Jenderassik and Grof (1938), standard kit was obtained from Aggappe Diagnostics.
6.3.2.1.3 Enzyme assay:

The serum was analysed for serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and serume alkaline phosphatase (SAP) levels using enzopak reagent kits by the method proposed by Reitman and Frankel (1957).

6.3.2.2 Analysis of \textit{in-vivo} Antioxidant activity:

The enzyme extracts for SOD, POD, CAT, GR, GRS and lipid peroxidation were prepared by small piece of liver tissue (200 mg) was homogenized with ice-chilled 10% KCl solution and centrifuged at 1200 rpm for 15 min at 4\(^\circ\) C. The supernatant was used for the assay of antioxidant parameters.

6.3.2.2.1 Assay of Super Oxide Dismutase (SOD):

The SOD activities in liver were estimated by recording decrease in the optical density of nitro-blue tetrazolium dye (Dhindsa et al., 1981). 3 ml of the reaction mixture contained 13 mM Methionine, 25 mM Nitro-blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 ml enzyme extract. Reaction was started by adding 2 \(\mu\text{M}\) riboflavine and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme with maximal color served as control. The reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the amount of
enzyme which reduces the absorbance reading at 50% in comparison with tubes lacking enzyme.

6.3.2.2.2 Assay of Peroxidase:

The peroxidase activity was assayed according to the method of Malick and Singh (1980). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol and 0.1 ml of enzyme extract in a total volume of 3 ml. The reaction was started with the addition of 0.03 ml of 0.1 mM H₂O₂ and the absorbance was recorded at 436 nm spectrophotometrically up to 10 min against the blank.

6.3.2.2.3 Assay of Catalase:

Catalase activity was assayed by estimating residual hydrogen peroxide by titanium reagent (Teranishi et al., 1974). The reaction mixture (3 ml) contained 0.1 mM phosphate buffer (pH 7.0), 6 mM H₂O₂ and 0.2 ml enzyme extract. The reaction was stopped after 5 min by the addition of 4 ml of titanium reagent, which form coloured complex with residual hydrogen peroxide. Aliquots were centrifuged at 10,000 rpm for 10 min. Absorbance of supernatant was recorded at 415 nm in spectrophotometer. The residual H₂O₂ content in samples were computed with the help of standard curve.

6.3.2.2.4 Assay of Glutathione Reductase (GR):

The glutathione reductase was assayed by recording the increase in absorbance in the presence of oxidized glutathione and 5, 5-dithio bis-2-nitrobenzoic acid (DTNB) (Smith et al., 1988). The 3 ml reaction mixture contained 200 µM phosphate buffer (pH 7.5) containing 1 µM EDTA,
1.5 µM DTNB, 0.2 µM NADPH, 0.1 ml enzyme extracts and distilled water. The reaction was initiated by adding 0.2 µM oxidized glutathione (GSSG). The increase in absorbance at 412 nm was recorded at 25°C over a period of 5 min spectrophotometrically. The activity was expressed as total absorbance (ΔA412) per min per mg protein.

6.3.2.2.4 Estimation of Glutathion (GSH):

Reduced glutathione (GSH) content was estimated as described by Dekok and Kuiper (1986). The samples (100 mg/ml) were homogenized in ice-cold 0.1 M KCl containing 30 mM EDTA. The homogenate (2 ml) was deproteinized by the addition of 3 ml of a solution containing 0.3 g/ml NaCl, 0.0167 g/ml metaphosphoric acid and 0.002 g/ml EDTA. After centrifugation at 10,000 rpm for 20 min at 4°C, 0.5 ml of the diluted supernatant was taken in 3 ml of reaction mixture containing 0.3 M Na₂HPO₄ and 0.04% of DTNB in 10% sodium citrate. The content of glutathione present in samples was calculated by standard calibrated curve of glutathione at 412 nm.

6.3.2.2.5 Estimation of Lipid peroxidation:

The level of lipid peroxidation was measured in terms of Malondialdehyde (MDA) content, a product of lipid peroxidation following the method of Heath and Packer (1968). A small piece of liver 100 mg/ml were homogenized in 0.1% trichloroacetic acid and the homogenate was centrifuged at 15,000 g for 10 min. The aliquot of supernatant was mixed with 0.5% thiobarbutyric acid in the ratio of 1:2. The mixture was heated at
95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using the absorption coefficient of 155 mMol^{-1} cm^{-1} and expressed as nMol (MDA) g^{-1}.

6.3.2.3 Histopathological Studies:

Processing of isolated liver:

The animals were sacrificed and the liver of each animal was isolated and was cut into small pieces, preserved and fixed in 10% formalin for two days. Then the liver piece was washed in running water for about 12 h to remove the formalin and was followed by dehydration with isopropyl alcohol of increasing strength (70%, 80% and 90%) for 12 h each. Then finally dehydration is done using absolute alcohol with about three changes for 12 h each.

Dehydration was performed to remove all traces of water:

Further, alcohol was removed by using chloroform and removed by paraffin infiltration. The clearing was done by using chloroform with two changes for 15 to 20 min each. After paraffin infiltration the liver pieces were subjected to automatic tissue processing.

Embedded paraffin vacuum: Hard paraffin was melted and then hot paraffin was poured into L-shaped blocks. The liver pieces were then dropped into the molten paraffin quickly and allow cooling.
Sectioning:

The blocks were cut using microtome to get sections of thickness of 5 µ. The sections were taken into a micro slide on which egg albumin i.e., sticking substance was applied. The sections were allowed to remain in an oven at 60°C for 1 h. Paraffin melts and egg albumin denatures, thereby fixing tissue to slide.

Staining:

Eosin is an acid stain, hence it stains all the cell constituents pink which are basic in nature i.e., cytoplasm. Hematoxylin is a basic stain which stains all the acidic cell components blue i.e., DNA in the nucleus.

The slides were observed under a light microscope for any histological damage or protection.

Statistical analysis

The data of all measurements are means from three replications. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey-Kramers multiple comparison tests.

6.4 RESULTS AND DISCUSSION:

The following results on effect of ethanolic extract of seed coat and cotyledon of *C. cajan* on acute toxicity and hepatoprotective activity in Albino mice. The results thus obtained are discussed in the light of the available literature.
6.4.1 Acute toxicity study:

In the present study it was observed that the ethanolic extract of seed coat treated animals at 50, 500, 1000 mg/kg b.w. (Group - II to IV) was normal up to 48 h of treatment. However, two animals are killed out of the four from the Group-IV (1000 mg/kg) after 48 h of treatment. Therefore, the lethal dose (LD 50) of the ethanolic extract of seed coat in mice was 1000 mg/kg b.w. Whereas, the ethanolic extract of cotyledon treated mice at the dose of 50, 500, 1000 mg/kg b.w. (Group- V to VI) were normal and healthy even seven days after treatment. Thus, the cotyledon extract of *C. cajan* is safer up to at the dose of 1000 mg/kg b.w. (Table-6.1).

6.4.2 Hepatoprotective activity:

The effect of ethanolic extract of seed coat and cotyledon on CCl₄ induced hepatotoxic Albino mice were studied on biochemical, *in vivo* antioxidants and histopatholgical parameters are as follows.

6.4.2.1 Biochemical parameters:

Effect of ethanolic extracts of seed coat and cotyledon of *C. cajan* on serum marker enzymes, total proteins and bilirubins level in CCl₄ induced hepatotoxic mice and their results are presented in Table - 6.2 - 6.4.

6.4.2.1.1 Effect on serum marker enzymes:

From the result it was observed that administration of CCl₄ led to significant increase in serum enzyme activities of SGPT, SGOT and SALP (54.32±0.4472, 44.70±0.178 and 143.4±0.0268 units/ml) in comparison with the normal control group (Table-6.2).
Table 6.1: Acute toxicity study of ethanol extract of Seed coat and Cotyledon of *C. cajan* in Albino mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group of animals</th>
<th>Dose of the drug (kg/b.w)</th>
<th>Number of animals in each group</th>
<th>Mortality of death of animals (72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gum acacia (Negative control)</td>
<td>I</td>
<td>-</td>
<td>4</td>
<td>Nil</td>
</tr>
<tr>
<td>Ethanolic extract of seed coat (EESC)</td>
<td>II</td>
<td>50</td>
<td>4</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>500</td>
<td>4</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1000</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ethanolic extract of cotyledon (EEC)</td>
<td>V</td>
<td>50</td>
<td>4</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>500</td>
<td>4</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>1000</td>
<td>4</td>
<td>Nil</td>
</tr>
</tbody>
</table>
Table – 6.2: Effect of ethanolic extract of seed coat and cotyledon of *C. cajan* on serum enzymes levels in CCl₄ induced hepatotoxic Albino mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>23.35±0.0447</td>
<td>34.65±0.0447</td>
<td>85.7±0.0408</td>
</tr>
<tr>
<td>B</td>
<td>Toxicant</td>
<td>44.70±0.178</td>
<td>54.32±0.4472</td>
<td>143.4±0.0268</td>
</tr>
<tr>
<td>C</td>
<td>Standard</td>
<td>29.70±0.044***</td>
<td>37.23±0.0268</td>
<td>102.3±0.0178***</td>
</tr>
<tr>
<td></td>
<td>Liv-52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>EESC (100mg)</td>
<td>36.34±0.0178***</td>
<td>46.61±0.01506***</td>
<td>99.56±0.0447***</td>
</tr>
<tr>
<td>E</td>
<td>EESC (500mg)</td>
<td>21.26±0.0268***</td>
<td>38.50±0.4082***</td>
<td>63.0±0.0447***</td>
</tr>
<tr>
<td>F</td>
<td>EEC (100mg)</td>
<td>32.42±0.0178***</td>
<td>42.01±4.899***</td>
<td>85.40±0.0447***</td>
</tr>
<tr>
<td>G</td>
<td>EEC (500mg)</td>
<td>25.01±1.366***</td>
<td>40.00±3.578***</td>
<td>74.70±0.0447***</td>
</tr>
</tbody>
</table>

- EESC - Ethanolic extract of seed coat, EEC - Ethanolic extract of cotyledon
- The values are mean±SD (Standard deviation), n=6mice/group one way ANOVA followed by Tukey-kramer’s test. Where * represents significant at P<0.05, ** represents significant at P<0.01, *** represents significant at P<0.001 mice treated with CCl₄ alone and NS (non significant).
Treatment of mice with the ethanolic extract of seed coat (EESC) at a dose 100 mg/kg b.w and 500 mg/kg b.w exhibited a significant reduction (P<0.01 and P<0.05) in CCl₄ induced elevation of serum enzymes SGOT, SGPT and SALP (36.34±0.0178 to 21.26±0.0268, 46.61±0.01506 to 38.50±0.04082, and 99.56±0.0447 to 63.0±0.0447 units/ml). Whereas, treatment of mice with the ethanolic extract of cotyledon (EEC) at a dose 100 mg/kg b.w and 500 mg/kg b.w. exhibited a moderate reduction in CCl₄ induced elevation of serum enzymes SGPT, SGOT, and SALP (42.01±4.899 to 40.00±3.578, 32.42±0.0178 to 25.01±1.366 and 85.40±0.0447 to 74.70±0.0447 units/ml). Treatment with Liv-52 also significantly reversed the hepatotoxicity. However, the extract produced hepatoprotective activity in a dose dependent manner. Administration of ethanolic seed coat extracts at higher dose (500 mg/kg b.w.) the drug effectively prevented CCl₄ induced reduction in elevated serum enzymes level. SGPT in serum increases due to leakage of this cellular enzyme into plasma by toxicants induced hepatic injury (Chenoweth and Hake, 1962). Serum levels of SGPT can increase due to damage of the tissues producing acute hepatic necrosis, such as viral hepatitis and acute cholestasis. In the current study treatment of mice seed coat extract significantly decreased the levels of SGPT in serum which is an indication of hepatoprotective activity. SGOT is a mitochondrial enzyme released from heart, liver, skeletal muscle kidney. Liver toxicity elevated the SGOT levels in serum due to the damage to the tissues producing acute necrosis, such as severe viral hepatitis and acute cholestasis. In the current study treatment of animals with seed coat extract significantly
decreased the levels of SGOT in serum which is an indicative of hepatoprotective activity compared to cotyledon extract. In case of toxic liver, alkaline phosphatase levels are very high, which may be due to defective hepatic excretion or by increased production of ALP by hepatic parenchymal or duct cells (Harsh Mohan, 2002). In the current study treatment of animals with seed coat and cotyledon extract significantly decreased the levels of ALP in serum which is an indicative of hepatoprotective.

6.4.2.1.2 Effect on Serum Bilirubin and Proteins:

The administration of CCl₄ led to significant hepatocellular damage as evident from the increased in total bilirubins (0.95±0.0447 units/ml) and decreased the level of proteins (2.56±0.752 units/ml) in comparison with the normal control group. Treatment of mice with the ethanolic extract of seed coat (EESC) at a dose 100 mg/kg b.w and 500 mg/kg b.w exhibited a significant reduction (P<0.01 and P<0.05) in CCl₄ induced elevation of total bilirubins (0.472±0.1623 to 0.55±0.0178 units/ml) and increase of total proteins level (3.38±0.025 to 6.03±0.031 units/ml). Whereas, treatment of mice with the ethanolic extract of cotyledon (EEC) at a dose 100 mg/kg b.w and 500 mg/kg b.w exhibited a moderate reduction of total bilirubins (0.56±0.0447 to 0.66±0.447 units/ml) and increase of total proteins level (5.2±0.1265 to 8.41±0.025 units/ml). Treatment with Liv-52 also significantly reversed the hepatotoxicity. Administration of ethanolic seed coat and cotyledon extract at higher dose (500 mg/kg b.w) the drug effectively prevented CCl₄ induced reduction in total bilirubins and increase of total proteins level (Table-6.3).
Table - 6.3: Effect of ethanolic extract of seed coat and cotyledon of *C. cajan* on total bilirubins and proteins levels in CCl₄ induced hepatotoxic Albino mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total bilirubins (mg/dl)</th>
<th>Total proteins (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>0.490±0.0268</td>
<td>9.85±0.632</td>
</tr>
<tr>
<td>B</td>
<td>Toxicant CCl₄</td>
<td>0.95±0.0447</td>
<td>2.56±0.752</td>
</tr>
<tr>
<td>C</td>
<td>Standard Liv-52</td>
<td>0.63±0.0089***</td>
<td>8.34±0.008***</td>
</tr>
<tr>
<td>D</td>
<td>EESC (100mg)</td>
<td>0.472±0.1623***</td>
<td>3.38±0.025**</td>
</tr>
<tr>
<td>E</td>
<td>EESC (500mg)</td>
<td>0.55±0.0178***</td>
<td>6.03±0.031</td>
</tr>
<tr>
<td>F</td>
<td>EEC (100mg)</td>
<td>0.56±0.0447***</td>
<td>5.2±0.1265***</td>
</tr>
<tr>
<td>G</td>
<td>EEC (500mg)</td>
<td>0.66±0.447***</td>
<td>8.41±0.025***</td>
</tr>
</tbody>
</table>

- EESC-Ethanolic extract of seed coat, EEC-Ethanolic extract of cotyledon
- The values are mean±SD (Standard deviation), n=6 mice/group one way ANOVA followed by Tukey-kramer’s test. Where * represents significant at P<0.05, ** represents significant at P<0.01, *** represents significant at P<0.001 versus mice treated with CCl₄ alone and NS (non significant).
Bilirubin level rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum, in haemolysis and defects of hepatic uptake and conjugation of bilirubin pigment such as in Gilbert’s disease (Rahman and Sultana, 2007). In the current study treatment of animals with seed coat and cotyledon extract significantly decreased the level of total bilirubin in serum which is an indicative of hepatoprotective. Rajib Ashan (2009) and Siddhartha Singh et al. (2011) have observed the significant hepatoprotective activity of ethanolic extract of C. cajan leaves.

**6.4.2.2 In vivo Antioxidant activity of C. cajan:**

The effect of ethanolic extract of seed coat and cotyledon of C. cajan on SOD, CAT, POX, GR, GSH and Lipid peroxidation in Liver of CCl₄ induced hepatotoxic mice as shown in Table-6.4.

In the liver tissues standard Liv-52 at a dose of 10 mg/kg also showed significant antioxidant activity. The results elicited by the ethanolic extracts are comparable with that of the standard Liv-52.

The present study showed that administration of ethanolic extract of seed coat (EESC) at doses of 100 and 500 mg/kg b.w to CCl₄ intoxicated mice caused a significant increase in the level of CAT (35.7±0.0163 to 50.5±0.0376 U/protein mg), POX (3.147±0.0225 to 6.245±0.0357 U/protein mg), SOD (8.4±0.0347 to 10.3±0.0447 U/protein mg), GR (5.102±0.0447 to 8.024±0.0075 U/protein mg) and GSH (22.6±0.9663 to 28.3±0.0316 U/protein mg) and a significant decrease in the level of TBARS (150.7±0.0316 to 132.5±1.265 nmol/mg protein) in comparison with the CCl₄ treated control group (Table-6.4).
Table 6.4: Effect of ethanolic extract of seed coat and cotyledon of *C. cajan* on *in vivo* antioxidants in CCl₄ induced hepatotoxic Alino mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Catalase (U/mg proteins)</th>
<th>Peroxidase (U/mg proteins)</th>
<th>Superoxide dismutase (U/mg proteins)</th>
<th>Glutathione reductase (U/mg proteins)</th>
<th>GSH (mg/100mg proteins)</th>
<th>LPO (nmol/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>72.4± 0.0178</td>
<td>4.523± 0.0178</td>
<td>14.2± 0.0447</td>
<td>8.874± 0.0163</td>
<td>34.6± 1.2313</td>
<td>185.3± 1.897</td>
</tr>
<tr>
<td>B</td>
<td>Toxicant</td>
<td>14.3± 0.0447</td>
<td>2.814± 0.0286</td>
<td>5.6± 0.0447</td>
<td>2.132± 0.0075</td>
<td>18.3± 0.0316</td>
<td>383.7± 0.0376</td>
</tr>
<tr>
<td></td>
<td>CCl₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Standard</td>
<td>40.2± 0.0408***</td>
<td>5.131± 0.0089***</td>
<td>13.4± 0.0376***</td>
<td>8.125± 0.0163***</td>
<td>23.2± 0.0376***</td>
<td>190.6± 0.0126***</td>
</tr>
<tr>
<td></td>
<td>Liv-52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>EESC (100mg)</td>
<td>35.7± 0.0163***</td>
<td>3.147± 0.0225***</td>
<td>8.4± 0.0347***</td>
<td>5.123± 0.268***</td>
<td>22.6± 0.9663</td>
<td>150.7± 0.0316***</td>
</tr>
<tr>
<td>E</td>
<td>EESC (500mg)</td>
<td>50.5± 0.0376***</td>
<td>6.245± 0.0357***</td>
<td>10.3± 0.0447***</td>
<td>8.024± 0.0075***</td>
<td>28.3± 0.0316*</td>
<td>132.5± 1.265***</td>
</tr>
<tr>
<td>F</td>
<td>EEC (100mg)</td>
<td>29.3± 0.0178***</td>
<td>3.456± 0.0268***</td>
<td>6.8± 0.0178***</td>
<td>5.106± 0.0633***</td>
<td>15.2± 0.0316***</td>
<td>121.6± 0.0316**</td>
</tr>
<tr>
<td>G</td>
<td>EEC (500mg)</td>
<td>42.3± 0.0408***</td>
<td>5.102± 0.0447***</td>
<td>8.5± 0.0247***</td>
<td>8.116± 0.0225***</td>
<td>18.6± 0.0178***</td>
<td>141.7± 1.265***</td>
</tr>
</tbody>
</table>

- EESC-Ethanolic extract of seed coat, EEC-Ethanolic extract of cotyledon
- The values are mean±SD (Standard deviation), n=6 mice/group one way ANOVA followed by Tukey-kramer’s test. Where * represents significant at P<0.05, ** represents significant at P<0.01, *** represents significant at P<0.001 versus mice treated with CCl₄ alone and NS (non significant).
In the present study, administration ethanolic extract of cotyledon (EEC) at doses of 100 and 500 mg/kg b.w to CCl$_4$ intoxicated mice caused a significant increase in the level of SOD (6.8± 0.0178 to 8.5± 0.0247 U/protein mg), CAT (29.3±0.0178 to 42.3± 0.0408 U/protein mg), POX (3.456±0.0268 to 5.102±0.0447 U/protein mg), GR (5.106±0.0633 to 8.116±0.0225 U/protein mg), GSH (15.2±0.0316 to 18.6±0.0178 U/protein mg) and a significant decrease in the level of TBAR ($\text{S}$ (121.6±0.0316 to 141.7±1.265 nmol/mg protein ) in comparison with the CCl$_4$ treated control group (Table 6.4).

Lipid peroxidative degradation of the biomembrane is one of the principle causes of CCl$_4$ toxicity. This is evidenced by the elevation of TBARS and a decrease in the activity of free radical scavenging enzymes such as SOD, POX, CAT and GR in CCl$_4$ intoxicated animals. These findings are in agreement with other reports by Gunjan Biswas et al. (2011). The increase in the level of MDA in liver induced by CCl$_4$ suggests enhanced lipid peroxidation. This leads to tissue damage and the failure of antioxidant defence mechanisms preventing the formation of excessive free radicals. Treatment with the ethanolic extract of seed coat at 500 mg/kg b.w significantly reduced the elevated level of TBARS (thiobarbituric acid reactive substances) in CCl$_4$ intoxicated liver tissues. It was also observed that SOD, POX, CAT and GR depletion due to the CCl$_4$ challenge was reversed by the test extract. The treatment of extract increased the hepatocyte SOD, POX, CAT, GR activities and reduced lipid peroxidation, which could effectively prevent radical mediated loss of membrane integrity.
GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals including CCl₄. The significant impairment of hepatic GSH status associated with a substantial hepatocellular damage induced by CCl₄ suggested the determinant role of hepatic GSH in the development of CCl₄ toxicity (Takate et al., 2010)

6.4.2.3 Histopathological Studies of Albino mice Liver

**Normal control group:** Histopathological examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central veins (Plate - 6.1a & 6.2a).

**CCl₄ treated group:** All the zones show areas of haemorrhage, cell necrosis, inflammation (Plate - 6.1b & 6.2b), degenerative hepatocytes, vacuolization of cytoplasm and fatty degeneration were observed in CCl₄ intoxicated animals.

**Liv-52 + CCl₄ treated group:** The liver sections of the mice treated with Liv-52 followed by CCl₄ intoxication showed a sign of protection (Plate - 6.1c & 6.2c).

**EESC (100 mg/kg) + CCl₄:** Section studied shows liver parenchyma with partially effaced architecture, mild fatty degeneration, mild chronic inflammation and also shows mild focal rearrangement of cells (Plate - 6.1d).
Plate – 6.1: Histopathological changes in the Liver of mice caused by CCl₄ and preventive effect of ethanolic seed coat extract of C.cajan in different groups: a-Control; b- CCl₄; c- Liv-52; d- 100 mg/kg b.w.; e- 500 mg/kg b.w.

Note: EESC – Ethanol extract of seed coat
Note: EEC - Ethanolic extract of cotyledon

Plate – 6.2: Histopathological changes in the Liver of mice caused by CCl₄ and preventive effect of ethanolic cotyledon extract of C.cajan in different groups: a-Control; b- CCl₄; c-Liv-52; d- 100 mg/kg b.w.; e- 500 mg/kg b.w.
EESC (500 mg/kg) + CCl₄: The animal treated with extract exhibited significant liver protection against the toxicant as evident by the presence of normal hepatic cords, absence of necrosis and lesser fatty infiltration (Plate - 6.1e).

EEC (100 mg/kg) + CCl₄: Section shows partially effaced liver architecture. Moderate accumulation of fatty lobules and mild chronic inflammation also shows mild focal rearrangement of cells (Plate - 6.2e).

EEC (500 mg/kg) + CCl₄: Section shows normal liver architecture similar to control group. Absence of necrosis, vacuoles is a sign of protection (Plate - 6.2f).

The present studies revealed that the ethanolic extracts of seed coat and cotyledon of C. cajan protects CCl₄ induced hepatotoxicity in mice by lowering serum marker enzymes, bilirubin, significant enhance of protein and restoring the liver antioxidant status and histopathological examination of liver. The results obtained in this investigation lend support for the previous reports. Siddharth Singh et al. (2011) have reported the hepatoprotective activity of ethanolic extracts of C. cajan leaf (at 400 mg/kg b.w) against CCl₄ induced hepatic damage in rats shows that lowering serum marker enzyme, bilirubin. Further, Kasturi Sarkar et al. (2006) have characterized the 43kD hepatoprotective protein from the seeds of C. indicus against various hepatic toxins.