

ASSESSMENT OF HEPATOPROTECTIVE ACTIVITY OF CHLOROFORM AND ETHANOL EXTRACTS OF WHOLE PLANT OF *CUSCUTA REFLEXA* IN CCL₄ TREATED RATS AND EFFECTIVENESS OF EXTRACTS ON LIPOPROTEIN SECRETION BY HEPATIC CELLS

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Summary

The aim of this study was to evaluate the hepatoprotective activity and effect on lipoprotein of liver by chloroform and ethanol extracts of whole plant of *Cuscuta reflexa* in rats by inducing liver damage by carbon tetrachloride. Liver damage was studied by assessing parameters such as serum glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, acid phosphatase and bilirubin in serum. The chloroform and ethanol extract at an oral doses of 200 mg/kg and 400 mg/kg exhibited a significant ($P < 0.05$) protective effect by lowering serum levels of above parameter. Silymarin was used as a standard drug at a dose of 25 mg/kg. The total cholesterol, triglyceride levels, ALPO₄, Lactate dehydrogenase, high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein in serum were observed to be elevated in CCl₄ treated group but reduced by extract treatment significantly as well as silymarin showing their beneficial effects. These biochemical observations were supplemented by histopathological examination of liver sections. Furthermore, the acute toxicity of the extracts showed no signs of toxicity up to a dose level of 2000 mg/kg. The results suggest that the chloroform and ethanol extract of *C. reflexa* exhibits significant hepatoprotective properties.

Keywords *Cuscuta reflexa* Carbon tetrachloride, marker enzymes, silymarin

Introduction

The liver is the vital organ in human body, has a wide range of functions, including detoxification, protein synthesis, fight against disease, nutrient supply, and production of biochemical necessary for digestion. The liver is chief site for intense metabolism and excretion; hence it has a surprising role in the maintenance, performance and regulating homeostasis of the body[1]. The liver is expected not only to perform physiological functions but also to protect against the hazards of harmful drugs and chemicals[2]. Liver diseases are the most serious ailment and are mainly caused by toxic chemicals (excess consumption of alcohol, high doses of paracetamol, carbon tetrachloride, chemotherapeutic agents, peroxidised oil, etc). Jaundice and hepatitis are two major hepatic disorders that account for a high death rate[3].

In spite of the tremendous advances made in allopathic medicine, no effective hepatoprotective medicine is available. Plant drugs are known to play a vital role in the management of liver diseases. In India, more than 93 medicinal plants are used in different combinations in the preparations of 40 patented herbal formulations. However, only a small proportion of hepatoprotective plants as well as formulations used in traditional medicine are pharmacologically evaluated for their safety and efficacy[4,5]. Presently number of medicinal plants is used for treatment of liver disease by tribal people, but is still not scientifically evaluated.

Cuscuta reflexa (Cuscutaceae) known in Hindi as Amarbela, are phanerogamic stem parasites, which are distributed world-wide. They are rootless, leafless twining annual parasites with yellowish stems, distributed on tropical and temperate regions, and in India about 6 species are found. It grows on thorny or other shrubs, sometimes completely covering bushes and trees[6]. *C. reflexa* spread from one host to another, on each victim they twine and cling tightly with special branching organs called houstoria, penetrating the host and connecting to the host xylem as well as to the host phloem and absorbing from it both water and elaborated food stuffs such as sugars and amino acids[7]. Various parts of this plant are used in tribal medicine for the disease like impotence, premature ejaculation, sperm leakage, frequent urination, ringing in the ears, lower back pain, sore knees, leucorrhea, dry eyes, blurred vision, and tired eyes. *Cuscuta* is one of nine herbs included in the manufacture of Equiguard, a Chinese herbal medicine recommended for kidney and prostate disorders. Research performed at New York Medical College indicates that the combination of ingredients in Equiguard may well be effective in the treatment of prostate cancer. The preparation inhibited the growth of cancer cells, increased the rate of self-destruction (apoptosis) of cancer cells, and prevented the surviving cells from forming colonies[8-10]. Phytochemical investigated on *Cuscuta reflexa* have reported the presence of kaempferol-3-O-glucoside, astragallic acid[11], myricetin, benzopyrones[12], glucopyranosides[13], propenamide, flavonols[14], quercetin and quercetin-3-O-glucoside, β -sitosterol, and bergenin[15]. The main objectives of this study were to assess the hepatoprotective potential of chloroform and ethanol extracts of *Cuscuta reflexa* and effectiveness on various biochemical parameters viz total cholesterol, triglycerides (TGL), high density lipoprotein, (HDL), low density lipoprotein, (LDL), very low density lipoprotein, (VLDL), ALP₀₄ and Lactate dehydrogenase (LDH).

Material and Methods

Plant materials: Whole plants were collected from PNT square, Jawahar Chowk, Bhopal (M.P.) India, during the months of January and February 2007. The species was identified by the local people during the time of collection and later on authentication was made by Dr. Pradeep Tiwari, Professor, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.), India. Vouchered herbarium specimen was prepared and preserved along with crude drug sample at the herbarium (BOT/412/123) of Department of Botany, Dr. H.S. Gour University, Sagar (M.P.), India. Whole plant was shade dried, reduced to coarse powder and stored in airtight container till further use.

Preparation of extract: 1 Kg of powdered drug was packed in soxhlet apparatus and extracted with petroleum ether (60-80°C) to defat the drug. Defatted powdered drug was then extracted with chloroform. The chloroform extract was separated and the marc was further extracted with ethanol (95%). The solvents were removed by distillation and the last traces of solvent being

removed under reduced pressure. Suspension of chloroform and ethanol extract was prepared and was divided in two doses 200 mg/kg and 400 mg/kg and subjected for hepatoprotective activity.

Experimental animals: Male wistar albino rats having weight 180-230gm were kept in quarantine for 10 days under standard husbandry conditions (27.3°C, Relative humidity 65 ±10%) for 12 hrs in dark and light cycle respectively and were given standard food and water *ad libitum*. The project proposal was approved by the Institutional Animal Ethical Committee (ref. no. IAEC/A/500/2001).

Acute oral toxicity study: Acute oral toxicity was performed by following OECD guideline – 420 fixed dose procedure for chloroform and ethanol extract and it was found that dose increasing up to 2000 mg/kg body wt. shown no toxicity or mortality in experimental rats. The LD₅₀ of the chloroform and ethanol extract as per OECD guidelines – 420 is greater than 2000 mg/kg[16,17].

Hepatoprotective activity: Assessment of hepatoprotective activity was carried out on wistar albino rats. The animals were segregated into seven groups of six animals each. Groups II to VII received CCl₄ (1ml /kg i.p.) with equal volume of olive oil (50% v/v) to induce acute toxicity, for two successive days. Group II animals were maintained as CCl₄ group, while Groups III & IV animals were treated orally for seven days with suspension of chloroform extract at the dose of 200 mg/kg and 400 mg/kg respectively. Groups V & VI animals were treated orally for seven days with suspension of ethanol extract at the dose of 200 mg/kg and 400 mg/kg respectively. Group VII animals were treated with standard drug silymarin (25 mg/kg). After the drug treatment all the animals were sacrificed by cervical dislocation. Blood was collected from the carotid artery and was allowed to clot for 45 min at room temperature; serum was separated by centrifugation at 2500 rpm for 15 min, used for the estimation of various biochemical parameters.

Serum analysis: Serum separated by centrifugation were used to determine serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (ALP), serum acid phosphatase (ACP) and serum bilirubin[18-23].

Lipoprotein estimation: The lipoprotein parameters were determined after the animals were sacrificed by cervical dislocation. Total cholesterol, triglycerides (TGL), ALPO₄, Lactate dehydrogenase (LDH), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), by using auto-analyzer[24-26].

Histopathology: The liver from each animal was removed after dissection, liver lobes were fixed for 48 hours in 10% formalin and were embedded in paraffin. Subsequently, seven sections (T.S.) were cut on a microtome and stained with haematoxylin and eosin. These sections were observed under microscope for histopathological changes and finally, compared with that of normal system[27].

Statistical analysis: Results of estimation of biochemical and functional parameters have been reported as mean value ± SEM. The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA). Individual comparisons of group mean values were done using Dunnett's test (Sigma stat 3.5). P values <0.05 were considered statistically significant.

Results

The result of acute toxicity study of chloroform and ethanol extracts of *C. reflexa* on laboratory animals showed that the animals were safe up to a maximum dose of 2000 mg/kg body weight. Hepatoprotective activity of chloroform and ethanol extracts at the dose of 200 mg/kg and 400 mg/kg body weight were determined in CCl₄ induced hepatotoxicity model. The rats treated with CCl₄, developed significantly liver damage, were observed from the alteration in the activities of serum enzyme (SGOT, SGPT, ALP and ACP), total bilirubin and direct bilirubin in serum (Table 1). The level of values of the SGOT, SGPT, ALP, ACP, total bilirubin and direct bilirubin were significantly increased in the CCl₄ treated rats as compared with the normal control group (P<0.05).

Treatment with chloroform and ethanol extract of *C. reflexa* at the different doses (200 mg/kg and 400 mg/kg) and also silymarin decreased the activity of serum transaminase, ALP, ACP and bilirubin in CCl₄ induced liver damaged rats compared to that of CCl₄ treated groups (P<0.05). It was found that the test samples offer protection against toxin as evidenced by remarkable reduction in all serum enzyme (P<0.05). It also revealed that decreased serum enzyme levels of both extracts are less compared to standard drug.

Abnormalities in lipoproteins are very common in liver hepatotoxicity. Although lipoprotein alterations appear to be an intrinsic part of these disorders, such alterations are also induced by liver injury. The total cholesterol, triglyceride levels, VLDL, LDL, HDL, ALPO₄ and LDH were observed to be elevated in CCl₄ treated group but reduced by extract treatment significantly as well as silymarin showing their beneficial effects (Table 2). These results suggest the beneficial effects of the natural extract in improving the imbalance in lipoprotein metabolism are also comparable to those of silymarin.

Histopathological studies provided supportive evidence to the biochemical analysis. The histopathological study from liver of normal group (Fig. a) showed a normal hepatic structure. Under CCl₄ conditions (Fig. b), it can be observed that the administration of this noxious agent produces focal necrosis, portal infiltration, fatty changes, Kupfer's cells hyperplasia and hydropic changes. After the administration of 200 mg/kg and 400 mg/kg of the chloroform extract (Fig. c & Fig. d), it was observed that necrosis and fatty change were prevented, a situation that was also observed after the administration of an oral dose of 200 mg/kg and 400 mg/kg of ethanol extract (Fig. e & Fig. f). The toxin mediated histological changes in the liver section of rats of test groups were much less intensity than those observed in the rats of CCl₄ treated group. Under silymarin conditions (Fig. g), it is observed that ballooning degeneration and fatty changes associated to hepatocyte necrosis, were prevented.

Table 1: Effect of different extracts of *C. reflexa* on SGOT, SGPT, ALP, ACP, total bilirubin and direct bilirubin in CCl₄ induced liver toxicity in rats

Treatment	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	ACP (U/L)	Bilirubin (mg/100 ml of blood)	
					Direct (mg/dl)	Total (mg/dl)
Control	64.25	114.6	179.1	144.3	0.27	0.55
	± 6.30	± 6.12	± 11.0	± 6.17	± 0.04	± 0.08
CCl ₄ (1ml/kg i.p)	202.2 ^a	319.7 ^a	385.0 ^a	266.7 ^a	1.98 ^a	2.85 ^a
	± 5.59	± 7.9	± 8.89	± 6.96	± 0.17	± 0.36
CHCl ₃ extarct (200 mg/kg)	142.1 ^b	257.8 ^b	324.3 ^b	228.1 ^b	1.15 ^b	2.04 ^b
	± 4.39	± 4.15	± 8.52	± 5.23	± 0.08	± 0.05
CHCl ₃ extarct (400 mg/kg)	99.67 ^b	162.2 ^b	216.3 ^b	163.4 ^b	0.59 ^b	0.99 ^b
	± 5.15	± 8.04	± 4.62	± 5.74	± 0.07	± 0.05
Ethanol extract (200 mg/kg oral)	125.4 ^b	220.3 ^b	302.1 ^b	206.4 ^b	0.90 ^b	1.58 ^b
	± 5.84	± 10.94	± 7.74	± 9.35	± 0.10	± 0.31
Ethanol extract (400 mg/kg oral)	72.59 ^b	192.2 ^b	186.1 ^b	138.6 ^b	0.25 ^b	0.50 ^b
	± 7.84	± 6.14	± 6.09	± 7.07	± 0.04	± 0.07
Silymarin (25 mg/kg oral)	67.43 ^b	107.0 ^b	172.1 ^b	140.2 ^b	0.23 ^b	0.52 ^b
	± 8.25	± 5.42	± 6.93	± 7.67	± 0.12	± 0.03

Values are expressed as mean ± SEM, n = 6 in each group. ^aP<0.05 when compared with normal control group, ^bP<0.05 when compared with CCl₄ treated group.

Table 2: Effect of different extracts of *C. reflexa* on total cholesterol, triglyceride levels, HDL, LDL, VLDL, ALPO₄ and LDH in CCl₄ induced liver toxicity in rats

Treatment	Cholesterol	Triglycerides	HDL	LDL	VLDL	ALPO ₄	LDH
Normal Control	62.33	109	18.48	23.67	21.57	571.9	975.2
10ml/kg	± 4.80	± 4.36	± 1.94	± 1.16	± 1.06	± 4.97	± 5.44
CCl ₄ 1ml/kg i.p	122.5 ^a	163.8 ^a	26.06 ^a	51.51 ^a	33.54 ^a	1185 ^a	1223 ^a
	± 5.12	± 6.87	± 1.99	± 2.25	± 1.97	± 8.90	± 11.12
CHCl ₃ extract (200 mg/kg)	97.61 ^b	152.8	25.29	47.3	33.11	1010 ^b	1106 ^b
	± 3.46	± 3.29	± 0.45	± 1.17	± 2.28	± 8.10	± 5.08
CHCl ₃ extract (400 mg/kg)	78.85 ^b	143.4 ^b	22.58 ^b	40.96	27.67 ^b	906.6 ^b	1027 ^b
	± 4.72	± 3.75	± 1.11	± 1.36	± 1.43	± 8.02	± 8.91
Ethanol extract (200 mg/kg oral)	89.17 ^b	145.4 ^b	23.46 ^b	39.69 ^b	26.13 ^b	903.9 ^b	1039 ^b
	± 3.48	± 3.20	± 0.86	± 1.12	± 0.75	± 5.36	± 5.52
Ethanol extract (400 mg/kg oral)	67.55 ^b	118.3 ^b	20.14 ^b	22.07 ^b	22.06 ^b	738 ^b	865.5 ^b
	± 4.00	± 4.10	± 0.45	± 0.64	± 0.97	± 9.48	± 7.31
Silymarin (25 mg/ kg oral)	60.24 ^b	107 ^b	21.63 ^b	24.33 ^b	20.95 ^b	632.6 ^b	781.8 ^b
	± 3.45	± 6.70	± 0.56	± 0.93	± 1.13	± 5.31	± 8.15

Values are expressed as mean ± SEM, n = 6 in each group. ^aP<0.05 when compared with normal control group, ^bP<0.05 when compared with CCl₄ treated group

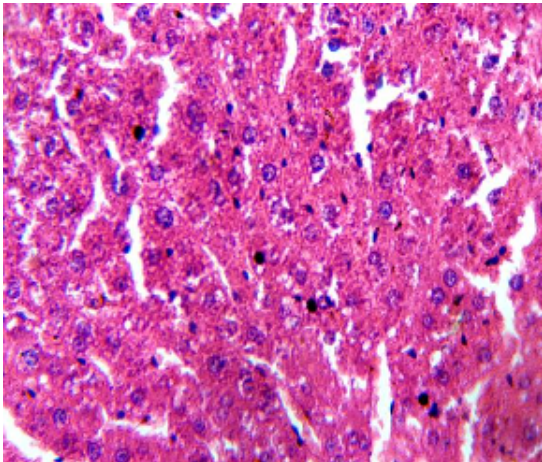


Fig. 4.16a- Liver tissue of control rats showing normal histology

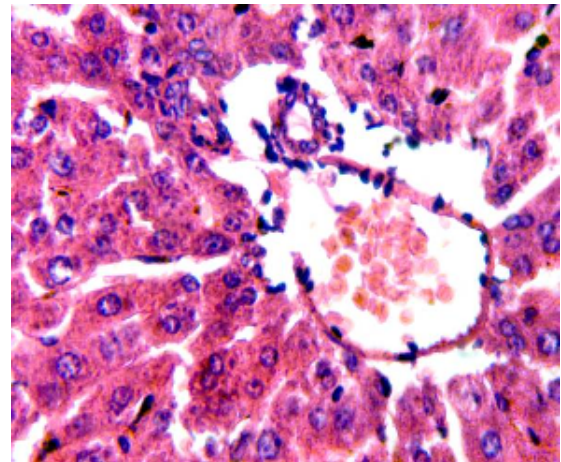


Fig. 4.16b - Liver tissue of CCl₄ treated rats showing necrosis of the hepatic cells

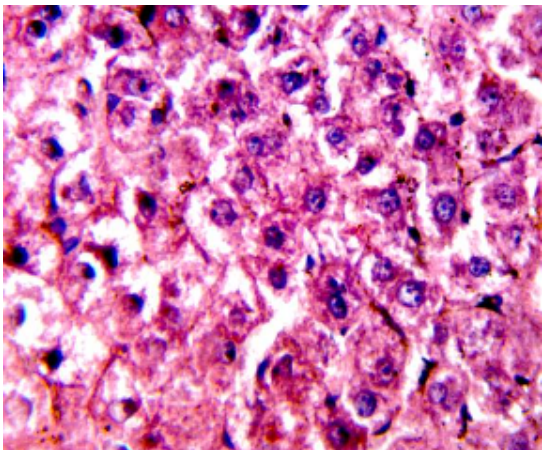


Fig. 4.16c- Liver tissue of CCl₄+ *C. reflexa* chloroform extract (200 mg/kg) treated rats showing necrosis of the hepatic is mild prevented

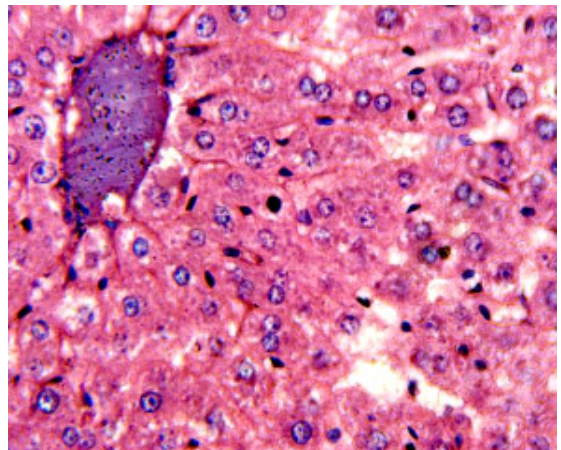


Fig. 4.16d- Liver tissue of CCl₄ + *C. reflexa* chloroform extract (400 mg/kg) treated rats showing necrosis of the hepatic cells almost prevented

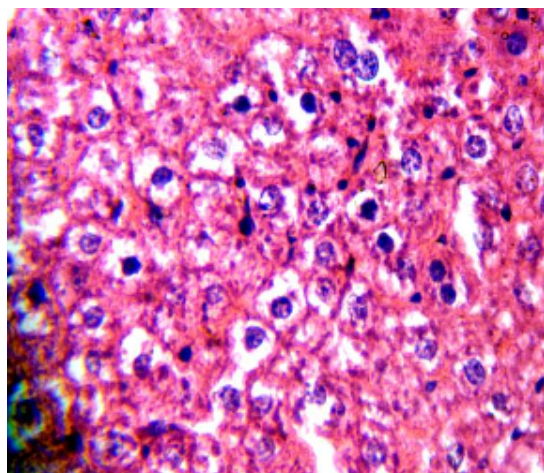


Fig. 4.16e- Liver tissue of CCl₄+ *C. reflexa* ethanol extract (200 mg/kg) treated rats showing necrosis of the hepatic is mild prevented

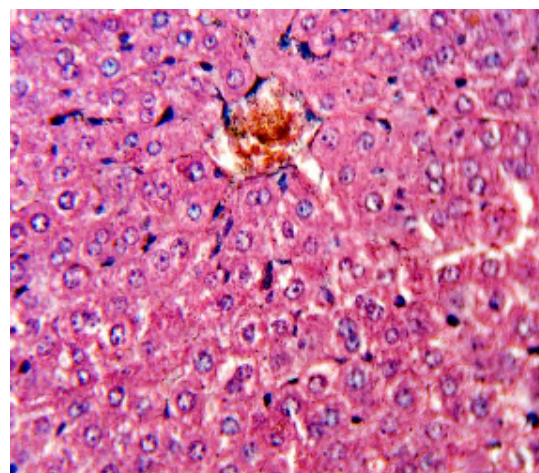


Fig. 4.16f- Liver tissue of CCl₄ + *C. reflexa* ethanol extract (400 mg/kg) treated rats showing necrosis of the hepatic cells almost prevented

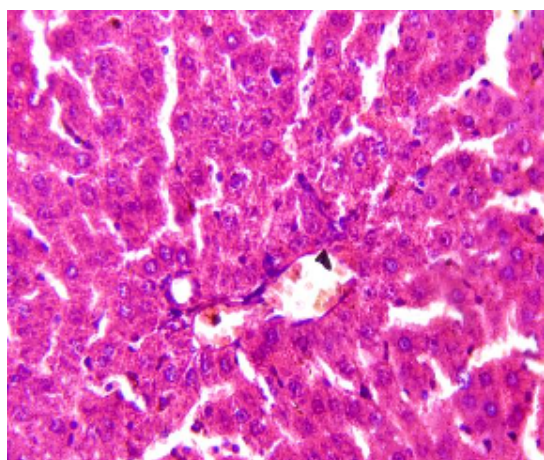


Fig. 4.16g- Liver section from CCl₄ + silymarin (25 mg/kg) treated rats showing ballooning degeneration and necrosis is markedly prevented

Discussion

In the assessment of liver damage by CCl₄ hepatotoxin, the determination of enzyme level such as SGPT and SGOT are largely used. Necrosis or membrane damage release the enzyme in to circulation, therefore it can be measured in serum. Higher level of SGOT indicates the liver damage, due to active metabolite trichloromethyl free radical produced from carbon tetrachloride

during metabolism by hepatic microsomes which in turn cause peroxidation of lipid of cellular membrane[28]. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in similar manner. Therefore SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury. Our results using the model of CCl₄-induced hepatotoxicity in the rats demonstrated that *C. reflexa* at different doses of chloroform and ethanol extracts caused significant inhibition of SGPT and SGOT levels. Serum ALP, ACP and bilirubin levels on other hand related to the function of hepatic cells. Increase in serum level of ALP ACP and bilirubin is due to increased synthesis, in presence of increasing biliary pressure. Our results using the model of CCl₄-induced hepatotoxicity in rats demonstrated that *C. reflexa* at different doses both extracts caused significant inhibition of ALP, ACP and bilirubin levels. Effective control of bilirubin level and alkaline phosphatase activity point towards on early improvement in the secretory mechanism of the hepatic cell.

All the tissues of body require cholesterol and triglycerides for their proper functions and liver play major role on producing these lipoprotein. Generally, for about 8 hours after a meal, liver takes up dietary cholesterol and triglycerides from the bloodstream. During times when dietary lipids are not available, liver produces cholesterol and triglycerides itself. About 75% of the cholesterol in your body is manufactured by the liver. Liver then places the cholesterol and triglycerides, along with special proteins, into tiny sphere-shaped packages called lipoproteins such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), which are released into the circulation. Cholesterol and triglycerides are removed from the lipoproteins and incorporated into body's cells, wherever they are needed. Liver diseases cause changes in blood lipoprotein levels. The significant increase in cholesterol, triglycerides, VLDL, LDL and HDL noted in serum might have been due to hepatocellular damage causes biochemical changes which affect transport of lipoprotein out of the liver[29].

Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate with simultaneously interconversion of NADH and NAD⁺ in liver. LDH converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply and it performs the reverse reaction during the Cori cycle in the liver. At high concentrations of lactate, the enzyme exhibits feedback inhibition and the rate of conversion of pyruvate to lactate is decreased. Here increase in LDH level in serum indicates that the liver damage[30]. The significant preservation of all of the above biochemical parameters towards normal values after administration of both extract at different doses indicates the protection of vital organs from damage induced by necrosis of hepatic cells.

Histopathological studies showed that CCl₄ caused focal necrosis, portal infiltration, fatty changes, Kupfer's cells hyperplasia and hydropic changes of the liver tissue. After administration of chloroform and ethanol extracts exhibited protection, this confirmed the results of biochemical studies. All the effects of extracts were comparable with those of silymarin, a proven hepatoprotective.

Hence, the results of serum enzyme prove the potent hepatoprotective activity and significant restoration of lipoprotein parameters towards normal confirms the protection action of chloroform and ethanol extracts of *C. reflexa* on liver against CCl₄ induced hepatotoxicity.

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