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In vitro and *in vivo* hepatoprotective activity of *Cissampelos pareira* against carbon-tetrachloride induced hepatic damage

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Administration of hydroalcoholic extract of *Cissampelos pareira* roots (CPRE) and standard drug silymarin in rats showed significant hepatoprotective action against CCl_4 induced hepatotoxicity. Elevated serum marker enzymes of AST, ALT, ALP and serum bilirubin were significantly reduced to near normal level in CPRE treated rats. Lipid peroxidation level was decreased significantly in CPRE 100, 200, 400 mg/kg doses treatment groups. In case of antioxidant enzymes SOD, catalase levels were increased significantly after CPRE 200, 400 mg/kg doses, similarly it increased the enzyme levels of GST, GPx, and GSH. CPRE 200, 400 mg/kg decreased cholesterol level, and increased triglyceride level. *In vitro* hepatoprotective activity of the extract was evaluated at 20, 40, 60, 80 and 100 µg/ml concentration against CCl_4 (1%) induced toxicity in freshly isolated rat hepatocytes. HepG2 cells showed significant dose dependent increase in percentage viability at the doses 20, 40, 60, 80 and 100 µg/ml of CPRE compared to CCl_4 exposed HepG2 cells. Results of this study strongly demonstrate *Cissampelos pariera* having good hepatoprotective potential.

Keywords: Antioxidant enzymes, Carbon tetrachloride, *Cissampelos pareira,* Hepatoprotective, HepG2 cells

Liver diseases are posing as a major health problem around the world. Hepatitis viral infection, toxic industrial chemicals, alcohol, aflatoxins, water pollutants are the major risk factors of liver diseases[1]. Plant based herbal medicines are viable alternative to synthetic drugs have been used since the dawn of civilization. *Cissampelos pareira* L. var. hirsuta (Menispermaceae) popularly known as Abuta in South America, and laghupatha in Ayurveda (Indian traditional system of medicine) found throughout tropical and subtropical regions of India, East Africa and the American tropics. This plant yields rich source of nutrient for breast milk secretion[2]. *C. Pariera* with other herbals as poly herbal formulation used for increasing milk yield in veterinary drugs[3]. Many marketed food supplement products in American continent contain *C. pariera* as one of its main ingredient. The roots of *C. pareira* also been used for treating tumors[4]. This plant has Cissampareine, a bis-benzyl-isoquinoline alkaloid that has inhibitory activity against human carcinoma cells of the naso-pharynx in cell culture[5]. Also it contains methiodide and methochloride derivatives of hayatin are the important compounds reported to be potent neuromuscular blocking agents and lower blood pressure[6]. The roots of *C. pareira* possess high concentration of bebeerine a pharmacologically active alkaloid[7]. Moreover it is being used by indigenous people of South America for the treatment of jaundice[8]. Based on its diversified pharmacological properties and its uses in liver diseases, the objective of the present study was to evaluate the hepatoprotective activity of *Cissampelos pareira* against carbon tetrachloride (CCl_4) toxicity.

Materials and Methods

Chemicals—All chemicals used were of analytical grade and procured from Sigma Chemicals Co., USA and Qualigens Fine Chemicals, Mumbai, India. Human hepatoma cell line (HepG2) was obtained from National Center for Cell Science (NCCS), Pune, India.

Plant collection and extract preparation—*Cissampelos pareira* plant was collected from the botanical garden of National Botanical Research Institute, Lucknow, India in the month of March 2010. The plant material was identified and authenticated by a taxonomist Dr A K S Rawat of the

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institute, and voucher specimen of the collected sample was deposited in the departmental herbarium (NAB 68004A) for future reference. The roots were removed and washed with double distilled water to eliminate dirt, then shade dried. The dried materials were powdered and passed through a 10-mesh sieve. The coarsely powdered material (1 kg) was extracted with petroleum ether thrice to remove the fatty material and further marc was extracted thrice with 50 % aqueous ethanol. The extracts were filtered, pooled and concentrated at 5°C on a rotary evaporator (Buchi, USA) and then freeze dried (Freezone® 4.5, Labconco, USA) at high vacuum (133×10^{-3} m Bar) at 47° ± 2°C, and yield of *C. pareira* root extract (CPRE) was 9.6% w/w. For the pharmacological tests CPRE was suspended in double distilled water containing carboxymethyl cellulose 1% w/v. For *in vitro* hepatoprotective studies CPRE was dissolved in DMSO and the volume was made up to 10 ml with Ham's F12/MEM. Further dilutions were made to obtain different concentrations and were used for *in vitro* investigations.

Experimental animals—Male Sprague-Dawley rats weighing 140-160 g were procured from the Central Drug Research Institute, Lucknow, and kept in conventional cages with free access to water *ad libitum* and standard rat feed (Dayal, India) at 27° ± 2°C, humidity 44-56%, light and dark cycles of 10 and 14 h, respectively. All the experiments were performed in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 222/2000/CPCSEA). The standard orogastric cannula was used for oral drug administration.

Experimental design—Animals were divided into six groups of six rats each. Group 1 received a single daily oral dose of CMC (1 ml of 1%, w/v) for 14 days. Group 2 received carbon tetrachloride (1.0 ml/kg body weight, i.p., 1:1 v/v mixture of CCl_4 and olive oil) once in every 72 h for 14 days. Groups 3, 4 and 5 received twice daily oral dose of 100, 200 and 400 mg/kg body weight of CPRE in 1%, w/v CMC respectively along with CCl_4. Group 6 were received daily oral dose of silymarin (25 mg/kg) along with CCl_4. In this study the role of silymarin was used as a positive control, as well as the hepatoprotective potential of different doses of CPRE was compared with the effect of silymarin. Rats were sacrificed 48 h after the last dose of the drug. Blood was collected by cardiac puncture for biochemical analysis[9].

The liver tissue was dissected out for histological investigation and fixed in 10% formalin, dehydrated in gradual ethanol (50-100%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin (H-E) dye for photomicroscopic observation, including cell necrosis, fatty change, hyaline regeneration, ballooning degeneration. All samples were observed and photographed with SZX12 Olympus microscope.

Determination of serum biochemical parameters—The collected blood was allowed to clot and centrifuged at 12000 rpm for 15 min to obtain the serum. The biochemical parameters like serum enzymes glutamate-pyruvate transaminase (ALT), glutamate oxaloacetate transaminase (AST)[10], serum alkaline phosphatase (ALP)[11], triglyceride (TG)[12], total cholesterol (TC)[13] and total bilirubin[14] were estimated using respected assay kits according to the methods described by the manufacturers.

Estimation of lipid peroxidation and antioxidant enzymes—The dissected livers were washed with 0.9% saline and homogenated (5%) in ice-cold phosphate buffer, and then centrifuged at 1000 rpm for 10 min followed by centrifugation of the supernatant at 12000 rpm for 15 min to get the mitochondrial fractions. These fractions were used for the following estimations. Lipid peroxidation (LPO) was estimated by the standard method[15] and expressed as nmol of malondialdehyde (MDA) formed/min/mg protein. Superoxide dismutase (SOD) activity was assayed by the inhibition of nicotinamide adenine dinucleotide (reduced) phenazine methosulphate nitrobluetetrazolium reaction system as adapted method[16] and the results have been expressed as units (U) of SOD activity/mg protein. Catalase (CAT) was assayed by the method[17] and results are expressed as units (U) of CAT activity/mg protein. Reduced glutathione (GSH) was determined according to the method[18] and expressed as GSH formed nmol/mg protein/min. Activities of glutathione S-transferase (GST) were determined by the method[19] and expressed as 1, 2-dichloro-4-nitrobenzene nmol/mg protein/min. Glutathione peroxidase (GPx) activity was determined by the method as described earlier[20] and expressed as mmol/mg tissue.

In vitro hepatoprotective effect of CPRE on freshly isolated rat hepatocytes—Liver cells were isolated by

the procedure described elsewhere[21]. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, $10^{-6}M$ dexamethasone and 10^{-8} *M* bovine insulin. The cell suspension was incubated at 37°C for 30 min in a humidified incubator under 5% CO_2. After incubation of 24 h, the hepatocytes were exposed to the fresh medium containing CCl_4 (1%) along with/without various concentrations of CPRE (20, 40, 60, 80 and 100 µg/ml) or medium alone (as vehicle). After 60 min of CCl_4 intoxification, concentrations of ALT, AST, and ALP, TGL (triglycerides), total bilirubin and direct bilirubin in the medium were measured as an indication of hepatocytes necrosis using diagnostic kits[22]. Cell viability was determined by the Trypan blue dye exclusion method[23].

Hepatoprotective effect in HepG2 cell line—Human liver HepG2 cells were exposed to a medium containing CCl_4 (1%) along with/without various concentrations of the CPRE (20, 40, 60, 80 and 100 µg/ml). Then cytotoxicity was assessed by estimating the viability of HepG2 cells by MTT reduction assay[24].

Statistical analysis—All the data were presented as mean ± SEM. Statistical analyses were performed by one way ANOVA followed by Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant at the value of probability less than 5% ($P < 0.05$).

Results

Effects on serum enzymes—A significant increase was observed in AST, ALT, ALP and serum bilirubin levels after exposed to CCl_4. However administration of CPRE at different dose levels (100, 200 and 400 mg/kg) recovered significantly the increased liver marker enzyme levels, and alkaline phosphatase. It indicated the stabilization of plasma membranes and repair of hepatic tissue (Table 1).

Effects on lipid peroxidation, antioxidant enzymes—Treatment with CPRE 100, 200, 400 mg/kg, showed significant reduction in LPO level compared to elevated level in CCl_4 induced group. CPRE at 200 and 400 mg/kg dose levels increased significantly the liver antioxidant enzymes levels SOD and CAT in CCl_4 administered rats group in which SOD, CAT were significantly brought down by CCl_4. However GPx, GSH and GST marker enzymes were also decreased after CCl_4 exposure, but showed increase levels at 200, 400 mg/kg doses of CPRE (Table 2).

Effect on serum cholesterol and protein levels—The elevated serum levels in cholesterol and decreased level of triglycerides, protein were noticed after CCl_4 treatment. CPRE 200, 400 mg/kg dose significant decreased cholesterol level as compared to increased level in CCl_4 treated rats. On contrary the

Table 1—Effect of CPRE on serum transaminases and bilirubin.
[Values are mean ± SEM of 6 animals in each group]

Treatment	ALT(U/L)	AST(U/L)	ALP(U/L)	Bilirubin (U/L)
Control (CMC 1%w/v,1 ml)	49.96 ± 5.88	53.41 ± 4.48	94.75 ± 6.85	0.46 ± 0.04
CCl_4 (1 ml/kg)	122.85 ± 4.88†	110.48 ± 8.41†	224.01 ± 8.42†	1.05 ± 0.05†
CPRE (100 mg/kg)+ CCl_4	110.11 ± 4.37*	90.41 ± 4.44**	190.02 ± 8.77**	0.87 ± 0.03**
CPRE (200 mg/kg)+ CCl_4	77.28 ± 2.5***	81.16 ± 4.33***	125.31 ± 4.41***	0.69 ± 0.05***
CPRE (400 mg/kg)+ CCl_4	58.13 ± 3.84***	68.08 ± 2.16***	100.88 ± 6.06***	0.52 ± 0.02***
Silymarin (25 mg/kg)+ CCl_4	54.3 ± 1.43***	58.21 ± 2.65***	96.78 ± 4.89***	0.49 ± 0.05***

Significant at $^{\dagger}P < 0.001$ compared to control group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to CCl_4 group.

triglyceride, protein levels increased in 200, 400 mg/kg doses of CPRE as compared to decreased level in CCl_4 induced rats (Table 3).

Hepatoprotective effect of CPRE in freshly isolated rat hepatocytes and HepG2 cell line—A significant increase in the levels of ALT, AST, ALP, total bilirubin and direct bilirubin and a significant reduction in TGL level as compare to control was observed in CCl_4 exposed hepatocytes (Table 4). These cells, when treated with different concentrations of CPRE (20, 40, 60, 80 and 100 µg/ml) showed a significant restoration in altered biochemical parameters towards the normal and was dose dependent manner. The CCl_4 exposed HepG2 cells showed a percentage viability of 24.30% compared to normal cell. This CCl_4 exposed HepG2 cells, when treated with different concentrations of the CPRE showed significant dose-dependent increase in percentage viability as 76.20, 83.64, 85.42, 87.81, 92.63% at the doses 20, 40, 60, 80 and 100 µg/ml of CPRE, respectively.

Histopathological observations—Livers of animals induced with CCl_4 on gross examination seen with scattered white areas attributed to fatty and necrotic changes. Examinations of liver tissues of animals treated with CPRE and silymarin exhibited recovery towards normal in a dose related manner. Light microscopic examination of H & E stained slides of liver sections of the animals induced with CCl_4 alone showed vacuolated hepatocytes, degenerated nuclei, and focal necrosis, scattered lymphomononuclear infiltrate in hepatic parenchyma, micro and macro vesicular fatty changes. Affected normal architecture in rats treated with CCl_4 was recovered by

Table 3— Effect of CPRE on protein, cholesterol, triglyceride levels
[Values are mean ± SEM of 6 animals in each group]

Treatment	Protein (g/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Control (CMC 1%w/v, 1 ml)	8.47 ± 0.42	45.35 ± 2.95	89.41 ± 3.73
CCl_4(1 ml/kg)	5.9 ± 0.28††	108.23 ± 6.43††	65.23 ± 6.02†
CPRE (100 mg/kg) + CCl_4	6.89 ± 0.38*	93.83 ± 5.97*	76.01 ± 3.49
CPRE (200 mg/kg) + CCl_4	7.77 ± 0.22**	74.58 ± 4.05***	80.96 ± 2.76*
CPRE (400 mg/kg) + CCl_4	8.17 ± 0.29***	70.45 ± 3.38***	87.4 ± 5.09**
Silymarin (25 mg/kg) + CCl_4	8.25 ± 0.4***	39.15 ± 3.48***	90.33 ± 4.22**

Significant at $^{\dagger}P$ <0.01, $^{\dagger\dagger}P$ <0.001 compared to control group; $^{*}P$ <0.05, $^{**}P$<0.01, $^{***}P$ <0.001 compared to CCl_4 group.

Table 2— Effect of CPRE on SOD, CAT, GPx, GSH and GST enzymes and LPO
[Values are mean ± SEM of 6 animals in each group]

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GPx (mmol/mg tissue)	GSH (n mol/mg protein/min)	GST (mmol/mg tissue)	LPO (U/mg protein)
Control (CMC 1%w/v, 1 ml)	110.3 ± 3.43	27.46 ± 2.22	4.13 ± 0.46	30.03 ± 1.37	239.06 ± 6.93	0.43 ± 0.02
CCl_4(1 ml/kg)	53.33 ± 2.77††	12.8 ± 1.21††	2.90 ± 0.18†	14.51 ± 0.71††	136.81 ± 2.45††	2.06 ± 0.34††
CPRE (100 mg/kg) + CCl_4	67.24 ± 3.48	16.42 ± 1.68	3.56 ± 0.36	14.33 ± 0.42	142.81 ± 4.87	1.21 ± 0.32**
CPRE (200 mg/kg) + CCl_4	82.83 ± 6.25***	20.98 ± 2.44**	4.5 ± 0.28*	24.06 ± 0.88***	161.90 ± 4.0***	0.52 ± 0.08***
CPRE (400 mg/kg) + CCl_4	93.5 ± 5.25***	24.83 ± 1.75***	5.05 ± 0.34***	27.20 ± 0.74***	199.98 ± 3.86***	0.39 ± 0.03***
Silymarin (25 mg/kg) + CCl_4	97.46 ± 6.82***	26.42 ± 2.14***	5.23 ± 0.35***	28.28 ± 0.60***	206.88 ± 1.93***	0.37 ± 0.01***

Significant at $^{\dagger}P$ <0.05, $^{\dagger\dagger}P$ <0.001 compared to control group; $^{*}P$ <0.05, $^{**}P$<0.01, $^{***}P$ <0.001 compared to CCl_4 group.

administration of CPRE and showing few foci of necrosis of hepatocytes and signs of regenerative activity. Liver sections of rats treated with CPRE (400 mg/kg) showed near normal hepatic cells, central vein, and portal triad, as well as mild degree of fatty change, and necrosis almost comparable to the control and silymarin treated groups (Fig. 1).

Discussion

The present work was undertaken to study the possible role of hepatoprotective and antioxidant potential of 50% ethanolic extract *C. pareira,* against CCl_4 induced hepatotoxicity. Phenolics and flavonoids normally scavenge free radicals and play an essential role in prevention and therapy of cancer[25].

Table 4— Effect of treatment of CPRE on the biochemical parameters of CCl_4 intoxicated freshly isolated rat hepatocytes. [Values are mean ± SEM, n = 3 replicates]

Treatments	ALT (U/l)	AST (U/l)	ALP (U/l)	TGL (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Control	14.00 ± 0.02	10.01 ± 0.35	30.00 ± 0.42	183.00 ± 8.01	0.209 ± 0.006	0.039 ± 0.002
CCl_4 (1%)	59.01 ± 0.64†	72.00 ± 0.54†	81.00 ± 2.61†	95.01± 3.08†	0.614 ± 0.01†	0.186 ± 0.05†
CPRE (20µg/ml) + CCl_4	33.00 ± 1.58*	28.00 ± 0.42*	38.01 ± 1.42*	170.00 ± 7.05*	0.426 ± 0.03*	0.092 ± 0.003*
CPRE (40µg/ml) + CCl_4	31.02 ± 0.94*	26.30 ± 0.38*	36.10 ± 1.32*	174.00 ± 5.41*	0.401 ± 0.03*	0.088 ± 0.002*
CPRE (60µg/ml) + CCl_4	27.00 ± 1.06*	21.00 ± 0.60*	34.00 ± 1.21*	180.20 ± 9.18*	0.340 ± 0.02*	0.070 ± 0.001*
CPRE (80µg/ml) + CCl_4	24.00 ± 0.45*	17.00 ± 0.79*	33.01 ± 1.28*	185.00 ± 8.72*	0.326 ± 0.01*	0.056 ± 0.002*
CPRE (100µg/ml) + CCl_4	20.00 ± 1.29*	15.00 ± 0.86*	31.00 ± 0.85*	186.30 ± 10.01*	0.267 ± 0.05*	0.049 ± 0.002*

Significant at $^{\dagger}P$ <0.001 compared to control group; $^{*}P$ <0.001 compared to CCl_4 group.

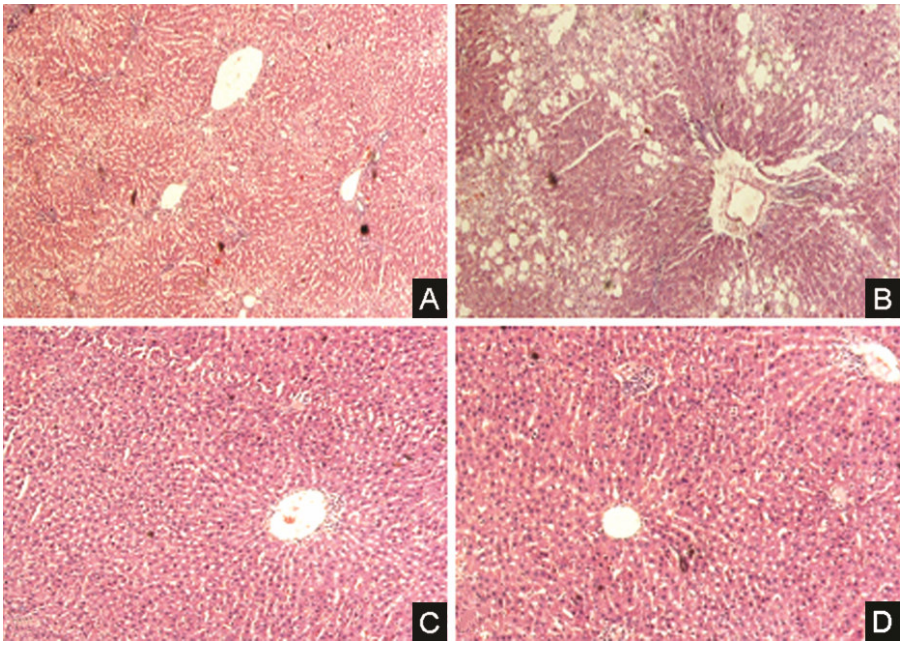

Fig. 1 — Effect of CPRE on histopathological study of rat liver in CCl_4 induced hepatotoxicity (a)-liver section of control rats showing normal liver architecture; (b)-liver section of CCl_4 alone (1ml/kg) treated rats showing patches of liver cell necrosis with inflammatory collections and the loss of cellular boundaries; (c)-Liver section of rats treated CCl_4 and CPRE (400 mg/kg) showing regeneration of hepatocytes with prominent nucleus, normal hepatic cells, no signs of necrosis and minimal inflammatory cellular infiltration; and (d)-liver section of rats treated with CCl_4 and silymarin (25 mg/kg) showing well brought out central vein, hepatic cell with well preserved cytoplasm, prominent nucleus.

In our earlier studies, we found *C. pareira* roots contain polyphenolics[26], which contributes free radical scavenging and antioxidant activities. This study deals with the status of serum marker enzymes, and antioxidant enzymes for prevention of CCl_4-induced hepatic damages by CPRE. Hepatic damage induced by CCl_4 causes instability of liver cell metabolism, inducing triacylglycerol accumulation, changes in serum transaminases activities, increased lipid peroxidation, membrane damage and depression of protein synthesis, these are the indicators of liver damage[27,28].

Present study demonstrates a significant increase in the activities of AST, ALT, ALP and serum bilirubin levels, that indicates increased permeability, severe damage to tissue membrane and necrosis of hepatocytes after exposing with CCl_4. Administration of CPRE at different dose levels (100, 200 and 400 mg/kg) prevented the rise in levels of above serum enzymes, alkaline phosphatase and serum bilirubin levels. It indicates a possible stabilization of plasma membranes as well as repair of hepatic tissue damages caused by CCl_4 exposure.

Hepatoprotective activity is associated with antioxidant activity, since it is free radical mediated damage[29]. Elevated level of MDA reflects an enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals[30]. The enzymatic antioxidant defense systems are the natural protector against lipid peroxidation. SOD, CAT enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage[31]. Earlier studies regarding mechanism of CCl_4- induced hepatotoxicity have shown that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 and that liver necrosis begins when the GSH stores are markedly in depleted state[32,33]. Administration of CPRE increased the content of GSH significantly at 200, 400 mg/kg doses when comparing CCl_4 alone group.

We concluded that a reduction in the activities of antioxidant enzymes (SOD and CAT) in CCl_4 treated group as compared to the control group. After CPRE administration, the above changes were reversed as compared to the group of rats which received only CCl_4. Those findings showed CPRE can scavenge reactive free radicals that could lead to the decrease in severity of oxidative damage in the liver.

CPRE at different concentrations (20, 40, 60, 80 and 100 µg/ml) exhibited significant restoration of the altered biochemical parameters towards normal in CCl_4 intoxicated rat hepatocytes. CPRE at 100µg/ml was found to be comparable to control group. It was carried out in human liver derived HepG2 cells against CCl_4 induced damage and CPRE markedly reduce cell viability in a concentration dependent manner. Inhibition of HepG2 cells showed cytotoxic nature of CPRE.

Histopathological findings clearly revealed that the hepatic cells, central vein, and portal triad are almost normal in CPRE (400 mg/kg) administered rats, compare with CCl_4 alone induced rats. This study finally confirms *C. pareira* having hepatoprotective effect on hepatic damage induced by CCl_4. Thus it can be concluded that mechanism of hepatoprotective activity of *C. pareira* roots may be due to its free radical-scavenging, antioxidant activity, and synergistic effect of the phytophenolics present in the root. The data of this finding could lead to the use of this plant as in potent hepatoprotective therapy.

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