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Research Article

THERAPEUTIC POTENTIAL OF *CASSIA FISTULA* POD EXTRACT IN AMELIORATION OF CARBON TETRACHLORIDE INDUCED LIVER TOXICITY

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ABSTRACT
The liver is a vital organ and impairment of its function can be lethal. *Cassia fistula* Linn. (Fabaceae) has long been used in traditional Indian medicine for the treatment of various ailments including liver disorders. This work evaluates the hepatoprotective activity of hydro-alcoholic pod extract of C. fistula (CFPE) against carbon tetrachloride (CCl_4) induced liver toxicity in rats. The animals were orally pre-treated with two different doses of CFPE (250 and 500 mg/kg body weight) or distilled water for 7 consecutive days. CCl_4 at a dose level of 1 mL/kg body weight (50% v/v in soya oil) was then administered orally on the 8th day in all the groups except the control group. Pre-treatment of rats with CFPE reduced the impact of CCl4 induced disturbances in liver metabolism as indicated by increased levels of protein, glycogen and cholesterol compared with CCl_4 treated rats. In addition, CFPE pre-treated rats showed a significant inhibition in the elevation of serum hepatic marker parameters like AST, ALT, ALP and bilirubin caused by CCl_4. Parallel with these changes, CFPE also prevented CCl_4 induced oxidative stress in the liver of rats by inhibiting lipid peroxidation and restoring the levels of antioxidants SOD, glutathione and ascorbic acid. Adverse histopathological changes induced by CCl_4 in liver of rats were also minimized by CFPE pre-treatment. The results of the study suggested that CFPE pre-treatment could protect hepatotoxicity induced by CCl_4 possibly by its antioxidative and membrane stabilizing activity.

Keywords: *Antioxidant, Hepatoprotective, Lipidperoxidation, Reactive Oxygen Species, CCl4*

INTRODUCTION
The liver is considered as one of the most vital organs which regulate several functions including metabolism, detoxification, excretion of xenobiotics from the body and maintaining biological equilibrium. Various xenobiotics can damage liver and hepatotoxicity induced by CCl_4 is one of the most commonly used model system for the screening of hepatoprotective activity of herbal drugs (Clawson, 1989; Srivastava and Shivanandappa, 2010). The hepatotoxicity induced by CCl_4 is due to transformation of CCl_4 to trichloromethyl radical (CCl_3) by the cytochrome P_{450} in liver microsomes, and consequently causes lipid peroxidation of membranes that leads to the liver injury (Weber *et al*., 2003; Demirdag *et al*., 2004). As reactive oxygen species (ROS) play central role in liver disease and pathology, antioxidants might prevent hepatic damage through scavenger activity and increase the activity of intracellular antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Kuo *et al*., 2010).
Documented evidences suggest that natural product derived antioxidants are effective in preventing oxidative stress related pathologies of liver due to particular interactions and synergisms (Vitaglione *et al*., 2004).
Since, free radicals play an important role in CCl_4 induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have a hepatoprotective effect. The present study was carried out to investigate the protective efficacy of hydro-alcoholic (70% alcohol) extract of the complete pod of *Cassia fistula* against CCl_4 induced hepatotoxicity in Wistar rats.

Cassia fistula Linn. [Fabaceae (Leguminosae)], also called amaltas in Hindi, is native to India. It has long been used in traditional Indian medicine for the treatment of various ailments (Chatterjee and Pakrashi, 1991). Its leaves are reported to possess hepatoprotective activity (Bhakta *et al*., 1999). Its seeds are reported to have antifertility activity in female rats (Yadav and Jain, 1999). Leaves and bark of the plant are reported to possess antidiabetic activity (Einstein *et al*., 2013). The hypolipidemic activity of the legumes of the plant has been reported by Gupta and Jain (2009). Almost all parts of the plant are reported to have antioxidative action (Luximon-Roma *et al*., 2002; Siddhuraju *et al*., 2002). It has been reported that the intraperitoneal infusion of *Cassia fistula* pods possessed very low levels of toxicity in mice, having LD_{50} 6600 mg/kg (Akanmu *et al*., 2004). Phytochemical studies revealed that the pulp of the pod is rich in antioxidants and contain, anthraquinone glycosides, sennosides A and B, rhein and its glucoside, barbaloin, aloin, formic acid, butyric acid and their ethyl esters and oxalic acid, pectin, and tannin (Agarwal and Paridhavi, 2005; Khare, 2007). Proanthocyanidins containing flavan-3-ol units with abnormal 2S configuration have also been observed in the pods of the plant, together with the common flavan-3-ols and proanthocyanidns like catechin, epicatechin, procyanidin B-2, and epiafzelechin (Kashiwada *et. al*., 1990). A new bioactive flavone glycoside 5,3′,4′-tri-hydroxy-6-methoxy-7-O-α-L-rhamnopyranosyl-(1→2)-O-β-D-galactopyranoside was reported by Yadav and Verma (2003). Oxyanthraquinones, chrysophanol and chrysophanein, were also isolated from the seeds of *Cassia fistula* by Kuo *et al*., (2002).

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride (CAS No.: 56-23-5, Batch No.: R155F04) was purchased from Ranbaxy Fine Chemicals Limited, New Delhi, India. All other chemicals were of analytical grade.

Preparation of Extract

Fresh pods of *C. fistula* were collected in the months April-June, 2013, from the campus of University of Rajasthan, Jaipur. The plant was taxonomically identified by Prof. K.P. Sharma, Incharge, Herbarium, Department of Botany, University of Rajasthan, Jaipur, India where a voucher specimen (Specimen no. RUBL21057) was deposited. The fresh pods were washed with distilled water, shade dried, and powdered in an electric grinder. The powder (300 g) was suspended in 70% ethanol and allowed to stand for 24 h.

The mixture was subjected to Soxhlet apparatus for extraction at 60°C-70°C for 35 h. It was then filtered using a filter paper and the filtrate was evaporated to dryness in an oven at 40°C. A brownish residue weighing 32.5 g (10.83% of dried powder) was obtained. This was kept in an air tight bottle in a refrigerator until used. The extract was suspended in water before administering to experimental animals.

Experimental Design

Twenty-four, adult, colony bred Wistar albino rats, weighing 150-180 g each, were left under normal healthy conditions at the animal house. Animals were fed with standard rat diet (Aashirwad Food Industries, Chandigarh) and with water *ad libitum*. They were housed in polypropylene cages. The animals were maintained as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations. The study was approved by the Institutional Ethical Committee, Department of Zoology, University of Rajasthan, Jaipur, India.

Animals of both sexes were equally divided into four groups each having six animals (3 each male and female). The rats of group III and IV were orally treated with CFPE at a dose of 250 and 500 mg/kg body weight respectively while group I and II rats received distilled water orally for 7 consecutive days. Group I served as control.

The rats in groups II, III and IV were orally administered with CCl_4 at a dose level of 1 mL/kg body weight diluted in soya oil (50% v/v solution) on the 8^{th} day while the rats in the control group were administered with equal volume of soya oil. At the end of the experimental period, animals were fasted overnight. Animals were then sacrificed under mild ether anesthesia. Blood samples were collected by cardiac puncture and serum was separated by centrifugation at 3000 rpm for 10 min. Liver was dissected

out and washed in ice cold saline solution, blotted and a small portion was cut and weighed for biochemical estimations. Rest of the portion of liver tissue was fixed in Bouin's fixative for histopathological studies.

Tissue Biochemistry

Quantitative biochemical estimations of total protein (Lowry *et al*., 1951), glycogen (Montgomary, 1957) and total cholesterol (Zlatkis *et al*., 1953) were made in the frozen liver samples.

Serum Biochemistry

Serum samples were analyzed for transaminases viz. aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954) and total bilirubin (Malloy and Evelyn, 1937) using standard kits (Accurex Biomedical Pvt. Ltd., Mumbai, India).

Lipid Peroxidation and Antioxidant Defense System

Lipid peroxidation in liver was estimated by employing the thiobarbituric acid reactive substances (TBARS) assay (Okhawa *et al*., 1979). Superoxide dismutase (SOD) activity, (Marklund and Marklund, 1974), glutathione level (GSH) (Moron *et al*., 1979) and ascorbic acid level (Roe and Kuether, 1943) were also determined in liver samples.

Histopathological Study

Pieces of fresh liver tissue were cut and fixed in Bouin's fixative and processed through an ascending series of alcohol solutions (30, 50, 70, 90 and 100%) and cleared in xylene. The tissues were then embedded in paraffin wax. Sections of 5 µ thick were cut, stained with haematoxylin and eosin and observed under light microscope for histopathological changes.

Statistical Analysis

All the values of biochemical estimations were averaged; standard error of the mean was calculated and compared by applying Student's *t*-test.

RESULTS AND DISCUSSION

Tissue Biochemistry

The tissue biochemical analysis reflects a significant decrease in protein ($P \leq 0.01$) and glycogen ($P \leq 0.05$) content in the liver of rats receiving CCl_4 (Group II), while their cholesterol content showed a significant increment ($P \leq 0.01$) compared to animals of the control group (Group I). However, pre-treatment with CFPE at a dose of 250 mg/kg body weight, significantly inhibited the decrease in protein level ($P \leq 0.05$) but there were non-significant changes in the levels of liver glycogen and cholesterol compared to control group (Group I).

Higher dose of CFPE was found to be more effective in restoring the levels of liver biochemical parameters. Alterations in the protein ($P \leq 0.01$), glycogen ($P \leq 0.05$) and cholesterol ($P \leq 0.05$) levels were significantly restored in the rats receiving extract at a dose level of 500 mg/kg body weight as compared to CCl_4 treated rats (Table 1).

Table 1: Effect of Hydralcoholic Pod Extract of *C. fistula* on Tissue Biochemical Parameters on CCl_4 Induced Hepatotoxicity in Rats

Treatments	Protein (mg/g)	Glycogen (mg/g)	Cholesterol (mg/g)
Group I	180.84 ± 4.43	5.3 ± 0.18	7.41 ± 0.19
Group II	152.2^{b} ± 4.88	4.56^{a} ± 0.19	8.46^{b} ± 0.24
Group III	166.16^{*} ± 3.95	5.01^{ns} ± 0.22	7.99^{ns} ± 0.29
Group IV	171.27^{**} ± 3.85	5.31^{*} ± 0.25	7.52^{*} ± 0.23

Values are mean ± SEM (n = 6) in each group
Levels of significance: $^{a}p \leq 0.05$; $^{b}p \leq 0.01$; $^{c}p \leq 0.001$ of group II compared with group I
$^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ group III and IV compared with group II.

Serum Biochemistry

The levels of serum markers of hepatic damage viz. AST, ALT, ALP and total bilirubin of control and treated groups are summarized in Table 2. After a single dose of CCl_4, serum levels of AST, ALT and ALP enzymes and of bilirubin in group II increased significantly ($P \leq 0.001$) compared to control group. This acute hepatotoxicity was significantly suppressed in all animals previously treated for 7 consecutive days with *C. fistula* pod extract at a dose of 250 or 500 mg/kg as indicated by the reduced levels of serum bilirubin ($P \leq 0.01$, $P \leq 0.001$) and the activities of AST ($P \leq 0.01$, $P \leq 0.001$), ALT ($P \leq 0.001$) and ALP ($P \leq 0.05$, $P \leq 0.01$) respectively.

Table 2: Effect of Hydralcoholic Pod Extract of *C. fistula* on Serum Biochemical Parameters of Rats Intoxicated with CCl_4

Treatments	AST (U/L)	ALT (U/L)	ALP (KA Units)	Bilirubin (mg/dl)
Group I	51.96 ± 2.02	40.24 ± 1.58	12.13 ± 0.34	0.95 ± 0.04
Group II	101.45^{c} ± 3.82	98.6^{c} ± 3.03	17.57^{c} ± 0.4	1.78^{c} ± 0.05
Group III	78.08^{**} ± 3.69	80.77^{**} ± 2.75	16.08^{*} ± 0.41	1.35^{**} ± 0.09
Group IV	61.44^{***} ± 2.57	61.64^{***} ± 2.29	15.1^{**} ± 0.42	1.03^{***} ± 0.06

Values are mean ± SEM (n = 6) in each group
Levels of significance: $^{a}p \leq 0.05$; $^{b}p \leq 0.01$; $^{c}p \leq 0.001$ of group II compared with group I
$^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ group III and IV compared with group II.

Table 3: Effect of Hydralcoholic Extract of *C. fistula* on Lipid Peroxidation, Superoxide Dismutase, Glutathione and Ascorbic Acid on CCl_4 Induced Hepatotoxicity in Rats

Treatments	Lipid Peroxides (LPO) (n mole/mg tissue)	Superoxide Dismutase (SOD) (U/mg protein)	Glutathione (GSH) (µ mole/g tissue)	Ascorbic Acid (mg/g tissue)
Group I	2.9 ± 0.09	14.86 ± 0.42	3.9 ± 0.16	1.78 ± 0.09
Group II	4.2^{c} ± 0.21	10.64^{c} ± 0.63	2.42^{c} ± 0.16	1.14^{c} ± 0.1
Group III	3.62^{*} ± 0.14	12.55^{*} ± 0.53	2.88^{*} ± 0.14	1.48^{*} ± 0.11
Group IV	3.38^{**} ± 0.14	13.07^{**} ± 0.46	3.26^{**} ± 0.18	1.71^{**} ± 0.14

Values are mean ± SEM (n = 6) in each group
Levels of significance: $^{a}p \leq 0.05$; $^{b}p \leq 0.01$; $^{c}p \leq 0.001$ of group II compared with group I
$^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ group III and IV compared with group II

Lipid Peroxidation

A marked increase ($P \leq 0.001$) in the TBARS concentration (a measure of lipid peroxidation) was observed in the liver of rats exposed to CCl_4 (Group II) relative to control rats (Group I). Pre-treatment with CFPE was found to result in a significant lowering of mean TBARS concentration in group III ($P \leq 0.05$) and group IV ($P \leq 0.01$) rats compared to group II rats.

Antioxidant Defense Marker Parameters

The antioxidant activity of SOD and content of glutathione (GSH) and ascorbic acid in the liver of CCl_4 treated rats (Group II) were found to be significantly decreased ($P \leq 0.001$) relative to the control group (Group I). However, CFPE pre-treatment significantly boosted the activity of SOD in rats of group III ($P \leq 0.05$) and group IV ($P \leq 0.01$) compared to group II. CFPE pre-treatment significantly prevented the decrease in content of GSH and ascorbic acid in the rats of group III ($P \leq 0.05$) and group IV ($P \leq 0.01$) compared to CCl_4 treated group.

Histopathology

Histopathological examination of liver of control rats revealed normal histoarchitecture showing normal hepatocytes radiating from the central vein with well defined nucleus and characteristic cord like

arrangement separated by sinusoids (Figure 1). In contrast, a complete loss of normal hepatic architecture with extensive vacuolization of hepatocytes, ballooning degeneration, fatty changes, pycnotic nuclei and centrilobular necrosis was observed in the liver of CCl_4 administered rats (Figure 2). Administration of CFPE at lower dose (Group III) improved the histological structure of liver, though there was some scattered vacuolization and degeneration of hepatic cells (Figure 3). The protective effect of pre-treatment of CFPE at higher dose (Group IV) is confirmed by preservation of hepatocellular architecture as compared to CCl_4 treated group. There was considerable reduction in vacuolization, ballooning degeneration, necrosis and fatty changes (Figure 4).

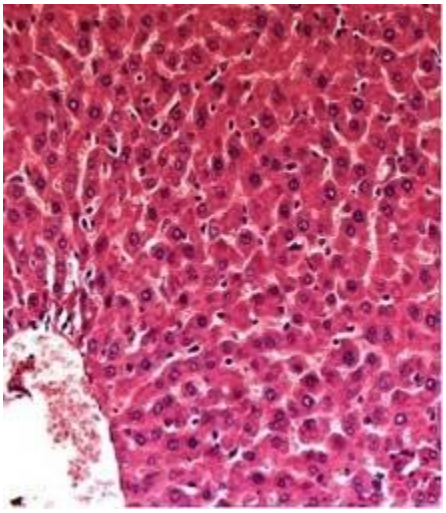

Figure: 1. Photomicrograph of Liver of a Rat of Group I (Vehicle Treated Control) Showing Characteristic Arrangement of Hepatocytes Around the Central Vein with Well Defined Nucleus and Cytoplasm; H & E x200

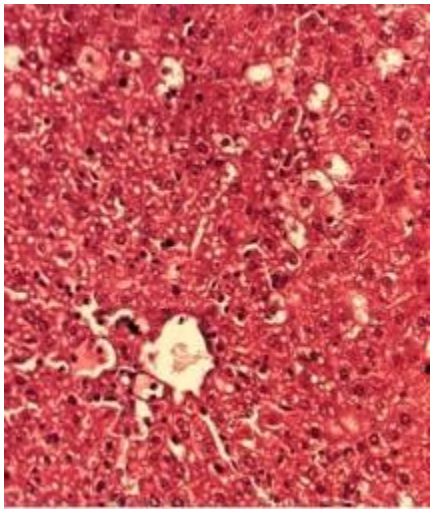

Figure: 2. Photomicrograph of Liver of a Rat of Group II (CCL_4 Treated) Showing Disturbed Arrangement of Hepatocytes, Vacuolization and Damaged Parenchymal Cells; H & E x200

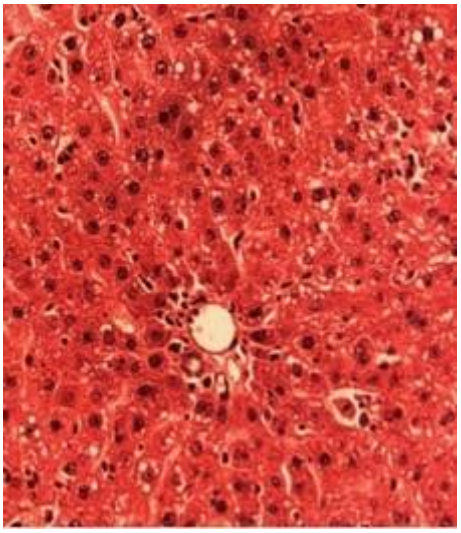

Figure: 3. Photomicrograph of Liver of a Rat of Group III (250 mg/kg b. wt. CFPE + CCL_4) Showing Reduced Vacuolization and Lessened Degenerative Changes; H & E x200

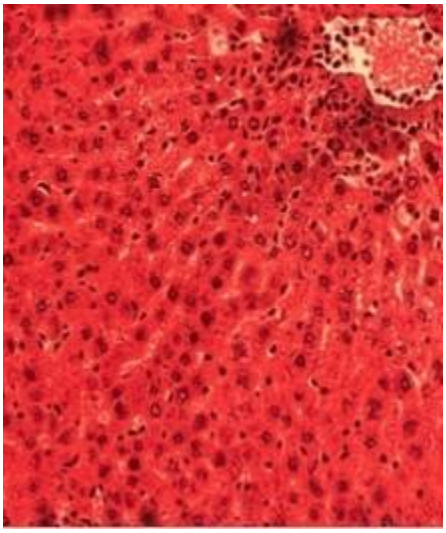

Figure 4: Photomicrograph of Liver of a Rat of Group IV (500 mg/kg b. wt. CFPE + CCL_4) Showing Histoarchitecture of Liver Very Much Similar to that of Control Group Rats; H & E x200

Discussion

Administration of CCl_4 significantly decreased the protein and glycogen contents of the liver in rats of hepatotoxic group which indicated poor liver function and impaired metabolism of proteins and carbohydrates. Earlier reports have also demonstrated decrease in hepatic glycogen content after treatment with CCl_4 (Muriel *et al*., 2001). However, pre-treatment of CFPE prevented the reduction in hepatic glycogen content probably due to hypoglycemic activity of the extract which might result in increased glycogenesis.

Hypoglycemic activity of the *C. fistula* seed extract has already been reported in normal young rats by Singh and Bharadwaj (1975). The results of the present study also exhibited that CCl_4 treatment induces increased accumulation of cholesterol in liver indicating disturbed cholesterol metabolism occurring due to damage of hepatic parenchymal cells (Havel, 1986; Althnaian *et al*., 2013). However, level of cholesterol in the liver of the extract pre-treated rats was comparable to that of control group suggesting modulatory influence on cholesterol metabolism (Gupta and Jain, 2009). Thus, CFPE pre-treatment was found to be effective in maintaining the protein, glycogen and cholesterol metabolism of liver against CCl_4 induced disturbances.

Elevated levels of AST, ALT and ALP (Liver function marker enzymes) indicate the cellular leakage and loss of functional integrity of hepatic membrane architecture which results in release of these enzymes from the cell cytosol into blood stream (Yogalakshmi *et al*., 2010; Sivaraj *et al*., 2011). Results of the present study revealed a significant increase in the serum activities of AST, ALT, ALP and total bilirubin level on exposure to CCl_4 indicating considerable hepatocellular injury which is also evident by the histopathological study of the liver. In an earlier report, Srivastava and Shivanandappa (2010) also observed a similar increase in the levels of AST, ALT and ALP after administration of single dose of CCl_4 in rats. Pre-treatment with CFPE inhibited the increase in activities of the serum AST, ALT, ALP and total bilirubin level induced by CCl_4. The protective effects of CFPE against CCl_4 induced hepatotoxicity might be due to prevention of membrane damage, loss of structural integrity and oxidative stress. Significant reduction in the serum level of total bilirubin and activities of AST, ALT and ALP in animals of group III and group IV further supports effectiveness of the extract in maintaining normal functional status of the liver.

Lipid peroxidation (TBARS) has been widely used as an indicator of ROS mediated oxidative damage to cell membranes. Vivek *et al*., (1994) have reported that CCl_4 causes significant increase in hepatic lipid peroxidation due to radical injury in liver of rats. In the present study, increased concentration of TBARS in liver of CCl_4 treated rats indicated excessive radical formation and activation of lipid peroxidation, resulting in hepatic damage. Pre-treatment of CFPE prevented increased lipid peroxidation which could be attributed to radical scavenging activity of the phytoconstituents present in *Cassia fistula* pods.

In our study, CCl_4 administration to rats showed decline in the antioxidant activity of SOD which is in agreement with earlier reports (Shahjahan *et al*., 2004; Srivastava and Shivanandappa, 2010). Decreased activity of SOD might be correlated with the presence of enhanced ROS level in CCl_4 treated rats. The animal groups pre-treated with CFPE showed an increase in the SOD activity which indicated the antioxidant activity of CFPE.

Significantly reduced levels of GSH due to administration of CCl_4 can be the important factor in CCl_4 induced hepatic damage. This reduction in GSH level might be due to excessive utilisation of GSH to alleviate free radicals or its reduced synthesis by damaged liver.

The significant increase in the GSH content observed in extract pre-treated rats might be due to *de novo* GSH synthesis or decreased utilization of GSH due to free radical scavenging activity of the phytoconstituents present in the extract.

Pradeep *et al*., (2010) also found decreased LPO, increased GSH content and enhanced SOD activity after the administration of ethanolic leaf extract of *C. fistula* for 30 days against diethyl nitrosamine induced hepatic injury and oxidative stress in ethanol pre-teated rats. Decreased level of ascorbic acid in rats of hepatotoxic group might be correlated with the decreased GSH level or increased utilisation of ascorbic acid in deactivation of increased ROS (Chatterjee and Nandi, 1991). Pre-treatment of rats with CFPE

inhibited the decrease in levels of ascorbic acid indicating the antioxidant activity of *Cassia fistula* extract.

The histopathological findings also confirmed the protective effect of CFPE against CCl_4 induced hepatotoxicity.

Photomicrographs of the liver of rats pre-treated with CFPE revealed dose related preventive effects. Das *et al*., (2008) also showed preventive effect of aqueous extract of the pulp part of the fruit of *Cassia fistula* on hepatic histoarchitecture of CCl_4 intoxicated rats.

The observed hepatoprotective effect of CFPE might be due to presence of polyphenolic and flavanoid phytoconstituents which act as antioxidants, thereby; scavenge free radicals and consequently show membrane stabilizing activity.

Conclusion

The present study confirms the protective action of the hydro-alcholic extract of pods of *C. fistula* against experimentally induced liver damage in rats which might be a result of antioxidative or free radical scavenging activity owing to the presence of polyphenolic and flavonoid phytoconstituents such as anthoquinones, flavon-3-ol, catechin, epicatechin, procyanidin B-2, epiafzelechin, chrysopanol and chrysophanein (Kashiwada *et al*., 1990; Kuo *et al*., 2002) present in the pods of *C. fistula*. Further studies are needed to identify and isolate active principles from *C. fistula* pod extract which possesses antioxidant and hepatoprotective properties.

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