Effect of pretreatment of *Cassia fistula* Linn. leaf extract against subacute CCl₄ induced hepatotoxicity in rats

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CCL₄ alone treatment (0.1ml of liquid paraffin/100g body weight, ip) for 7 days followed by 0.1ml of CCl₄ in liquid paraffin/100g body weight, id from day 8 till day 14 caused a 16 fold increase in lipid peroxidation and a 50% reduction in catalase and glutathione reductase in liver tissue of rats accompanied by an increase in the activities of transaminases, alkaline phosphatase, lactate dehydrogenase and γ-glutamyl transpeptidase in serum as compared to liquid paraffin treated control. Pretreatment of ethanolic leaf extract of *C. fistula* (500mg/kg body weight/day for 7 days) followed by CCl₄ treatment (0.1ml/100g body weight from day 8 till day 14) completely reversed back lipid peroxidation and the activities of catalase and glutathione reductase in the liver tissue towards normalcy. This treatment also reversed the elevated levels of the enzymes in the serum. Ethanolic leaf extract alone treatment did not produce any change in all the parameters studied. The results suggest antioxidant and hepatoprotective properties of *C. fistula* during its pretreatment against CCl₄ induced hepatotoxicity.

Keywords: Antioxidants; *Cassia fistula* CCL₄; Hepatotoxicity; Hepatoprotective

*Cassia fistula* Linn. (Family: Caesalpinaceae) is a medium sized deciduous tree, widely cultivated throughout India as an ornamental plant. Various parts of the plant are used for the treatment of several ailments, the leaves are used as laxative, anti-periodic in rheumatism. Though leaves and pods are reported to be used in the treatment of jaundice by some people of North Eastern India, the antioxidant and hepatoprotective properties of leaf extract are not well established. Free radical generation and lipid peroxidation of hepatocellular membrane are often implicated as positive factors for the onset of carbon tetrachloride (CCl₄) induced hepatocellular damage. Antioxidants play a crucial role in hepatoprotective activity and hence, search for crude drugs of plant origin with this property has become a central focus of studies of hepatoprotection today.

Thus hepatoprotective activity of leaf extract of *C. fistula* is reported, the antioxidant properties of the leaf extract against hepatocellular damage needs to be substantiated. Hence, the present study has been undertaken to investigate the antioxidant and hepatoprotective activity of ethanolic leaf extract of *C. fistula* against subacute CCl₄ induced hepatotoxicity in rats.

**Materials and Methods**

The leaves of *Cassia fistula* Linn. were collected from Tamil Nadu Medicinal Plants Farm and Herbal Concentrates Ltd. (TAMPCOL), Chennai, during July and August. The plant was authenticated by Dr. Narayanappa, Chief Botanist, TAMPCOL and a voucher specimen of this plant is deposited in the Department of Botany, Presidency College, Chennai (Herbarium No: 507).

The leaves were washed, shade dried and powdered coarsely by hand. The particle size of the powdered leaf ranged between 0.5-1 cm. About 100g dry weight of the powdered leaf was soaked in 1 liter of 90% redistilled ethanol for 1 month, as it is an ideal medium for the extraction of both polar and non-polar active principles. Extraction of active principles was allowed to undergo by natural percolation under occasional shaking by swirling movement of the container for about 20-30 times every day at an interval of approximately 7-8 hr. The ethanolic leaf extract was filtered using Whatmann No: 1 filter paper and the filtrate was evaporated to dryness at

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About 19-20g of crude extract was obtained after evaporation, which corresponds to 19-20% of 10g of dried leaf. The qualitative phytochemical screening of the ethanolic leaf extract was performed at the plant extract showed positive for the presence of alkaloids, glycosides, flavonoids, saponins and tannins.

After getting approval from the Institutional Animal Ethical Committee, Wistar Albino rats of the sex weighing between 180-200g obtained from Nal Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai were housed in polypropylene cages and acclimatized for 10 days and were fed pellet diet and water ad libitum.

Rats were divided at random into 4 groups of 6 animals each. Group I (normal control) received paraffin (0.1ml/100g body weight, ip), daily for 7 days. Group II (CCl₄ control) received liquid paraffin (0.1ml/100g body weight, ip), daily for 7 days and from 8th day, it was followed by treatment with CCl₄ in liquid paraffin (1:1; 0.2ml/100g body weight, ip), upto 14th day. Group III (pretreatment group) animals were pretreated with 500mg/kg body weight of ethanolic leaf extract of C. fistula orally (as a suspension in distilled water) on day 1 till day 7 and from 8th day they were treated with CCl₄ in liquid paraffin (1:1; 0.2ml/100g body weight, ip), upto 14 days. Group IV (control for extract alone treatment) animals received ethanolic leaf extract (500mg/kg body weight, orally) on day 1 till day 7 and was followed by liquid paraffin from day 8 till day 14.

Animals were sacrificed 24 hr after last injection. Blood collected into clean tubes from retro orbital venous of ether anaesthetized rats was allowed to clot and serum separated. The liver was dissected out after hepaticization of rats and 1% liver homogenate was prepared in tris-HCl buffer (0.1M; pH 7.4), which was used for all biochemical assay. Serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma glutamyl transpeptidase (γ-GT) were measured in serum. Lipid peroxidation (LPO) in terms of thiobarbituric acid reacting substances (TBARS), catalase (CAT), glutathione reductase (GR) and protein were estimated in liver tissue homogenate. A piece of liver tissue was fixed in 10% formalin and was subsequently wax mounted. The hematoxyline and Eosin stained sections (10 μm thick) were observed under microscope for evaluation of histopathological changes.

The data were subjected to One Way Analysis of Variance (ANOVA) and the significance of the difference between the means of various treatment groups was performed by employing Tukey’s multiple comparison test, using SPSS statistical package (Version 7.5). The values are expressed as mean ± SE and P value < 0.05 was considered significant.

Results and Discussion

CCl₄ alone (Group II) induced hepatocellular damage was evident by an increase in the levels of marker enzymes of liver toxicity i.e., AST (2 folds), ALT (4 folds), ALP, LDH and γ-GT in serum (Table 1), as compared to liquid paraffin alone treated control (Group I). It is postulated that administration of CCl₄ could cause cell lysis, resulting in the release of cytoplasmic enzymes of the liver into the blood circulation, leading to their increase in levels in serum and this property is often implicated to assess the extent of CCl₄ induced hepatocellular damage. The observations of the present study are in accordance with these reports. Pretreatment of rats with ethanolic leaf extract (Group III) partially inhibited the increase in the levels of all the above marker enzymes of liver toxicity in the serum (Table 1), indicating the hepatoprotective property of the extract.

Hepatocellular membrane damage, consequent to administration of CCl₄ (Group II) was evident by a 16 fold increase in the LPO and 50% reduction in the activities of CAT and GR in the liver tissue (Table 1) as compared to control (Group I). Pretreatment of ethanolic leaf extract for 7 days prior to CCl₄ administration (Group III) completely inhibited the elevated levels of LPO and reversed the decrease in the levels of CAT and GR towards normalcy in the liver tissue. CCl₄ induced liver injury is reported to cause lipid peroxidation resulting in membrane damage and the present observations are in accordance with these reports. It is also hypothesized that CCl₄ is metabolically activated by Cytochrome P450 dependent mixed function oxidases to form trichloromethyl free radical (CCl₃) and peroxide radical (OCCl₃), which are highly reactive and are capable of combining with cellular and membrane lipids in presence of oxygen to induce lipid peroxidation by hydrogen abstraction. The complete inhibition of 16 fold increase in LPO and
Reversal of 50% reduction in the activities of CAT and GR (Table 1) observed in the present study, only demonstrate the strong antioxidant property of ethanolic leaf extract. It is likely that the leaf extract preserves the activity of GR, which maintains the levels of GSH and inhibits LPO by reducing the generation of free radicals derived from CCl₄, thereby accelerating the repair mechanism. 23 Thus exhibit significant antioxidant and hepatoprotective effect. Administration of ethanolic leaf extract alone (Group A) did not produce any alteration in all the parameters studied in the serum and liver tissue and they do not differ from liquid paraffin treated control (Table 1).

Histopathological profiles of the liver from liquid paraffin:CCl₄ treated rats (Group II) showed hepatocellular necrosis, fatty degeneration and extensive vacuolation (Fig. 1b). The protective effect with pretreatment of leaf extract (Group III) is confirmed by significant improvement of hepatocellular architecture over CCl₄ alone treated groups and it is evident by considerable reduction in necrosis and fatty changes (Fig. 1c). The liver sections of rat treated with leaf extract alone (Group 1) showed the presence of normal hepatocellular architecture and absence of necrosis and steatosis (Fig. 1d) and these were comparable with those of liquid paraffin treated control (Fig. 1a).

In conclusion, the present study demonstrates the hepatoprotective and antioxidant properties of ethanolic leaf extract of C. fistula during its treatment against CCl₄ induced hepatocellular damage. The antioxidant potential and hepatoprotective effect of ethanolic leaf extract could have been brought about by various phytochemical principles i.e., flavonoids, saponins, tannins and tannoids that are present in the ethanolic leaf extract. In this regard, it is pertinent to point out that flavonoids and tannins have been suggested to act as antioxidants and exert their antioxidant activity by scavenging lipid peroxidation. 24 Thus, the plausible mechanism of the hepatoprotective effect of ethanolic leaf extract that is observed in this study may be due to its antioxidant effect. Further study is warranted to identify and isolate the active biomolecule of ethanolic leaf extract, which offer antioxidant and hepatoprotective properties.

Acknowledgement
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Table I—Effect of pretreatment of ethanolic leaf extract of *C. fistula* on various biochemical parameters in liver and serum of rats. [Values are mean ± SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>One Way ANOVA (df = 3,20)</th>
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<td></td>
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<tr>
<td>g¹</td>
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<td>0.532 ±</td>
<td>0.036 ±</td>
<td>0.029 ±</td>
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<td></td>
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<td>0.01 *</td>
<td>0.003 **</td>
<td>0.005 ***</td>
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<td>g²</td>
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<td>26.26 ±</td>
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<td>1.46 **</td>
<td>0.86 **</td>
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<tr>
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<td>11.76 ±</td>
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<tr>
<td></td>
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<td>0.57 *</td>
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<td>0.62 **</td>
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<td>M (IU/Lit)</td>
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<td>110.02 ±</td>
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<td>4.23</td>
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*µes: <0.001; *compared to Group I; **compared to Group II; *** = non significant compared to Group I.

Table I—Histopathology of liver tissue on pretreatment of ethanolic leaf extract of *C. fistula* against CCl₄ induced hepatocellular damage. (Group I) — normal liver architecture; (b) (Group II) — liver tissue shows hepatocellular necrosis, fatty degeneration and extensive infiltration; (c) (Group III) — reduction in necrosis and fatty changes; (d) (Group IV) — normal liver architecture, comparable to Group I. (50X)
EFFECT OF POST-TREATMENT OF ETHANOLIC LEAF EXTRACT OF CASSIA FISTULA (LINN) ON CARBONTETRACHLORIDE-INDUCED HEPATOTOXICITY IN RATS


ABSTRACT

In this study the efficacy of ethanolic extract leaf of Cassia fistula (Linn) was investigated in CCl$_4$ pre-treated rats. Administration of 0.1 ml of CCl$_4$ in 0.1 ml of liquid paraffin for 7 days (Group-I) produces an increase in the activities of Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and Bilirubin (BLN) in the serum followed by a decrease in the activities of AST, ALT and ALP in the liver tissue compared to liquid paraffin alone treated animals for 7 days (Group-I). On the contrary, treatment of rats with CCl$_4$ in liquid paraffin for 7 days followed by ethanolic leaf extract (500mg/kg/day) of Cassia fistula (Linn) from 8th to 14th day (Group-III) caused 60-70% reversal of increase in the activities of AST, ALT, ALP and BLN in serum followed by 95-100% reversal of fall in the activities of AST, ALT and ALP in the liver tissue when compared to CCl$_4$ alone treated rats (Group-II), indicating hepatoprotective effect of the extract during post-treatment. Administration of liquid paraffin alone for 7 days (Group-I) or liquid paraffin for 7 days followed by ethanolic leaf extract from 8th to 14th (Group-IV) did not produce any alteration in the activities of above parameters studied and they do not differ from each other. This preliminary investigation clearly demonstrates that the post-treatment of ethanolic leaf extract of Cassia fistula (Linn) could effectively prevent the liver injury induced by CCl$_4$ pre-treatment.
INTRODUCTION

Cassia fistula (Linn) commonly called "Konrai" in Tamil (Family: Caesalpiniaceae) is a medium sized tree, bearing yellow blossoms and it is widely cultivated throughout India as an ornamental and deciduous plant. This plant has been used in the treatment of various ailments dating back to Sushruta and Charaka. The leaves of Cassia fistula (Linn) are used as laxative, anti-periodic and in rheumatism and its juice is given in erysipelas and skin diseases (Chopra et al 1992). This plant is also used in skeletal fractures, soar throat, swollen throat and jaundice (Ekanayake 1980; Asolkar et al 1992) and pods and leaves of this plant were said to be used as anti-allergic and hepatoprotective agent by the urban people of North Eastern Region of India. Bhakta et al (1999, 2001), reported hepatoprotective effect of methanolic leaf extract of Cassia fistula (Linn) against carbon tetrachloride-induced hepatotoxicity. These reports demonstrate the hepatoprotective properties of leaf extract of Cassia fistula (Linn). However, studies regarding the hepatoprotective effect of ethanolic leaf of extract of Cassia fistula (Linn) during post-treatment in CCl₄ pre-treated animal model are not available. This preliminary study investigates the hepatoprotective efficacy of ethanolic leaf extract of Cassia fistula (Linn) during post-treatment of CCl₄ induced hepatotoxicity in rats and the observations are presented hereunder.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh leaves of Cassia fistula (Linn) were collected from Arignar Anna Government Siddha College and Hospital, Arumbakkam, Chennai. The plant was identified and authenticated by Chief Botanist of Arignar Anna Government Siddha College and Hospital, Arumbakkam, Chennai.

PREPARATION OF EXTRACT

The leaves were shade dried at room temperature for at least a week and was powdered mechanically for use in extraction. The powdered leaves were soaked in ethanol (70%) to enable extraction of active principles. The extract was filtered and was evaporated to dryness at 60°C. The concentrated extract was
stored in dark container. The yield was around 20% and this was used to evaluate the hepatoprotective effect against CCl₄ induced hepatotoxicity. Phytochemical screening of the extract showed positive reactivity to the test for saponins, glycosides and tannins.

ANIMALS

In bread white albino rats (Wistar strain) of both the sexes weighing 180±20 gm, which were maintained in polypropylene cages over husk beadings, and provided pellet feed (Hindustan Lever, India Ltd., Bangalore) and water ad libitum were used for this study.

EXPERIMENTAL DESIGN

Twenty four albino rats of both the sexes were divided into four groups of six animals each. Group-I animal received only liquid paraffin for 7 days and it served as control. Animals of Group-II were treated with 1:1 ratio-of CCl₄ in liquid paraffin for 7 days. Group-III animals (also called post-treatment group) received CCl₄ in liquid paraffin (1:1 ratio) initially for a period of 7 days followed by ethanolic leaf extract of Cassia fistula (Linn) from 8th to 14th day.

Group-IV animals were treated with liquid paraffin initially for 7 days and then from 8th day to 14th day, they were treated with ethanolic leaf extract of Cassia fistula (Linn). For all CCl₄ treatment, 0.1 ml of CCl₄ was mixed with 0.1 ml of liquid paraffin per 100 gm body weight of the animal and was injected subcutaneously. The ethanolic leaf extract was dissolved in distilled water and administered at the dose of 500 mg/kg/day orally. All the administration was performed between 9 to 10 am every day.

COLLECTION OF SAMPLES

All the animals were sacrificed under mild ether anesthesia at the end of the study period and blood and liver samples were collected for use in enzyme assays. Prior to sacrifice, blood was collected in clear tubes and serum was separated immediately and stored at cold till further analysis. Immediately after sacrifice the liver was quickly excised and washed in saline to remove excess of blood and about 100 mg of liver tissue was homogenized in phosphate buffer (0.1M; pH 7.4) and centrifuged. The clear supernatant was used as the enzyme source.
ASSAY OF PARAMETERS

The activities of transaminases Aspartate Transaminase (AST) and Alanine Transaminase (ALT) was estimated according to the method of Wooten (1964). While the activity of Alkaline Phosphatase (ALP) was assayed as described by King (1965) that of Bilirubin (BLN) was carried out as described by Malloy and Evelyn (1936). The activities of AST, ALT and ALP were estimated in both the serum and liver sample and BLN was estimated only in serum.

STATISTICAL ANALYSIS

The results were subjected to one-way analysis of variance (ANOVA) and test of significance between the means were performed employing Tukey’s test. The values are expressed as mean ± S.D. and P value < 0.05 was considered significant. SPSS statistical package was used to perform statistical analysis.

RESULTS

Rats administered with CCl₄: liquid paraffin (1:1) for 7 days (Group-II) developed significant hepatocellular damage as evident from a highly significant (P<0.001) elevation in the activities of AST (2 fold), ALT (4 fold), ALP and BLN (4 fold) in the serum when compared to control (Group-I). Post-treatment of leaf extract of Cassia fistula (Linn) (Group-III) significantly reduce the increase in the levels of AST, ALT (p<0.01), ALP and Bilirubin (p<0.001) in serum compared to CCl₄ and liquid paraffin treated rats (Group-II), indicating partial recovery from CCl₄ induced hepatotoxicity. Administration of liquid paraffin followed by ethanolic leaf extract (Group-IV) did not produce any alteration in all the parameters studied in the serum (Fig. 1). The ethanolic leaf extract when administered at a dose of 500 mg/kg thus exhibits a significant protection against CCl₄ induced hepatotoxicity in post-treatment i.e. (Group-III), and it is evident by the reduction in the activities of marker enzymes of hepatotoxicity i.e. AST, ALT, ALP and BLN in the serum when this group is compared to the CCl₄ alone treated (Group-II) rats (Fig. 1).

Seven days after daily administration of CCl₄ in liquid paraffin (Group-II) the liver tissue exhibits a significant reduction (P<0.001) in the activities of AST, ALT and ALP indicating extensive liver damage,
compared to liquid paraffin alone treated (Group-I) control rats. Post-treatment of ethanolic extract in CCl₄ pre-treated rats (Group-III), completely prevented the reduction in the activities of AST, ALT and ALP in the liver tissue, indicating an almost complete protection (about 95 to 100%) against CCl₄ induced liver damage. Administration of ethanolic extract alone (Group-IV) or liquid paraffin alone (Group-I) did not produce any alteration in the activities of above parameters in the liver tissue and they do not differ from control (Fig.2).

DISCUSSION

In view of the report that leaf extract of Cassia fistula (Linn) have been used in traditional medicinal practice to cure liver ailments like jaundice in Northern region of India, attempts have been made previously to study the hepatoprotective effect of leaf extract of Cassia fistula (Linn) and it was reported that simultaneous treatment of methanolic leaf extract of Cassia fistula (Linn) protects liver against CCl₄ induced hepatotoxicity (Bhakta et al 1999; 2001). Consequently, in this study it was considered of interest to evaluate the hepatoprotective efficacy Cassia fistula (Linn) following post-treatment in CCl₄ pre-treated rats.

CCl₄ has been shown to produce an experimental liver damage, which histologically resembles viral hepatitis, showing an elevation in the levels of indicators of liver toxicity i.e. AST, ALT, ALP and Bilirubin (BLN) (Recknagel 1983; Mahendale et al 1985). Preventive action of liver damage induced by CCl₄ has widely been used as an indicator of the liver protective activity of drugs and medicinal preparations in general. In our present investigation, rats treated with CCl₄ for 7 days developed significant hepatic damage, which was evident from a highly significant increase in the activities of AST, ALT, ALP and BLN in the serum. This observation is in agreement with the reports of Bhakta et al (1999; 2001), who has shown the elevation of these parameters in rats treated with CCl₄ for 8 weeks. In the present investigation, post-treatment of ethanolic extract of Cassia fistula (Linn) leaves reduced the elevated levels of the above parameters in CCl₄ pre-treated rats, indicating hepatoprotective effect of the extract (Fig.1). Inhibition of increased levels
of AST, ALT, ALP and BLN was also reported during simultaneous administration of methanolic leaf extract of Cassia fistula (Linn) and CCl₄ (Bhakta et al 1999; 2001). In this study, CCl₄ alone administration caused a fall in the activities of AST, ALT and ALP in the liver tissue and this adverse effect, indicating hepatotoxicity has been shown to be completely protected by post-treatment of ethanolic leaf extract (Fig.2). The post-treatment hepatoprotective effect of ethanolic leaf extract of Cassia fistula (Linn) has not been reported in the previous literature and in this regard, this preliminary study gains immense importance.

The increased levels of AST and ALT in the serum of CCl₄ pretreated rats is an indicative of cellular leakage and loss of functional integrity of cell membrane in the liver (Drotman and Lowhorm 1978). The loss of functional integrity and cellular leakage is also evident in the liver tissue, which is indicated by a fall in the levels of AST and ALT (Fig.2). Administration of ethanolic extract of Cassia fistula (Linn) for 7 days following pretreatment with CCl₄ has been shown to cause a reduction in the elevated levels of transaminases in the serum to an extent of 60-70% (Fig.1). Similarly, the above treatment caused a complete reversal of fall in the activities of transaminases to an extent of 95-100% in the liver tissue (Fig.2). It could be inferred from these results that post-treatment with the ethanolic leaf extract of Cassia fistula (Linn) could cause stabilization of liver cell membrane as well as repair of hepatic tissue damage caused by CCl₄ by unknown mechanism. This effect is an agreement with the commonly accepted view that serum levels of transaminases returned to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew 1987).

Alkaline phosphatase (ALP) is a prototype enzyme that reflects the pathological alterations in biliary flow (Plaa and Hewitt 1989). In this study, administration of CCl₄ alone caused an elevation in the activity of this enzyme in serum along with high level of serum bilirubin content (Fig.1). The extract mediated suppression of increased ALP activity with concurrent depletion of raised bilirubin level in the serum suggests the
possibility that the post-treatment of ethanolic extract is able to stabilize biliary function in the liver during hepatic injury caused by CCl₄ pre-treatment.

The present study clearly demonstrates that post-treatment of ethanolic leaf extract of Cassia fistula (Linn) could effectively prevent the experimental liver injury induced by CCl₄ pre-treatment. It is interesting to observe that the post treatment of ethanolic leaf extract of Azadirachta indica does not seem to protect the liver against CCl₄ induced hepatotoxicity in rats (Mujumdar et al 1998). In this context it would be pertinent to mention that the ethanolic leaf extract of Cassia fistula (Linn) is a better alternative and is effective in preventing well developed hepatotoxicity, indicating that this extract can be used as an effective hepatoprotective agent. However, further studies are needed to find out the possible mechanism of action of this extract and the active principle that is responsible for hepatoprotective effect.

**REFERENCE**


**Figure - 1**

Effect of post treatment of ethanolic leaf extract of Cassia Fistula (Linn) on CCl₄ induced hepatotoxicity in the serum of rats. Group - I to Group IV were treated as mentioned under "Experimental Design" of Materials and Methods section. ** p<0.01; *** p<0.001 compared to Group-I (Control). $$ p<0.01; $$$ p<0.001 compared to Group II (CCl₄ alone treated). NS-Non-Significant compared to Group-I. The values presented are mean ± S.D. of 6 nos. of animals. One-way analysis of variance (ANOVA) was performed to calculate mean variables and P value was calculated by Tukey's test. AST - Aspartate Transaminase, ALT - Alanine Transaminase, ALP - Alkaline Phosphatase, BLN - Bilirubin.
Effect of post-treatment of ethanolic leaf extract of Cassia Fistula (Linn) on $\text{CCl}_4$ induced hepatotoxicity in the liver tissue of rats. Group - I to Group - IV were treated as mentioned under "Experimental Design" of materials and methods section. ** $p<0.01$; *** $p<0.001$ compared to Group-I (Control). $$$ p<0.01$; $$$ p<0.001$ compared to Group II ($\text{CCl}_4$ alone treated). NS-Non-Significant compared to Group-I. The values presented are mean ± S.D. of 6 nos. of animals. One-way analysis of variance (ANOVA) was performed to calculate mean variables and $P$ value was calculated by Tukey's test. AST - Aspartate Transaminase, ALT - Alanine Transaminase, ALP - Alkaline Phosphatase, BLN - Bilirubin.