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Research Article
Pharmacological Evaluation of *Chrozophora tinctoria* as Hepatoprotective Potential in CCl_4 Induced Liver Damage in Rat

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Abstract

Background and Objective: *Chrozophora tinctoria* is a monoecious shrubby herb traditionally used as food supplement and in the treatment of various infectious diseases. Leaves were shown high content diterpenes, flavonoids and chromone glycosides possessing antioxidant potential. The study was designed to evaluate hepatoprotective effect of *Chrozophora tinctoria* leaves in carbon tetrachloride (CCl_4) induced liver damage in Wistar rats and to validate its traditional claims. **Materials and Methods:** Thirty Wistar rats (180-200 g) of either sex were randomly divided into 5 groups (n = 6) such as Group 1 served as the normal control (0.9% Saline); Group 2 rats were administered with CCl_4 1.5 mL kg^{-1} i.p. prepared in 20% olive oil for induction of hepatotoxicity, Group 3 and 4 rats were orally administered with the hydro-alcoholic leaves extract of *C. tinctoria* at a low dose 50 mg kg^{-1} and a high dose 100 mg kg^{-1}, respectively; Group 5 rats treated with Silymarin 25 mg kg^{-1} orally as standard drug for a period of 28 days. At the end of the study, the blood samples were collected for biochemical parameters in liver, antioxidant analysis and the liver tissue for histological analysis. **Results:** The CCl_4 treated rats developed hepatotoxicity that evident by the elevated level of AST, ALT, ALP, bilirubin, proteins, albumin and the antioxidant enzymes like CAT, SOD, GGT and GST. Treatment with hydro-alcoholic leaves extract of *C. tinctoria* 50 mg kg^{-1} shows less significant (**$p<0.05$) improvement, while the higher dose of *C. tinctoria* 100 mg kg^{-1} shows highly significant (**$p<0.05$) improvement in AST, ALT, ALP, bilirubin, proteins and albumin against disease control and almost comparable to normal and standard control. **Conclusion:** The hydro-alcoholic extract of *C. tinctoria* 100 mg kg^{-1} protect liver injuries due to the presence of potent phytoconstituents and antioxidant enzymes.

Key words: *Chrozophora tinctoria*, Hepatoprotective, antioxidant enzymes, ALT, AST

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatotoxicity is a major health problem causing rigorous morbidity and mortality in developing countries and become the hottest challenges not only health care professionals but also in drug regulatory agencies[1,2]. It was reported that the hepatic injury caused by various drugs and chemicals such as antibiotics, chemotherapeutic agents, heavy metals (CCl_4), thioacetamide, excessive consumption of alcohol and some microbes are highly responsible in young and old population[3].

Hepatotoxicity induced by CCl_4 is one of the commonest models used for the screening of hepatoprotective activities. The acute hepatotoxicity caused by the generation of free radicals that induce oxidative stress and leads to damage hepatic membrane[4]. These free radicals generate lipid peroxidation that leads to cellular damage and enhances formation of inflamed tissues that leads to necrosis or failure of the blood supply to the liver[5]. The raised serum levels of AST, ALT, lipids and protein have been attributed to the injured structural integrity of the liver because, these are cytoplasmic in nature and released into blood circulation after cellular damages[6].

Presently the allopathic medicines used for the treatment of liver disorders might be highly damaging liver tissues, whereas the herbal medicines are not causing any adverse effect and widely used for the management of human ailments science ancient time[7]. In this regards, the study mainly focused on the use of herbal medicines in the treatment of liver diseases intended for longer duration[8].

Chrozophora tinctoria is an annual, monoecious herb or undershrub belonging to family Euphorbiaceae. The plant is widely distributed to dry waste spaces found in Europe, Africa and Asia. Leaves consist of sessile and peduncle stellate or lepidote hairs[9]. Leaves contains biflavones, such as amentoflavone in addition to many flavonoids including apigenin, rutin, quercetin and acacetin possessing antioxidant and wound healing effects[10]. Naturally available flavonoids and their derivative have played important role as an antioxidant potential effectively controlling tumors and cell differentiation, thus acting as protective control over various organs. This study was designed for investigation of the hepatoprotective potential of *Chrozophora tinctoria* leaves extract in CCl_4-induced hepatotoxicity in the rat to evaluate their protective effect and explain the mechanism underlying these properties.

MATERIALS AND METHODS

Plant materials: The *C. tinctoria* plant has been collected in the month of May-June from the district of Ambedkar Nagar, Uttar Pradesh, India. Fresh, shade dried leaves have been collected accordingly and authenticated by Botanical Survey of India, northern regional center 192, Kaulagarh Road, Dehradun, the plant Acc. Number is 114546.

Experimental animals: Healthy Wistar rats having weight 150-250 g of either sex were selected for the study. The animals were kept in a well-ventilated animal house under natural conditions with 12 h in light and dark cycle at 25±2°C room temperature and followed by acclimatization for a period of one week by maintaining standard environmental conditions and fed with standard pellet diet and water *ad libitum*. The experiment was carried out after IAEC clearance (Approval No: HIPER/IAEC/18/18/04) followed by CPCSEA guidelines.

Chemicals and reagents: Chemicals and reagents were used in the study such as ethanol, chloroform, olive oil, formaldehyde, sodium phosphate (monobasic and dibasic), 0.9% normal saline, carbon tetrachloride, Dragendorff's reagent, concentrated hydrochloric acid, sodium hydroxide, copper sulfate solution, Ninhydrin's solution, alpha-naphthol, ferric chloride solution, ethyl acetate, acetic anhydride, potassium hydroxide procured from departmental store. The standard drug used in the experiment was Silymarin purchased from medical shope[11].

Preparation of extract: The extract of *Chrozophora tinctoria* was prepared by using hydro-alcoholic (60% water and 40% methanol) solvent was used for cold maceration method. The shade dried leaves crushed and blended into smaller pieces to enhance the penetration of the extracting solvents into the plant cells[12]. An accurate weight of 200 g powdered leaves was poured into 1000 mL prepared solvent in a conical flask with gentle shaking for 72 h after that filtered using a muslin cloth and then filtered again using suction pressure with the aid of a vacuum pump. The collected extract was dried by using rotatory evaporator at a temperature of 45±5°C until the entire solvent gets evaporated. The collected dried extract powder was stored in a well tightly closed container for phytochemical and pharmacological screening[13,14].

Experimental design: Thirty male rats were randomly divided into five groups (n = 6). Group 1 served as the normal control and orally administered with saline 0.9%; Group 2 served as disease control and administered with CCl_4 at a single dose of 1.5 mL kg^{-1} b.wt., i.p. prepared in 20% olive oil for induction of hepatotoxicity, Group 3 and 4 rats were orally treated with hydro-alcoholic leaves extract of *C. tinctoria* at a dose 50 and 100 mg kg^{-1}, respectively; Group 5 served as standard control and orally treated with Silymarin 25 mg kg^{-1} for a period of 28 days. Estimation of average body weight, liver weight, biochemical parameters and anti-oxidant potential were carried out[15].

Histology of liver: The rats were sacrificed at the end of the study and the liver of each animal were isolated and preserved in 10% formalin for biopsy. The liver pieces were processed under automatic tissue processing unit[16]. Tissues were cut using a microtome to get the thin section of 5 µm. The sections were taken on a micro slide on which egg albumin i.e., a sticking substance was applied. The sections were allowed to remain in an oven at 60°C for 1 h. Paraffin melts and egg albumin denatures, thereby fixing of tissue to slide. Eosin and Haematoxylin dyes were used for staining of liver sections[17].

Statistical analysis: All the data were expressed as Mean±SEM followed by one way ANOVA with Bonferroni's multiple comparison tests (n = 6). $^{\#}p<0.05$ represent statistical significance against normal control, while $^{**}p<0.05$ statistical significance against disease control and NS shows non-significance comparison between the groups.

RESULTS

Phytochemical investigation: In order to identify chemical constituents of the extracts preliminary phytochemical tests were performed on the leaves extract. Preliminary qualitative tests were carried out to evaluate the presence or absence of various phytoconstituents in *C. tinctoria* leaves, which showed the presence of phenols, amino acids, proteins, fatty acids, carbohydrates, alkaloids, sterols, flavonoids, glycosides and saponins (Table 1).

Pharmacological investigation: Physical parameter: The animal body weight were measured for all groups and the liver was excised after scarification of animal then liver weight also measured for all group of animals at the end of the study (Table 2).

Table 1: Phytochemical investigation of extracts

Phytoconstituents	Name of the test	Inference
Phenols	Ferric chloride test	+++
Amino acids	Biuret test	+++
Proteins	Biuret test	+
Fatty acids	Spot test	++
Carbohydrates	Fehling test	+
Alkaloids	Dragendorff's test	++
Sterols	Liebermann-Burchards test	+++
Flavonoids	Shinoda test	+++
Glycosides	Legal's test	+
Saponins	Foam test	++

+++: Highly present, ++: Moderately present, +: Slightly present

Table 2: Estimation of average body weight and liver weight at the end of study

Group descriptions	Body weight (g)	Liver weight (g)
Normal control (Saline 0.9%)	194.93±7.48	4.11±0.13
Disease control (CCl_4 1.5 mL kg^{-1})	174.84±8.56	5.43±0.23
CCl_4 + *C. tinctoria* 50 mg kg^{-1}	191.54±7.98	4.47±0.23
CCl_4 + *C. tinctoria* 100 mg kg^{-1}	176.90±7.87	4.70±0.15
CCl_4 + Silymarin 25 mg kg^{-1}	185.86±6.71	4.37±0.07

Values are given as Mean±SEM of animal groups (n = 6) and denoted in gram

The observed body weight and liver weight at the end of study did not show any significant changes as compared to disease group and normal control group. Treatment with higher dose of *C. tinctoria* leaves extract (100 mg kg^{-1}) shows the average body weight (176.90±7.87 g) and liver weight (4.70±0.15 g) that was rated as good indication of hepatoprotective activity (Table 2).

Biochemical parameters: Serum samples collected from different groups were analyzed for the evaluation of the elevated level of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphate (ALP). The estimation of liver function tests such as ALT, AST and ALP in serum sample showed that the level of ALT and AST significantly ($^{\#}p<0.05$) increases after the administration of CCl_4 (1.5 mL kg^{-1}), while the ALP level observed was significantly decreased as compared to normal control. The treatment groups show the statistically significant ($^{**}p<0.05$) improvement in these elevated parameters at the end of the study. The high dose of *C. tinctoria* 100 mg kg^{-1} showed highly significant improvement in the reported level and observed that slightly increased this level as compared to disease control and other treatment groups including standard control (Table 3).

The estimation of total bilirubin and total protein in experimental groups reveals that the i.p. administration of a

Table 3: Estimation of liver function test ALT, AST and ALP in the serum sample of rats

Group descriptions	ALT (IU L^{-1})	AST (IU L^{-1})	ALP(IU L^{-1})
Normal control (Saline 0.9%)	27.50±3.80	104.67±15.86	275.17±56.60
Disease control (CCl_4 1.5 mL kg^{-1})	41.67±7.74#	194.50±19.39#	206.00±48.29#
CCl_4+*C. tinctoria* 50 mg kg^{-1}	27.50±4.78**	150.17±41.95**	229.50±27.55**
CCl_4+*C. tinctoria* 100 mg kg^{-1}	35.67±5.91**	141.50±26.69**	247.50±70.04**
CCl_4+Silymarin 25 mg kg^{-1}	25.17±3.13**	100.33±19.92**	194.67±49.18**

Values are given as Mean±SEM of animal groups (n = 6), #p<0.05 statistical significance against normal control and **p<0.05 statistical significance against disease control

Table 4: Estimation of total bilirubin, protein and albumin in experimental groups

Group descriptions	Total bilirubin (mg dL^{-1})	Total protein (g dL^{-1})	Albumin (g dL^{-1})
Normal control (Saline 0.9%)	0.180±0.040	7.880±0.190	4.28±0.21
Disease control (CCl_4 1.5 mL kg^{-1})	1.050±0.115#	3.450±0.305#	2.33±0.18#
CCl_4+*C. tinctoria* 50 mg kg^{-1}	0.210±0.092**	6.620±0.322**	3.65±0.16**
CCl_4+*C. tinctoria* 100 mg kg^{-1}	0.140±0.040**	5.480±0.450**	3.88±0.20**
CCl_4+Silymarin 25 mg kg^{-1}	0.170±0.040**	6.630±0.270**	3.70±0.19**

Values are given as Mean±SEM of animal groups (n = 6), #p<0.05 statistical significance against normal control and **p<0.05 statistical significance against disease control

Table 5: Estimation of antioxidant enzyme SOD, CAT, GGT and GST in liver homogenate

Group descriptions	SOD (U mg^{-1} of protein)	CAT (U mg^{-1} of protein)	GGT (nmol min^{-1})	GST (nmol min^{-1})
Normal control (Saline 0.9%)	4.52±0.40	35.62±3.92	23.16±2.70	114.26±5.69
Disease control (CCl_4 1.5 mL kg^{-1})	1.83±0.13#	22.24±2.01#	26.71±2.00	90.92±4.02#
CCl_4+ *C. tinctoria* 50 mg kg^{-1}	2.86±0.15	29.99±2.01	26.85±2.26	96.68±4.00
CCl_4 + *C. tinctoria* 100 mg kg^{-1}	3.54±0.35**	34.01±2.69**	24.04±2.65**	106.96±5.36**
CCl_4 + Silymarin 25 mg kg^{-1}	3.07±0.23**	33.48±2.58**	26.27±2.91	107.05±5.86**

Values are given as Mean±SEM of animal groups (n = 6), #p<0.05 statistical significance against normal control and **p<0.05 statistical significance against disease control

single dose of CCl_4 1.5 mL kg^{-1} significantly (#p<0.05) increases the level of total bilirubin (1.050±0.115 mg dL^{-1}) and significantly (#p<0.05) decreases the level of total protein (3.450±0.305 g dL^{-1}) compared to normal control. The treatment groups show the statistically significant (**p<0.05) improvement in these elevated parameters at the end of the study. The high dose of *C. tinctoria* 100 mg kg^{-1} showed highly significant improvement in the reported level and observed that approximately comparable as compared to normal control and standard control (Table 4).

Antioxidant parameter: The estimation of the antioxidant enzyme such as SOD, CAT, GGT and GST in liver homogenate showed that the values of this parameter significantly (#p<0.05) decrease after administration of CCl_4. The treatment and standard groups show statistically significant (**p<0.05) improvement as compared to disease control. The standard control animal shows non-significant (NS) improvement in GGT level, while the low dose of *C. tinctoria* 50 mg kg^{-1} also indicated non-significant (NS) improvement in the antioxidant enzymes as compared to disease control. The animal treated with a high dose of extract *C. tinctoria* 100 mg kg^{-1} indicated the approximately comparable improvement in the elevated parameters as normal control (Table 5).

Histological investigation: The histological analysis of the liver tissues shows that the normal control group presenting normal hepatic cells, well-preserved cytoplasm, prominent nucleolus and well brought out central hepatic veins. Whereas the CCl_4 intoxicated rats have shown the fatty degeneration of hepatocytes, hepatic cell necrosis, portal tract fibrosis and presence of a fatty cyst, congestion in sinusoids of the globule. The animals treated with standard drug Silymarin (25 mg kg^{-1}) showed the similar hepatic architecture as compared to normal control.

The animal treated with hydro-alcoholic extract of *C. tinctoria* (50 mg kg^{-1}) exhibited significant liver protection against the toxic substance as evident by normal lobular pattern with a mild degree of fatty change, absence of necrosis and lymphocyte infiltration mild comparable to the standard and control and *C. tinctoria* (100 mg kg^{-1}) showed the more effective and cleared biopsy than low dose and almost comparable to the standard and control group of animal. However, accumulation of fatty lobules, necrosis and scattered lymph mononuclear (LMN) cell infiltrate in hepatic parenchyma were noticed with hydro-alcoholic extract of *C. tinctoria* treatment group (Fig. 1).

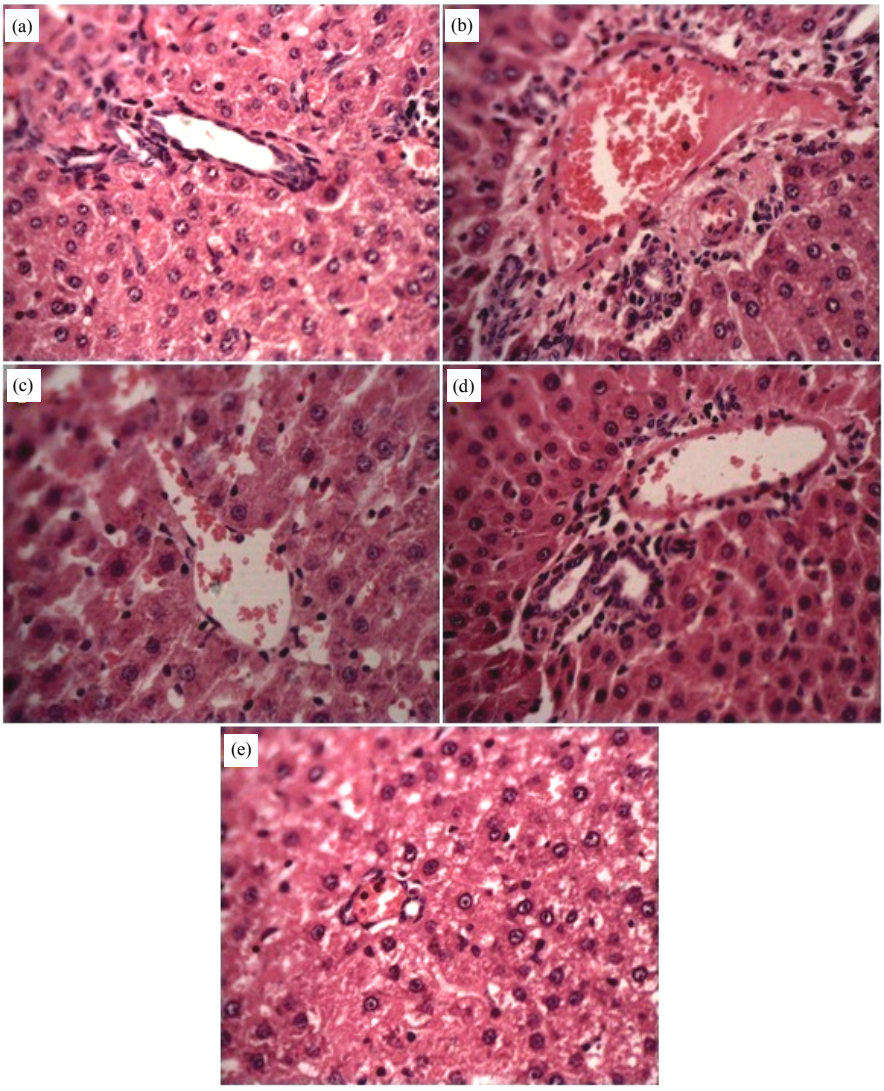

Fig. 1(a-e): Histological section of the rat's liver shows in different experiment group, (a) Normal control (Saline 0.9%), (b) Disease control (CCl_4 1.5 mL kg^{-1}), (c) Treatment with CCl_4+*C. tinctoria* 50 mg kg^{-1}, (d) Treatment with CCl_4+*C. tinctoria* 100 mg kg^{-1} and (e) Standard control (CCl_4+Silymarin 25 mg kg^{-1})

DISCUSSION

The hepatotoxicity has been developed from the biotransformation of CCl_4 through the enzyme cytochrome P450 2E1 in the direction of the trichloromethyl free radical ($^{\bullet}CCl_3$) generation. Further the alteration of this free radical into a highly reactive species (CCl_3O_2.) through a reaction with an oxygen molecule[18]. Trichloromethyl peroxy radical covalently binds to macromolecules of cells and leads to the formation of a polyunsaturated fatty acids chain reaction in the cytoplasmic membrane phospholipids, inducing functional and morphological changes in the cell membrane and leads to cell necrosis[19].

Hepatocytic damage is analyzed by different hepatic enzymes markers such as ALT, AST, ALP and the levels of bilirubin, protein and albumin. While liver cells injured, these enzymes leak into the bloodstream and create markedly elevated serum levels[20]. Both ALT and AST are associated with liver parenchymal cells, where ALT is a more specific indicator of liver intoxication as levels of AST, while the serum ALP and bilirubin levels are related to the functions of the hepatic cell[21]. This experiment reported that the CCL_4 induced significant and comprehensible liver necrosis evidenced by increased levels of hepatic enzymes marker (ALT, AST and ALP) and the levels of total bilirubin, whereas levels of total protein and albumin were decreased due to liver injury.

The study also evaluated the effect of *C. tinctoria* leave extract on hepatic antioxidant enzymes such as SOD, CAT, GGT and GST levels that was impaired due to the oxidative stress caused by CCl_4. An important mechanism involved in the protection against CCl_4-induced liver damage is the inhibition of excessive ROS production. The MDA is widely used as a marker of lipid peroxidation and a major parameter for the status of oxidative stress[22]. The hepatic MDA level increases under the enhancement of oxidative stress in a rodent model[23]. Treatment with extract significantly reduced the CCl4-induced hepatic MDA elevation. Thus *C. tinctoria* provides a protective effect against CCl_4-induced liver damage in terms of preventing lipid peroxide formation and blocking oxidative chain reaction[24,25].

The above inferences were further confirmed by histopathological studies which showed that the administration of CCl_4 caused severe liver damage characterized by hepatic cell necrosis, ballooning degeneration, fatty changes or inflammatory cell infiltration and other histological manifestations, which were consistent with previous findings[26,27]. After treatment with *C. tinctoria* extract hepatic injury was significantly improved and the structure of hepatocytes was almost restored to the normal level.

The study observed that the *C. tinctoria* have a high total phenolic, flavonoid, saponins and tannins, which exert antioxidant and anti-inflammatory activities. Higher total phenolic content has been known to contribute to the antioxidant activity of extracts, while antioxidant activity has been linked to the hepatoprotective effect[28]. Hepatoprotective potential of *C. tinctoria* might be due to the presence of phenols, amino acids, proteins, fatty acids, carbohydrates, alkaloids, sterols, flavonoids, glycosides and saponins which was determined during phytochemical screening. Flavonoids have been reported to exhibit antioxidant, anti-inflammatory and hepatoprotective activities[29-31]. Furthermore, tannins have been suggested to possess free radical scavenging and antioxidant, anti-inflammatory and hepatoprotective activities[32], while saponins have been also reported to exhibit hepatoprotective activity via modulation of its antioxidant[33] and anti-inflammatory activities[34].

CONCLUSION

The result revealed that the biochemical analysis like AST, ALT, ALP, bilirubin, protein and albumin shows statistically significant (**$p<0.05$) improvement in the elevated values after administration of *C. tinctoria* leave extract 100 mg kg^{-1}. Histopathological study on the basis of the result it was observed that the higher dose administration of *C. tinctoria* liver architecture more effective and almost comparable to the standard and control. However, accumulation of fatty lobules (steatosis), necrosis and scattered lymph mononuclear (LMN) cell infiltrate in hepatic parenchyma were noticed. Finally, it is concluded that high dose of hydroalcoholic extract of *C. tinctoria* (100 mg kg^{-1} b.wt.,) was more effective and low dose also give hepatoprotective effect but it shows less effective in comparison to standard and control group.

SIGNIFICANCE STATEMENT

Chrozophora tinctoria is highly used as colouring agent for food and beverage, especially for jellies. It is a diverse array of active phytoconstituents (phenols, amino acids, proteins, fatty acids, carbohydrates, alkaloids, sterols, flavonoids, glycosides and saponins) that able to target several mechanisms in which phenolics, flavonoids, saponins and tannins exert anti-oxidant and anti-inflammatory activities. The uses of hydro-alcoholic extract of *C. tinctoria* play a significant protective role against chemically induced hepatotoxicity.

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