

HEPATOPROTECTIVE ACTION OF *CORDIA DICHOTOMA* AGAINST CARBON TETRACHLORIDE INDUCED LIVER INJURY IN RATS

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Key words: *Cordia dichotoma* Linn., Boraginaceae, carbon tetrachloride; serum enzyme levels; antioxidants; hepatoprotective activity.

ABSTRACT

The methanolic extract of the leaves of *Cordia dichotoma* at 300 mg/kg and 500 mg/kg were studied for hepatoprotective action in male Wistar rats by inducing liver damage using carbon tetrachloride (CCl₄). Silymarin was used as standard drug. The extract (300 mg/kg) significantly reduced the AST (P<0.001), ALT (P<0.001) and TBR levels (P<0.01) and at 500 mg/kg dose significantly reduced the AST (P<0.001), ALT (P<0.001), TBR (P<0.01) and lipid peroxide levels (P<0.05). The antioxidant parameters like glutathione and total antioxidant levels increased considerably although they were statistically insignificant. The hepatoprotective activity of *C. dichotoma* was comparable with silymarin (100mg/kg).

INTRODUCTION

Liver is the seat of xenobiotic metabolism. Various agents like drugs, alcohol, viruses and many other toxic agents damage the cells of liver. Since ancient periods many herbal medicines were used for treating the liver diseases. In modern medical practice also various herbal-based drugs like Silymarin from *Silybum marianum* and Phyllanthins which are polyphenols from *Phyllanthus amarus* have been successfully used [1]. Hence it becomes essential to explore the plant kingdom for development of new phytotherapeutic agents for liver diseases.

C. dichotoma Linn. (Boraginaceae) is an evergreen tree widely distributed in India and Srilanka [2]. It exhibits juvenomimetic [3] antifertility [4] and anti-inflammatory activities [5]. Traditionally it was being used as an astringent, anthelmintic, diuretic, demulcent, anti-diabetic and expectorant. The leaves of *C. dichotoma* are traditionally used for treatment of jaundice at Dandakaranya area, Andhra Pradesh in India.

The objective of the present study is to evaluate the protective effect of MCD against the toxicity of CCl₄ in rodents.

RESULTS

The effect of the extract on Aspartate amino transferase (AST), Alanine amino transferase (ALT), Alkaline phosphatase (ALP), serum bilirubin(TBR), glutathione, lipid peroxide(MDA) and total antioxidants levels in CCl₄ induced liver damage in rats are summarized in Table 1. There was a significant increase in AST, ALT, ALP, serum bilirubin and lipid peroxide levels in the test group and a sharp decrease in Glutathione (GSH), total antioxidants (TAO) levels in rats treated with CCl₄ [6] alone when compared to normal rats. These parameters were positively altered on treatment with extract at 300 and 500mg/Kg rat. The therapeutic effects exhibited by these extracts were comparable to standard drug Silymarin (100 mg/kg).

Table 1. Effect of methanolic extract of leaves of *Cordia dichotoma* on serum enzymes Aspartate amino transferase (AST), Alanine amino transferase (ALT), and Alkaline phosphatase (ALP), Total Bilurubin(TBR), Glutathione (GSH), lipid peroxide (MDA) and total antioxidants (TAO) levels in CCl₄ induced liver damage in rats.

| Groups | AST(U/L) | ALT(U/L) | ALP(KAU/ml) | TBR(mg %) | MDA(nM/ml) | GSH(μM/ml) | TAO(μM/ml) |
|----------|-----------------|----------------|----------------|---------------|----------------|-------------------|----------------|
| Normal | 37.48(± 3.05) | 53.38(±8.52) | 83.63(±19.54) | 0.26(±0.04) | 1.12(± 0.16) | 149.7(± 42.58) | 18.13(± 3.52) |
| Control | 60.72 (± 2.55)* | 82.59 (±1.90)* | 128.2 (±2.94) | 1.29 (±0.02)* | 2.54 (± 0.45)* | 112.8 (± 15.59)** | 13.82(±0.77)** |
| Standard | 43.48 (± 1.13) | 58.46 (±1.80) | 89.41 (±20.17) | 0.66 (±0.04) | 1.41 (± 0.38) | 144.2 (± 4.20) | 17.93(±3.50) |
| MCD300 | 52.01 (± 1.25)* | 68.85 (±1.12)* | 114.1 (±5.77) | 0.79 (±0.50)* | 2.10(± 0.48) | 132.4 (± 6.96) | 15.22(±0.50) |
| MCD500 | 47.15 (± 1.08)* | 63.86 (±0.67)* | 102.5 (±23.33) | 0.70 (±0.30)* | 1.98 (± 0.26)* | 139.4 (± 8.76) | 16.99 (± 1.01) |

Values are mean ± S.E.M. number of rats=6. Control group compared with normal group * p<0.001. Experimental groups compared with CCl₄ control group ** p<0.05.

The AST levels were increased to 60.72 U/L in CCl₄ treated rats and are considerably reduced on treatment with the extract at 300mg/Kg rat (52.01U/L) and 500mg/kg rat (47.15 U/L). A significant reduction on (p<0.001) was observed in AST levels when compared with toxic group. Dose dependency was exhibited by the extracts. The ALT levels of the rats treated with CCl₄ were found to be increased (82.59U/L) and there was a significant reduction to 68.85 U/L and 63.86 U/L by the extract at 300 and 500mg/kg respectively.

The raise in ALP levels due to induction of hepatotoxicity by CCl₄ were reduced, but the values were found to be statistically insignificant (p>0.05). The serum bilirubin levels of hepatotoxicity-induced rats (1.29 mg% units) were considerably reduced on treatment at both 300 (0.79 mg% units) and 500mg/kg (0.70 mg% units). The observed decrease in glutathione and total antioxidant levels due to induction of hepatotoxicity by CCl₄ were increased, but the values were found to be statistically insignificant (p>0.05).

The plant extract also exhibited satisfactory antioxidant property by reducing the lipid peroxides in CCl₄ induced hepatotoxic rats. The reduction in lipid peroxide level was found to be statistically significant (p<0.05) at 500mg/kg but insignificant (p>0.05) at 300.

DISCUSSION

The hepatotoxicity induced by CCl₄ is due to its metabolite CCl₃•, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum [7]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by carbon tetrachloride administration was observed by recording AST, ALT, ALP and serum bilirubin levels in treated, toxic and normal groups because serum transaminases, serum alkaline phosphatase and serum bilirubin have been reported to be sensitive indicators of liver injury [8]. The level of lipid peroxidase is a measure of membrane damage and alterations in structure and function of cellular membranes. The level of thiobarbituric acid reactive substance (TBARS) is an indirect measurement of lipid peroxidation. Lipid peroxide levels in tissue were found to be significantly elevated in CCl₄- challenged rats [9]. GSH is one of the most abundant tripeptide non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a peroxidation in a liver homogenate can proceed in a non-enzymatic way [10].

In our present study, the CCl₄ treated group exhibited increased serum enzyme levels. This indicated the damage of hepatocytes by CCl₄. There was a decrease in GSH and TAO levels which may be due to the oxidative stress induced by CCl₄. The reversal of serum enzyme levels would indicate that damaged hepatocytes were repaired and supported their regeneration.

The hepatoprotective properties of Silymarin have been related to the inhibition of lipid peroxide formation or scavenging of free radicals generated by microsomal ethanol oxidations. The extract

of *C. dichotoma* also produced hepatoprotective activity similar to silymarin and so its action may also be due to inhibition of lipid peroxide formation. It also increased the total antioxidant levels to some extent. The hepatoprotective action combined with antioxidant activity may have a synergistic effect in preventing the initiation and progress of hepatocellular diseases [11].

MATERIALS AND METHODS

Plant Material

The leaves of *C. dichotoma* were collected from the campus of Kakatiya University, Warangal District, Andhra Pradesh, India. They were identified by Dr. Raju S. Vastavaya, Department of Botany, Kakatiya University, Warangal. Voucher specimens are being maintained in the herbarium (No.PLB-047) of University College of Pharmaceutical Sciences, Kakatiya University, Warangal. Fresh leaves were washed with tap water to make them free of dust and later dried under shade for 4 days. Dried leaves were ground to coarse powder and stored in an airtight container.

Extraction

The methanolic extract of *C. dichotoma* (MCD) was prepared by the maceration of leaf powder (1 Kg) with methanol (3L) at room temperature in a round bottom flask for 7 days with intermittent stirring. Then filtered to collect the extract and concentrated under reduced pressure using a rotary flash evaporator. The extract obtained was preserved in a desiccator to prevent degradation by moisture.

Drugs and Chemicals

Silymarin was obtained as a gift sample from Micro Labs (Hosur, Tamilnadu, India). ALT test kit, AST test kit, ALP test kit (Asclepius Immunotek Private Limited, Hyderabad, India), bilirubin test kit (M/s Excel Diagnostics Pvt. Ltd. Hyderabad, India), thiobarbituric acid (Hi Media Laboratories Ltd; Mumbai, India), 1, 1, 3, 3-tetraethoxy Propane (Sigma, St. Louis, USA), trichloro acetic acid (Qualigens Fine chemicals, Mumbai, India), 5-5'-Dithiobis-2-Nitro benzoic acid (Hi Media Laboratories Ltd, Mumbai, India), sodium dihydrogen Phosphate (S.D. Fine chemicals; Mumbai, India), glutathione (Hi Media Laboratories Ltd; Mumbai, India), ascorbic acid (S.D. Fine chemicals; Mumbai, India), diphenyl picryl hydrazyl (Sigma, St. Louis, USA) are various chemicals and reagents which were used in different stages of the study. They were used as received without any further purification.

Animal

Studies were carried out using male Wistar albino rats (180–220g). They were obtained from the animal house of Mahaveera Enterprises (Reg. No.146/1999/CPCSEA), Ranga Reddy District, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 °C) with dark and light cycle (12/12 h). They were allowed free access to standard dry pellet diet and water *ad libitum*. All procedures described were reviewed and approved by the Institutional animal ethical committee.

Animal grouping

Healthy albino rats were divided into 5 groups each containing six animals. Group 1, which served as normal, received 10 ml / kg body weight, orally (p.o) of 5% w/v gum acacia in water daily for seven days. Group 2 served as toxic and they received 1.5 ml/kg body weight of 25% v/v CCl₄ in olive oil on seventh day only. Group 3 received silymarin 100 mg/kg body weight of rats per day, Group 4 and 5 received MCD (300 and 500 mg/kg body weight) daily p.o for seven days respectively.

On the seventh day, 25%v/v CCl₄ in olive oil was given by oral route at a dose of 1.5 ml/kg body weight 30 minutes after the administration of silymarin and extracts. After 36 hours of administration of CCl₄ the rats were anaesthetized with thiopentone sodium (60 mg/ kg body weight i.p) and the blood samples were collected from common carotid artery by carefully opening the neck region.

Biochemical Studies

The blood samples were allowed to coagulate at room temperature for at least one hour. Serum was separated by centrifugation at 3000 rpm for 20 minutes and analyzed for AST, ALT, ALP, serum bilirubin, Glutathione, lipid peroxide and total antioxidants levels [6,12-16].

Estimation of AST Levels

Into a clean dry test tube, 0.25 ml of buffered substrate aspartate keto-glutarate (AKG) was taken and incubated at 37°C in a water bath for 30 minutes. Then 0.25 ml of dinitrophenyl hydrazine (DNPH) colour reagent was added, mixed well and allowed to stand at room temperature for 20 minutes. To this, 0.05 ml of serum was added and then 2.5 ml of 0.4 NaOH solution in water was added, mixed well and allowed to stand at room temperature for further 10 minutes for complete development of brown colour. Optical density of the solution was read in a spectrophotometer at 505 nm against blank.

Estimation of ALT levels

Into a clean dry test tube, 0.25 ml of buffered substrate Alanine ketoglutarate (AKG) was taken and incubated at 37°C in a water bath for 30 minutes. Then 0.25 ml of DNPH reagent was added, mixed well and allowed to stand at room temperature for 20 minutes.

To this, 0.05 ml of serum was added and then 2.5 ml of 0.4 M NaOH solution in water was added, mixed well and allowed to stand at room temperature for further 10 minutes for complete development of brown colour. Optical density of the solution was read in a spectrophotometer at 505 nm against blank. ALT levels were obtained by extrapolation from the standard curve and expressed in Karamen units (KU) per dl.

Estimation of Serum Alkaline Phosphatase levels

Into a clean dry test tube, 1.0 ml each of buffered substrate (phenyl phosphate) and distilled water were taken, mixed well. To this, 0.1ml of serum was added, mixed well and incubated at 37°C for 15 minutes in the water-bath. Then 2.0 ml of colour reagent was added and thoroughly mixed. Corresponding to each of the test samples, a control is run in which serum is added to the contents after the

incubation period, all other additions being the same. A standard was run in the same way as for test in which 1.0 ml of 1% phenol standard was added in place of serum. Similarly, a blank was run to which buffered substrate, distilled water, and colour reagents were only added. Optical densities of blank, standard, control and test were measured at 510 nm in spectrophotometer against distilled water. Serum alkaline phosphatase activity was calculated based on the following formula and expressed in Kind and Anderson units per ml.

Serum Alkaline Phosphatase activity,

$$KA \text{ U/ml} = \frac{(O.D. \text{ test} - O.D. \text{ control})}{(O.D. \text{ S tandard} - O.D. \text{ blank}) \times 10}$$

Estimation of Total Serum Bilirubin

Into a clean test tube, 1 ml of Diazo A and 0.1 ml of Diazo B reagents, 1.0 ml of activator, 2.5 ml of distilled water and 0.2 ml of serum were added (test solution). Corresponding to each test a control was run in the same way, the only difference being Diazo B was not added. The tubes were mixed well and kept in dark for 5 minutes. The optical density of test and control was read at 540 nm in a spectrophotometer against distilled water. Similarly the optical density of the artificial standard was read against distilled water. Serum bilirubin levels were calculated based on the following formula and expressed in mg per ml.

Total Serum Bilirubin in mg% =

$$\frac{\text{Optical density of test} - \text{Optical density of control}}{\text{Optical density of artificial standard}} \times 10$$

Estimation of Lipid Peroxides

The amount of lipid peroxidation products present in the serum samples/pleural fluid was estimated by the thiobarbituric acid reactive substances (TBARS) method, which measures the malondialdehyde (MDA) reactive products by using High Pressure Liquid Chromatography (HPLC). To 0.5 ml of serum/pleural fluid 0.5 ml of 30% trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant 50 µl of 1%TBA solution was added and the solution was heated for 1hr at 98°C. 20 µl of the mixture, which is pink in colour, was injected into HPLC.

The HPLC Conditions are Mobile phase :Methanol: Water (70:100) containing 250 µl of H₃PO₄ with 80 nM of NaOH; Column : Altec C18 (25 cm length, 4.6 mm diameter, 5 µsize); Wavelength was fixed at λ_{max} 540 nm. The temperature : Ambient; Flow rate : 1ml/min.; Injection volume : 20µl. Standard graph was plotted using TEP (1, 1, 3, 3-tetra ethoxy propane).

Estimation of Glutathione

Glutathione forms a coloured complex with DTNB, which is measured spectrophotometrically. To 0.5 ml of citrated blood, 0.5 ml of 5% TCA solution was added to precipitate the proteins

and centrifuged at 3000 rpm for 20 minutes. To 0.1 ml of supernatant, 1 ml of sodium phosphate buffer and 0.5 ml of DTNB reagent were added. The absorbance of the yellow colours developed was measured at λ_{\max} 421 nm. The glutathione content was determined from standard graph by using pure glutathione.

Estimation of Total Antioxidant Status

For the estimation of total antioxidant status, we used a stable free radical α, α -diphenyl- α -picryl hydrazyl (DPPH), at the concentration of 0.2 mM in methanol. 0.1 ml of serum / pleural fluid was deproteinated by the addition of 1 ml of methanol, vortexed for 30 sec. Then centrifuged at 3000 rpm for 30 minutes to separate the proteins. To the clear supernatant 1.5 ml of methanol and 0.5 ml of DPPH solution were added, mixed thoroughly and absorbance was read at λ_{\max} 517nm against blank, prepared in an identical way but without the addition of serum / pleural fluid. Ascorbic acid was used as a reference standard. The standard graph was plotted using different concentrations of ascorbic acid and the antioxidant status values were expressed in terms of nM of ascorbic acid. Standard graphs were also prepared with known concentrations for tests where.

Statistical analysis

The results obtained were statistically analyzed by Student's T-test followed by Newman-Keul's multiple comparison tests.

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