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Mechanistic Insights into the Cytoprotective Effects of 3, 4, 5-Trihydroxybenzoic Acid on Carbon Tetrachloride-Induced Liver Injury

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Abstract

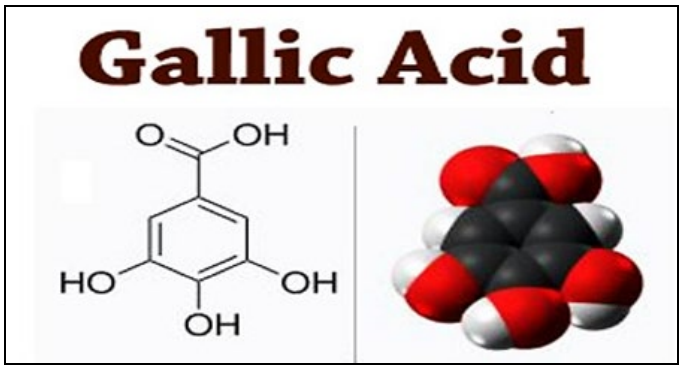
The hepatoprotective activity of the alcoholic extract of *Terminalia belerica* (TB) and its active principle i.e. 3, 4, 5-trihydroxybenzoic acid: Gallic acid (GA) has been studied against carbon tetrachloride induced liver toxicity. Carbon tetrachloride (1.5 ml/kg *i.p.*) caused significant increase in the activities of serum alkaline phosphatase and serum transaminases. Serum protein content elevated significantly after CCl_4 administration. A significant increased was observed in hepatic lipid peroxidation while a considerable fall was found in reduced glutathione after toxicant administration. These altered parameters were significantly restored with the therapy of active principle (200 mg/kg *p.o.*) whereas extract therapy (400 mg/kg *p.o.*) comparatively less effective. The effects of therapeutic agents on BSP clearance test indicated its protective role. Carbon tetrachloride caused severe degeneration, disturbed chord arrangement and focal necrosis in hepatocytes were also seen. Recoupment were seen in histological alterations by therapy with extract and active principle but the degree of protection conferred by active principle was more as compared to extract. The extract *per se* and active principle *per se* did not show any sign of toxicity.

Keywords: *Terminalia belerica* (TB), carbon tetrachloride, Active principle (AP).

Introduction

Herbal medicines have been used since the down of civilization to maintain health and to treat diseases. The World Health Organization estimates that about three quarter of the world's population currently use herbs and other forms of traditional medicines to treat their diseases (Kuruvilla, 2002). In the present world scenario, herbal medicines are gaining popularity in day to day life because they are cheap, easily available, and have no side effects (Rai, 1994). Ayurveda, Siddha and Unani systems of medicine are widely used in India. Faith in these traditional systems of medicine is related to cultural practices and beliefs. Inspite of the wide spread interest, knowledge regarding the scientific basis of the use of the herbal medicines is not known to large majority of physicians and user of these preparations. *Terminalia belerica* Roxb. (Combretaceae, 'Bahera' TB) is distributed throughout the forest of India. Among the various medicinal properties attributed to its significance, one is its therapeutic value is the treatment of liver disorders and digestion (Nandkarni, 1954). The fruit of TB is reported to have purgative (Chakravarti and Tayal, 1947) cardiac depressant, hypotensive and choleretic effects (Siddiqui, 1967). It is one of the ingredients of ayurvedic purgative medicament Triphala. Gallic acid (3,4,5-trihydroxy benzoic acid) is an active principle of *Termialia belerica* Roxb.

Therefore present investigation aims in identifying the hepatoprotective potential of *Terminalia belerica* and comparison with its active principle on experimental liver injury induced by carbon tetrachloride.



3, 4, 5 – trihydroxybenzoic acid (Gallic acid)

Materials and Methods

Preparation of the Extract: Fruits of *Terminalia belerica* were procured from authenticated ayurvedic dealer and were identified by the taxonomist of Botany Department of Jiwaji University, Gwalior. A voucher specimen (No. 336) has been deposited in Herbarium of Jiwaji University, Gwalior. Fruits were dried, chopped and ethanolic extract was prepared (17.6% w/v). An aqueous suspension of crude extract in 2% gum acacia was be administered to the animals orally.

Chemicals: Active principle of *Terminalia belerica i.e* gallic acid was procured from Sigma Aldrich, carbon tetrachloride was procured from Ranbaxy and other chemicals used in the study were procured from Sigma Aldrich and E- Merck.

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Animals: Female albino rats of *Sprague Dawley* strain (130±10 g b.w.) were used for hepatoprotective studies. Animals were housed under standard conditions (25±2 °C, 60%-70% relative humidity and 14 h light and 10 h dark. Animals were fed on standard pellet diet (Hindustan Liver Ltd., India) and water *ad libitum*. Animals used in this study were treated and cared for in accordance with the guidelines recommended by the Control and Supervision of Experiments on Animals (CPCSEA), Chennai. Experimental protocol for treating animals was approved by our ethical committee of School of Studies in Zoology, Jiwaji University, Gwalior.

Hepatotoxin: Carbon tetrachloride 1.5 ml/kg was prepared in liquid paraffin (1:1) and was administered intraperitoneally once only (Sharma *et al*., 1989, Janbaz and Gilani, 1995). Equal amount of liquid paraffin was given as vehicle.

Experimental Design: Animals were divided into six groups of five animals each. Group 1 served as normal control and group 2 and 3 received only extract and active principle respectively and served as extract *per se* group and active principle *per se* group. Other three groups were administered CCl_4 (1.5 ml/kg, *i.p.).* Group 4 was treated as experimental control. Groups 5-6 were administered *Terminalia belerica* (fruit) extract (400 mg/kg *p.o*.) and active principle (200 mg/kg *p.o*.) respectively after 24 h of CCl_4 administration. All the animals were sacrificed after 24 h of last treatment.

Just before the necropsy, blood was collected by puncturing the retero-orbital sinus and various haematological parameters *viz* -serum alkaline phosphatase (Fiske and Subbarow, 1925), serum protein (Lowry *et al.,* 1951), serum transaminases (Reitmen and Frankel, 1957) were processed. Immediately after necropsy, liver was excised and homogenate were prepared for the estimation of lipid peroxidation (Sharma and Krishnamurthy, 1968) and reduced glutathione (Brehe and Burch, 1976). The quantitative measurement of lipid peroxidation was done by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in liver. Reduced glutathione was estimated in the liver homogenate using dithio nitrobenzoic acid (DTNB) and excretory capacity of liver was estimated by bromosulphalien (BSP) clearance test. Blood was collected in heparinized tubes exactly 30 min after BSP administration and dye concentration was estimated in the plasma (Kutab and Plaa, 1962).

Histopathological Study: For histopathological studies, liver was immediately excised after necropsy and small pieces of liver were fixed in Bouin's solution for a period of 24 h. and embedded in paraffin wax. 5µm thick paraffin sections were obtained after sectioning. They were then stained with heamatoxylin - eosin and mounted in Dibutylpolystyrene Xylene (DPX). Sections were observed under light microscope.

Statistical Analysis: Data were expressed as means ± S.E. Comparison between two groups was made by Student's't' test and variation among groups was made by one way analysis of variance (ANOVA). Significance of this statistical analysis was set at $P \leq 0.05$ (Snedecor and Cochran, 1994).

Results

The results of the present study clearly demonstrate that the various biochemical alterations produced by carbon tetrachloride in the serum and tissue were reversed significantly by the administration of the extract and active principle. Administration of CCl_4 led to increase the activity of ALT upto 10 folds whereas 3-4 folds increase in AST was observed when compared to control group. Treatment of rats with extract and active principle significantly prevented CCl_4 induced elevation of serum transaminases but the effect was more pronounced by active principle. Toxicant exposure provoked sharp elevation in the activity of serum alkaline phosphatase. Therapeutic agents prevent the leakage of enzymatic activity thereby conferring its protective effect. Serum protein contents elevated significantly ($P \leq 0.05$) after toxicant administration. Therapeutic agents showed significant reversal in these parameters (Table-1).

Table 1: Effect of Terminalia belerica (fruit) extract and its active principle (AP) on marker enzymes of liver against carbon tetrachloride induced hepatotoxicity in rats.

Treatments	AST (IU/L)	ALT (IU/L)	SALP (mg Pi/100 g/h)	Serum protein (mg/100 ml)
Control	65.50±3.84	43.40±2.85	206.00±12.04	38.04±2.75
Extract *per se*	66.60±3.91	41.90±3.03	210.00±11.18	40.00±2.42
AP *per se*	69.30±3.73	39.00±3.25	207.00±12.94	41.00±2.85
CCl4	180.00±9.67*	390.00±21.50*	1077.00±54.53*	62.25±3.28*
CCl4 + Extract	115.60±6.97#	190.80±10.19#	474.20±36.70#	49.00±3.37#
CCl4 + AP	74.00±4.51#	124.00±6.81#	324.60±27.95#	42.20±3.04#
F-values at 5% level	75.71@	221.33@	156.74@	11.59@

Values: Mean ± S.E. (Standard Error), N=5.
*$P < 0.05$: vs.**Normal Control** group.
#**$P < 0.05$:** vs. **CCl_4 treated** group.
ns: Not significant, **@:** Significant.
AST: Aspartate transaminase
ALT: Alanine transaminase
SALP: Serum alkaline phosphatase

Lipid peroxidation is a well-known marker of liver injury induced by CCl_4 and MDA is one of its end products. Thus, MDA is a good indicator of degree of lipid peroxidation. There was a significant ($P \leq 0.05$) increase in lipid peroxidation on the contrary decrease was observed in the glutathione content after carbon tetrachloride administration. Treatment of 3, 4, 5 -trihydroxybenzoic acid completely prevented the increase in MDA leves and also effective in recouping GSH level (Table-2).

Table 2: Effect of therapeutic agents on oxidative stress biomarkers against CCl4 induced toxicity.

Treatments	Lipid peroxidation (n moles of MDA/mg protein)	Glutathione (µ moles/gm)
Control	0.25 ± 0.01	8.00 ± 0.45
Extract per se	0.36 ± 0.02	7.40 ± 0.45
AP per se	0.34 ± 0.01	7.26 ± 0.37
CCl4	1.50 ± 0.09*	4.32 ± 0.26*
CCl_4+Extract	0.67 ± 0.03#	5.30 ± 0.32^{#}
CCl_4 + AP	0.34 ± 0.02#	6.90 ± 0.39#
F-values at 5% level	155.67@	24.72@

- Values are mean ± S.E., N = 5.
- ns = not significant, @ = significant
- * P < 0.05 vs. normal control gp.
- # P < 0.05 vs. CCl_4 treated gp.

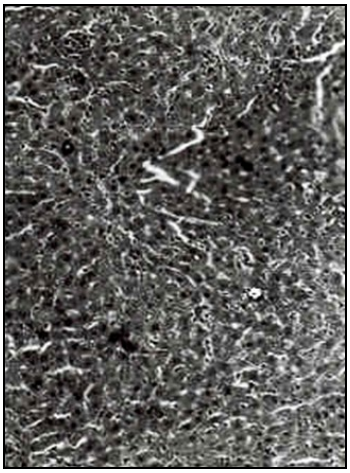
BSP retention time was significantly increased by CCl_4 intoxication after 30 min of its administration in normal animals ($P \leq 0.05$). In mice, treated with extract and active principle this increase of BSP retention with CCl_4 was significantly reduced. Active principle was found to be very effective (Table-3). In these parameters no significant effect was observed in the extract *per se* group and active principle *per se* group.

Table 3: Effect of extract and active principle (AP) on BSP retention in mice after CCl4 administration.

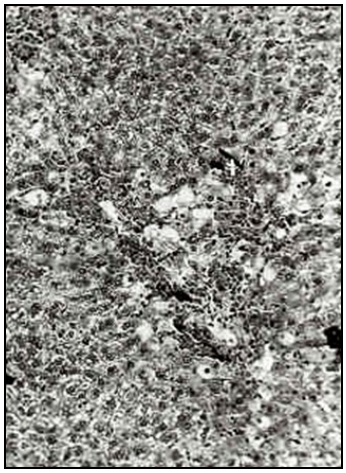
Treatment	BSP retention (mg/100 ml)
Control	2.203±0.16
Extract *per se*	2.49±0.17
AP *per se*	2.25±0.16
CCl4	6.77±0.53*
CCl4+Extract	5.38±0.41#
CCl4+ AP	3.78±0.26#
Fvalues at 5% level	42.46@

Values are mean ± S.E., N = 5.
ns = not significant, @ = significant
*P < 0.05 vs. normal control gp.
# P < 0.05 vs. CCl_4 treated gp.

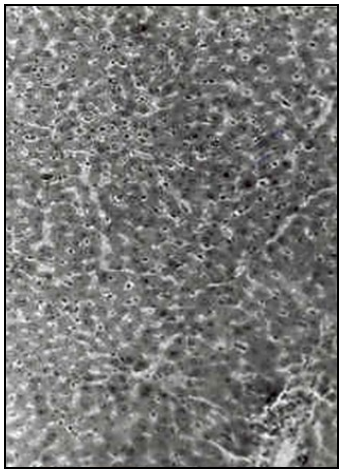
Light Microscopical Changes: Liver of control depicted normal histoarchitecture (Fig. 1). Administration of CCl_4 produced enlargement of liver with degenerative changes. Fatty accumulation, ballooning of hepatocytes and necrosis was observed along with hyperchromatic nuclei. Heavy infiltration of kupffer cells was also noted along with increased number of karyorrhectic and pyknotic nuclei. Some nuclei were enormously enlarged and deformed (Fig. 2 & 3). Administrations of extract showed maintained sinusoidal spaces. Chord arrangement was disturbed to some extent and less degree of vacuolation and granulation were seen (Fig. 4). With the treatment of active principle maintained histoarchitecture was seen. Sinusoidal spaces were normal and well-formed nuclei were also seen. Normal kupffer cells were also noted (figs 5 & 6).



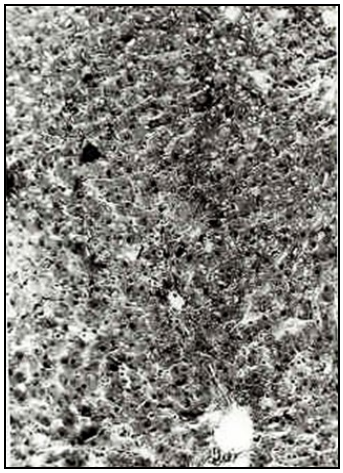
Control (120X)



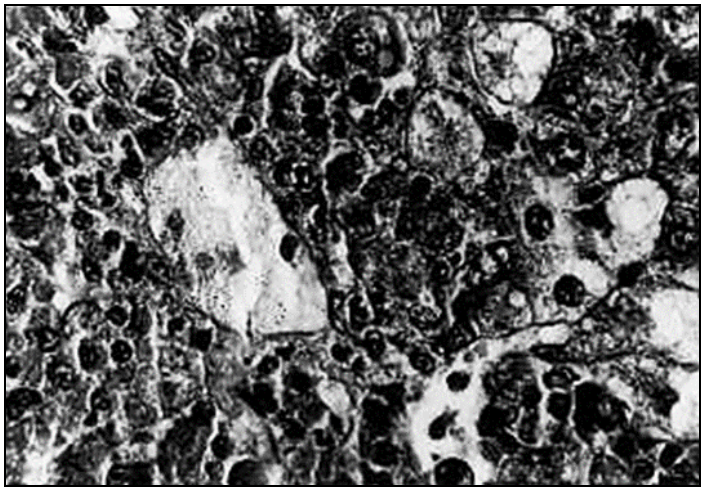
CCl_4 (120X)



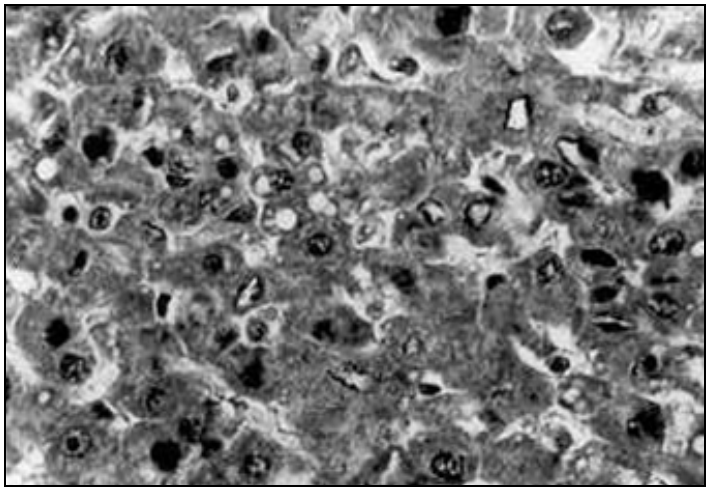
CCl_4 + TB extract (120X)



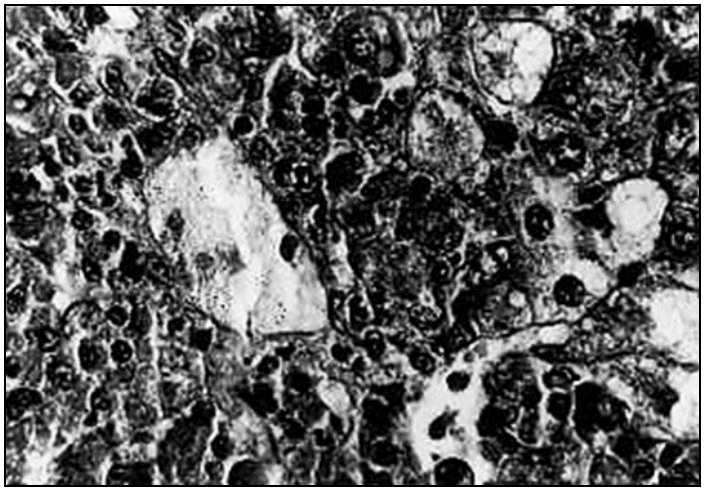
CCl_4 + G.A. (120X)



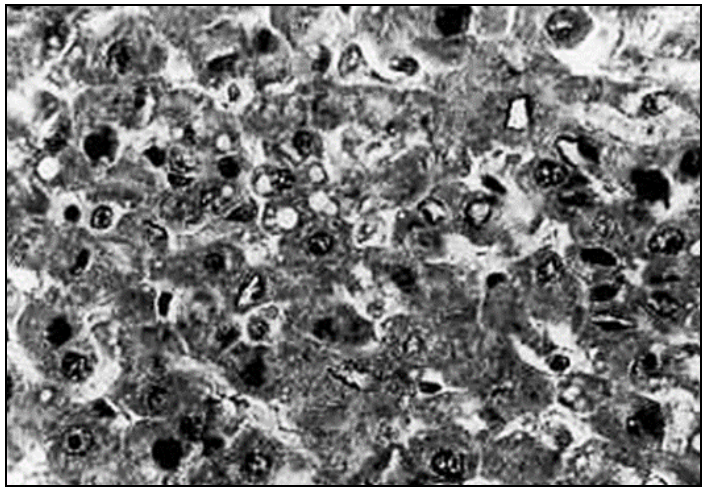
CCl_4 (400X)



CCl_4 + G.A. (400X)



CCl_4 (400X)



CCl_4 + G.A. (400X)

Explanation of Figures:

Fig 1: Photomicrograph of control rat's liver shows normal histoarchitecture (120 X).

Fig.-2: Carbon tetrachloride caused disturbed chord arrangement, hypertrophy and vacuolation in hepatocytes (120 X).

Fig 3: Carbon tetrachloride induced ballooning of hepatocytes and hypertrophy of nuclei, pycknotic nuclei were also observed (400 X).

Fig 4: With the treatment of extract showed maintain sinusoidal spaces (120 X).

Fig 5: Treatment of active principle showed improvement in chord arrangement with clear sinus (120 X).

Fig 6: Well-formed hepatocytes were observed after gallic acid treatment when compared with carbon tetrachloride treated rats (400 X).

Discussion

In the present investigation, therapeutic potential of *Terminalia belerica* extract and its active principle against carbon tetrachloride induced liver injury in rats has been studies. Serum level of transaminases, alkaline phosphatase, hepatic lipid peroxidation and glutathione were used as indicators of hepatoprotection.

Carbon tetrachloride induced hepatotoxicity via the formation of free radicals (Janbaz and Gilani, 1995). Significant elevation in transaminases can be attributed to the damaged structure integrity of the liver because these are cytoplasmic in location and are released into blood circulation after cellular damage (Tiwari and Madhusudan, 2002; Muriel and Escobar, 2003). Serum alkaline phosphatase is an energy producing enzyme and is involved in active transportation of metabolites. Due to liver injury, the transport function of the hepatocytes gets disturbed, thereby increased in enzymatic activity (Jafri *et al*., 1999). 3, 4, 5-trihydroxy benzoic acid an active principle of *Terminalia belerica* may combine with free radicals and lead to inactivate them, which may suppress the intracellular concentration of free radicals. Thus it may prevent the acute organ dysfunction and cellular injury thereby inhibiting the rapid leakage of these enzymes. A number of investigators have previously demonstrated that antioxidants prevent CCl_4 induced hepatotoxicity showed by lowering these enzymatic activities (Ohata *et al*., 1998; Lin and Hung, 2000).

Increased lipid peroxidation is an important cause of the initiation of tissue injury and cell death. During the CCl_4 toxicity, free radicals appear to attack the adjacent lipid in the tissue to induce lipid peroxidation (Basu, 2003). These radicals may also attract membrane proteins. This reaction of peroxyl radical to generate a carbon centered radical, which inturn can react with oxygen to form another peroxyradical. The increased accumulation of lipid peroxidative products might be the consequences of a progressive degradation of necrotic tissue and also and indication of free radical induced tissue damage (Seki *et al.*, 2000; Roy *et al.,* 2000; Trivedi and Raval, 2000). During the conversion of potentially reversible cell injury the mitochondria often passes through a transient condensation phase, lose their metrical granules and eventually undergo swelling. This severe damage in mitochondria was clearly visible after toxicant administration.

Glutathione is the first line of defense against peroxidant status (Ahmad *et al*., 2000). It plays a key role in the liver in detoxification include thiol transfer reactions that protect cell membranes and proteins (Rana *et al*., 2002). In the present study, treatment with crude extract and active principle significantly increased the GSH levels. The preventive effect of 3, 4, 5-trihydroxybenzoic acid may be due to inhibition of lipid peroxidation by its antioxidant nature. These findings also supported by antioxidant nature of *Gymnema montanum* (Ananthan *et al*., 2003) and *Cardusintybus* (Aktey *et al*., 2000). Studies suggest that there exists an inverse relationship between peroxidative decomposition of membrane PUFA and GSH levels (Singh *et al*., 2003; Tripathi and Pandey, 1999; Shenoy and Bairy 1999; Lin, 1998; Vijayapadma, 1998).

Active principle also restored the serum protein contents altered by CCl_4 toxicity. BSP clearance is an important and sensitive test for the functional integrity of the liver (Klassen and Plaa, 1968). Active principle has significantly improved the capacity of damaged liver to excrete BSP, as is clearly evident from the plasma levels of the dye which is lower in drug treated animals.

Histopathological studies demonstrated that CCl_4 caused steatosis, degeneration in hepatocytes, disturbed chord arrangement, vacuolation in hepatocytes, hypertrophy of nuclei, pyknotic nuclei were also observed. These findings are further supported by earlier reports (Shenoy *et al.*, 2001; Aktay *et al.*, 2000). Significant recoupment in histoarchitecture was seen with the therapy of active principle. Presence of flavonoides in the fruits of *Terminalia belerica* significantly contributes to its therapeutic efficacy. Thus hepatoprotective effect of the active principle may be due to its strong free radical scavenging activity thereby conferring its protective effect.

Conclusion

Thus it may conclude that administration of carbon tetrachloride resulted in profound hepatotoxicity, evidenced by severe cellular degeneration, disrupted hepatic chord architecture, and focal necrosis. While both the crude extract and the isolated active principle facilitated histological recovery, the active principle exhibited a higher degree of therapeutic efficacy. Notably, neither the extract nor the active principle induced intrinsic toxicity, confirming their safety profiles as hepatoprotective agents.

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