Comparative studies on mitigation of the deteriorative effect of carbon tetrachloride in rates: Effect of *Terminalia belerica* and Gallic acid – Lipoic acid combination

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Abstract
Terminalia belerica fruit extract (TB 400 mg / kg, po for five days) and its active principle: gallic acid (GA 200 mg / kg, po for five days) were studied against carbon tetrachloride (CCl_4 0.15 ml / kg, ip for 21 days) induced liver injury in rats. Toxicants increased blood sugar and serum protein (P 0.05). Induced liver necrosis increased liver-maker enzyme activity (AST, ALT, SALP and LDH), oxidative stress parameters and histopathology including necrosis, hepatocyte degeneration and inflammatory cell infiltration. CCl_4 increased albumin and bilirubin. Carbon tetrachloride intoxication altered urea and creatinine. GA and LA therapy was effective, with values close to control and comparable to silymarin. At 0.15 ml/kg i.p. for 21 days, carbon tetrachloride increased hepatic triglycerides and serum cholesterol (P 0.05).

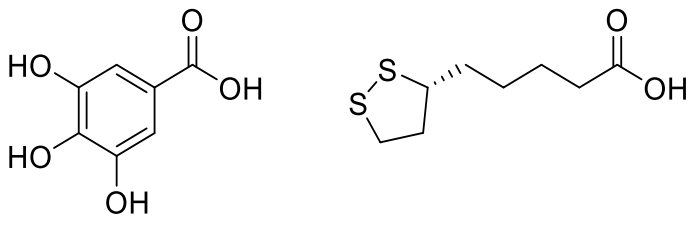
Hepatic lipid peroxidation increased. CCl_4 decreased reduced glutathione. CCl^4 harmed succinic dehydrogenase, adenosine triphosphatase, acid and alkaline phosphatases. GA and LA reduced oxidative damage from CCl_4 gallic acid and lipoic acid synergized. GA and LA acid improved liver function and oxidative stress markers. The current findings suggest that five-day GA and LA treatment could prevent liver injury in rats.

Keywords: Hepatoprotection, Carbon tetrachloride, *Terminalia belerica*, Gallic Acid, Lipoic acid.

Introduction
The liver is one of the vital organs performing physiological functions and protects against harmful substances such as drugs and chemicals. Most chemicals damage liver cells by inducing lipid peroxidation and oxidative stress[11,45]. Despite tremendous advances in modern medicines, there are no effective and reliable drugs available to stimulate liver function and protect liver cells from damage, or help regenerate hepatic cells[12,22,23]. The use of herbal remedies these days has become more popular in part due to scientific validation of some of their traditional medicinal values[2,5,13,14,19,25,30,39]. *Terminalia belerica* Roxb. is a medicinal plant and commonly known as *Bahera*[24]. Fruits of this plant are used to treat piles, dropsy, leprosy, diarrhea, biliousness, dyspepsia and headache[16,24,27].

Gallic acid (GA: 3,4,5-trihydroxybenzoic acid) is an active principle of fruit extract of this plant[6]. Lipoic acid [LA: *R*-5-(1,2-Dithiolan-3-yl)pentanoic acid, Figure 1] is a powerful antioxidant that has varied effects within the cell, due to its unique molecular structure. It has high reactivity to specific free radicals including oxygen radicals and ionized metals and interacts synergistically with other antioxidants[51]. Carbon tetrachloride-induced liver necrosis is an exemplary model for experimental liver necrosis caused by oxygen free radicals.

Recent studies have shown beneficial effects of *Terminalia belerica* extract[9,19,24,28]. The current investigation attempts to study the hepatoprotective effect of *Terminalia belerica*, gallic acid (one of the active principles of *Terminalia belerica) and* a combination of gallic acid with lipoic acid against carbon tetrachloride-induced subchronic toxicity in rats.



Gallic Acid (**GA**) Lipoic Acid (**LA**)

Figure 1: Chemical structure of gallic acid and lipoic acid

Gallic acid is considered an antioxidant and is present in several medicinal plants[4]. Lipoic acid was chosen to supplement gallic acid based on these facts: (i) Lipoic acid has a radical scavenging and chelating capability[32]. (ii) It acts as an essential cofactor for many enzyme complexes[18,48]. (iii) It is lipophilic and hydrophilic, thus addresses both fat- and water-soluble free radical species[34,40]. (iv) It recycles antioxidants and helps to reduce vitamins C and E from their oxidized forms and repairs protein damage due to oxidative stress either in the cytosol or hydrophobic domains[49].

Material and Methods

Preparation of the extract (TB extract): Fruits of *Terminalia belerica* were obtained from the authenticated ayurvedic dealer and were identified by the taxonomist of the Department of Botany of Jiwaji University, Gwalior. A voucher specimen (No. 336) has been deposited in the Herbarium (Acronym JUG) of Jiwaji University, Gwalior. The fruits were dried and chopped. An aqueous suspension of crude extract in 2% gum acacia was administered to the animals orally.

Chemicals: Gallic acid (GA) was obtained from Sigma-Aldrich and lipoic acid (LA) was obtained from SRL (Sisco Research Laboratories Pvt. Ltd). CCl_4 (Ranbaxy), Silymarin (Sigma-Aldrich) and other chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich, E-Merck and Loba Chemicals Pvt. Ltd. All diagnostic kits used in the experiments were procured from E-Merck.

Animals: Albino rats of the *Sprague Dawley* strain (130±10 g b.w.) were used for this study. They were housed under standard conditions of light (14L: 10D), temperature (25±2 °C) and 60%-70% relative humidity. The animals were fed a standard pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*. The experimental protocol for treating animals was approved by the departmental animal ethics committee.

Toxicant: Toxicity was induced by carbon tetrachloride (0.15 ml/kg, *i.p.*)[25]. Control animals received equal vehicle.

Therapeutic agents: The doses of TB extract (400 mg/kg, *p.o)* and GA (200 mg/kg, *p.o.*) were selected after screening the dose[24]. Lipoic acid (100 mg/kg, *p.o.)*[35] and silymarin (50 mg/kg, *p.o.)* were reference standard[6,41].

Experimental design: Albino rats were divided into six groups of five animals each and treated:

Group 1: Normal control (vehicle only)
Group 2: Experimental control (CCl_4 0.15 ml/kg, *i.p.* for 21 days and 5 days rest)
Group 3: CCl_4 + TB (400 mg/kg, *p.o.* for 5 days)
Group 4: CCl_4 + GA (200 mg/kg, *p.o.* for 5 days)
Group 5: CCl_4 + GA (200 mg/kg, *p.o.* for 5 days) + LA (100 mg/kg, *p.o.* for 5 days)
Group 6: CCl_4 + Sily (50 mg/kg, *p.o.* for 5 days)

24 h after the final administration, the rats were sacrificed under light anesthesia to collect blood (in heparinized tubes) and the large lobe of the liver was excised. Standard techniques were employed to determine blood sugar,[7] serum protein contents,[26] the activities of transaminases,[37] lactate dehydrogenases,[52] tissue and serum alkaline phosphatase and acid phosphatase,[20] activities of adenosine triphosphatase[42] and succinic dehydrogenase,[46] liver lipid peroxidation[43] and reduced glutathione.[10] Total bilirubin, triglycerides, cholesterol, albumin, urea and creatinine were also determined (Kit methods, E-Merck) using Autoanalyzer (Microlab 200).

Statistical analysis: Data were statistically expressed as mean ± S.E. The difference between the treated and control groups was calculated by Student's 't' test. The P value at the 5% level was significant[47]. One-way analysis of variance (ANOVA) was done to compare the mean levels of various parameters of the different experimental groups and the F-ratios were computed. All biostatistical analysis of this experiment was calculated using a statistical programme (Graph Pad software).

Light microscopical study: After necropsy, the liver tissues of the rats were collected from the large lobe and middle portion respectively and fixed in Bouin solution for 24 h. The tissues were dehydrated and embedded in paraffin. The tissue blocks were prepared and cut into 5 mm thick sections and cross-sections were stained using haematoxylin and eosin dyes (H&E) (E-Merck) and mounted with dibutyl polystyrine xylene (DPX) for photomicroscopic observations.

Results

The results revealed that the biochemical alterations produced by carbon tetrachloride were significantly reversed by the combined therapy GA+LA. The results obtained from this study were also compared with the reference drug silymarin. The toxicant caused a significant increase in blood sugar level and serum protein content after 21 days of exposure (Table 1).

The restoration was maximal with combined therapy with lipoic acid and gallic acid, while extract therapy was minimally effective in both parameters. F values were statistically significant at a level of 5% in both parameters. Figure 2 showed that subchronic exposure of carbon tetrachloride led to an increase in ALT activity up to ten times, while a 3-4 fold increase in AST was observed compared to the control group. Carbon tetrachloride also caused severe toxic responses by significantly increasing the activities of serum alkaline phosphatase and lactate dehydrogenase. Combined therapy for 5 days was very effective in reducing elevated levels of these enzymes. However, other therapeutic agents were not very useful at this level. Animals treated with silymarin showed values close to the control group ($P \leq 0.05$).

Table 1
Effect of therapeutic agents on blood sugar and serum protein content against CCl_4 intoxication.

Treatments	Blood sugar (mg glucose/100 ml)	Serum Proteins (mg/100 ml)
Normal control	99.94 ± 7.40	38.60 ± 2.79
CCl_4	156.00 ± 12.55#	54.50 ± 4.16#
CCl_4 + TB	135.20 ± 9.44	48.40 ± 3.51
CCl_4 + GA	116.00 ± 10.78*	45.40 ± 2.36
CCl_4 + GA + LA	105.00 ± 7.63*	40.00 ± 3.14*
CCl_4 + Sily	110.20 ± 7.83*	42.20 ± 2.30*
One-way ANOVA F-values	6.32@	4.52@

CCl_4 = carbon tetrachloride, TB = *Terminalia belerica* extract, GA = gallic acid, LA = lipoic acid, Sily = Silymarin. Data are mean ± S.E., N = 5.
P ≤ 0.05 vs. Normal control group, * P ≤ 0.05 vs. CCl_4 administered group.
ANOVA 'F' values, @ = Significant at 5% level.

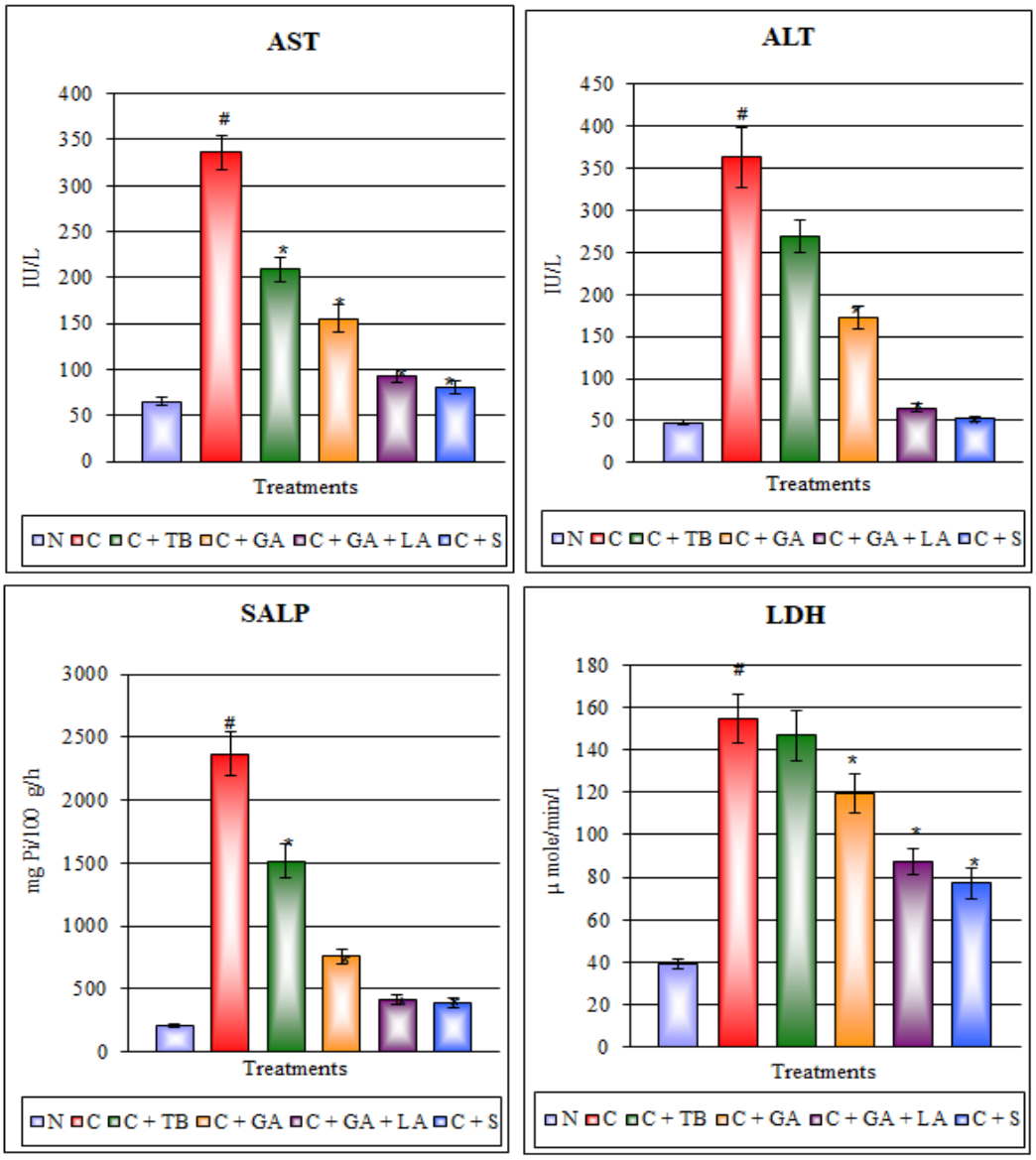


Figure 2: N = Normal control, C = CCl_4, TB = *Terminalia belerica* extract, GA = Gallic acid, LA = Lipoic acid, S = Silymarin. Data are mean ± S.E., N = 5, # P ≤ 0.05 vs. normal control, * P ≤0.05 vs. Group treated with CCl_4. ANOVA 'F' values, @ = Significant at 5% level. F values: AST = 90.17@, ALT = 67.44@, SALP = 95.61@, LDH = 33.43@

Carbon tetrachloride caused a significant increase in albumin and total bilirubin levels, which were significantly restored with GA therapy in both parameters. GA+LA was the most effective agent in conferring their therapeutic effectiveness ($P \leq 0.05$). The F values were significant when analyzed by one-way ANOVA. Urea and creatinine are kidney function tests, which were altered after intoxication with carbon tetrachloride. These parameters were significantly restored with GA + LA therapy (Figure 3). Figure 4 summarizes the effect of therapeutic agents on fat content, lipid peroxidation and reduced glutathione level followed by subsequent treatment with therapeutic agents. The toxicant caused a significant elevation in liver triglycerides and serum cholesterol level ($P \leq 0.05$).

GA therapy was very effective in reducing elevated levels followed by lipoic acid. The production of MDA in the CCl_4 treated group increased 5 folds, while the toxicant significantly depleted GSH content compared to the control. The combination of GA+LA was the best; GA intermediate and extract therapy were least effective in recovering these variables (Figure 4). Subchronic exposure of CCl_4 caused liver damage as revealed by an increase in acid phosphatase activity and subsequently decreased the activities of ALPase, ATPase and succinic dehydrogenase. GA+LA was effective in reviving the enzymatic activity, which was close to the control values ($P \leq 0.05$). Data were statistically analyzed by one-way ANOVA and F values were significantly effective at the 5% level (Figure 5).

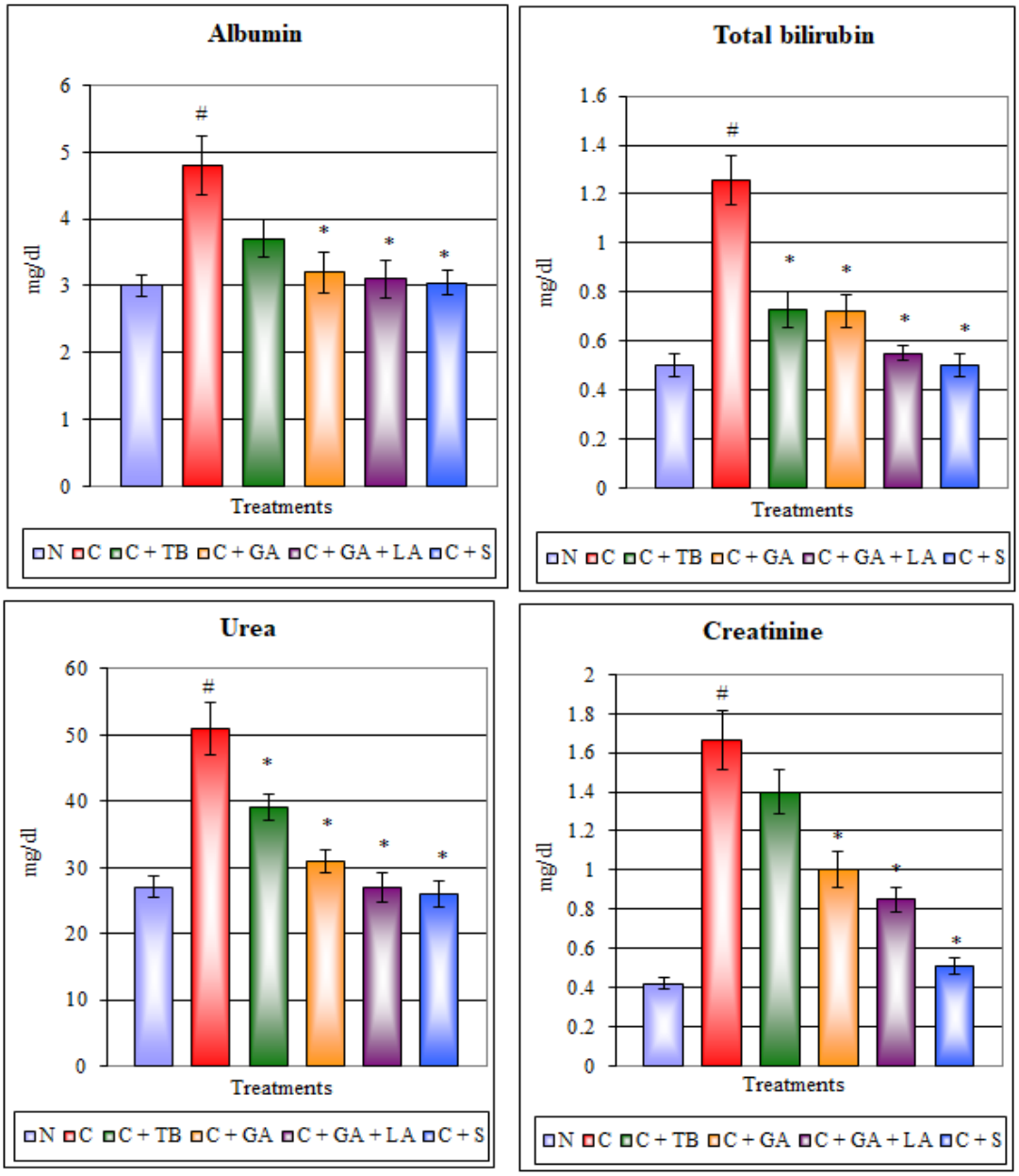


Figure 3: N = Normal control, C = CCl_4, TB = *Terminalia belerica* extract, GA = Gallic acid, LA = Lipoic acid, S = Silymarin. Data are mean ± S.E., N = 5, # P ≤ 0.05 vs. normal control, * P ≤ 0.05 vs. CCl_4 treated group. ANOVA 'F' values, @ = Significant at 5% level, F values: Albumin = 6.76@, T. Bilirubin = 24.10@, Urea = 20.94@, Creatinine = 34.41@

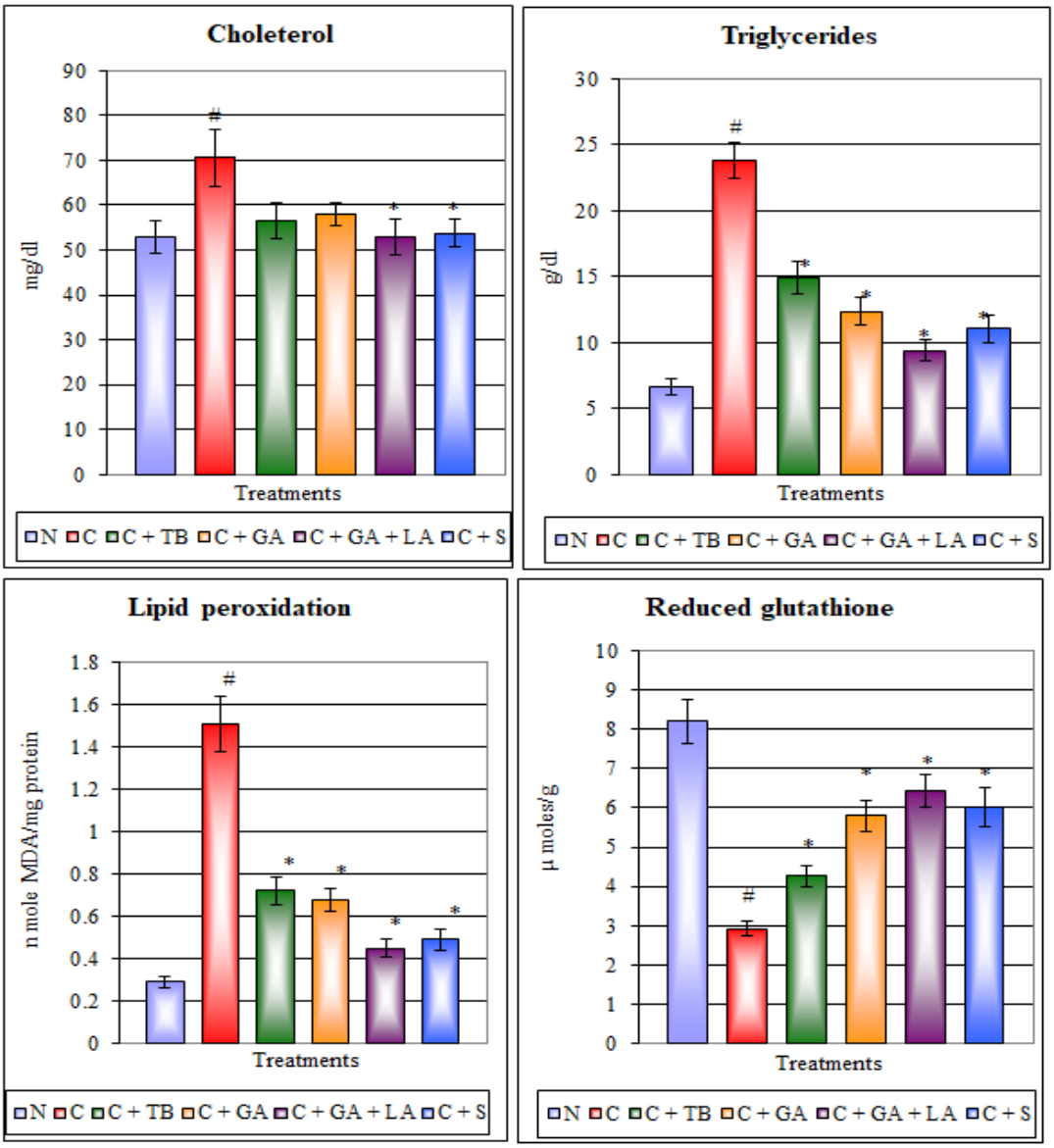


Figure 4: N = Normal control, C = CCl_4, TB = *Terminalia belerica* extract, GA = Gallic acid, LA = Lipoic acid, S = Silymarin. Data are mean ± S.E., N = 5, # P ≤ 0.05 vs. normal control, * P ≤ 0.05 vs. CCl_4 treated group. ANOVA 'F' values, @ = Significant at 5% level. F values: Cholesterol = 3.40@, Trigycerides = 39.95@, LPO = 47.27@, GSH = 23.93@

Histopathological observations: CCl_4 at a dose of 0.15 ml/kg, *i.p.* for 21 days showed hydropic degeneration (Figure 6 A). Toxicant also caused hyperplasia of the bile duct. There was bridging necrosis and early fibrosis between the portal tract and the central veins. Heavy infiltration of Kupffer cells and increase in the number of pyknotic nuclei showed that some nuclei were enormously enlarged (Figure 6 B, C). Treatment with TB extract protected to less extent the liver lesions produced by CCl_4. The arrangement of the chords was slightly disturbed compared to gallic acid (Figure 6 D).

There was a significant reduction in liver necrosis, although few necrotic cells were observed (Figure 6 E). GA+LA showed a remarkable improvement in the histoarchitecture of the liver. Hexagonal hepatocytes were observed and the nuclei showed little degeneration. Canaliculi proliferation was normal with reestablishing chord arrangement (Figure 6 F, G).

Discussion

Carbon tetrachloride is a commonly used standard hepatotoxin and CCl_4 damage is regarded an analogue of liver damage caused by a variety of hepatotoxicants in humans[9,31]. It is converted by the liver drug metabolizing enzyme system into the CCl_3 radical, which attacks unsaturated fatty acids of membranes in the presence of oxygen to give lipid peroxides. The functional integrity of hepatic mitochondria is altered. These events ultimately lead to liver damage[21]. The transaminases, serum alkaline phosphatase and lactate dehydrogenase enzymes are found in the highest concentration in the cytoplasm and are regularly used to evaluate liver function[17]. These cytosolic enzymes are released into the circulation because of

hepatocellular damage. CCl_4 may contribute to hepatotoxicity and subsequent increase in hepatic enzymes[22].

GA showed a more pronounced effect with lipoic acid because GA can combine with reactive metabolites of carbon tetrachloride. Lipoic acid is a dithiol compound that protects cell membranes by a possible interaction with the antioxidant glutathione. Thus, it may prevent acute organ dysfunction and cellular injury, thus inhibiting the rapid leakage of these enzymes. Several investigators have shown that antioxidants prevent carbon tetrachloride-induced hepatotoxicity by lowering these enzymatic activities[15,22].

Hyperglycemia was observed after carbon tetrachloride administration. It may be due to enhanced glycogenolysis, which is well correlated with decreased tissue glycogen levels[38]. In the present study, the vulnerable effect of carbon tetrachloride on carbohydrate metabolism was protected by combined therapy because lipoic acid was effective against diabetes[33]. Accelerated lipid peroxidation and a drastic fall in liver glutathione contents after carbon tetrachloride exposure have been demonstrated in the present study and also agree on studies by various researchers[44,50]. Here the role of TB extract and its active principle in reversing these features by removing free radicals is by a quenching mechanism.

The effectiveness of therapeutic agents increased with lipoic acid because it is rapidly taken up by the cell and converted to DHLA, which reduces cystine to cysteine and accelerates GSH biosynthesis. Significant improvement in cholesterol and liver triglyceride concentration was observed in animals receiving CCl_4. Restoration with GA+LA in the lipid profile, bile obstruction and renal damage was evident. Histological findings also showed that GA+LA treatment significantly recouped fat deposition in the liver. A remarkable increase in the level of bilirubin was observed in the serum. Depletion of the elevated bilirubin level, with suppression of ALP activity in the serum of rats treated with GA+LA, suggests the possibility that lipoic acid and gallic acid can stabilize biliary dysfunction of the rat liver.

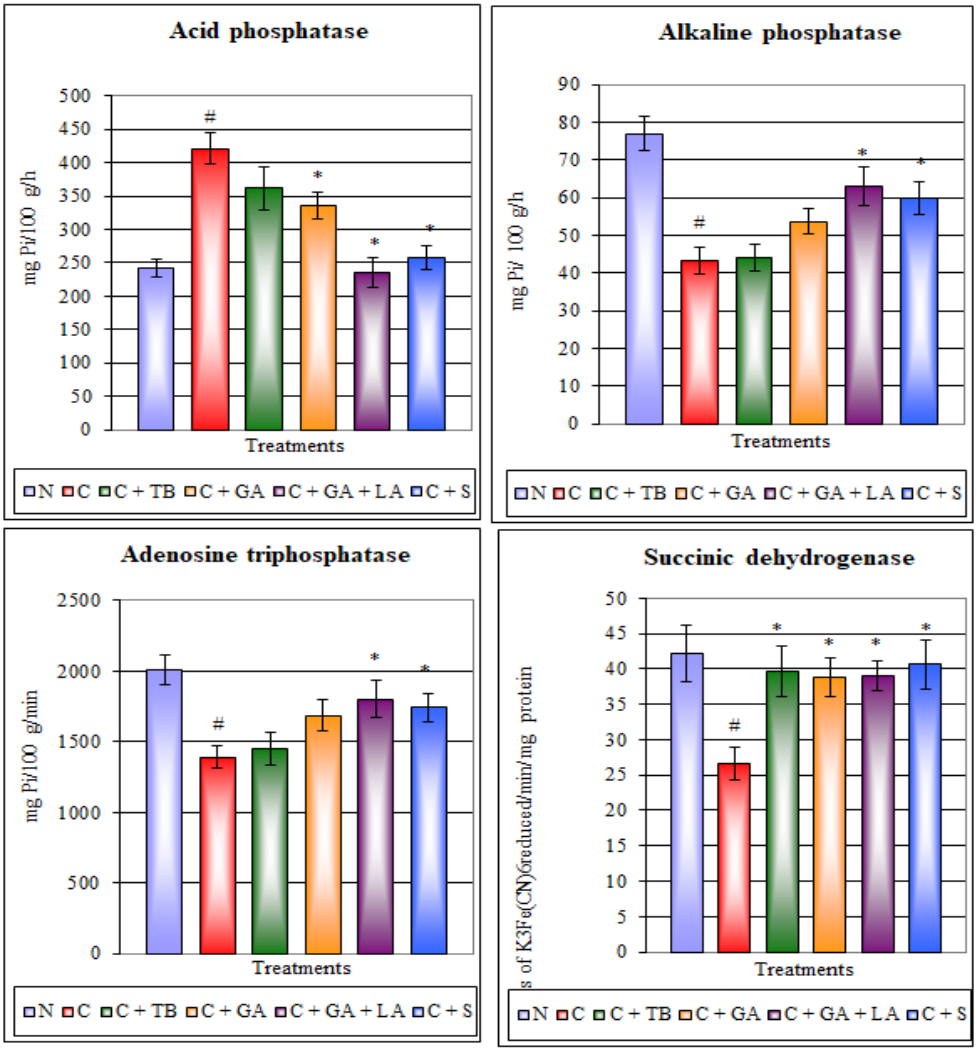


Figure 5: N = Normal control, C = CCl_4, TB = *Terminalia belerica* extract, GA = Gallic acid, LA = Lipoic acid, S = Silymarin. Data are mean ± S.E., N = 5, # P ≤ 0.05 vs. normal control, * P ≤ 0.05 vs. CCl_4 treated group. ANOVA 'F' values, @ = Significant at 5% level. F values: ACPase = 14.16@, ALPase = 11.73@, ATPase = 5.59@, SDH = 3.50@

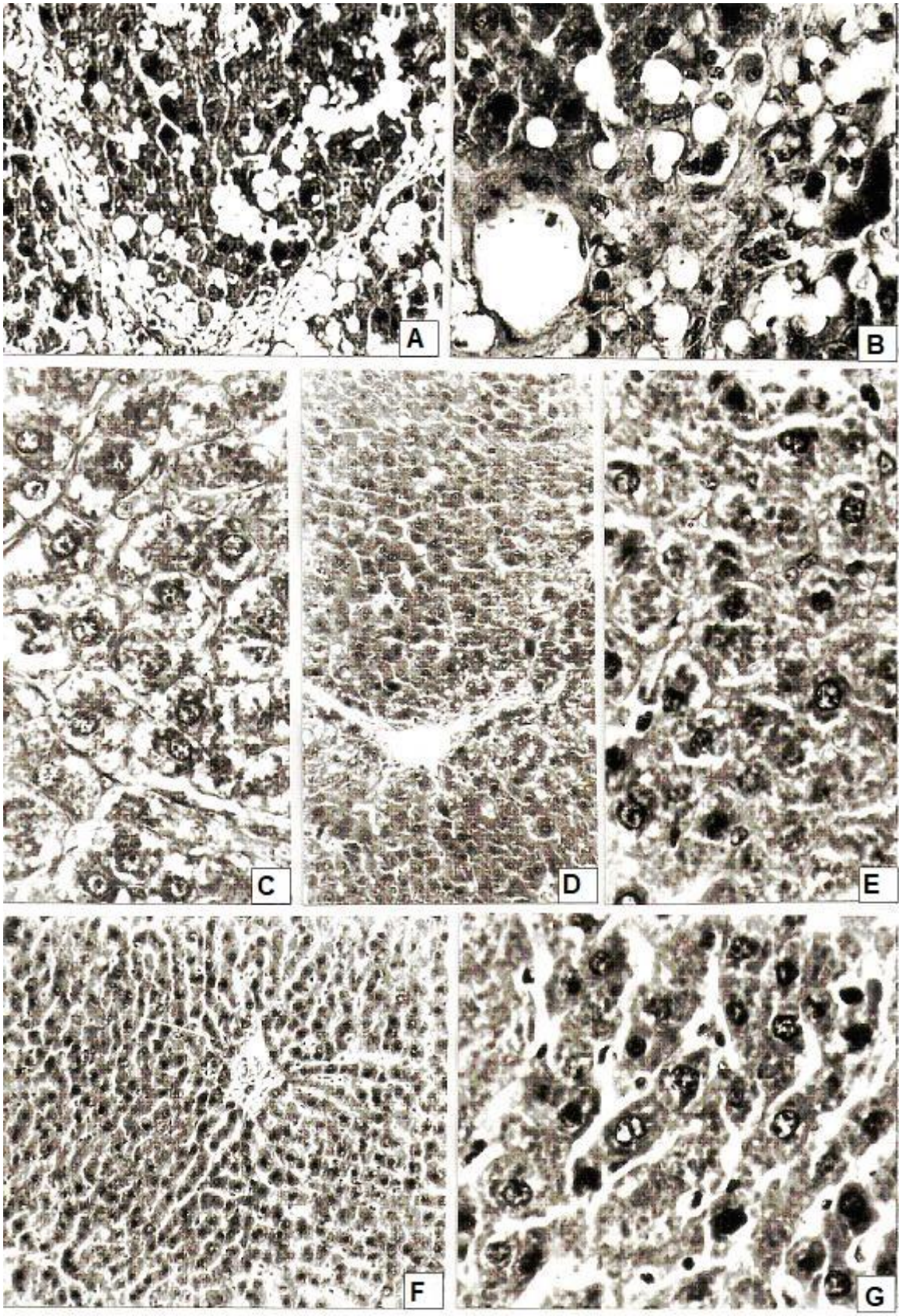


Figure 6: (A-C) Treated with CCl_4. Note hydropic changes and steatosis, massive fatty changes along with necrosis in hepatocytes (X 140, 400). Collapsed cell membrane with hyperchromatic nuclei (X 400). (D) After 5 days with treatment of *Terminalia belerica* extract: Note improved hepatocytes (c.f.1) (X 140). (E) Treatment with gallic acid shows recovery in hepatocytes but perinuclear vacuolation is visible (X 400). (F-G) Combination therapy of GA+LA showing almost normal hepatocytes with recovery in portal triad, hexagonal hepatocytes with well-formed nuclei (X 140, 400).

It also caused a significant increase in the level of urea and creatinine, which may be due to renal failure secondary to liver disease[29]. Degeneration in renal architecture was prevented with GA+LA, thus recouping the above parameters[29]. Carbon tetrachloride also caused a significant increase in acid phosphatase activities[1]. This may be due to the lysosomal imbalance resulting in destruction of the intact membranes. The extract and the active principle may possess anti-inflammatory and lysosomal stability properties and obstruct the rise in the enzymatic activity. Alkaline phosphatase, adenosine triphosphatase and succinic dehydrogenase are energy-producing enzymes and are altered after CCl_4 exposure[36]. This may be due to the structural and functional disorganization of mitochondrial assembly.

Combination therapy was most effective because GA may directly interact with free radicals and lipoic acid may improve mitochondrial dysfunctions by recycling GSH, vitamin E and vitamin C through a redox reaction. Carbon tetrachloride caused steatosis, vacuolation in hepatocytes, disturbed chord arrangement, hypertrophy of the nuclei and pyknotic nuclei[3,8].

In the present study, carbon tetrachloride exhibited extensive degenerative lesions in all cell organelles of the liver. Significant recoupment in histoarchitecture was observed with GA+LA. Hepatic fibrosis is a critical consequence of subchronic liver damage caused by carbon tetrachloride. Replacement of normal hepatic parenchymal tissue with connective tissue compromises of the functional capacity of the liver and disrupts the normal architecture of the organ.

An important feature of liver fibrosis is the deposition of connective tissue around the hepatic sinusoids, so vascular diffusion barriers are disrupted and sinusoidal blood flow is narrowed. Thus, from histological studies, it is shown that combined therapy of GA+LA significantly protected the liver against carbon tetrachloride toxicity, as evidenced by biochemical data.

Conclusion

Thus, it may be concluded that the active principle of *Terminalia belerica* showed a synergistic role with lipoic acid in ameliorating the deteriorative biochemical effects induced by carbon tetrachloride in rats. Gallic acid may have the ability to block CCl_4 bioactivation by inhibiting P450 2E1 activity, or it may combine directly with free radicals and may hinder the formation of these radicals. Lipoic acid may also prevent the peroxidation of lipids of the endoplasmic reticulum by scavenging the superoxide anion, hydroxyl radical and chelating the ferrous ions involved in the production of free radicals.

Therefore, lipoic acid can synergistically enhance the antioxidant activity of gallic acid by scavenging toxic radicals, possibly through the 'free radical reductase' mechanism and glutathione recycling.

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