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Synergistic Modulation of Radical Induced Oxidative Stress Via a Redox Coupling of 3,4,5-Trihydroxybenzoic Acid and Thioctic Acid

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Abstract- ***The present study was undertaken to investigate the hepatoprotective activity of Terminalia belerica fruits extract (400 mg/kg p.o.) Gallic Acid (200 mg/kg p.o.) supplemented with an antioxidant lipoic acid (100 mg/kg p.o.) on sub-chronic exposure of carbon tetrachloride (0.15 ml/kg i.p. for 21 days) induced toxicity. Liver damage by carbon tetrachloride was evident by the increase in the level of hepatic marker enzymes such as transaminases, serum alkaline phosphatase and lactate dehydrogenase. Toxicant exposure caused significant increase in blood sugar level where as significant fall was found in glycogen contents (P¬<0.01). Significant elevation was observed in hepatic lipid peroxidation on the contrary a considerable fall was found in reduced glutathione after CCl4 administration. Co-administration of active principle and lipoic acid completely ameliorated the CCl4 induced oxidative damage. Combination of active principle and lipoic acid significantly (P<0.01) ameliorated the liver function test and markers of oxidative stress. Thus it may be concluded that combination therapy was effective against subchronic toxicity of carbon tetrachloride.***

Keywords: Hepatoprotection, CCl4, Terminalia belerica, Gallic Acid, Lipoic acid.

I. INTRODUCTION

Terminalia belerica, commonly known as Bahera or Bhibitaki, is a large deciduous tree belonging to the family Combretaceae. It is native to the plains and lower hills of Southeast Asia and is found extensively throughout the Indian subcontinent. Renowned for its extensive medicinal properties, it is most famous for being one of the three constituent fruits of Triphala, a cornerstone herbal formulation in Ayurvedic medicine.

Lipoic acid (LA), also known as α-lipoic acid and alpha lipoic acid (ALA) and thioctic acid is an organosulfur compound derived from caprylic acid (octanoic acid). ALA is made in animals normally, and is essential for aerobic metabolism. It is also manufactured and is available as a dietary supplement in some countries where it is marketed as an antioxidant, and is available as a pharmaceutical drug in other countries. Lipoic acid (LA), also known as α-lipoic acid, alpha lipoic acid (ALA) and thioctic acid is an organosulfur compound derived from octanoic acid. LA contains two sulfur atoms (at C6 and C8) connected by a disulfide bond and is thus considered to be oxidized although either sulfur atom can exist in higher oxidation states. The carbon atom at C6 is chiral and the molecule exists as two enantiomers (R)-(+)-lipoic acid (RLA) and (S)-(-)-lipoic acid (SLA) and as a racemic mixture (R/S)-lipoic acid (R/S-LA). LA appears physically as a yellow solid and structurally contains a terminal carboxylic acid and a terminal dithiolane ring. For use in dietary supplement materials and compounding pharmacies, the USP has established an official monograph for R/S-LA.

Chemical structure:

Alpha Lipoic Acid

Chemical Formula	$C8H14O_2S_2$
Molecular Weight	206.318 g/mol

Traditional Name	Lipoic acid, α-lipoic acid, alpha lipoic acid, thioctic acid

Gallic acid (GA: 3,4,5-trihydroxybenzoic acid) is an active principle of Termialia belerica Roxb (Anand et al., 1997). Therefore present investigation aims to evaluate the hepatoprotective potential of Gallic Acid supplemented with lipoic acid (1,2-dithiolane-3-pentonoic acid) against carbon tetrachloride induced sub-chronic toxicity in rats. Gallic acid is a naturally occurring trihydroxybenzoic acid, a type of phenolic acid found in various plants, fruits, and herbs. It serves as a primary bioactive secondary metabolite, notably present in tea leaves, oak bark, and the Ayurvedic medicinal plant Terminalia belerica (Bahera). In its chemical structure, it consists of a benzene ring with three hydroxyl groups and one carboxylic acid group. This specific arrangement of hydroxyl groups makes it an exceptionally potent antioxidant, as it can easily donate electrons to neutralize free radicals. Gallic acid is widely recognized in pharmacology and toxicology for its diverse therapeutic benefits:

- Antioxidant Power: It acts as a powerful scavenger of reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals.
- Hepatoprotective Activity: It protects liver cells from xenobiotic-induced damage (like CCl_4 or acetaminophen) by stabilizing cell membranes and preventing lipid peroxidation.
- Anti-inflammatory Effects: It inhibits the expression of pro-inflammatory cytokines and enzymes like COX-2.
- Antimicrobial & Antiviral: It disrupts the cell walls of various bacteria and inhibits viral replication.
- Synergistic Potential: When combined with other antioxidants like alpha-lipoic acid, gallic acid's efficacy is enhanced through a "redox recycling" mechanism, where the thiol-based antioxidant helps regenerate the gallic acid molecules after they have neutralized a radical.

Beyond its health benefits, gallic acid is a critical industrial precursor. It is used:

1. As a Standard: It is the primary reference standard for the Folins-Ciocalteu assay, used to measure the total phenolic content of various substances.
2. In Synthetic Chemistry: It is used to synthesize propyl gallate, a common food preservative, and trimethoprim, an antibiotic.
3. In Traditional Medicine: It is the active principle behind the liver-toning effects of many herbal formulations in Ayurveda and Traditional Chinese Medicine (TCM).

Given its ability to modulate oxidative stress pathways, Gallic acid remains a focal point for research in preventing chronic diseases, including cancer, diabetes, and cardiovascular disorders.

Chemical structure:

GALLIC ACID

Chemical Formula	C7H6O5
Molecular Weight	170.12 g/mol
Traditional Name	3,4,5-trihydroxybenzoic acid

Chemicals: Gallic acid was procured from Sigma Aldrich and other chemicals used in the study were procured from Sigma Aldrich and E- Merck.

Animals: Albino rats of Sprague Dawley strain (130+10 g b.w.) and swiss albino mice (25+5 g b.w.) were used for hepatoprotective studies. Animals were housed under standard conditions of light (14L: 10D), temperature (25+2 □C) and 60%-70% relative humidity. Animals were fed on standard pellet diet (Hindustan Lever Ltd., India) and water ad libitum. Experimental protocol for treating animals was approved by departmental animal ethical committee.

Toxicant: Hepatic injury was induced by the administration of CCl4 in liquid paraffin. For sub-chronic toxicity the dose of CCl4 is recommended ie. 0.15 ml/kg i.p. (Jose & Kuttan, 2000)

Therapeutic agents:
Terminalia belerica fruits extract (TB): 400 mg/kg, p.o. for 5 days

Active principle (AP: Gallic acid): 200 mg/kg p.o. for 5 days

Lipoic acid (LA): 100 mg/kg p.o. for 5 days (Pari and Murugvel, 2004)

Silymarin: 50 mg/kg p.o. was used as a reference standard for 5 days (Anand et al., 1997).

Carbon tetrachloride CCl4 (0.15 ml/kg, i.p.) was administered to the animals for 21 days followed by therapy for 5 days. They were divided into six groups of five animals each and were treated as follows:

Group 1: Normal control (vehicle only).
Group 2: Exp. control (CCl4 0.15 ml/kg, i.p. for 21 days & 5 days rest).
Group 3: CCl4 (0.15 ml/kg, i.p. for 21 days) + TB (400 mg/kg, p.o. for 5 days)
Group 4: CCl4 (0.15 ml/kg, i.p. for 21 days + GA (200 mg/kg, p.o. for 5 days)
Group 5: CCl4 (0.15 ml/kg, i.p. for 21 days + GA (200 mg/kg, p.o.) + LA (100 mg/kg, p.o.) for 5 days.
Group 6: CCl4 (0.15 ml/kg, i.p. for 21 days + Sily (50 mg/kg, p.o. for 5 days)

All the animals were sacrificed after 24 h of last treatment and various biochemical parameters were observed as shown under.

- Aspartate Aminotransferase (Reitman & Frankel, 1957)
- Alanine Aminotransferase (Reitman & Frankel, 1957)
- Lactate dehydrogenases (Wrobleski & Due, 1955)
- Serum alkaline phosphatase (Hawk et al., 1954)
- Total Bilirubin (Kit method)
- Blood sugar (Asatoor & King, 1954)
- Lipid peroxidation (Sharma & Krishnamurthy, 1968)
- Reduced glutathione (GSH) (Brehe & Burch, 1976)
- Urea (Kit method)
- Creatinine (Kit method)
- Colesterol (Kit method)
- Trigycerides (Kit method)

Statistical analysis: The data were subjected for statistical analysis using student's't' test and Analysis of variance (ANOVA) (Snedecor and Cochran, 1994) by the statistical programme, Graph pad software.

II. RESULTS:

The results revealed that the various biochemical alterations produced by carbon tetrachloride were reversed significantly by combined therapy. Carbon tetrachloride exposure at 21 days led to increase the activity of ALT upto 10 folds whereas 3-4 folds increase in AST was observed when compared to control group. Toxicant exposure provoked sharp elevation in the activity of lactate dehydrogenase, total bilirubin level and hepatic lipid peroxidation where as significant fall was found in reduced glutathione level ($P<0.01$). All these parameters were significantly recovered by the therapy. The protective effects of these therapeutic agents were also compared with silymarin treated animals, which is used as a reference drug. Liver damage by carbon tetrachloride was evident by the increase in the level of hepatic marker enzymes such as transaminases, serum alkaline phosphatase and lactate dehydrogenase. Toxicant exposure caused significant increase in blood sugar level where as significant fall was found in glycogen contents ($P\neg<0.01$). Significant elevation was observed in hepatic lipid peroxidation on the contrary a considerable fall was found in reduced glutathione after CCl4 administration. Co-administration of active principle and lipoic acid completely ameliorated the CCl4 induced oxidative damage. Combination of active principle and lipoic acid significantly ($P<0.01$) ameliorated the liver function test and markers of oxidative stress. Thus it may be concluded that combination therapy was effective against subchronic toxicity of carbon tetrachloride.

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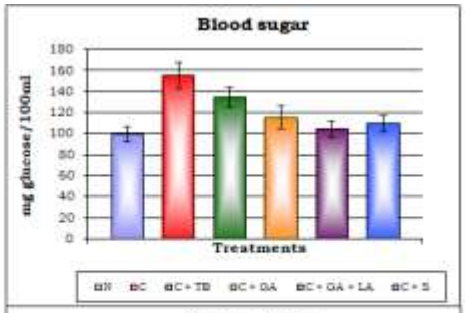
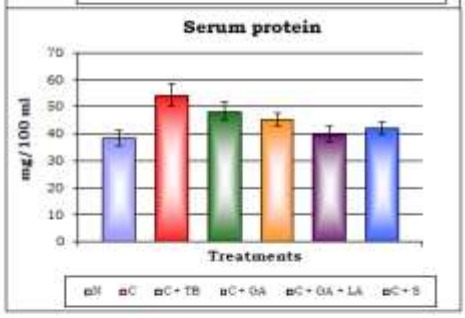
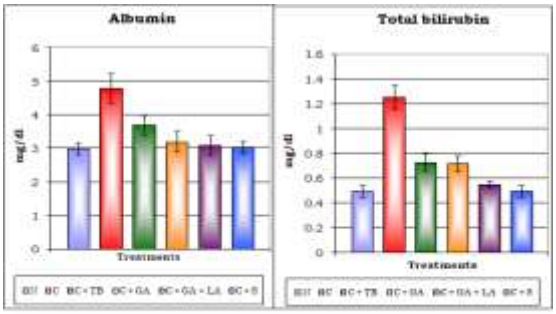
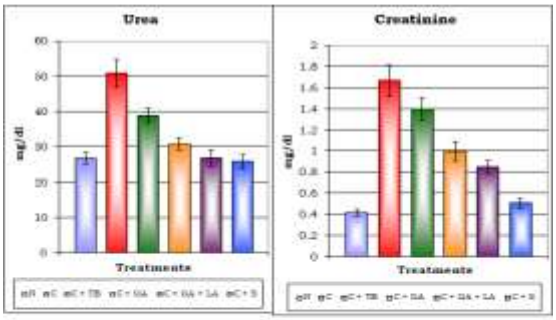




N = Normal control, C = CCl_4, TB = *Terminalia belerica* extract,
GA = Gallic acid, LA = Lipoic acid, S = Silymarin.
Data are mean ± S.E., N = 5
P ≤ 0.05 vs. normal control
* P ≤ 0.05 vs. Carbon tetrachloride treated group

Parameters	Blood Sugar	Serum Protein
F values	6.32#	4.52#





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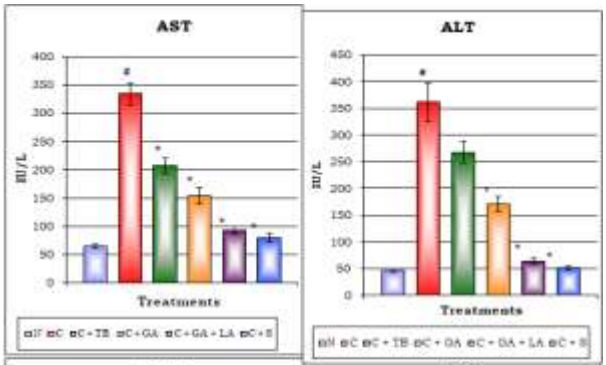
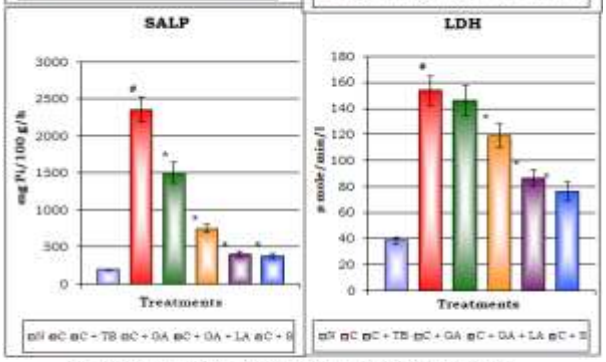
Data are mean ± S.E., N = 5
P ≤ 0.05 vs. normal control
* P ≤ 0.05 vs. Carbon tetrachloride treated group

Parameters	Albumin	T. Bilirubin	Urea	Creatinine
F values	6.70#	24.10#	20.94#	34.41#

@= Significant at 5% level





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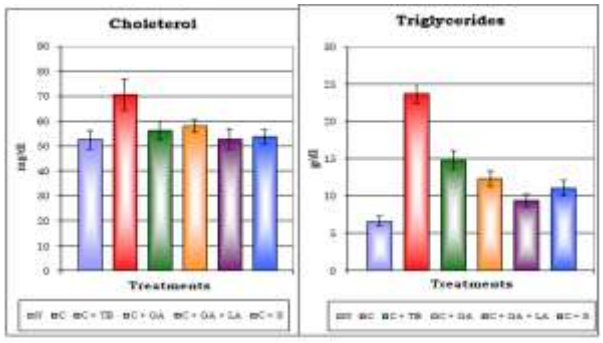
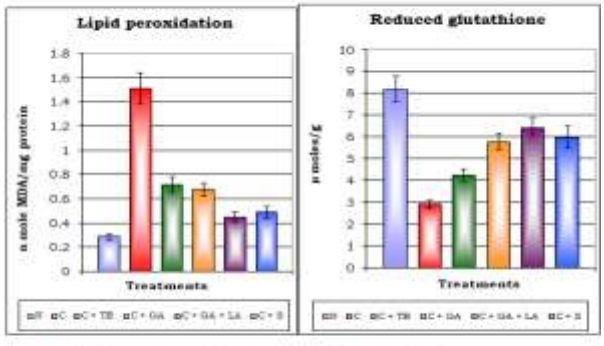
GA = Gallic acid, LA = Lipoic acid, S = Silymarin.
Data are mean ± S.E., N = 5
P ≤ 0.05 vs. normal control
* P ≤ 0.05 vs. Carbon tetrachloride treated group

Parameters	AST	ALT	SALP	LDH
F values	90.17#	67.44#	95.61#	33.43#

@= Significant at 5% level





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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. Carbon tetrachloride treated group

Parameters	Cholesterol	TG	LPO	GSH
F values	3.40#	39.95#	47.27#	23.93#

@= Significant at 5% level

III. DISCUSSION:

Carbon tetrachloride is a commonly used standard hepatotoxin (Janbaz and Gilani, 1999) and damage caused by CCl4 is regarded as an analogue of liver damage caused by a variety of hepatotoxicants in humans (Basu, 2003; Muriel and Escobar 2003). It is converted by the liver drug metabolizing enzyme system into CCl3 radical, which attacks unsaturated fatty acids of membranes in the presence of oxygen to give lipid peroxides. Consequently, the functional integrity of hepatic mitochondria is altered. All these events ultimately lead to liver damage (Rajesh and Latha, 2001). The enzymes transaminases, serum alkaline phosphatase and lactate dehydrogenase are found in the higher concentration in the cytoplasm. These cytosolic enzymes are released into the circulation as a result of hepatocellular damage and are regularly used in the assessment of liver function (Clarke et. al., 1997). CCl4 may contribute to the hepatotoxicity and subsequent increase in hepatic enzymes (Ahmed et. al., 2001). Active principle showed protective effect with lipoic acid against toxicant whereas individual treatment of active principle and lipoic acid is not significantly effective. This may be due to synergistic effect of lipoic acid. Active principle may combine with reactive metabolites of toxicant. Lipoic acid, a dithiol compound protected cell membranes by possible interaction with the antioxidant glutathione and ascorbate by the vitamin E cycle. 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 CCl4 induced hepatotoxicity by lowering these enzymatic activities (Ramanathan et al., 2003; Maritim et al., 2003)

Hyperglycemia was observed after carbon tetrachloride administration. It may be due to enhanced glycogenolysis which is well correlated with the decreased tissue glycogen levels. These findings are also supported by Revathi and Amasivayam, 2000. In the present study the vulnerable effect of CCl4 on carbohydrate metabolism was protected by therapy of active principle where as extract therapy was found to be less effective. Combination with lipoic acid significantly decreases the glucose level because lipoic acid was found effective against diabetic (Ziegler et al., 1995). An accelerated lipid peroxidation and a drastic fall in hepatic glutathione content after CCl4 exposure has been demonstrated in our present study. This is also in agreement by Tripathi and Pandey, 1999; Shenoy and Bairy, 1999. Here the role of TB extract and its active principle in reversing these features in the elimination of hydro-peroxide by reduction of free radicals by quenching may be visualized as a form of adaptation on the part of GSH dependent defence system against lipid peroxidation. But the effectiveness of following therapeutic agents increased along with lipoic acid. Lipoic acid is taken up rapidly by the cell and reduced to DHLA, which inturn reduces cystine to cysteine and accelerates the biosynthesis of GSH. Because alpha lipoic acid helps to conserve and increase production of glutathione, it may be beneficial in treating certain diseases that affect the liver. Thus treatment of lipoic acid shows a significant increase in the tissue GSH level.

Carbon tetrachloride also caused significant increase in acid phosphatase activities (Abraham and Wilfred, 2000). This may be due to the lysosomal imbalance resulting in destruction of the intact membranes. Extract and active principle may possess anti-inflammatory and lysosomal stability properties and obstructs the rise in the enzymatic activity. Alkaline phosphatase, adenosine triphosphatase and succinic dehydrogenase are energy producing enzymes and are altered after CCl4 exposure (Rastogi and Rana, 1990). This may be due to the structural and functional disorganization of the mitochondrial assembly. Combination therapy was found to be most effective because active principle may directly interact with free radicals and lipoic acid may improve mitochondrial dysfunctions through recycles of GSH, Vit E and Vit C by redox reaction. CCl4 caused steatosis, vacuolation in hepatocytes, disturbed chord arrangement, hypertrophy of nuclei and pycknotic nuclei (Shenoy et al., 2001; Aktay et al., 2000). In the present study, CCl4 exhibited extensive degenerative lesions in all the cell organelles of liver. Significant recoupment in histoarchitecture was seen with the combination therapy of active principle and lipoic acid.

IV. CONCLUSION:

Thus it may conclude that combined therapy was found to be most effective when compared to individual treatments. Gallic acid may possess the ability to block the bioactivation of carbon tetrachloride by inhibiting P450 2E1 activity and its expression or it may directly combine with free radicals and hinder the formation of these radicals. Lipoic acid may be protecting the liver by preventing lipid peroxidation of the endoplasmic reticulum because it scavenges the superoxide anion, hydroxyl radical and also chelates the ferrous ion involved in the production of free radicals. Thus lipoic acid can synergistically enhance antioxidant activity of gallic acid by reducing the toxic radicals possibly via "free radical reductase" mechanism and recycling of glutathione.

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