

HEPATOPROTECTIVE ACTION OF ETHANOLIC EXTRACTS OF *ECLIPTA ALBA* AND *PIPER LONGUM* LINN AND THEIR COMBINATION ON CCl₄ INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

A comparative analysis in evaluating the hepatoprotective action of ethanolic extract of *Eclipta alba* (EAE) and *Piper longum* (PLE) with their combination *Biherbal extract* (BHE) against carbon tetrachloride (CCl₄) induced hepatic damage is reported in albino rats. The three ethanolic extracts at a dose level of 50 mg/kg body weight each were administered to three different groups of rats orally once daily for 14 days. The degree of liver protection was determined by estimating the levels of serum marker enzymes such as Alanine amino transferase, Aspartate amino transferase, Alkaline phosphatase, Acid phosphatase, Lactate dehydrogenase, γ -Glutamyl transferase and 5'Nucleotidase. The biochemical parameters like total protein, total bilirubin, total cholesterol, triglycerides and urea were also estimated. There was marked elevation of serum marker enzyme levels in CCl₄ treated rats, which were restored towards normalization in these drug treated animals. The biochemical parameters were also restored towards normal levels. The combined BHE has shown more significant reduction of these enzymes than EAE or PLE against CCl₄ induced hepatotoxicity. The results strongly indicate that BHE has more potent hepatoprotective action than EAE or PLE individually against CCl₄ induced hepatic damage in rats. Among these extracts, BHE showed similar hepatoprotective action to silymarin, which was the positive control in this study.

Keywords: Biherbal extract (BHE), Carbon tetra chloride, Hepatoprotective, Silymarin.

INTRODUCTION

Liver, an important organ actively involved in many metabolic functions and is the frequent target for a number of toxicants¹. Hepatic damage is associated with distortion of these metabolic functions. The disorders associated with the liver are numerous and varied². Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects³. In the traditional system of Ayurvedic treatment, medicines consists of plant products either single drug or in combination with others are considered to be less toxic and free from side effects when compared to synthetic drugs⁴. CCl₄-induced hepatotoxicity in rats represents an adequate experimental model of cirrhosis in man and it is used for the screening of hepatoprotective drugs⁵. Carbon tetra chloride is toxic to the liver and its toxicity is dose dependent and time of exposure^{6,7}. In the liver, CCl₄ is metabolized in to the highly reactive trichloro methyl radical. This free radical generated would lead to auto oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and cause functional and morphological changes in the cell membrane⁸. The metabolism of CCl₃ free radical released from CCl₄, initiates peroxidative and cleavage of fatty acids in membranes. Thus, trichloromethyl peroxy free radical leads to elicit lipid peroxidation, the destruction of Ca²⁺ homeostasis, and finally, results in cell death⁹. In absence of a reliable liver protective drug in the modern medicine, there are number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders^{10,11}. A single drug cannot be effective against all types of severe liver diseases. Therefore effective formulations have to be developed using indigenous medicinal plants, with proper pharmacological experiments and clinical trials.

In this context, Biherbal ethanolic extract (BHE) made up of equal quantities of leaves of *Eclipta alba* seeds of *Piper longum* Linn was subjected to various assays in order to evaluate its hepatoprotective effect against CCl₄ toxicity in albino rats. These plants have traditional claim against liver disorders¹² and all of them are scientifically

evaluated for their potency individually¹³. The plant *E. alba* has been extensively studied for its hepatoprotective activity and a number of herbal preparations comprising of *E. alba* are available for the treatment of jaundice and viral hepatitis¹⁴. *Piper longum* Linn, an important medicinal plant belonging to the family Piperaceae is been used in traditional medicine by many people in Asia and Pacific islands especially in Indian medicine¹⁵. *Piper longum* is a component of medicines reported as good remedy for treating gonorrhoea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions¹⁶. In the present study, the hepatoprotective action of ethanolic extract of *E.alba* (EAE) and *P.longum* (PLE) was compared with that of its combination biherbal ethanolic extract (BHE) against CCl₄ induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

All routine chemicals were obtained from SD Fine Chemicals Mumbai. CCl₄ was obtained from Merck Ltd, Ambemath India. Standard Silymarin was obtained from Ranbaxy (India) Ltd, New Delhi. All the chemicals used were of analytical grade.

Collection of plant material

The leaves of *E.alba* and seeds of *P. longum* were collected from the center for Advanced Studies in Botany Field Research Laboratory, University of Madras, Chennai, India, and were authenticated by Prof. P.T. Kalaichelvan (Advanced Studies in Botany, University of Madras, Chennai, India). The voucher specimen is available in the herbarium file of the Studies in Botany Field Research Laboratory, University of Madras, Chennai, India.

Preparation of plant extract

The leaves (1Kg) of *E.alba* and seeds (1kg) of *P. longum* each were shade-dried and pulverized to a coarse powder. Equal quantities of the

powder was passed through 40 mesh sieve and exhaustively extracted with 90% (v/v) ethanol in Soxhlet apparatus at 60 °C. The extract was evaporated under pressure till all the solvent had been removed and further removal of water was carried out by freeze drying to give an extract sample with the yield of 19.6% (w/w). Similarly the EAE and PLE were also prepared separately. The EAE extract yield was 9.6% (w/w) and the PLE sample yield was 8.6% (w/w). The extracts were stored in refrigerator and a weighed amount of the three extracts were dissolved in 2% (v/v) aqueous Tween- 80 and used for the present investigation.

Animals

Adult albino male rats of Wistar strain, weighing 200-250g were used. The inbred animals were obtained from the animal house of Madras Medical College, Chennai, India. The animals were maintained in propylene cages in well-ventilated room with natural 12h ± 1h day-night cycle. They were fed balanced rodent pellet diet (Poultry Research Station, Nandam, Chennai-35) and tap water *ad libitum*, throughout the experimental period. The animals were housed for one week, prior to the experiments to acclimatize to laboratory conditions. The protocol was approved by Animal Ethics Committee constituted for the purpose, as per CPCSEA Guidelines.

Acute toxicity study

Acute toxicity studies were conducted with the plant extracts in Wistar albino mice by staircase method¹⁷. First group served as normal control. BHE, EAE and PLE were administered orally to different groups at the dose level of 250, 500, 1000 and 2000 mg/kg body weight, po. All animals were observed for toxic symptoms and LD₅₀ doses were selected for the evaluation of hepatoprotective activity.

Experimental groups

The rats were divided into following 6 groups of 6 animals each: Group I: Animals were given a single administration of 0.5 ml vehicle (2% v/v aqueous Tween 80) po for 14 days. This group served as control. Group II, III, IV, V and VI: Animals were given a single dose of CCl₄ (2ml/kg, po for 7 days) according to the method of Shivaipandey *et al*¹⁸. Group III: Animals were pre treated with BHE (50mg/kg, po for 7 days) and simultaneously received the same during CCl₄ treatment for next 7 days. Group IV: Animals were pre treated with EAE (50mg/kg, po for 7 days) and received the same along with CCl₄ treatment for next 7 days. Group V: Animals were pre treated with PLE (50mg/kg, po for 7 days) and received the same along with CCl₄ treatment for next 7 days. Group VI: Animals were pre treated with Silymarin (50mg/kg, po for 7 days) and received the same along with CCl₄ treatment for next 7 days.

On the 15th day, the animals were sacrificed by cervical decapitation and various biochemical parameters were analyzed.

Biochemical analysis

At the end of the experimental period, animals were sacrificed by cervical decapitation under light ether anesthesia and blood was collected, serum was separated by centrifuging at 3,000 rpm for 10 min. The serum was used for the assay of marker enzymes, such as alanine amino transferase (ALT)¹⁹, aspartate amino transferase (AST)¹⁹, alkaline phosphatase (ALP)²⁰, acid phosphatase (ACP)²⁰, lactate dehydrogenase (LDH)²¹, gamma glutamyl transferase (γGT)²² and 5' nucleotidase (5' NT)²³. The biochemical parameters such as total protein²⁴, total cholesterol²⁵, total bilirubin²⁶, triglycerides²⁷ and urea²⁸ were also estimated. All the enzymatic and biochemical assays were read at specific wavelength using Shimadzu spectrophotometer, UV-1601 model.

Histopathological investigations

The rats were sacrificed and the liver was dissected out and cleaned well with cold physiological saline to remove blood and adhering tissues. The samples were then fixed in 10% formalin- saline and embedded in paraffin. Serial sections (5µm thick) were stained with haematoxylin and eosin. The sections were examined under light microscope and photographs were taken.

Statistical Analysis

Values reported are mean ± S.E. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnet's 't' test. P values <0.05 were considered as significant²⁹.

RESULTS

In the acute toxicity studies death was recorded during the treatment period in treated groups receiving 500mg/kg po of BHE orally. The animals showed changes in general behavior and other physiological activities like giddiness, sniffing, aggressiveness, tachypnoea, and finally convulsion. From the above toxicity studies the ED₅₀ dose of the BHE was calculated and it was fixed as 50 mg/kg body weight.

A significant increase in the serum enzyme levels were seen in the Group II CCl₄ intoxicated animals (Table-1). These enzymes were brought back to near normal levels in BHE (50mg/kg body weight) pretreated Group III animals (*P*<0.001). These levels were brought back to the near normal levels in BHE pretreated Group III animals more than the Group IV and Group V animals, which received the individual plant extracts such as EAE and PLE. All the parameters were under normal limits in the silymarin treated group, which acted as a positive control.

Table1: Effect of BHE/EAE/PLE/Silymarin on various enzymatic parameters in CCl₄ intoxicated rats

Groups	AST(U/L)	ALT (U/L)	ALP (IU/L)	ACP(K.A Units)	LDH (U/L)	γ GT (U/L)	5'NT (U/L)
I (Control)	46.10 ± 1.10	46.00 ± 1.03	76.60 ± 0.53	4.11±0.23	145.90±1.87	13.28 ± 0.57	5.35 ± 0.34
II(Toxicant) a	143.79±4.50*	145.50±1.08*	172.68±0.64*	12.25±1.06***	435.38±1.84***	45.03± 1.50*	7.60± 0.40*
III(BHE+CCl ₄ treated) b	75.30± .40**	75.89± 0.98**	122.38±0.61*	8.28±0.30***	244.36±1.90***	18.30 ±0.46**	5.60 ±0.24*
IV(EAE+ CCl ₄ treated) b	89.11±2.45*	90.86±3.04 *	143.44±2.05 *	8.87±0.32 *	324.22±3.87**	25.46±1.08 *	6.60± 0.40 *
V(PLE+ CCl ₄ treated) b	87.67±2.70*	90.16±1.50*	146.28±3.00 *	9.60±0.71*	299.89±3.34*	24.67±2.44 *	6.77±0.76 *
VI(Positive control) c	76.92± 3.60 ^{NS}	78.16±0.54 ^{NS}	121.28±1.00 ^{NS}	6.70±0.20 ^{NS}	240.71±2.94 ^{NS}	21.34±1.07 ^{NS}	5.84±0.37 ^{NS}

Values are mean ± SEM from 6 animals in each group Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test. Comparison between: a- Group I and Group II, b- Group II vs Groups III, IV, and V and c - Group III vs Group VI. P Values: * <0.05, ** <0.01, *** <0.001 NS- Non significant.

Table2: Effect of BHE/EAE/PLE/Silymarin on various Biochemical parameters in CCl₄ intoxicated rats

Groups	Total protein (g/dl)	Total cholesterol (mg/dl)	Triglycerides(mg/dl)	Urea (mg/dl)	Bilirubin(mg/dl)
I (Control)	6.9 ± 0.24	144.16±2.3	163.0±2.05	19.00±1.50	0.51±0.03

II(Toxicant) a	5.25±0.18 *	115.33±2.90*	125.0±2.10**	45.00±2.40 ***	2.47±0.09***
BHE+CCl ₄ treated) b	6.10 ± 0.32 *	128.16±3.34*	187.33±7.00**	36.16±1.86 **	1.57±0.10**
IV(EAE+ CCl ₄ treated) b	5.68±2.27 *	128.23±2.27 *	140.8±3.02*	38.66 ±1.32 *	1.45 ± 0.58 *
V(PLE+ CCl ₄ treated) b	5.43±3.17*	123.56±2.30*	146.8±3.02b*	37.69±1.79 *	1.53±0.24 *
VI(Positive control) c	6.2 ± 0.32 ^{NS}	139.0±3.10 ^{NS}	148.8±1.49 ^{NS}	32.33±2.40 ^{NS}	1.46±0.04 ^{NS}

Values are mean ± SEM from 6 animals in each group Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test. Comparison between: a- Group I and Group II, b- Group II vs Groups III, IV, and V and c - Group III vs Group VI. P Values: * <0.05, ** <0.01, *** <0.001 NS-Non significant.

The biochemical parameters such as serum bilirubin and urea levels were also lowered significantly in Group III BHE treated animals ($P<0.001$), when compared with the CCl₄ intoxicated Group II animals which had an increased level of total bilirubin and urea respectively (Table-2). Whereas there was a significant increase in total protein, total cholesterol and triglyceride levels in the CCl₄ intoxicated and BHE treated animals ($P<0.001$) when compared with to CCl₄ intoxicated animals. BHE was effective in correcting these biochemical parameters, when compared with its individual preparations like EAE and PLE extracts. Group comparison between Group III and Group VI showed no significant variation in these parameters indicating that BHE had effects similar to silymarin, which was the positive control in this study.

Histopathological examination of liver sections showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein (Fig 1A). The liver sections of rats of the CCl₄ treated group showed dilatation of sinusoids and presence of destructive alterations in the parenchyma, extensive fatty changes, disarrangement of normal hepatic cells with high degree of damage characterized by centrilobular necrosis and cells with pycnotic nuclei (Fig 1B). The sections of the liver treated with plant extracts such as BHE, EAE and PLE (Fig 1C, D, and E) and intoxicated with CCl₄ exhibited less centrilobular necrosis and fatty changes compared to the CCl₄ treated group. However the standard Silymarin (50mg/kg body weight) and CCl₄ treated animals revealed normal cellular architecture and demonstrated some cellular damage and centrilobular congestion with no infiltration of inflammatory cells. Most notably, no evidence of cirrhosis was noted in these livers. However the treatment with BHE exhibited less centrilobular fatty changes, necrosis and numerous hepatocytes without infiltration indicating its pronounced hepatoprotective activity when compared with its individual preparations like EAE and PLE extracts.

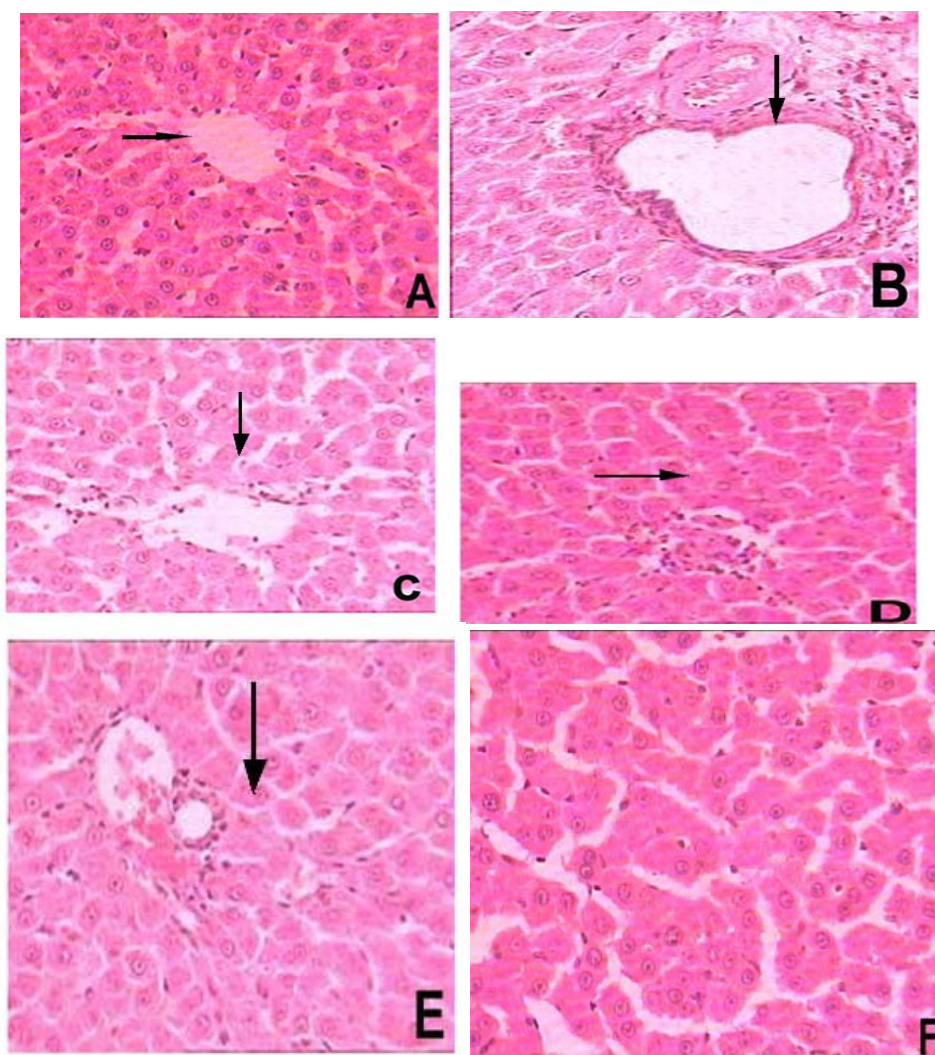


Fig. 1: Histopathological changes occurred in the liver after CCl₄ intoxication and prevention by the treatment with the plant extracts. (haematoxylin and eosin ,400x) (A) Normal with normal cellular architecture (B) CCl₄ control with extensive fatty changes (C) Biherbal Extract (BHE) (50mg/kg)+ CCl₄ showing less fatty changes (D) E. alba Extract (EAE)(50mg/kg) + CCl₄ (E) P. longum extract(PLE) (50mg/kg) + CCl₄ (F) Silymarin (50mg/kg) + CCl₄.

DISCUSSION

It is well established that CCl₄ induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function³⁰. CCl₄ is bio-transformed by the cytochrome P450 system in the endoplasmic reticulum to produce trichloromethyl free radical ($\bullet\text{CCl}_3$). Trichloromethyl free radical combines with cellular lipids and proteins in presence of oxygen to form trichloromethyl peroxy radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethylperoxy free radical elicits lipid peroxidation, the destruction of Ca²⁺ homeostasis, and finally, results in cell death³¹.

Assessment of liver damage can be made by estimating the activities of serum enzymes ALT, AST, ALP, LDH, 5' NT, and γ GT which are originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage³². The elevated level of these marker enzymes observed in the Group II CCl₄ treated rats in the present study correspond to the extensive liver damage induced by the toxin. The reduced concentrations of ALT and AST as a result of plant extract administration observed during the present study may probably be due in part to the presence of catechins in the extract³³. The tendency of these marker enzymes to return towards near normalcy in Group III (BHE treated) rats was a clear manifestation of anti-hepatotoxic effect of BHE. Treatment with BHE (50mg/kg) significantly prevented ($P < 0.001$) the rise in the levels of marker enzymes than EAE or PLE when compared to CCl₄ treated group. These investigations suggest the highest hepatoprotective activity of BHE when compared with EAE or PLE. The results were found comparable to silymarin. Silymarin contains three flavonoids and is isolated from milk thistle *Silybum maritimum*. It is used as hepatoprotective against experimental hepatotoxicity of various chemicals including CCl₄³⁴.

In the present study it was noted that the administration of CCl₄ decreased the levels of total protein, total cholesterol, and triglycerides. These parameters were brought back to normal levels in Group III BHE treated animals. BHE treatment showed a protection against the injurious effects of CCl₄ that may result from the interference with cytochrome P450, resulting in the hindrance to the formation of hepatotoxic free radicals. Numerous physiological and biochemical processes in the human body may produce oxygen centered free radical and other reactive oxygen species and by products³⁵. The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis. Attainment of near normalcy in protein, cholesterol, and triglycerides levels in CCl₄ intoxicated and BHE treated rats confirms the hepatoprotective effect of the plant. BHE was more effective in correcting these biochemical parameters when compared with its individual preparations like EAE and PLE. Moreover, the hepatoprotective activity of BHE was much stronger than that of the reference drug silymarin, administered at the same concentrations.

Histopathological examination of the livers provided supportive evidence for the study. Liver of rats administered with CCl₄ showed centrilobular necrosis with mononuclear infiltration in the portal area, fatty deposition and loss of cell boundaries. In animals treated with the BHE, EAE and PLE there were much lesser hepatocellular necrosis, mononuclear infiltration and loss of cell architecture. Faster regeneration of the hepatic cells in rats treated with BHE seems to

suggest the possibility of BHE being able to condition the hepatic cells towards accelerated regeneration. Similar histopathological observations observed with silymarin seem to suggest that the ability to cause accelerated regeneration may be a feature common to certain medicinal plants to protect against liver dysfunction.

On the basis of the results obtained in the present investigation it can be concluded that the combined ethanolic extract of *E.alba* and *P.longum* (BHE) exerts more hepatoprotective activity than when they were administered separately and may serve as a useful adjuvant in several clinical conditions associated with liver damage. This may be attributed to the synergistic activity of both the herbal drugs when given in combination.

Possible mechanism that may be responsible for the protection of CCl₄ induced liver damage by BHE may be that it could act as a free radical scavenger intercepting those radicals involved in CCl₄ metabolism by microsomal enzymes. By trapping oxygen related free radicals the extract could hinder their interaction with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes³⁶. Flavonoids and glycosides are known strong antioxidants³⁷. Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet.

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