

## Hepatoprotective action of ethanolic extracts of *Melia azedarach* Linn. and *Piper longum* Linn and their combination on CCl<sub>4</sub> induced hepatotoxicity in rats

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A comparison of analysis in evaluating the hepatoprotective action of ethanolic extract of *M. azedarach* (MAE) and *P. longum* (PLE) with their combination biherbal extract (BHE) against carbon tetrachloride (CCl<sub>4</sub>) induced hepatic damage is reported in albino rats. There was a marked elevation of serum marker enzyme levels in CCl<sub>4</sub> treated rats, which were restored towards normalization in the drug (MAE and/or PLE:50 mg/kg body weight po, once daily for 14 days) treated animals. The biochemical parameters like total protein, total bilirubin, total cholesterol, triglycerides, and urea were also restored towards normal levels. The combined BHE showed more significant reduction of the enzymes than MAE or PLE against CCl<sub>4</sub> induced hepatotoxicity. The results strongly indicate that BHE has more potent hepatoprotective action than MAE or PLE individually against CCl<sub>4</sub> 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, CCl<sub>4</sub>, Hepatoprotective, Marker enzymes

In the traditional system of Ayurvedic treatment, medicines consisting of plant products, either singly or in combination with others are considered to be less toxic and free from side effects when compared to synthetic drugs<sup>1</sup>. The disorders associated with the liver are also numerous and varied<sup>2</sup>. CCl<sub>4</sub> is toxic to the liver and its toxicity is dose dependent and based on time of exposure<sup>3,4</sup>. In the liver, CCl<sub>4</sub> is metabolized in to the highly reactive trichloromethyl radical. This free radical causes autooxidation of the fatty acids present in the cytoplasmic membrane phospholipids resulting in functional and morphological changes in the cell membrane<sup>5</sup>. The metabolism of CCl<sub>3</sub> free radical released from CCl<sub>4</sub>, initiates peroxidation and cleavage of fatty acids in membranes. Thus, trichloromethylperoxyl free radical elicits lipid peroxidation, the destruction of Ca<sup>2+</sup> homeostasis, and finally, results in cell death<sup>6</sup>. In absence of a reliable liver protective drug in modern

medicine, there are a number of medicinal preparations in Ayurveda that are recommended for the treatment of liver disorders<sup>7,8</sup>. 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 *Melia azedarach* Linn. and seeds of *Piper longum* Linn. was subjected to various assays in order to evaluate its hepatoprotective effect against CCl<sub>4</sub> toxicity in albino rats. *M. azedarach*, commonly called as "pride of India" and "Persian lilac" in English, "*Bakain*" in Hindi and "Malaivembu" in Tamil, a member of the family *Meliaceae* is widely grown as an ornamental tree, and is being used against intestinal worms, in skin diseases, stomach ache, intestinal disorders, uterine illnesses, cystitis, as diuretic and febrifuge<sup>9,10</sup>. It has got antiviral, antimalarial, anthelmintic and cytotoxic activities<sup>11</sup>. *P. longum*, known as *Pipli* (Hindi) and *Thippali* (Tamil) an

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important medicinal plant (Piperaceae) is used in traditional systems of medicine in Asia and Pacific islands especially in Indian medicine<sup>12</sup>. *P. longum* is a component of medicines reported as a good remedy for treating gonorrhoea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions<sup>13</sup>. In the present study, the hepatoprotective action of ethanolic extract of *M. azedarach* (MAE) and *P. longum* (PLE) was compared with that of its combination biherbal ethanolic extract (BHE) against CCl<sub>4</sub> induced hepatotoxicity in rats.

### Materials and Methods

**Chemicals**—All routine chemicals were obtained from SD Fine Chemicals Mumbai. CCl<sub>4</sub> 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 *M. azedarach* 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 *M. azedarach* 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 MAE and PLE were also prepared separately. The MAE 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 200-250 g 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 12 ± 1 h day–night cycle. They were fed balanced rodent pellet diet (Poultry Research Station, Nandam, Chennai) 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 method<sup>14</sup>. First group served as normal control. BHE, MAE 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 LD<sub>50</sub> 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 CCl<sub>4</sub> (2 ml/kg, po for 7 days) according to the method of Shivaipandey *et al.*<sup>15</sup>. Group III: animals were pre-treated with BHE (50 mg/kg, po for 7 days) and simultaneously received the same during CCl<sub>4</sub> treatment for next 7 days. Group IV: animals were pre-treated with MAE (50 mg/kg, po for 7 days) and received the same along with CCl<sub>4</sub> treatment for next 7 days. Group V: animals were pre-treated with PLE (50 mg/kg, po for 7 days) and received the same along with CCl<sub>4</sub> treatment for next 7 days. Group VI: animals were pre-treated with Silymarin (50 mg/kg, po for 7 days) and received the same along with CCl<sub>4</sub> treatment for next 7 days.

On the 15<sup>th</sup> 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)<sup>16</sup>, aspartate amino transferase (AST)<sup>16</sup>, alkaline phosphatase (ALP)<sup>17</sup>, acid phosphatase (ACP)<sup>17</sup>, lactate dehydrogenase (LDH)<sup>18</sup>, gamma glutamyl transferase (γGT)<sup>19</sup> and

5' nucleotidase (5 'NT)<sup>20</sup>. The biochemical parameters such as total protein<sup>21</sup>, total cholesterol<sup>22</sup>, total bilirubin<sup>23</sup>, triglycerides<sup>24</sup>, and urea<sup>25</sup> 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 (5 µm thick) were stained with haematoxylin and eosin. The sections were examined under light microscope and photographed.

**Statistical analysis**—Values reported are mean ± SE. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnet's 't' test. *P* values <0.05 were considered as significant<sup>26</sup>.

## Results

In the acute toxicity studies death was recorded during the treatment period in treated groups receiving

500 mg/kg, po of BHE orally. The animals showed changes in general behaviour and other physiological activities like giddiness, sniffing, aggressiveness, tachypnoea, and finally convulsion. From the above toxicity studies the ED<sub>50</sub> 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 Gr. II CCl<sub>4</sub> intoxicated animals (Table 1). These enzymes were brought back to near normal levels in BHE (50 mg/kg body weight) pretreated Gr. III animals (*P*<0.001) more than the Gr. IV and Gr. V animals, which received the individual plant extracts such as MAE and PLE. All the parameters were under normal limits in the silymarin treated group, which acted as a positive control.

The biochemical parameters such as serum bilirubin and urea levels were also lowered significantly in Gr. III BHE treated animals (*P*<0.001), when compared with the CCl<sub>4</sub> intoxicated Gr. II animals which had a increased level of total bilirubin and urea respectively (Table 1). Where as there was a significant increase in total protein, total cholesterol and triglyceride levels in the CCl<sub>4</sub>

Table 1—Effect of BHE/MAE/PLE/Silymarin on various enzymatic and biochemical parameters in CCl<sub>4</sub> intoxicated rats [Values are mean ± SE from 6 animals in each group]

Parameters	Group I Tween 80 control	Group II CCl <sub>4</sub> treated	Group III BHE+CCl <sub>4</sub> treated	Group IV MAE+ CCl <sub>4</sub> treated	Group V PLE+ CCl <sub>4</sub> treated	Group VI Silymarin CCl <sub>4</sub> treated
<i>Enzymatic parameters</i>						
AST(U/L)	46.1 ± 1.10	143.79±4.50a*	87.30± 3.40b**	92.11±2.45b*	87.67±2.70 b*	76.92± 3.60 c <sup>NS</sup>
ALT (U/L)	46.0 ± 1.03	145.50±1.08a*	75.00± 0.98b**	92.86±3.04 b *	90.16±1.50 b *	78.16±0.54 c <sup>NS</sup>
ALP (IU/L)	76.6 ± 0.53	172.68±0.64a*	121.75±0.72.b**	140.91±3.7 b *	146.28±3.0 b *	121.28±1.00c <sup>NS</sup>
ACP (K.A Units)	4.11±0.23	12.25±1.06a***	6.76±0.24b***	9.15±0.41 b *	9.60±0.71 b*	6.70±0.2 c <sup>NS</sup>
LDH (U/L)	145.90±1.87	435.38±1.84a***	248.05±4.70b***	324.22±3.87b**	299.89±3.34b*	240.71±2.94 c <sup>NS</sup>
γ GT (U/L)	13.28 ± 0.57	45.03± 1.59a*	20.41 ±1.04b**	25.46±1.08 b*	24.67±2.44 b*	21.34±1.07 c <sup>NS</sup>
5'NT (U/L)	5.35 ± 0.57	7.60± 0.40a*	5.80 ±0.28b*	6.60± 0.40 b*	6.77±0.76 b*	5.84±0.37 c <sup>NS</sup>
<i>Biochemical parameters</i>						
Total protein (g/dl)	6.9 ± 0.24	5.25±0.18 a*	6.10 ± 0.32 b*	5.68±2.27 b*	5.43±3.17b*	6.2 ± 0.32c <sup>NS</sup>
Total cholesterol (mg/dl)	144.16±2.3	115.33±2.90a*	128.16±3.34b*	128.23±2.27 b**	123.56±2.3b*	139.0±3.1 c <sup>NS</sup>
Triglycerides (mg/dl)	163.0±2.05	125.0±2.10a**	187.33±7 .00b**	140.8±3.02b*	146.8±3.02b*	148.8±1.49 c <sup>NS</sup>
Urea (mg/dl)	18.33±1.40	45.00±2.4 a***	32.16±2.75 b**	38.66 ±1.32 b*	37.69±1.79 b*	32.33±2.40 c <sup>NS</sup>
Bilirubin (mg/dl)	0.51±0.03	2.47±0.09 a***	1.57±0.10 b***	1.45 ± 0.58 b*	1.53±0.24 b*	1.46±0.04 c <sup>NS</sup>

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

c - Group III vs Group VI

*P* Values: \* <0.05, \*\* <0.01, \*\*\* <0.001

intoxicated and BHE treated animals ( $P < 0.001$ ) when compared with  $\text{CCl}_4$  intoxicated animals. BHE was effective in correcting these biochemical parameters, when compared with its individual preparations like MAE and PLE extracts. Group comparison between Gr. III and Gr. 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  $\text{CCl}_4$  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, MAE and PLE (Fig. 1C, D, and E) and intoxicated with  $\text{CCl}_4$  exhibited less centrilobular necrosis and fatty changes compared to the  $\text{CCl}_4$  treated group. However the standard Silymarin (50 mg/kg body weight) and  $\text{CCl}_4$  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 MAE and PLE extracts.

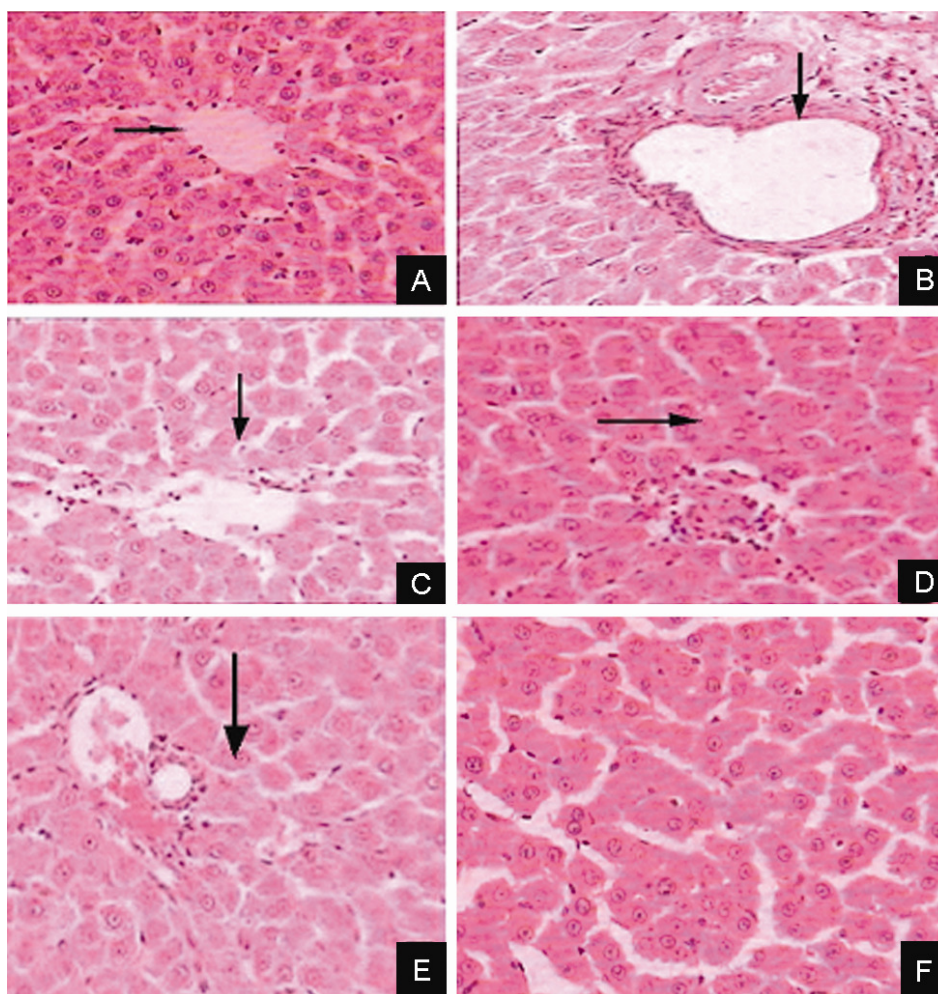


Fig. 1—Histopathological changes occurred in the liver after  $\text{CCl}_4$  intoxication and prevention by the treatment with the plant extracts (Haematoxylin and Eosin, 400 $\times$ ) (A) normal with normal cellular architecture, (B)  $\text{CCl}_4$  control with extensive fatty changes, (C) biherbal extract (BHE) (50 mg/kg) +  $\text{CCl}_4$  showing less fatty changes, (D) *M. azedarach* extract (MAE) (50 mg/kg) +  $\text{CCl}_4$ , (E) *P. longum* extract (PLE) (50 mg/kg) +  $\text{CCl}_4$ , (F) Silymarin (50 mg/kg) +  $\text{CCl}_4$ .

## Discussion

It is well established that  $\text{CCl}_4$  induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function<sup>27</sup>.  $\text{CCl}_4$  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, trichloromethyl peroxy free radical elicits lipid peroxidation, the destruction of  $\text{Ca}^{2+}$  homeostasis, and finally, results in cell death<sup>28</sup>.

Assessment of liver damage can be made by estimating the activities of serum enzymes ALT, AST, ALP, LDH, 5' NT, and  $\gamma\text{GT}$  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 damage<sup>29</sup>. The elevated level of these marker enzymes observed in the Gr. II  $\text{CCl}_4$  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 extract<sup>30</sup>. The tendency of these marker enzymes to return towards near normalcy in Gr. III (BHE treated) rats was a clear manifestation of anti-hepatotoxic effect of BHE. Treatment with BHE (50 mg/kg) significantly prevented ( $P < 0.001$ ) the rise in the levels of marker enzymes than MAE or PLE when compared to  $\text{CCl}_4$  treated group. These investigations suggest the highest hepatoprotective activity of BHE when compared with MAE 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 a hepatoprotective agent against experimental hepatotoxicity of various chemicals including  $\text{CCl}_4$ <sup>31</sup>.

In the present study the administration of  $\text{CCl}_4$  decreased the levels of total protein, total cholesterol, and triglycerides. These parameters were brought back to normal levels in Gr. III BHE treated animals. BHE treatment showed a protection against the injurious effects of  $\text{CCl}_4$  that may result from the interference with cytochrome P450, resulting in the hindrance to the formation of hepatotoxic free radicals.

The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis<sup>32</sup>. Attainment of near normalcy in protein, cholesterol, and triglycerides levels in  $\text{CCl}_4$  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 MAE 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  $\text{CCl}_4$  showed centrilobular necrosis with mononuclear infiltration in the portal area, fatty deposition and loss of cell boundaries. In animals treated with the BHE, MAE 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 *M.azedarach* 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  $\text{CCl}_4$  induced liver damage by BHE may be that it could act as a free radical scavenger intercepting those radicals involved in  $\text{CCl}_4$  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 processes<sup>33</sup>. Flavonoids and glycosides are known strong antioxidants<sup>34</sup>. 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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