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Hepatoprotective Activity of Bi-Herbal Ethanolic Extract on CCl₄ Induced Hepatic Damage in Rats

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Abstract: The combined hepatoprotective effect of Bi-Herbal Ethanolic Extract (BHEE) pretreatment against carbon tetra chloride (CCl₄) induced hepatic damage in rats was investigated. Ethanolic extract from the leaves of *Melia azedarach* and seeds of *Piper longum* at a dose level of 50 mg kg⁻¹ body weight was administered orally daily once for 14 days, prior to the administration of 0.2 mL of CCl₄ kg⁻¹ body weight for 7 days. The substantially elevated serum marker enzymes such as Serum glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), Lactate dehydrogenase (LDH), Gamma glutamyl Transferase (γGT) and 5' Nucleotidase (5'NT), due to CCl₄ treatment were restored towards normalization in rats pretreated with BHEE. The determination of liver weight and biochemical parameters such as serum bilirubin and blood urea showed a remarkable decrease in rats pre-treated with the BHEE when compared with those administered CCl₄ alone. In addition BHEE significantly increased the total protein, total cholesterol and triglyceride also towards normal levels. Silymarin at a dose level of 50 mg kg⁻¹ was used as a standard reference also exhibited significant hepatoprotective activity against CCl₄ induced hepatotoxicity. The results of this study strongly indicate that BHEE has got a potent hepatoprotective action against CCl₄ induced hepatic damage in rats.

Key words: Hepatoprotective, marker enzymes, bi-herbal ethanolic extract, carbon tetra chloride

INTRODUCTION

The use of herbal medicine in the treatment of diseases is increasing worldwide. Indeed, the effectiveness of some medicinal herbs in the treatment of diseases has been validated by rough research and clinical studies (Oguntola, 2007). Liver, an important organ actively involved in many metabolic functions, is the frequent target for number of toxicants (Meyer and Kulkarni, 2001). The disorders associated with the organ are numerous and varied (Wolf, 1999). Serum or plasma enzyme levels have been employed as markers for monitoring chemically induced tissue damages (Hukkeri *et al.*, 2002). Carbon tetra chloride is toxic to the liver and its toxicity is dose dependent and time of exposure (Junnila *et al.*, 2000). In the liver, CCl₄ is metabolized into the highly reactive trichloromethyl radical. The 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 membrane (Pandit *et al.*, 2004).

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In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function and offer protection to the liver from the damage or help in the regeneration of hepatic cells (Guntupalli *et al.*, 2006). 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 (Chatterjee, 2000). A single drug cannot be effective against all types of severe liver diseases (Shahani, 1999). Therefore effective formulations have to be developed using indigenous medicinal plants, with proper pharmacological experiments and clinical trials.

With the above scenario, the Bi-Herbal Ethanolic Extract (BHHE) made up equal quantities of leaves of *Melia azedarach* and seeds of *Piper longum* were subjected to various assays in order to evaluate their hepatoprotective effect from mixture of these herbs against CCl₄ toxicity in albino rats. *Melia azedarach*, a member of the family Meliaceae is widely grown as an ornamental tree, is being used against intestinal worms, in skin diseases, stomach ache, intestinal disorders, uterine illnesses, cystitis, as diuretic and febrifuge (Senthil Nathan *et al.*, 2005; Alche *et al.*, 2002). It has got antiviral, antimalarial, anthelmintic and cytotoxic activities (Khan *et al.*, 2001). *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 medicine (Guido and David, 1998). *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 conditions (Singh, 1992).

MATERIALS AND METHODS

Plant Material

The leaves of *Melia azedarach* and seeds of *Piper longum* were collected in the month of November 2005 from center for Advanced Studies in Botany Field Research Laboratory, University of Madras, Chennai, India and were authenticated by Dr. P.T. Kalaichelvan at the same centre. The voucher specimen is also available in herbarium file of the Studies in Botany Field Research Laboratory, University of Madras and Chennai, India.

Extraction

The leaves of *Melia azedarach* (1 kg) and seeds of *P. longum* (1 kg) 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 (Chattopadhyay, 2003). The extract was evaporated under pressure until all the solvent had been removed and further removal the of water was carried out by freeze drying to give an extract sample with the yield of 19.7% (w/w). The extract was stored in refrigerator; weighed amount was dissolved in Tween-80 and used for present investigation.

Animals

Adult albino male rats of wistar strain weighing 150-175 g were used in the pharmacological and toxicological studies. The inbred animals were taken from animal house in Madras Medical College, Chennai, India. The animals were maintained in well-ventilated room temperature with natural 12±1 h day-night cycle in the propylene cages. They were with fed balanced rodent pellet diet from Poultry Research Station, Nandam, Chennai, India and tap water *ad libitum* was provided throughout the experimental period. The animals were sheltered for one week and prior to the experiment they were acclimatized to laboratory temperature. Acute toxicity studies were carried out as per up and down or staircase method (Ghosh, 1984). The protocol was approved by Animal Ethics Committee constituted for the purpose as per CPCSEA Guideline.

Experimental Groups

The rats were divided into five groups with six animals in each group and were given dose schedule as:

- Group 1: Animals were given a single administration of 0.5 mL vehicle (2% (v/v) aqueous Tween- 80) p.o for 14 days. This group served as control.
- Group 2, 3 and 5: Animals were given a single dose of 2 mL kg⁻¹, p.o CCl₄ (2% (v/v) aqueous Tween-80) daily for 7 days.
- Group 3: Animals were treated with 50 mg kg⁻¹, p.o of (BHEE) daily for 14 days.
- Group 4: Animals received only 50 mg kg⁻¹, p.o of BHEE daily for 14 days.
- Group 5: Animals received 50 mg kg⁻¹ p.o Silymarin in 2 % (v/v) aqueous Tween-80 daily for 14 days and this group served as positive control.

On the 15th day the animals were sacrificed and various biochemical parameters were analyzed.

Liver Weight and Biochemical Parameters

At the end of the experimental period animals were sacrificed by cervical decapitation under mild pentobarbitone anesthesia, blood was collected and the serum was separated by centrifuging at 3,000 rpm for 10 min. Immediately after the sacrifice, the liver was excised from the animals, washed in ice-cold saline. The weight of the liver is measured and expressed as (mg g⁻¹) wet tissue. The biochemical parameter such as total protein was estimated by the method of Gornall *et al.* (1949). The total cholesterol was estimated by the method of Wybenga *et al.* (1970). The total bilirubin was estimated by Malloy and Evelyn (1937) method. Triglyceride was estimated by the method of Fossati and Lorenzo (1982) and urea concentration was determined by the method of Varley (1967).

Marker Enzymes

The above collected serum was also used for the assay of marker enzymes. The Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) were estimated by the method of Reitman and Frankel (1957). Alkaline Phosphatase (ALP) and Acidphosphatase (ACP) were determined by the method of Kind and King (1954). The enzyme Lactatedehydrogenase (LDH) was analyzed by the method of King (1965). The gamma glutamyl transferase (γ GT) enzyme was determined by the method of Szasz (1969) and 5' nucleotidase (5' NT) enzyme by Luly *et al.* (1972). All the enzymatic and biochemical assays were taken at particular nm using Shimadzu spectrophotometer, UV-1601 model.

Statistical Analysis

Values reported are the mean \pm SEM. The statistical analysis was carried out using Analysis of Variance (ANOVA) followed by Dunnet's t-test. $p < 0.05$ were considered as significant.

RESULTS

Liver Weight and Biochemical Parameters

In the present investigation a significant reduction in the liver weight ($p < 0.001$) was shown in group 3 BHEE pretreated animals when compared to that of the group 2 CCl₄ intoxicated animals. Table 1 shows that in group 3 there was a significant increase in total protein (6.1 ± 0.32 g dL⁻¹), total cholesterol (128.16 ± 3.34 mg dL⁻¹) and triglyceride (187.33 ± 7.0 mg dL⁻¹) levels in the CCl₄ intoxicated and BHEE pretreated animals ($p < 0.001$) when compared with group 2 CCl₄ intoxicated animals which has the total protein (5.25 ± 0.18 g dL⁻¹), total cholesterol (125.33 ± 2.901 mg dL⁻¹) and

Table 1: The average values of weight and biochemical parameters of the liver under different experimental conditions

Parameters	Group 1 control	Group 2 CCl ₄ treated	Group 3 BHEE+ CCl ₄ treated	Group 4 BHEE treated	Group 5 Silymarin treated
Liver weight (mg g ⁻¹ wt.)	38.67±0.41	69.56±0.23a*	52.69±0.57b*	49.87±0.86b*	33.780±0.92 ^{NS}
Total protein (g dL ⁻¹)	6.90±0.24	5.25±0.18a*	6.10±0.32b*	7.10±0.21b*	6.200±0.32 ^{NS}
Total cholesterol (mg dL ⁻¹)	144.16±2.30	125.33±2.90a*	128.16±3.34b*	142.30±2.01b*	139.000±3.10 ^{NS}
Total bilirubin (mg dL ⁻¹)	0.52±0.02	2.47±0.09a*	1.57±0.10b*	0.87±0.13b*	0.564±0.01 ^{cNS}
Urea (mg dL ⁻¹)	19.00±1.50	45.00±2.40a*	32.16±2.75b*	21.00±1.90b*	33.000±2.00 ^{cNS}
Triglycerides (mg dL ⁻¹)	163.00±2.05	125.00±2.101a*	187.33±7.00b*	148.80±1.49b*	157.800±3.11 ^{cNS}

Values are Mean±SEM of 6 animals each in a group. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's t-test (n = 6) Comparison between: a: Group 1 vs Group 2, b: Group 2 vs. Group 3 and 4, c: Group 1 vs Group 5, *p<0.001, NS: Not Significant

Table 2: The average values of liver marker enzymes under different experimental conditions

Parameters	Group 1 Tween-80	Group 2 CCl ₄	Group 3 BHE+ CCl ₄	Group 4 BH	Group 5 Silymarin
SGPT (U L ⁻¹)	46.15±1.10	143.79±4.50a*	87.30±3.40b*	38.75±1.46b*	76.92±3.60 ^{NS}
SGOT (U L ⁻¹)	46.00±1.03	145.50±1.08a*	75.16±3.40b*	45.50±1.66b*	78.16±0.54 ^{NS}
ALP (KA)	76.66±0.53	172.68±0.64a*	121.75±0.72b*	76.16±0.38b*	121.28±1.00 ^{cNS}
ACP (KA)	4.11±0.05	12.25±1.06a*	6.76±0.24b*	3.20±0.15b*	6.70±0.29 ^{cNS}
LDH (U L ⁻¹)	145.90±1.87	435.38±1.84a*	253.00±1.50b*	135.26±0.87b*	240.70±2.90 ^{cNS}
γ GT (U L ⁻¹)	13.28±0.57	45.03±1.59a*	20.41±1.04b*	10.30±1.06b*	11.30±0.32 ^{cNS}
5'NT (U L ⁻¹)	5.35±0.57	7.60±0.40a*	5.85±0.28b*	4.88±0.30b*	5.50±0.23 ^{cNS}

Values are Mean±SEM of 6 animals each in a group. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's t-test (n = 6) Comparison between: a: Group 1 vs Group 2, b: Group 2 vs. Group 3 and 4, c: Group 1 vs Group 5, *p<0.001, NS: Not Significant

triglyceride (125±2.01 mg dL⁻¹), respectively. The biochemical parameters such as serum bilirubin (1.57±0.10 mg dL⁻¹) and urea (32.16±2.75 mg dL⁻¹) levels were also decreased in the BHEE (at a dose level of 50 mg kg⁻¹ of body wt) pretreated group 3 animals, (p<0.001) When compared with the CCl₄ intoxicated group 2 animals which has the total bilirubin and urea (2.47±0.09 and 45.0±2.4 mg dL⁻¹), respectively. Group Comparison between group 1 and 4 shows no significant variation in liver weight and biochemical parameter levels indicates no appreciable adverse side effect due to the administration of Tween-80 and BHEE alone. Group comparison between group 3 and group 5 shows no significant variation in these parameters indicating that BHEE has got the same effect as that of the Silymarin, which was considered as the positive control in this study.

Marker Enzymes Level

A significant increase in the serum GOT (145.50±1.08 U L⁻¹) and GPT (143.79±4.5 U L⁻¹) levels were seen in the group II CCl₄ intoxicated animals. These enzymes were brought back to near normal levels such as (75±1.46 U L⁻¹) and (87.30±3.4 U L⁻¹), respectively in BHEE (50 mg kg⁻¹ body weight) pretreated group 3 animals (p<0.001). Similarly the elevated ALP (172.68±0.64 KA) and ACP (12.25±1.06 KA) enzyme levels in group 2 CCl₄ intoxicated animals were also significantly decreased to (121.75±0.72 KA) and (6.76±0.24 KA), respectively in the group 3 BHEE pretreated and CCl₄ intoxicated animals (Table 2). The enzymes such as LDH (235.0±1.50 U L⁻¹), γ-GT (20.41±1.04 U L⁻¹) and 5'NT (5.85±0.28 U L⁻¹) were also significantly decreased in the group 3 CCl₄ intoxicated and BHEE pretreated, animals when compared with the group 2 CCl₄ intoxicated animals which showed the elevated enzyme levels of LDH (435.38±1.84 U L⁻¹), γ-GT (45.03±1.59 U L⁻¹) and 5'NT (7.6±0.4 U L⁻¹) (p<0.001), respectively. Group comparison between Group 1 control rats and the animals of group 4 which received only BHEE shows no significant variation in the marker enzyme levels indicating no adverse side effects due to the administration of Tween-80 and BHEE alone. All the parameters were under normal limits in the group 5 animals that acted as a positive control, which were intoxicated by CCl₄ and treated by silymarin.

DISCUSSION

It is well established that CCl₄ induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl₄ is bio-transformed by the cytochrome p450 system in the endoplasmic reticulum to produce trichloromethyl free radical (CCl₃). Trichloromethyl free radical when combined with cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxy radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical (Reckengel *et al.*, 1989). Thus, trichloromethylperoxy free radical leads to elicit lipid peroxidation, the destruction of Ca²⁺ homeostasis and finally, results in cell death (Opuku *et al.*, 2007).

In this 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 the normal levels in BHEE pretreated group 3 and silymarin treated group 5 animals. BHEE treatment showed a protection against the injuries effects of carbon tetra chloride that may result from the interference with cytochrome p450, resulting in the hindrance of the formation of hepatotoxic free radicals (Cavin *et al.*, 2001). The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis. (Bandyopadhyay *et al.*, 1999). Attainment of near normalcy in protein, cholesterol and triglyceride levels in CCl₄ intoxicated and BHEE treated rats confirms the hepatoprotective effect of the plant extract. It can be said that BHEE had played a crucial role in synthesizing newer cytoplasm thus enabling regeneration of more and more hepatocytes, which in turn will take over the liver function with improved vigor and vitality. (Venukumar and Latha, 2002). It has been reported that the antioxidant activity or inhibition of the generation of free radicals is important in the protection against CCl₄ induced liver lesion.

The marked elevation of bilirubin and urea level in the serum of group 2 CCl₄ intoxicated group rats were significantly decreased in the group III BHEE pretreated animals. Bilirubin is the conventional indicator of liver diseases (Girish *et al.*, 2004). Bilirubin an endogenous organic anion binds reversibly to albumin and it is transported to the liver, conjugates with the glucouronic acid and excreted in the bile. Hepatobiliary disease is indicated when total bilirubin exceeds the upper limit of normal (Rosen and Keefe, 1998) The bilirubin lowering ability of the extract in pretreated rats further indicate the hepatoprotective nature of the BHEE on hepatocytes when compared with rats administered CCl₄ alone.

Estimating the activities of serum GOT, GPT, ALP, LDH, 5' NT, can make assessment of liver damage. These are the enzymes 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 (Nkosi *et al.*, 2005). The elevated level of these entire marker enzymes observed in the group 2 CCl₄ treated rats in the present investigation corresponded to the extensive liver damage induced by the toxin. These marker enzymes are cytoplasmic in origin and are released into the circulation after cellular damage (Lin *et al.*, 2000). The tendency of these marker enzymes to return towards a near- normalcy in-group III BHEE pretreated rats was a clear manifestation of anti- hepatotoxic effect of BHEE. The reduced concentrations of GOT and GPT as a result of plant extract administration observed during the present study might probably be due to the presence of catechins in the extract (Naidoo *et al.*, 2006). The results were found comparable to Silymarin. Silymarin that is composite name of three flavonoids isolated from milk thistle *Silybum marimum* and are used as hepatoprotectives against experimental hepatotoxicity of various chemicals including CCl₄ (Gadgoli and Mishra, 1999).

In conclusion the Bi herbal ethanolic extract afforded protection from CCl₄ induced liver damage. The protections against liver damage by the BHEE were found comparable to silymarin. Possible mechanism that may be responsible for the protection of CCl₄ induced liver damage by BHEE may be 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 processes (Upadhyay *et al.*, 2001). It is well documented that flavonoids and glycosides are strong antioxidants (Kavithalakshmi *et al.*, 2006). 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. Thus, from the foregoing findings, it was observed that BHEE is a promising hepatoprotective agent and this hepatoprotective activity of BHEE may be due to its antioxidant chemicals present in it. Work is in progress here to identify the antioxidant ability of this Bi-herbal extract.

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