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Evaluation of Combined hepatoprotective effect of Bi - herbal ethanolic extract on CCl_4 induced hepatic damage in rats

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Abstract

The combined hepatoprotective effect of Bi- herbal ethanolic extract (BHEE) was evaluated against carbon tetra chloride (CCl_4) induced hepatic damage in rats. Ethanolic extract from the leaves of *Eclipta alba* and seeds of *Piper longum* at a dose level of 50 mg/kg body weight was administered orally daily once for 14 days. The substantially elevated serum marker enzymes such as SGOT, SGPT, ALP, LDH, ACP, γGT and 5' Nucleotidase, due to CCl_4 treatment were restored towards normalization. The biochemical parameters like total protein, total bilirubin, total cholesterol, triglycerides, and urea were also restored towards normal levels. In addition, BHEE significantly decreased the liver weight of CCl_4 intoxicated rats. Silymarin at a dose level of 50 mg/kg was used as a standard reference also exhibited significant hepatoprotective activity against CCl_4 induced hepatotoxicity. The results of this study strongly indicate that BHEE has got a potent hepatoprotective action against CCl_4 induced hepatic damage in rats.

Keywords: Hepatoprotective, marker enzymes, Bi-herbal ethanolic extract, carbon tetra chloride.

INTRODUCTION

Liver, an important organ actively involved in many metabolic functions and is the frequent target for a number of toxicants (Meyer et al., 2001) . Hepatic damage is associated with distortion of these metabolic functions (Wolf, 1999). 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 effects (Guntupalli et al., 2006). In the absence of a reliable liver protective drug in modern medicine there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders (Chatterjee, 2000). In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity. A single drug cannot be effective for all types of severe liver diseases (Shahani, 1999). Therefore an effective formulation has to be developed using indigenous medicinal plants, with proper pharmacological experiments and clinical trials.

With the above scenario, the Biherbal ethanolic extract (BHEE) made up equal quantities of leaves of *E. alba* and seeds of *P. longum* were subjected to various assays in order to evaluate their hepatoprotective effect from mixture of these herbs against CCl_4 toxicity in albino rats. These plants have traditional claim against Liver disorders (Sathyavathi et al., 1988) and all of them are scientifically evaluated for their potency individually (Kulshrestha et al., 1971). 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 hepatitis (Wagner et al., 1986; Singh et al., 1993; Singh et al., 2001). *P. longum* is a component in medicines reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions (Singh, 1992). Preliminary phytochemical analysis of the BHEE reveals the presence of flavonoids and glycosides (Christina et al., 2006). The activity of the BHEE against CCl_4 toxicity was compared with silymarin a well-known

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

Parameters	Group I	Group II	Group III	Group IV	Group V
Liverweight (mg/gram.wt)	38.67±0.41	69.56±0.23a*	52.68±0.53b*	49.87±0.86b*	33.78±0.92c^{NS}
Total Protein (g/dl)	6.9 ± 0.24	5.25±0.18 a*	6.2 ± 0.27 b*	7.1±0.21. b*	6.2 ± 0.32c^{NS}
Total cholesterol (mg/dl)	144.16±2.3	125.33±2.9a*	130.8±3.002b	142.3±2.01b*	139.0±3.1 c^{NS}
Total bilirubin (mg/dl)	0.52± 0.02	2.54± 0.01a*	1.6 ± 0.02b*	0.87±0.13b*	0.564±0.01c^{Nc}
Urea (mg/dl)	19 ±1.5	45± 2.4 a*	32.3±2.7 b*	21±1.9b*	33.0±2.0 c^{NS}
Triglycerides (mg/dl)	163.0±2.05	125.0±2.101a	186.0± .6b*	148.8±1.49b*	157.8±3.11c^{NS}

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 I vs Group II, b–Group II vs. Group III or IV, c–Group Group V vs I. *P<0.001, NS–Not Significant.

antihepatotoxic agent.

MATERIALS AND METHODS

The leaves of *E. alba* and seeds of *P. longum* were collected 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 Center. The voucher specimen is also available in herbarium file of the Studies in Botany Field Research Laboratory, University of Madras, Chennai, India.

The leaves of *E. alba* (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^{O}C (Chattopadhyay, 2003). The extract was evaporated under pressure until all the solvent had been removed and further removal of the 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 2% (v/v) aqueous Tween–80 (2 ml of Tween 80 dissolved in 98 ml of water) and used for present investigation

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 ± 1h 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 study was carried out as per "Up and down" or "Stair case" method (Ghosh, 1984). The protocol was approved by Animal Ethics Committee constituted for the purpose as per CPCSEA Guideline.

The rats were divided into five groups with six animals in each group and were given dose schedule as: Group I: Ani-mals 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 II, III, and V: Animals were given a sin- gle dose of 2 ml/kg, p.o CCl_4 daily for 7 days; Group III: Animals were treated with 50mg/kg, p.o of BHEE daily for 14 days;

Group IV: Animals received only 50 mg/kg, p.o of BHEE) daily for 14 days; Group V: Animals received 50 mg/kg p.o. silymarin in 2% (v/v) aqueous Tween –80 daily for 14 days. This group served as positive control. On the 15th day the animals were sacrificed and various biochemical parameters were analyzed.

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. The above collected serum was used for the assay of marker enzymes. The glutamate oxaloacetate trans-aminase (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 lactate-dehydrogenase (LDH) was analyzed by the method of King (1965). The gamma glutamyl transferase (γGT) enzyme was determined by the method of Szasz (1969) and 5' nucleotidase (5 'NT) enzyme by Luly (1972).

The biochemical parameter such as total protein was estimated by the method of Gornall (1949). The total cholesterol was estima-ted by the method of Wybenga (1980). The total bilirubin was esti-mated by Malloy and Evelyn (1937) method. Triglyceride was estimated by the method of Fossati and Lorenzo (1983) and urea concentration was determined by the method of Bousquet (1971). Immediately after the sacrification, the liver was excised from the animals, washed in ice-cold saline, and the weight of the liver was calculated. All the enzymatic and biochemical assays were taken at particular nm using Shimadzu spectrophotometer, UV-1601 model. Values reported are the mean ± S.E.M. 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 (Woodson, 1983).

RESULTS

In the present investigation a significant reduction in the liver weight (P<0.001) was seen in-group III BHEE treat-ed animals when compared to that of group II CCl_4 intoxi-cated animals. The biochemical parameters such as serum bilirubin (1.6 ± 0.02 mg/dl) and urea (32.3 ± 2.7 mg/dl) levels were also decreased significantly in-group III BHEE (at a dose level of 50 mg/kg of body wt) treated animals (P<0.001), when compared with the CCl_4 intoxicated group II animals which had the total bilirubin and urea (2.54 ± 0.01 mg/dl) and (45.0 ± 2.4 mg/dl) respectively. Table 1 shows that in-group III there was a significant increase in total protein (6.2 ± 0.27 g/dl), total cholesterol (130.8 ± 3.00 mg/dl), and triglyceride (186 ± 3.6 mg/dl) levels in the CCl_4 intoxicated and BHEE treated animals (P<0.001) when compared with the group II CCl_4 intoxicated animals, which has the total protein (5.25 ± 0.18), total cholesterol (125.33 ± 2.901) and triglyceride (125 ± 2.01) respectively. Group Comparison

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

Parameters	Group I Control	Group II CCl_4 treated	Group III CCl_4 + BHEE treated	Group IV BHEE treated	Group V Silymarin treated
GPT (U/L)	46.15 ± 1.10	143.79 ± 4.50a*	87.30 ± 3.40b*	38.75 ±1.46b*	76.92± 3.6c^{NS}
GOT (U/L)	46.00 ± 1.03	145.50±1.08a*	75.00± 0.98b*	45.50 ± 1.66b*	78.16±0.54c^{NS}
ALP (K.A)	76.66 ± 0.53	172.68±0.64a*	121.75±0.72b*	76.16±0.38b*	121.28±1.0c^{NS}
ACP (K.A)	4.11 ± 0.05	12.20± 1.06a*	6.76 ±0.24b*	3.2 0 ± 0.15b*	6.70 ±0 .29c^{NS}
LDH (U/L)	145.9 ± 1.87	435.38±1.84a*	253.00 ±1.50b*	135.26±0.87b*	240.70± 2.90c^{NS}
γ GT (U/L)	13.28 ± 0.57	45.03± 1.59a*	20.41 ±1.04b*	10.30± 1.06b*	11.30±0 .32c^{NS}
5'NT (U/L)	5.35 ± 0.57	7.60± 0.40a*	5.85 ±0.28b*	4.88 ± 0.30b*	5.50 ±0 .23c^{NS}

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 I vs Group II, b–Group II vs. Group III or IV, c–Group I vs Group V. *P<0.001, NS–Not Significant.

between Group I and IV 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 III and Group V 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.

A significant increase in the serum GOT (145.50±1.08 U/L) and GPT (143.79 ± 4.5 U/L) levels were seen in the group II CCl_4 intoxicated animals. These enzymes were reduced to near normal levels such as (75±0.98 U/L) and (87.30 ± 3.4 U/L) respectively in-group III BHEE (50mg/kg body weight) treated animals *(P*<0.001). Similarly the elevated ALP (172.68±0.64K.A) and ACP (12.2 ± 1.06 K.A) enzyme levels in-group II CCl_4 intoxica-ted animals were also decreased to (121.75 ± 0.72 K.A) and (6.76±0.24 K.A) respectively in the group III BHEE treated rats (Table 2). The enzymes such as LDH (235.0 ± 1.50 U/L), γ-GT (20.41±1.04 U/L) and 5'NT(5.85 ± 0.28 U/L) were also significantly decreased in the group III BHEE treated animals when compared with the group II CCl_4 intoxicated animals that showed the elevated enzyme levels of LDH (435.38±1.84 U/L), γ-GT (45.03±1.59 U/L) and 5' NT (7.6±0.4 U/L) respectively (*P*<0.001). Group comparison between Group I control rats and the animals of group IV 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 V animals that acted as a positive control, which were intoxicated by CCl_4 and treated by silymarin.

DISCUSSION

It is well established that CCl_4 induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function (Mujumddar et al., 1998). CCl_4 is bio-transformed by the cytochrome *P*450 system in the endoplasmic reticulum to produce trichloromethyl free radical ($\bullet CCl_3$). Trichloromethyl free radical when combined with cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxyl radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethylperoxyl free radical leads to elicit lipidperoxidation, the destruction of Ca^{2+} homeostasis, and finally, results in cell death (Opoku et al., 2007).

In this present study it was noted that the administration of CCl_4 decreased the levels of total protein, total cholesterol, and triglycerides. These para-meters were brought back to the normal levels in the group III BHEE treated animals. BHEE treatment showed a protection against the injurious 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. The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis (Uday et al., 1999). Attainment of near normalcy in protein, cholesterol, and triglycerides levels in CCl_4 intoxicated and BHEE treated rats confirms the hepatoprotective effect of the plant extract.

The marked elevation of bilirubin and urea level in the serum of group II CCl_4 intoxicated rats were significantly decreased in the group III BHEE treated animals. Bilirubin is the conventional indicator of liver diseases (Girish, 2004). These biochemical restorations may be due to the inhibitory effects on cytochrome P450 or/and promotion of its glucuronidation (Cavin et al., 2001).

Assessment of liver can be made by estimating the activities of serum GOT, GPT, ALP, LDH, 5' Nucleotidase, and GT which are enzymes originally present higher concentration in cytoplasm. When there is heaptopathy, 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 II CCl_4 treated rats in this present study corresponded to the extensive liver damage induced by toxin. The reduced concentrations of ALT and AST as a result of plant extract administration observed during the present study might probably be due in part to the presence of catechins in the extract (Naidoo et al., 2006). The tendency of these marker enzymes to return towards a near- normalcy in-group III BHEE treated rats was a clear manifestation of anti- hepatotoxic effect of BHEE. The results were found comparable to silymarin. Silymarin that is composite name of three flavonoids isolated from milk thistle *Silybum marinum* and are used as hepatoprotectives against experimental hepatotoxicity of various chemicals including CCl_4 (Chhaya and Mishra, 1999).

In conclusion the Bi-herbal ethanolic extract afforded protection from CCl_4 induced liver damage. The protecttions against liver damage by the BHEE were found comparable to silymarin. Possible mechanism that may be responsible for the protection of CCl_4 induced liver damage by BHEE may be it could act as a free radical scavenger intercepting those radicals involved in 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 (Upadhyay et al., 2001). . It is well documented that flavonoids and glycosides are strong antioxidants (Natarajan 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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