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Hepatoprotective Activity of Ethanolic Extract of *Bacopa monnieri* Linn. Aerial Parts Against CCl_4-induced Hepatotoxicity in Rats

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Abstract – The ethanolic extract of *Bacopa monnieri* Linn. aerial parts were studied for its hepatoprotective effect on CCl_4-induced hepatotoxic rats. The extract was found to decrease significantly CCl_4-induced elevation of SGOT, SGPT, ALP, bilirubin and total cholesterol. But, it increased HDL-cholesterol level and liver weight with respect to CCl_4 toxic rats. The extract was also found to decrease significantly the CCl_4-induced elevation of lipid peroxidation and increase the activity of antioxidant enzymes (SOD and CAT) and GSH level in the liver of extract treated rats when compared with CCl_4 induced rats. Histopathological profiles showed that the extract had significant protective effect against CCl_4-induced liver injury, which corroborates the above findings. Hence it may be possible that the mechanism of hepatoprotection of the extract is due to its antioxidant effect.
Keywords – *Bacopa monnieri* L., ethanolic extract, hepatoprotective activity, antioxidant activity, histopathology.

Introduction

Liver diseases remain one of the serious health problems. It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals (Osawa *et al.,* 1990). Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional Indian medicines (Babu *et al.,* 2001).

Bacopa monnieri Linn. (Scrophulariaceae) is a creeping, glabrous, succulent herb, rooting at nodes, distributed throughout India in all plain districts, ascending to an altitude of 1,320 m (Anonymous, 1998). In Ayurveda, the plant has been used in the treatment of insanity, epilepsy, and hysteria. The other reported activities include sedative, antiepileptic, vasoconstrictor, and anti-inflammatory (Chopra *et al.,* 1986). The ethanolic extract of the aerial parts of the plant has been reported to possess significant anthelmintic activity (Ghosh *et al.,* 2005). The plant is reported to contain tetracyclic triterpenoid saponins, bacosides A and B (Chatterjee *et al.,* 1965; Basu *et al.,* 1967), hersaponin, alkaloids viz. herpestine and brahmine and flavonoids (Anonymous, 1998).

Saponins are natural products, which have been shown to possess antioxidant property (Yoshiki and Okubo, 1995; Hu *et al.,* 2002). Studies have confirmed that oxidative stress also plays an important role in the initiation and progression of liver disease (Bautista, 2001; Arteel, 2003). As *B. monnieri* contains large amounts of saponins it was thought worthwhile to investigate the hepatoprotective activity of the aerial parts of *Bacopa monnieri* Linn. in a scientific manner.

Experimental

Plant materials – The plant was identified by the taxonomists of the Botanical Survey of India, Govt. of India, Shibpur, Howrah. After authentication, fresh aerial parts of the young and matured plants were collected in bulk from the rural belt of Salipur, Orissa, India during early summer, washed, shade dried and then milled in to coarse powder by a mechanical grinder. A voucher specimen has been preserved in our laboratory at the Department of Pharmaceutical Technology, Jadavpur University for future reference.

Preparation of the extract – The powdered plant

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material (1000 g) was defatted with petroleum ether (60 - 80 °C) and then extracted with 3.5 litre of ethanol (95%) in a soxhlet apparatus. The solvent was removed under reduced pressure, which obtained a greenish-black sticky residue (yield: 11.6% w/w with respect to dried plant material). The dried extract (EBM) was stored in a desiccator till further study.

Preliminary phytochemical studies – The test samples were subjected to preliminary phytochemical studies using standard procedures (Harborne, 1984; Trease and Evans, 1983) to find out the nature of the phytoconstituents present with in them.

Drugs and chemicals – Silymarin was purchased from Micro labs, Tamilnadu, India, 1-Chloro-2, 4-dinitrobenzene [CDNB], Bovine serum albumin (Sigma chemical St. Louis, MO, USA), Thiobarbituric acid, Nitroblue tetrazolium chloride (NBT) (Loba Chemie, Mumbai, India), 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB), Carbon tetrachloride (SICCO Research Laboratory, Bombay). The solvents and/or reagents were of analytical grade.

Animals – Studies were carried out using Wistar albino rats (150 - 180 g) of male sex. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions. They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of experiment. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Acute toxicity study - The test was carried out as suggested by Seth *et al.*, 1972. Forty eight Swiss albino mice of either sex weighing between 25 - 30 g were divided into eight groups of six animals in each. The control group received normal saline (2 ml/kg, p.o.). The other groups received 100 - 3000 mg/kg, p.o. of the test extract, respectively. Immediately after dosing, the animals were observed continuously for the first 4 hours for any behavioral changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any.

Carbon tetrachloride-induced liver damage in rats – Healthy albino rats were divided into 4 groups each containing 6 animals. Group II (Control) received 30% CCl_4 in liquid paraffin (1 ml/kg body weight, i.p.). Group III received EBM 300 mg/kg, p.o. and Group IV received standard drug Silymarin (25 mg/kg, p.o) once in a day and CCl_4 as mentioned above. Treatment duration was 10 days and the dose of CCl_4 was administered after every 72 h (Manoj and Aqueed, 2003). Animals were sacrificed 24 h after the last injection. Blood was collected, allowed to clot and serum separated. The liver was dissected out weighed and used for *in vivo* antioxidant studies.

Serum analysis – After 24 h of the last injection, the animals of all groups were anaesthetized and sacrificed. Blood was drawn from heart and serum was separated for the assay of serum glutamate oxaloacetate transaminase (SGOT) (Rietman and Frankel, 1957), serum glutamate pyruvate transaminase (SGPT) (Rietman and Frankel, 1957), alkaline phosphatase (ALP) (King, 1965), bilirubin (direct and total) (Malloy and Evelyn, 1937) and cholesterol (total and HDL) (Warnick *et al.*, 1985) using analytical kits from Span Diagnostics Ltd., Surat, India.

***In vivo* antioxidant activity** – After the treatment period following study, the animals were deprived of food overnight and sacrificed by cervical dislocation. The livers were dissected out, washed in ice-cold saline, patted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation (LPO) (Fraga *et al.*, 1988). A part of homogenate after precipitating proteins with trichloro acetic acid (TCA) was used for estimation of reduced glutathione (GSH) (Ellman, 1959). The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant thus obtained was used for the estimation of superoxide dismutase (SOD) (Kakkar *et al.*, 1984) and catalase (CAT) activity (Maehly and Chance, 1954). Protein estimation was done as per the method of Lowry *et al.*, 1951.

Statistical analysis – Statistical significance was determined by One Way Analysis of Variance (ANOVA) followed by Dunnet's t-test to compare group means. The level of significance was $P < 0.001$.

Results

In acute toxicity study, it was found that the extract induced sedation and temporary postural defect at all tested doses. However, there was no mortality at any of the tested doses till the end of 14 days of observation. Therefore the LD_{50} value was more than 3000 mg/kg body weight.

Preliminary phytochemical study showed the presence of saponin, alkaloid, and flavonoids in the ethanolic extract of *B. monnieri*.

Rats subjected to CCl_4 only, developed significant ($P < 0.001$) hepatocellular damage as evident from significant increase in serum activities of GOT, GPT, ALP and bilirubin concentration as compared to normal control

Table 1. Effect of EBM (300 mg/kg, p.o.) on SGOT, SGPT, ALP, bilirubin, cholesterol and liver weight on CCl_4-induced hepatotoxicity in rats

group	treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (KA units)	bilirubin (mg/dl)		cholesterol (mg/dl)		liver weight (g)
					Total	Direct	Total	HDL	
I	Control	46.33 ± 0.95	55.33 ± 0.67	78.00 ± 1.79	0.57 ± 0.02	0.08 ± 0.01	116.42 ± 1.90	9.98 ± 0.61	10.90 ± 0.45
II	CCl_4 treated	138.33 ± 3.44#	124.22 ± 2.58#	173.33 ± 3.41#	7.26 ± 0.54#	1.72 ± 0.09#	179.58 ± 4.81#	1.97 ± 0.21#	7.42 ± 0.28#
III	CCl_4 + EBM	67.33 ± 2.95*	70.33 ± 2.22*	125.67 ± 3.16*	1.74 ± 0.05*	0.21 ± 0.01*	142.78 ± 3.25*	6.45 ± 0.39*	10.15 ± 0.31*
IV	CCl_4 + Silymarin	57.00 ± 2.57*	60.67 ± 2.56*	88.00 ± 3.27*	0.84 ± 0.03*	0.15 ± 0.02*	121.75 ± 2.17*	22.21 ± 1.75*	10.10 ± 0.15*

All values are Mean ± SEM, n = 6 rats in each group
#$P < 0.001$ as compared with Group I and *$P < 0.001$ as compared with Group II.

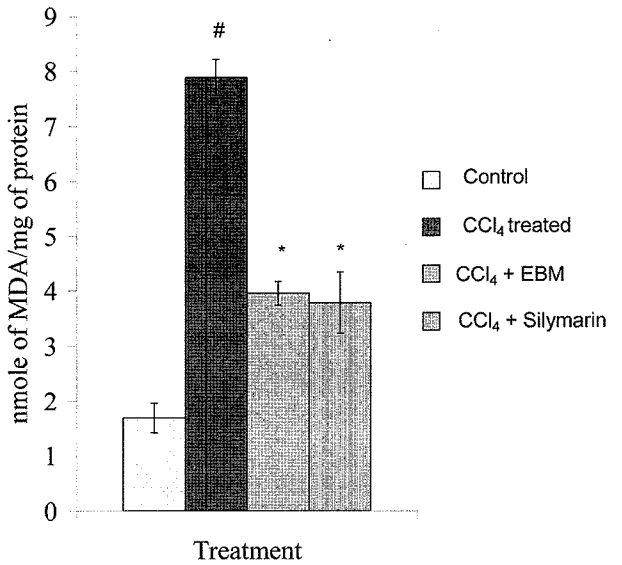

Fig. 1. Effect of EBM (300 mg/kg, p.o.) on TBARS level in liver of CCl_4-induced hepatotoxic rats *in vivo*.
All values are Mean ± SEM, n = 6 rats in each group.
#$P < 0.001$ as compared with control group and *$P < 0.001$ as compared with CCl_4- treated group.

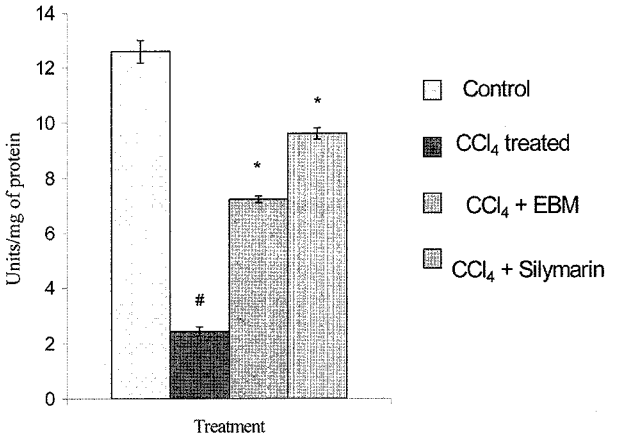

Fig. 2. Effect of EBM (300 mg/kg, p.o.) on SOD activity in liver of CCl_4-induced hepatotoxic rats *in vivo*.
All values are Mean ± SEM, n = 6 rats in each group.
#$P < 0.001$ as compared with control group and *$P < 0.001$ as compared with CCl_4- treated group.

group, which has been used as reliable markers of hepatotoxicity (Table 1). Oral administration of EBM (300 mg/kg, p.o.) exhibited significant reduction ($P < 0.001$) in CCl_4-induced increase in levels of GOT, GPT, ALP and bilirubin (Total and Direct) concentration. Treatment with silymarin (25 mg/kg, p.o.) also reversed the hepatotoxicity significantly ($P < 0.001$).

Table 1 also reveals that total cholesterol level of serum of rats treated only with CCl_4 increased significantly ($P < 0.001$) while HDL level decreased significantly ($P < 0.001$) with respect to control group. But, EBM was successful in blunting this CCl_4-induced increase in serum cholesterol level and decrease in HDL level, which is comparable with the reference drug silymarin. The liver weight of rats treated with CCl_4 only decreased significantly ($P < 0.001$), which is blunted by EBM and silymarin.

In vivo lipid peroxidation study reveals that rats of CCl_4 treated group showed significant increase ($P < 0.001$) in malondialdehyde (MDA) when compared with rats of normal control group. EBM and silymarin were able to significantly reduce ($P < 0.001$) this rise in MDA level (Fig. 1).

There was a marked decrease in the level of GSH and the activities of SOD and CAT in CCl_4 treated group when compared with normal control group. The GSH level and activities of SOD and CAT were significantly increased ($P < 0.001$) in EBM and silymarin treated

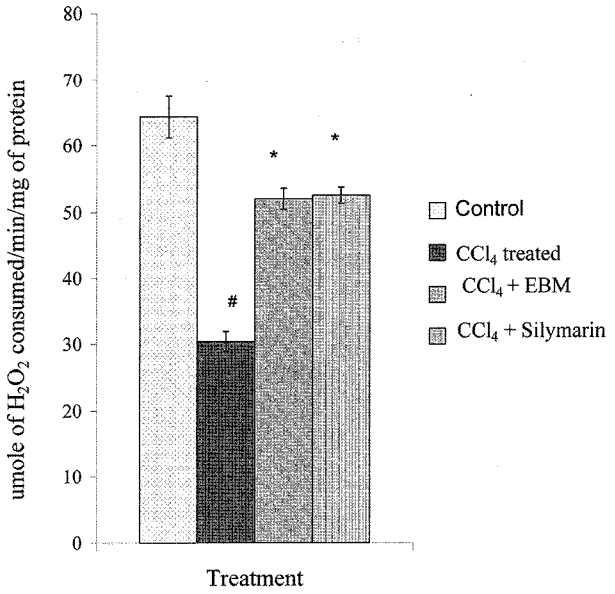

Fig. 3. Effect of EBM (300 mg/kg, p.o.) on CAT activity in liver of CCl_4-induced hepatotoxic rats *in vivo*.
All values are Mean ± SEM, n = 6 rats in each group.
$^{\#}P < 0.001$ as compared with control group and $^{*}P < 0.001$ as compared with CCl_4- treated group.

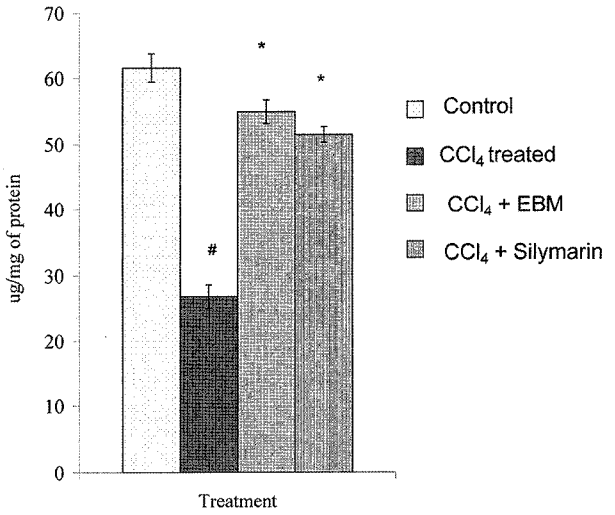

Fig. 4. Effect of EBM (300 mg/kg, p.o.) on GSH level in liver of CCl_4-induced hepatotoxic rats *in vivo*.
All values are Mean ± SEM, n = 6 rats in each group.
$^{\#}P < 0.001$ as compared with control group and $^{*}P < 0.001$ as compared with CCl_4- treated group.

groups (Fig. 2 - 4).

Histopathological profiles of CCl_4-induced hepatotoxic liver revealed extensive centrilobular necrosis extending to other necrotic areas, ballooning degeneration with steatosis (Fig. 6). The protective effect of EBM (300 mg/kg, p.o.) was confirmed by histopathological examination

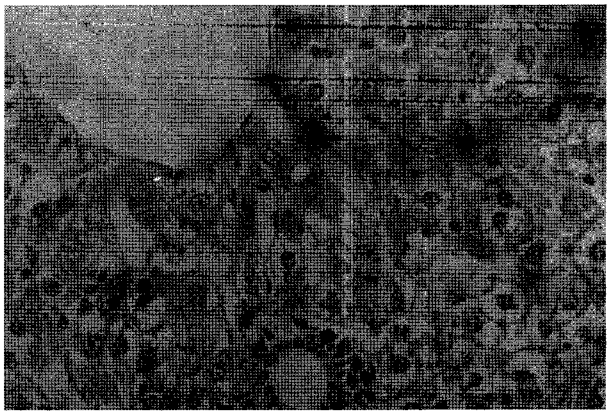

Fig. 5. Section of liver tissue of normal rat showing normal liver architecture with central vein and portal triads (H & E, 400×).

Fig. 6. Section of liver tissue of rat induced with CCl_4 showing extensive centrilobular necrosis, extending to other necrotic areas, ballooning degeneration along with fatty degeneration or steatosis (H & E, 400×).

Fig. 7. Section of liver tissue of EBM treated (300 mg/kg, p.o.) rat showing almost no necrosis, diffuse steatosis and mild increase in inflammatory cells in portal tract (H & E, 400×).

of liver section of control, CCl_4-induced and extract treated groups of rats (Fig. 5 - 7). EBM treated rats exhibited

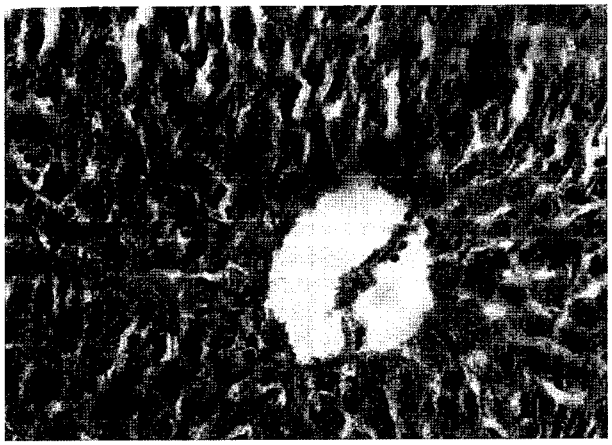

Fig. 8. Section of liver tissue of rat treated with silymarin (25 mg/kg, p.o.) showing almost normal liver architecture with no necrosis, no steatosis and mild sinusoidal congestion (H & E, 400×).

a significant improvement of hepatocellular architecture over CCl_4-induced group as evident from considerable reduction in necrosis and steatosis (Fig. 7). Liver section of rats treated with silymarin (25 mg/kg, p.o.) showed significant signs of amelioration of CCl_4-induced liver injury which is evident from normal liver architecture and absence of necrosis and steatosis (Fig. 8). The study showed that EBM and silymarin showed significant protective effect against CCl_4-induced liver injury which is evident from their histopathological examination (Fig. 5-8).

Discussion

Carbon tetrachloride is one of the most commonly used hepatotoxin. It is well documented that carbon tetrachloride is biotransformed under the action of cytochrome P_{450} in the microsomal compartment of liver to trichloromethyl radical which readily reacts with molecular oxygen to form trichloromethylperoxy radical (Raucy *et al.,* 1993). Both the radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides followed by pathological changes such as elevated levels of serum marker enzymes such as SGOT, SGPT, bilirubin and ALP (Zimmerman and Seeff, 1970), depletion of glutathione content and SOD and CAT activity (Kamiyama *et al.,* 1993).

The hepatoprotective activity of EBM was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which give a good idea about the functional state of the liver (Rao and Mishra, 1997). Necrosis or membrane damage releases the enzymes into circulation and therefore, it can be measured in serum. The increase in the level of serum bilirubin reflected the depth of jaundice and increase of serum transaminases and alkaline phosphatase was a clear indication of cellular leakage and loss of functional integrity of cell membrane (Saraswat *et al.,* 1993). Our results demonstrate that EBM caused significant inhibition of SGOT, SGPT, ALP and bilirubin levels when compared to CCl_4-induced hepatotoxic rats. Effective control of levels of serum transaminases, alkaline phosphatase activity and bilirubin level points towards an early improvement in secretory mechanism of hepatic cells.

The free radicals produced *in vivo* from CCl_4 toxicity attack the cell membrane and leads to membrane damage, alteration in the structure and function of cellular membrane. Thus increased level of lipid peroxides and decreased level of SOD and CAT activity as well as reduced glutathione are the indications of liver damage due to high oxidative stress in CCl_4 intoxicated rats (Mondal *et al.,* 2005).

Lipid peroxidation is an autocatalytic process in which MDA is one of the end products (Kurata *et al.,* 1993). In our *in vivo* study elevation in levels of end products of lipid peroxidation in liver of rats treated with CCl_4 were observed. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage. Pretreatment with EBM significantly reversed these changes. Moreover, cells have a number of mechanisms to protect themselves from the toxic effects of ROS. SOD removes superoxide (O^-_2) by converting it to H_2O_2, which can be rapidly converted to water by CAT (Halliwell *et al.,* 1992). It is well documented that hepatocellular enzymes (SOD, CAT) serve as biomarkers of hepatocellular injury due to alcohol and chemical toxicity (Chottopadhyay and Bondyopadhyay, 2005). So the studies on antioxidant enzymes (SOD, CAT) have been found to be of great importance in assessment of liver damage. The observed increase of SOD and CAT activity suggests that the ethanolic extract of *B. monnieri* have an efficient protective mechanism in response to ROS. GSH is a naturally occurring substance that is abundant in many living creatures. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and liver injury (Leeuwenburgh and Ji, 1995). From this point of view, exogenous ethanolic extract of *B. monnieri* supplementation might provide a mean to recover GSH levels and to prevent tissue disorders and injuries. In the present study, we have demonstrated the effectiveness of the extract by using CCl_4-induced rats, which is a known

model for both hepatic GSH depletion and injury. Our results are in line with this earlier report because we found that after EBM supplementation, elevated GSH level in rats with CCl_4 could be blunted to normal level. Hence it may be possible that the mechanism of hepatoprotection of extract is due to its antioxidant effect.

Results of the present studies, suggest that EBM has an ability to protect the liver from CCl_4-induced liver damage. The extract may be associated with decreased oxidative stress and free radical- mediated tissue damage. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Thus the hepatoprotective activity of EBM is most likely to be through its direct antioxidative effect. Further studies are needed to isolate and characterize the compounds responsible for the above effect.

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