RESEARCH ARTICLE

In *Vitro* Antioxidant and Hepatoprotective Activity of Ethanolic Extract of *Bacopa monnieri* Linn. Aerial Parts

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ABSTRACT

The ethanol extract of *Bacopa monnieri* Linn. (Schrophulariaceae) aerial parts (EBM) was investigated for any in vitro and in vivo antioxidant and hepatoprotective effects. EBM was prepared and estimation of total phenolics was carried out. Further, the antioxidant activity of EBM was studied using four *in vitro* models. The amount of total phenolics was estimated to be 47.7g of pyrocatechol equivalent per mg of extract. The reducing power of the extract was found to be concentration dependant. The antioxidant activity, nitric oxide scavenging activity and superoxide radical scavenging activity were also concentration dependant with IC_{50} value being 238.22g/ml, 29.17g/ml and 22.92g/ml respectively. The activities were found to be comparable with the reference drugs. Different groups of animals (Wistar albino rats) were administered with paracetamol (500 mg/kg, p.o., once in a day for 7 days). EBM at the dose of 300 mg/kg/day and silymarin at 25 mg/kg/day were administered to the paracetamol treated rats for seven days. The effects of EBM and silymarin on serum transaminases (SGOT, SGPT), alkaline phosphatase (ALP), bilirubin (Direct and Total), cholesterol (HDL and Total) and total protein were measured in the paracetamol-induced hepatotoxic rats. Further, the effects of the extract on lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were estimated. EBM and silymarin produced significant ($p < 0.05$) hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, total cholesterol and in vivo lipid peroxidation and significantly ($p < 0.05$) increasing the levels of GSH, SOD, CAT and HDL cholesterol. EBM also showed antioxidant effects on $FeCl_2$-ascorbate-induced lipid peroxidation in rat liver homogenate. From these results, it was suggested that EBM could protect the liver cells from paracetamol-induced liver damage perhaps, by its antioxidative effect on hepatocytes, hence eliminating the deleterious effects of toxic metabolites of paracetamol.

Keywords: *Bacopa monnieri, Hepatoprotective effect, Antioxidants, Paracetamol*

The role of free radical reactions in disease pathology is well established. It suggests that these reactions are necessary for normal metabolism but can be detrimental to health as well including outcome of various diseases like diabetes, immunosupression, neurodegenerative diseases and others [1]. Free radicals lead to cellular necrosis, which is implicated in some membrane pathophysiological conditions, including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotics [2].

Liver diseases remain a serious health problem. 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 [3]. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India [4].

Bacopa monnieri Linn. (Family-*Scrophulariaceae*) is a creeping, glabrous, succulent herb, rooting at nodes, distributed throughout India in all plain districts, ascending to an altitude of 1,320m [5]. 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 [6]. The ethanolic extract of the aerial parts of the plant has been reported to possess signify-

Reducing property of ethanol extract of B. monnieri
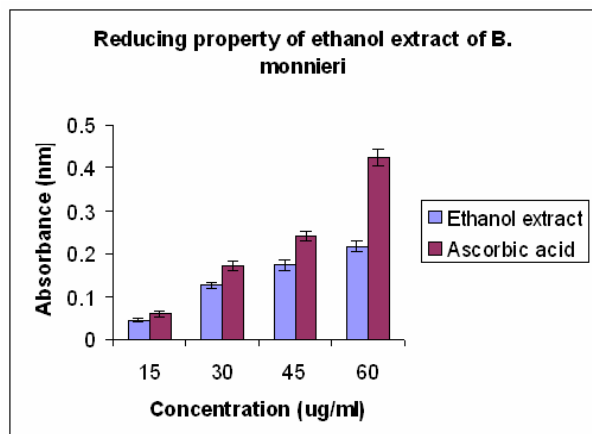
cant anthelmintic activity by some authors [7]. The plant is reported to contain tetracyclic triterpenoid saponins, bacosides A and B [8, 9], hersaponin, alkaloids viz. herpestine and brahmine and flavonoids [5].

Saponins are natural products, which have been shown to possess antioxidant property [10-12]. Studies have confirmed that indeed oxidative stress plays an important role in the initiation and progression of liver disease [13-15]. As *B. monnieri* contains large amounts of saponins it is thought worthwhile to investigate the antioxidant and hepatoprotective activity of the aerial parts of *Bacopa monnieri* Linn. in a scientific manner.

MATERIALS AND METHODS

Plant Material

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. The powdered plant material (400 g) was defatted with petroleum ether (60-80(C) and then extracted with 1.5 litre of ethanol (95%) in a soxhlet apparatus. The solvent was removed under reduced pressure, leaving 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 needed.

Preliminary Phytochemical Studies

The test samples were subjected to preliminary phytochemical studies, using standard procedures [16-17], to find out the nature of the phytoconstituents present with in them.

Chemicals Used

All the chemicals and reagents used in the study 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*. Swiss albino mice of either sex weighing between 25 – 30 g were acclimatized to laboratory condition for 10 days before commencement of experiment. They were also allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Estimation of Total Phenolic Compounds

The method of Naczk *et al*, 1989 [18] was followed. 0.1ml of 10mg/ml aqueous solution of the extract was diluted with 46 ml of distilled water in an Erlen meyer flask. Afterwards, 1 ml of Folin – Ciocalteu Reactive (FCR) was added into this mixture followed by addition of 3 ml of $Na_2 CO_3$ (2%) after 3 min. Subsequently, mixture was shaken on a shaker for 2 h at room temperature and then absorbance was measured at 760 nm. The concentration of total phenolic compounds was determined as micrograms of pyrocatechol equivalent by using the equation that was obtained from the standard pyrocatechol graph

In Vitro Antioxidant Activity

Reducing Power

The reducing power of the ethanolic extract of *B. monnieri* was determined according to the method of Oyaizu, 1986 [19]. Accurately weighed 10 mg of the extract in 1 ml of distilled water were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50^oC for 20 min. Following incubation, 2.5 ml of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

Antioxidant Activity

The antioxidant activity was determined according to the thiocyanate method of Gulchin *et al*, 2002 [20]. 1 ml of different concentrations of the extract of *B. monnieri* was added to linoleic acid in potassium phosphate buffer (2.5 ml, 0.04 M, pH 7.0). The mixed solution was incubated at 37^oC. The peroxide value was determined by reading the absorbance at 500 nm, after reaction with ferrous chloride and thiocyanate after two hours of incubation. The solutions without added extracts were used as blank samples. Percent inhibition was calculated from the formula:

% inhibition = $(A_c – A_t)/ A_c$ x 100

where A_c and A_t are absorbance of control and test samples respectively.

Table 1. Antioxidant activity of ethanolic extract of Bacopa monnieri aerial parts

Sample	Concentration (μg/ml)	% inhibition	IC_{50}	r^*
	100	22.20 ± 1.80		
	200	41.73 ± 2.71		
ETHANOLIC EXTRACT	300	59.13 ± 3.75	238.22	0.9944
	400	86.90 ± 4.97		
α-tocopherol			311.94	0.9699

n=3, Values are Mean ± S.D.

r^* = regression co- efficient

Assay for Nitric Oxide Scavenging Activity

The method of Sreejayan *et al*, 1997 [21] was followed. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of the extract dissolved in methanol and incubated at room temperature for 2½ h. The same reaction without the sample but equivalent amount of methanol served as control. After incubation period, 0.5 ml of Griess reagent was added. Absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as positive control.

The procedure is based on the principle that, Sodium nitroprusside solution spontaneously generates nitric oxides, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxides compete with oxygen leading to reduce production of nitrite ions

Assay for Superoxide Radical Scavenging Activity

The assay for superoxide radical scavenging activity was performed as per standard procedure [22]. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 μg riboflavin, 12 mM EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all added in chronological sequence. Reaction was started by illuminating the reaction mixture containing different concentrations of the sample extract for 90 sec and then measuring the absorbance at 590nm. Ascorbic acid was taken as the positive standard.

Acute Toxicity Studies

The test was carried out as suggested by Seth *et. al.*, 1972 [23]. Swiss albino mice of either sex weighing between 25 – 30 g were divided into different groups of six animals each. The control group received normal saline (2 ml/kg, p.o.). The other groups received 100, 200, 300, 600, 800, 1000, 2000, 3000 mg/kg of EBM respectively through oral route. 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.

Hepatoprotective Activity

Paracetamol – Induced Liver Damage in Rats (Acute Model) [24]

Four groups each comprising of six male Wistar albino rats weighing in the range of 150-180 g were selected. Group I served as control and fed orally with normal saline 5 ml/kg daily for seven days. Group II rats were similarly treated as Group I. Group III was treated with ethanolic extract 300 mg/kg/day orally for seven days, while Group IV was fed Silymarin 25mg/kg [25] as standard daily for seven days. Paracetamol suspension was given by oral route in a dose of 500 mg/kg/day to all rats except rats in Group I for seven days. The biochemical parameters were determined after 18 hours of fasting of the last dose. Daily records of body weight of all groups of animals were maintained during the whole experimental period.

Biochemical Studies

After the treatment period, the animals of all groups were anaesthetized and killed. Blood was drawn from heart and serum was separated for the assay of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase ALP, Bilirubin (Direct and total) and Cholesterol (Total and HDL). Analysis of SGOT, SGPT, ALP, Bilirubin (Direct and total) and Cholesterol (Total and HDL) was performed using analytical kits from Span Diagnostics Ltd., Surat, India. Serum GOT and GPT were measured according to the method of Rietman and Frankel, 1975 [26], serum ALP was measured according to the method of King, 1965 [27], serum bilirubin was estimated following Malloy and Evelyn method, 1937 [28]. Serum cholesterol (Total and HDL) was measured according to Warnick *et. al.*, 1985 [29]

FeCl₂-Ascorbic Acid Stimulated Lipid Peroxidation in Liver Homogenate

The Wister albino rats weighing 175-200 g were killed by decapitation and their liver tissues were

Table 2. Nitric oxide radical scavenging activity of ethanolic extract of Bacopa monnieri aerial parts

Sample	Concentration (μg/ml)	% inhibition	IC_{50}	r^*
	4.17	15.95 ± 0.66		
	8.33	27.27 ± 0.91		
ETHANOLIC EXTRACT	16.67	41.15 ± 1.02	29.17	0.9831
	33.33	51.70 ± 1.42		
	50.00	74.72 ± 1.61		
Ascorbic acid			15.02	0.9861

n=3, Values are Mean ± S.D.

r^* – regression co- efficient

Table 3. Superoxide radical scavengng activity of ethanolic extract of Bacopa monnieri aerial parts

Sample	Concentration (µg/ml)	% inhibition	IC$_{50}$	r*
ETHANOLIC EXTRACT	12.50	33.03 ± 2.06		
	18.75	40.40 ± 1.73	22.92	0.9873
	25.00	50.11 ± 1.76		
	37.50	80.85 ± 4.17		
Ascorbic acid			34.24	0.9868

n=3, Values are Mean ± S.D.

r* – regression co- efficient

quickly removed. A 2 g portion of liver tissue was sliced and then homogenized with 10 ml of 150 mM KCl Tris-HCl buffer (pH 7.4). The reaction mixture was composed of 0.25 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.4), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM FeCl$_2$ and 0.05 ml of various concentrations of EBM. The products of lipid peroxidation were quantified by the formation of the thiobarbituric acid-reactive material, MDA [30]. 1,1,3,3 Tetraethoxypropane was used as a standard for calibration of MDA. Appropriate controls were used to eliminate any possible interference with the thiobarbituric acid assay

Determination of In Vivo Antioxidant Activity

After collection of blood samples the rats were killed and their livers were excised, rinsed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.2) blotted 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 by the method of Fraga *et al.*, (1981)[31]. A part of homogenate after precipitating proteins was used for estimation of glutathione by the method of Ellman et al (1959)[32]. 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 SOD by the method described by Kakkar et al. (1984) [33]. CAT activity was measured by the method of (Maehly et al., 1954) [34]. Protein was estimated according to Lowry et al [35].

Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS in tissues was estimated by the method of Fraga *et al.*, (1981). To 0.5 ml tissue homogenate, 0.5 ml saline and 1.0 ml 10% TCA were added, mixed well and centrifuged at 3000 rpm for 20 min. To 1.0 ml of the protein-free supernatant, 0.25 ml of thiobarbituric acid (TBA) reagent was added; the contents were mixed well and boiled for 1 h at 95^{0}C. The tubes were then cooled to room temperature under running water and absorbance measured at 532 nm.

Determination of Reduced Glutathione (GSH)

GSH was determined by the method of Ellman *et al* (1958). 0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml precipitating reagent (1.67 g of meta phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2.0 ml of the filtrate, 4.0 ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5, 5-dithio- bis- 2-nitrobenzoic acid) reagent were added and read at 412 nm.

Determination of Super Oxide Dismutase (SOD)

The activity of SOD in tissue was assayed by the method of Kakkar *et al.* (1984). The assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1 ml phenazine methosulphate (186 mM/L), 0.3 ml NBT (300 mM/L), 0.2 ml NADH (780

Table 4. Effect of ethanolic extract of B. monnieri aerial parts (300 mg/kg p.o.) on paracetamol-induced hepatotoxicity in rats

Group	Treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (KA units)	Bilirubin (mg/dl) Total	Bilirubin (mg/dl) Direct	Cholesterol (mg/dl) Total	Cholesterol (mg/dl) HDL	Liver weight (g)
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	Paracetamol treated	126.50 ± 2.92*	117.67 ± 3.20*	153.67 ± 4.39*	5.43 ± 0.31*	0.96 ± 0.05*	175.46 ± 5.37*	1.71 ± 0.29*	7.28 ± 0.49*
III	Paracetamol + Extract	64.67 ± 3.00**	67.67 ± 3.24**	115.33 ± 2.11**	0.94 ± 0.05**	0.18 ± 0.03**	144.94 ± 5.64**	10.94 ± 0.98**	10.07 ± 0.50**
IV	Paracetamol + Silymarin	57.00 ± 2.24**	67.00 ± 4.72**	93.33 ± 2.62**	0.91 ± 0.04**	0.26 ± 0.03**	152.28 ± 4.83**	35.42 ± 2.05**	10.25 ± 0.34**

All values are Mean ± SEM, n=6 rats in each group

*p < 0.05 as compared with Group I and **p < 0.05 as compared with Group II.

Table 5. Effect of ethanolic extract of Bacopa monnieri aerial parts on in vitro lipid

Sample	Concentration (μg/ml)	% inhibition	IC_{50} (μg/ml)	r[*]
ETHANOLIC EXTRACT	50	16.33 ± 0.61		
	100	25.72 ± 1.95	154.51	0.9771
	150	43.90 ± 1.59		
	200	71.02 ± 2.32		
α-tocopherol			133.86	0.9947

n=3, Values are Mean ± S.D.

 r[*] = regression co- efficient.

mM/L) and approximately diluted enzyme preparation and water in a total volume of 3 ml. After incubation at 30^0C for 90 sec, the reaction was terminated by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The color intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol.

Determination of Catalase (CAT)

Catalase was assayed according to the method of Maehly and Chance, 1954. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The tissue was homogenized in M/150 phosphate buffer (pH 7.0) at $1–4^0C$ and centrifuged at 5000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The specific activity of catalase is expressed in terms of (mole of H_2O_2 consumed/min/mg of protein.

Statistical Analysis

Data for the *in vitro* antioxidant activity was expressed as Mean ± SD from three separate observations. Data for hepatoprotective activity and *in vivo* antioxidant activity were expressed as Mean ± SEM from 6 rats in each group. Hepatoprotective and *in vivo* antioxidant activity were analysed statistically using one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test. The minimum level of significance was fixed at $p<0.05$.

RESULTS

Determination of Total Phenolics

The study revealed that 1mg of ethanolic extract of *Bacopa monnieri* contains 47.7 μg of pyrocatechol equivalent.

Reducing Power of B. monnieri

Fig. 1 shows the reductive capacity of EBM as compared with ascorbic acid. The reducing power of *B. monnieri* was found to increase with increasing concentration of the extract, which is comparable with the standard drug ascorbic acid.

Antioxidant Activity

Table 1 reveals that the extract possessed significant antioxidant activity with IC_{50} value being 238.22 (μg/ml) and is comparable with the reference drug tocopherol.

NO Scavenging Activity

Ethanolic extract of *B. monnieri* significantly decreased with IC_{50} value 29.17 μg/ml, in a dose- dependent fashion, the concentration of nitrite after spontaneous decomposition of sodium nitroprusside, indicating that the ethanolic extract may contain compounds that are able to scavenge nitric oxide (Table 2).

Superoxide Radical Scavenging Activity

We have observed that the extract reduced the absorbance in a dose dependent manner, and the IC_{50} value was calculated to be 22.92 μg/ml from the regressional line, which is comparable with the reference drug (Table 3).

Acute Toxicity Study

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.

Serum Analysis

Rats subjected to paracetamol only, developed significant ($p<0.05$) hepatocellular damage as evident from significant increase in serum activities of GOT, GPT,

Table 6. Effect of ethanolic extract of B. monnieri aerial parts on LPO, antioxidant enzymes and GSH in liver of paracetamol-induced hepatotoxic rats in vivo

Group	Treatment	LPO[a]	SOD[b]	CAT[c]	GSH[d]
I	Control	1.69 ± 0.27	12.59 ± 0.42	64.40 ± 3.25	61.59 ± 2.11
II	Paracetamol treated	$8.09 \pm 0.42^*$	$4.49 \pm 0.28^*$	$43.31 \pm 1.97^*$	$32.42 \pm 0.79^*$
III	Paracetamol + Extract	$3.71 \pm 0.40^{**}$	$9.17 \pm 0.35^{**}$	$58.48 \pm 2.31^{**}$	$54.66 \pm 1.11^{**}$
IV	Paracetamol + Silymarin	$4.28 \pm 0.30^{**}$	$10.39 \pm 0.37^{**}$	$56.20 \pm 1.92^{**}$	$50.78 \pm 0.95^{**}$

All values are Mean ± SEM, n=6 rats in each group

[*]$p < 0.05$ as compared with Group I

[*]$p < 0.05$ as compared with Group II.

a= nmole of MDA/mg of protein.

b= Units/mg of protein

c= μmole of H_2O_2 consumed/min/mg of protein.

d= μg/mg of protein.

ALP and bilirubin concentration as compared to normal control group, which has been used as reliable markers of hepatotoxicity (Table 4). Oral administration of EBM (300 mg/kg, p.o.) exhibited significant reduction ($p<0.05$) in paracetamol-induced increase in levels of GOT, GPT, ALP and bilirubin (Total and Direct) concentration. Treatment with silymarin (200mg/kg, p.o.) also reversed the hepatotoxicity significantly ($p<0.05$).

Table 4 also reveals that total cholesterol level of serum of rats treated only with paracetamol increased significantly ($p<0.05$) while HDL level decreased significantly ($p<0.05$) with respect to control group. But, EBM was successful in blunting this paracetamol-induced increase in serum cholesterol level and decrease in HDL level, which was comparable with the reference drug silymarin.

Liver Weight

Table 4 also reveals that the liver weight of rats treated with paracetamol only decreased significantly ($p<0.05$), which was blunted by EBM and silymarin.

In Vitro Lipid Peroxidation

$FeCl_2$-ascorbic acid induced *in vitro* lipid peroxidation study revealed that EBM had significant anti lipid peroxidation potential with IC_{50} value being 154.31 g/ml, which was comparable with the reference drug α-tocopherol (Table 5).

In Vivo Antioxidant Activity

In vivo lipid peroxidation study reveals that rats of paracetamol treated group showed significant increase ($p<0.05$) in Malondialdehyde (MDA) when compared with rats of normal control group. EBM and silymarin were able to significantly blunt ($p<0.05$) this rise in MDA level (Table 6).

There was a marked decrease in the level of GSH and the activities of SOD and CAT in paracetamol treated group when compared with normal control group. The GSH level and activities of SOD and CAT were significantly increased ($p<0.05$) in EBM and silymarin treated groups (Table 6).

DISCUSSION

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS) may provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemicals or by other means. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and some other mechanism [36].

Phenols are very important plant constituents because of their free radical scavenging ability due to their hydroxyl groups [37]. The phenolic compounds may contribute directly to antioxidant action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human beings [38]. Ethanolic extract of *B. monnieri* is found to contain phenolic compound in significant amount, which attributes to its rationality of possessing antioxidant activity.

For measurements of the reductive ability, we investigated Fe^{+3} to Fe^{+2} transformation in the presence of ethanolic extract using the method discussed earlier. The reducing capacity of the extract may serve as a significant indicator of its potential antioxidant activity [39].

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states [40]. However, the specificity of this assay has been questioned since nitrite is one final product of the reaction of nitric oxide with oxygen, through intermediates such as NO_3, N_2O_4 and N_2O_3 [41]. Therefore the decrease in the nitrite production could also be due to interaction of the extract with other nitrogen oxides [42].

The *in vitro* superoxide radical scavenging activity is measured by riboflavin/ light/ NBT (Nitroblue tetrazoline) system reduction. The method is based on generation of superoxide radicals by auto oxidation of riboflavin in the presence of light. The superoxide radical reduces NBT to a blue colored formazone that can be measured at 560 nm [23]. The capacity of the ethanolic extract of *B. monnieri* to inhibit the colour to 50% is measured in terms of IC_{50}. Superoxide radical is known to be very harmful to the cellular components as a precursor of more ROS [43]. The extract has been found to have significant superoxide radical scavenging activity, which ultimately adds to its antioxidant potential.

Paracetamol (Acetaminophen) is a widely used antipyretic and analgesic, produces acute liver damage if overdoses are consumed. Paracetamol is mainly metabolized in liver to excretable glucuronide and sulphate conjugates [44, 45]. However, the hepatotoxicity of Paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-450 [46], to a highly reactive metabolite N-acetyl-P-benzoquinone imine (NAPQI) [47]. NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid [48]. However, when the rate of NAPQI formation exceeds the rate of detoxification by GSH, it oxidizes tissue macromolecules such as lipid or SH group of protein and alters the homeostasis of calcium after depleting GSH.

Paracetomol produces hepatic necrosis when ingested in very large doses. The hepatoprotective activity of the ethanolic extract was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which give a good idea about the functional state of the liver [49]. Necrosis or membrane damage releases the enzyme into circulation and therefore, it can be measured in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury [50]. Our results demonstrate that the ethanolic extract of *B. monnieri* caused signifi-

cant inhibition of SGOT and SGPT levels. Serum ALP and bilirubin levels on the other hand, are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure [51]. Our results also demonstrate that the ethanolic extract of *B. monnieri* caused significant inhibition of serum ALP and bilirubin levels. Effective control of alkaline phosphatase activity and bilirubin level points towards an early improvement in secretory mechanism of hepatic cells.

The effects of the extract on liver weights of rats are shown in Table 4. The paracetamol treated rats showed a significant loss in liver weight. But the extract administration significantly prevented this paracetamol-induced weight loss of liver in rats, which is comparable with that of standard drug administration.

Most hepatotoxic chemicals including paracetamol and alcohol damage liver by inducing, directly or indirectly, lipid peroxidation [52]. So, the studies on *in vitro* and *in vivo* lipid peroxidation of rats are performed. *In vitro* lipid peroxidation in liver homogenate can proceed in a non-enzymatic way. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, and it has been reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. In order to clarify the mode of action of ethanolic extract, *in vitro* lipid peroxidation experiments were carried out. According to the result obtained, the extract inhibited $FeCl_2$-ascorbic acid-stimulated lipid peroxidation in liver homogenate (Table 2).

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 [53]. In addition, a large reserve of reduced glutathione is present in hepatocytes and red blood cells for detoxification of xenobiotics or free radicals. However, oxidative stress results in toxicity when the rate at which the ROS are generated exceeds the cell capacity for their removal. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the lipid peroxidation process [54]. In our *in vivo* study elevation in levels of end products of lipid peroxidation in liver of rats treated with paracetamol were observed. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage. Pretreatment with EBM significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of extract is due to its antioxidant effect. GSH is widely distributed in cells. GSH is an intracellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. Indeed, GSH depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects, for example, a decrease in the rate of gluconeogenesis or an increase in glycogenolysis. The concept of a glutathione- SH threshold for drug detoxification was discussed by Jollow [55]. 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 injury. For example, liver injury included by consuming alcohol or by taking drugs like acetaminophen, lung injury by smoking and muscle injury by intense physical activity [56], all are known to be correlated with low tissue levels of GSH. From this point of view, exogenous ethanolic extract of *B. monnieri* supplementation might provide a mean of recover reduced GSH levels and to prevent tissue disorders and injuries. In the present study, we have demonstrated the effectiveness of the extract by using paracetamol-induced rats, which is a known model for both hepatic GSH depletion and injury. Therefore, the levels of glutathione are of critical importance in liver injury caused by paracetamol. Our results are in line with this earlier report because we found that after EBM -supplementation elevated GSH level in rats with paracetamol could be blunted to normal level. Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators, enzymes such as SOD and CAT. [57]. The SOD dismutases superoxide radicals O^-_2 into H_2O_2 plus O_2, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The observed increase of SOD activity suggests that the ethanolic extract of *B. monnieri* have an efficient protective mechanism in response to ROS. And also, these findings indicate that the extract may be associated with decreased oxidative stress and free radical- mediated tissue damage. CAT is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of ethanolic extract of *B. monnieri* increases the activities of catalase in paracetamol-induced liver damage rats to prevent the accumulation of excessive free radicals and protects the liver from paracetamol intoxication. This ability of EBM to protect the liver from paracetamol-induced damage might be attributed to its ability to restore the activity of antioxidative enzymes. Thus, results of these studies together with those of earlier ones, suggest that EBM has an ability to protect the liver from paracetamol-induced damage through its direct antioxidative effect.

It is well documented that hepatocellular enzymes (SOD, CAT) serve as biomarkers of hepatocellular injury due to alcohol and drug toxicity [58]. So the studies on antioxidant enzymes (SOD, CAT) have been found to be of great importance in assessment of liver damage.

In summary, we demonstrate that EBM prevents paracetamol-induced oxidative stress and hepatic injury. Since these models of hepatic damage in the rat simu-

late many of the features of human liver pathology, we suggest that natural antioxidants and scavenging agents in BME might be effective as plant hepatoprotectors and thus may have some obvious therapeutic implications. Therefore, it seems logical to infer that EBM, because of its antioxidant property, might be capable of protecting the hepatic tissue from paracetamol-induced injury and inflammatory changes.

The ethanolic extract of *B. monnieri* is reported to be rich in saponins [59]. Presence of saponins in the ethanolic extract was confirmed through our preliminary phytochemical screening also. Saponins are natural products, which have been shown to possess antioxidant property [10-12]. Studies have confirmed that indeed oxidative stress plays an important role in the initiation and progression of liver disease [13-15]. As *B. monnieri* contains large amounts of saponins it may be suspected that the hepatoprotective activity may be due to the presence of saponins in the extract. The activity may be attributed to their protective action on lipid peroxidation and at the same time the enhancing effects on cellular antioxidant defense contributing to the protection against oxidative damage in paracetamol-induced hepatotoxicity.

Further studies regarding the isolation and characterisation of the active principles responsible for hepatoprotective activity is currently under progress.

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