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Protective role of *Bacopa monniera* L. against hepatic oxidative stress in wistar albino rats

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Abstract

The protective role of ethanol extract of *Bacopa monniera* against alcohol plus carbon tetrachloride (CCl₄) induced hepatic oxidative stress in male wistar albino rats has been evaluated. Hepatic oxidative stress was significantly ($P \leq 0.001$) induced by 30% alcohol (3.0 ml/100g b. wt/day, po) and carbon tetrachloride (1:1 groundnut oil, 0.5 ml/kg b. wt, ip, on day 20th only) for 21 days. Simultaneously, the oral administration of *Bacopa monniera* extract, at the doses of 100 mg and 200 mg/kg b. wt /day) for 21 days, significantly ($P \leq 0.001$) restored the enzymatic activities of gamma glutamyl transpeptidase (γ -GTP), aspartate and alanine aminotransferases (AST, ALT), alkaline phosphatase (ALP), total bilirubin, total protein and albumin in serum. Further, the levels of cholesterol, triglycerides, lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), cytochrome-P-450 and ascorbic acid in the liver also returned near to the normal level in alcohol plus carbon tetrachloride-intoxicated rats. These biochemical observations were also supported by histopathological findings. In conclusion, *Bacopa monniera* showed liver protection in wistar albino rats possibly by its free radical scavenging activity along with reduction of fat metabolism might be due to the presence of triterpenoid saponins, alkaloids, total phenolics and flavonoids in the extract and/or the purified compounds- betulinic acid, wogonin and oroxindin, which were isolated from the ethanol extract of *Bacopa monniera*.

Keywords: *Bacopa monniera* Linn, oxidative stress, marker enzymes, antioxidants

1. Introduction

Liver is the major site of intense metabolism and prone to various disorders as consequence of exposure to toxins of extrinsic as well as intrinsic origin which are produced hepatic oxidative stress. The oxidative stress plays an important role as a fundamental factor in the pathogenesis of various liver diseases^[1,2]. Especially in alcoholic liver disease and viral hepatitis, oxidative stress plays an important role in the pathophysiological changes that progress to liver cirrhosis and finally to hepatocellular death^[3,4].

Conventional drugs used in the treatment of liver disease are sometimes inadequate and can have serious adverse effects^[5]. In the absence of reliable liver protecting drug in modern medical practice, recently search for crude drugs of the plant origin with antioxidant activity has become a central focus of the study of liver protection in all over the world^[6-8].

Although herbal medicines are effective in the treatment of various ailments, but unscientifically exploited and/or improperly used. Therefore, these plant medicines deserve detailed studies in the light of modern science. A number of indigenous plants have been described in the literature to exhibit liver protection with antioxidant status^[9].

Bacopa monniera Linn. (Scrophulariaceae) known as "Brahmi" in Hindi, is an indigenous plant used for a large number of diseases^[10,11]. Traditionally, the plant has been used since times immemorial as a nerve tonic to improve intellect and memory^[12] and also reported to be useful in the treatment of cardiac ailments, respiratory problems and neuropharmacological disorders^[10,13].

The ethanol extract of *Bacopa monniera* is recognized to exhibit anticancer^[14], neuroprotective^[15], liver and kidney protective^[6,16,17], antioxidant and behavioral^[18], and also hypocholesterolemic^[13] activities.

In view of the medicinal use of *Bacopa monniera* in various ailments, no attention has been paid to its solely liver curing role along with antioxidant action. Therefore, the present study has been under taken.

2. Materials and methods

2.1 Plant material

The aerial parts of *Bacopa monniera* Linn. was collected from near the Jal Mahal area, Jaipur, in the month of Aug–Sep. 2013. The plant was identified and authenticated from the Department of Botany, University of Rajasthan, Jaipur (Herbarium Sheet No. RUBL–19878).

2.2 Plant extraction and isolation of compounds

The plant material was shade dried, ground to a coarse powder and treated with petroleum ether for defatting as well as to remove chlorophyll. The powder was packed into a soxhlet apparatus and subjected to hot continuous percolation using alcohol (70% v/v) as solvent for 48 h at 58–60 °C. The extract was concentrated under vacuum, dried in a vacuum desiccator, and yielded 5.5% w/w as a dark greenish-brown solid mass. The solid mass was then powdered, and washed with chloroform to remove the remaining content of chlorophyll presented in the extract. Half of the extract was suspended in a proper volume of normal saline to prepare the desired concentration for oral administration to rats and rest of the extract (35 g) was subjected to traditional column chromatography for fractionation with different solvents. For this purpose, a column (1.4 m × 5 cm) filled with 800g Si-gel (60–120 mesh) was used. The purity of fractions was checked by qualitative thin layer chromatography using different solvent systems. After determining the purity of compounds, it was subjected to detailed spectral analysis (IR, 1H NMR, 13C NMR and MS) to establish the structures of yielded compounds. As a result, the compounds (A, B & C) were isolated, purified and characterized.

2.2.1 Characterization of compound A

Compound A was obtained when the column was eluted with chloroform and methanol in the ratio 9:1. After removal of solvent, the compound A was obtained as colourless crystals (35 mg), m.p. 316 °C -318 °C. IR (KBr, cm⁻¹): 3320-2800 (OH, COOH), 1715 (C=O); ¹HNMR (CDCl₃, 400MHz): δ 4.36 and 4.48 (=CH₂), 1.68 (s, =C-CH₃), 2.30 (m, H-19), 3.27 (m, H-3α), 0.76 (s, 3H), 0.78 (s, 3H), 0.82 (s, 3H), 0.96 (s, 3H), 1.03 (s, 3H) for five tertiary methyl groups; ¹³CNMR (CDCl₃, 125MHz): δ 36.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.3 (C-7), 40.7 (C-8), 50.5 (C-9), 36.9 (C-9), 36.9 (C-10), 20.8 (C-11), 25.5 (C-12), 38.8 (C-13), 42.4 (C-14), 30.5 (C-15), 32.1 (C-16), 56.3 (C-17), 46.8 (C-18), 49.2 (C-19), 150.3 (C-20), 29.7 (C-21), 36.9 (C-22), 27.9 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.7 (C-27), 180.5 (C-28), 09.6 (C-29), 19.6 (C-30); MS (m/z): 456[M]⁺, 422, 410, 409, 341, 247, 219, 206, 203, 189, 143, 59, etc.

On the basis of ¹HNMR, ¹³CNMR and mass spectral studies, the compound A was characterized as betulinic acid (Figure 1) with molecular formula C₃₀H₄₈O₃ [19-21].

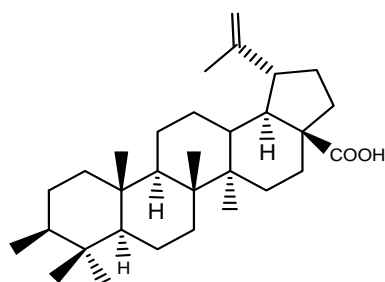


Fig 1: Chemical Structure of Compound A

2.2.2 Characterization of compound B

Compound B was obtained when the column was eluted with chloroform and methanol in the ratio 7:3. After the crystallization with acetone, compound B was obtained as crystalline solid (60 mg), m.p. 202 °C -204 °C. ¹HNMR (CDCl₃, 400MHz): δ 3.6 (3H, s, -OMe), 6.21 (1H, s, H-6), 6.7 (1H, s, H-3), 7.3 (3H, d, J=8Hz, H-3', 4', 5'), 8.0 (2H, d, J=8Hz, H-2', 6'), 8.2 (OH), 12.1 (5-OH); ¹³CNMR (CDCl₃, 125MHz): δ 61.43 (OMe), 99.7 (C-6), 104.9 (C-10), 105.3 (C-3), 126 (C-2', 6'), 127.3 (C-8), 129.1 (C-3', 5'), 131.1 (C-4'), 131.9 (C-1'), 149.3 (C-9), 156.0 (C-5), 156.3 (C-7), 163.1 (C-2), 182.3 (C-4). On the basis of ¹HNMR, ¹³CNMR spectral studies of compound B, it was characterized as wogonin (Figure 2) with molecular formula C₁₆H₁₂O₅ [19, 22, 23].

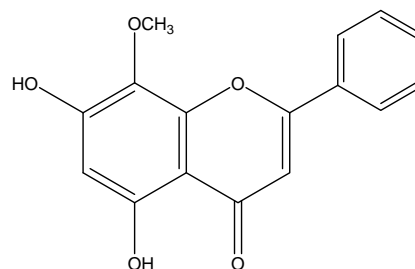


Fig 2: Chemical Structure of Compound B

2.2.3 Characterization of compound C

Compound C was obtained when the column was eluted with chloroform and methanol in the ratio 7:3. After the crystallization with acetone, compound C was obtained as light yellow crystals (40 mg), m.p. 212 °C -215 °C. IR (KBr) ν_{max} (cm⁻¹): 3400 (OH), 1720 (carboxyl) and 1640 (bonded carboxyl); ¹HNMR (400MHz): δ 3.2-3.6 (br.m, sugar protons), 3.6 (3H, s, -OMe), 5.2 (1H, br.s, H-1''), 6.3 (1H, s, H-3), 7.1 (1H, s, H-6), 7.8 (3H, d, J=7,8Hz, H-3', 4' & 5'), 8.3 (2H, d, J=7,8Hz, H-2', 6'), 12.4 (br, 5-OH & -COOH); ¹³CNMR (CDCl₃, 125MHz): δ 61.43 (OMe), 67.23 (C-4''), 71.89 (C-2''), 73.13 (C-3''), 76.61 (C-5''), 99.11 (C-6), 100.31 (C-1''), 105.20 (C-10), 105.21 (C-3), 126.41 (C-2', 6'), 129.31 (C-3', 5', 8), 130.75 (C-4'), 132.86 (C-1'), 149.11 (C-9), 156.65 (C-7), 163.53 (C-2), 180.36 (C-4, 6'').

On the basis of ¹HNMR, ¹³CNMR and IR spectral studies of compound C, it was characterized as oroxindin (Figure 3) with molecular formula C₂₂H₂₀O₁₁ [19, 24].

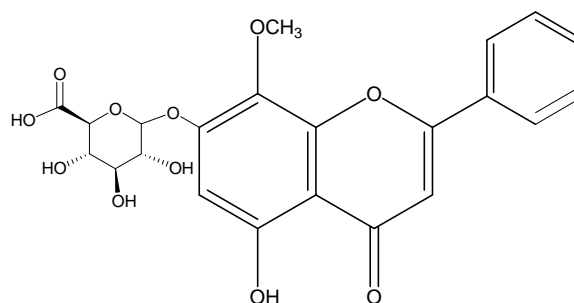


Fig 3: Chemical Structure of Compound C

2.3 Animals

Colony bred healthy, adult male albino rats (wistar strain) (*Rattus norvegicus*) weighing 150–160 g, were used in the present study. The rats were housed in polypropylene cages under controlled conditions of temperature (25±3 °C), humidity (60%–70%) and light (12h light/dark cycle). They were provided with a nutritionally adequate standard laboratory diet (Lipton, India Ltd., Bangalore, India) and tap water *ad libitum*.

2.4 Ethical aspects

The study was approved by the ethical committee (Protocol No. 1678/Go/a/12/CPCSEA/97) of the University Department of Zoology, Jaipur, India. Indian National Science Academy, New Delhi, (INSA, 2000) guidelines were followed for maintenance and use of the experimental animals.

2.5 Toxicological study

The extract of plant material was administered to all the test groups in graded doses ranging up to 2 g/kg body weight and the rats were observed for signs of toxicity and mortality for 30 days afterward. The extract was found to be practically non-toxic when given orally to rats and its LD₅₀ value was found to be higher than 2 g/kg body weight [data not shown]. The minimum dose levels viz. 100 and 200 mg/kg body weight were used for oral administration to rats during experimentation [7].

2.6 Chemicals

All chemicals were analytical grade and chemicals required for all biochemical assays were obtained from Sigma Chemicals Co., St. Louis, MO, USA.

2.7 Experimental design

After acclimatization of 15 days, the animals were divided into the following groups containing 06 animals in each group:

Group I: Vehicle (normal saline 3.0 ml/kg body weight) treated rats were kept on normal diet and served as control for 21 days.

Group II: Rats treated with hepatotoxins i.e. 30% alcohol (3.0 ml/100g b wt/three times in a day, orally) for 21 days plus a single injection of carbon tetrachloride (0.5 ml/kg b wt, dissolve in groundnut oil, 1:1, intra-peritoneally) on 20th day.

Group III: Rats orally received *Bacopa monniera* extract at 100 mg/kg b wt/day, and 30% alcohol plus CCl₄ as Group II for 21 days, simultaneously.

Group IV: Rats orally received *Bacopa monniera* extract at 200 mg/kg b wt/day, and 30% alcohol plus CCl₄ as Group II for 21 days, simultaneously.

2.8 Autopsy Schedule

After the last dose-delivery, rats of each group were kept on starvation for 24 h and after that anaesthetized under mild ether anesthesia. Blood samples were collected by cardiac puncture of each animal in sterilized vials and allowed to clot at 37 °C. Serum was separated by centrifugation then stored at 4 °C until assayed.

After the collection of blood, The entire liver was perfused immediately with cold physiological saline and thereafter carefully removed, trimmed free of extraneous tissues and weighed on an electrical balance. Half of the liver was fixed in Bouin's fixative for histopathological studies and the remaining half was immediately frozen (at -20°/-70 °C) for biochemical analysis.

2.9 Serum biochemistry

Biochemical assay of enzymes, viz. γ -GTP (gamma glutamyl transpeptidase), AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase) alongwith total bilirubin, total protein and albumin were performed by using colorimetrically kit methods. AST, ALT, ALP and total bilirubin kits were purchased from sigma Chemicals Co. (St. Louis, Mo), USA. Total protein and albumin kits were obtained from span diagnostics (India) Pvt. Ltd. and γ -GTP kit was also obtained from HUMAN, Germany.

2.10 Liver biochemistry

The quantitative estimation of total cholesterol [25], triglycerides [26], Lipid peroxidation (TBARS) [27], reduced glutathione [28], superoxide dismutase [29], catalase [30], and ascorbic acid [31] were performed in liver homogenate. A liver microsomal fraction was also prepared [32] and the cytochrome P-450 content in this fraction was measured from a reduced carbon monoxide difference spectrum [33], respectively.

2.11 Percentage of protection

The liver protection (LP), expressed as percentage (%), was calculated using the following equation:

$$LP (\%) = \frac{T_1 - T_2}{T_1 - T_3} \times 100$$

Where, T₁ is the mean value of only oxidative stressed animals; T₂ is the mean value of oxidative stressed plus *B. monniera* extract treated animals and T₃ is the mean value of normal control animals.

2.12 Histopathology

Liver was fixed in Bouin's fixative for 24 h and after that dehydrated in ethanol series (50%–100%), cleared in xylene, and embedded in paraffin using the standard microtechnique. Sections of the liver (5 μ m) were stained with alum haematoxylin and eosin (H-E) for histopathological changes.

2.13 Statistical analysis

The results obtained in the present study were expressed as the mean \pm SEM for each parameter and statistically processed by applying student "t" test. *p* values ≤ 0.05 were considered as significant.

3. Results

The findings of present investigation indicate that the 70% ethanol extract of *Bacopa monniera* possesses hepatoprotective, antioxidant and hypolipidemic action in a dose-dependent manner against chemically induced hepatic oxidative stress in the experimental rats at the dose levels of 100 mg and 200 mg/kg body weight/day for 21 days, respectively.

3.1 Determination of serum profile

The 30% alcohol plus CCl₄ were used as the oxidative stress agents which were induced-severe liver damage in the experimental rats. As shown in Table-1, 30% alcohol plus CCl₄ administration resulted the significant ($P \leq 0.001$) elevation in serum γ -GTP, AST, ALT, ALP, total bilirubin whereas serum total protein and albumin levels were depleted (Group II), when compared with normal controls (Group I). Concurrent oral administration of *Bacopa monniera* extract at the dose levels of 100 mg and 200 mg/kg b wt/day for 21 days, brought about a remarkable dose-dependent restoration in the levels of γ -GTP: 53.49% ($P \leq 0.001$) & 83.33% ($P \leq 0.001$); AST: 55.40% ($P \leq 0.001$) & 85.69% ($P \leq 0.001$); ALT: 58.21% ($P \leq 0.001$) & 90.11% ($P \leq 0.001$); ALP: 60.18% ($P \leq 0.001$) & 87.96% ($P \leq 0.001$); total bilirubin: 58.64% ($P \leq 0.001$) & 82.10% ($P \leq 0.001$); total protein: 37.03% ($P \leq 0.001$) & 70.37% ($P \leq 0.001$); and albumin: 35.71% ($P \leq 0.001$) & 71.43% ($P \leq 0.001$), respectively (Group III & IV).

Table 1: Showing serum analysis following 30% alcohol plus CCl₄-induced hepatic oxidative stress and *B. monniera* extract treatment in wistar albino rats

Group	γ -GTP		AST		ALT		ALP		Bilirubin		Total protein		Albumin	
	(IU/l)	(%) Liver Protection	(U/ml)	(%) Liver Protection	(U/ml)	(%) Liver Protection	(KAU)	(%) Liver Protection	(mg/dl)	(%) Liver Protection	(g/dL)	(%) Liver Protection	(g/dL)	(%) Liver Protection
Normal (vehicle treated) (Group I)	5.24 ± 0.31		55.4 ± 1.65		44.7 ± 1.53		9.5 ± 0.19		0.70 ± 0.04		6.6 ± 0.18		3.5 ± 0.11	
30% alcohol + CCl ₄ (Group II)	23.0 ± 1.40 ^a		272 ± 2.73 ^a		256 ± 2.31 ^a		20.3 ± 0.79 ^a		2.32 ± 0.17 ^a		3.9 ± 0.10 ^a		2.1 ± 0.07 ^a	
30% alcohol + CCl ₄ + <i>B. monniera</i> extract (100 mg/kg b wt) (Group III)	13.5 ± 0.52 ^{***}	53.49	152 ± 2.09 ^{***}	55.40	133 ± 1.52 ^{***}	58.21	13.8 ± 0.33 ^{***}	60.18	1.37 ± 0.14 ^{***}	58.64	4.9 ± 0.15 ^{***}	37.03	2.6 ± 0.09 ^{***}	35.71
30% alcohol + CCl ₄ + <i>B. monniera</i> extract (200 mg/kg b wt) (Group IV)	8.2 ± 0.27 ^{***}	83.33	86.4 ± 2.35 ^{***}	85.69	65.6 ± 1.65 ^{***}	90.11	10.8 ± 0.38 ^{***}	87.96	0.99 ± 0.02 ^{***}	82.10	5.8 ± 0.14 ^{***}	70.37	3.1 ± 0.10 ^{***}	71.43

Levels of significance:

Data are mean ± SEM (n=6)

a = $p < 0.001$ Group II compared with control (Group I).*** = $p < 0.001$ Group III and IV compared with group II.**Table 2:** Showing antioxidant and lipid parameters following 30% alcohol plus CCl₄-induced hepatic oxidative stress and *B. monniera* extract treatment in wistar albino rats

Group	Cytochrome P-450		Lipid peroxidation		GSH		SOD		Catalase		Ascorbic Acid		Total Cholesterol		Triglycerides	
	n mole/mg protein	(%) Liver Protection	TBARS, n mole/g tissue	(%) Liver Protection	n mole/g tissue	(%) Liver Protection	μ mole/mg protein	(%) Liver Protection	μ mole H ₂ O ₂ consumed/min/mg protein	(%) Liver Protection	mg/g tissue	(%) Liver Protection	mg/g tissue	(%) Liver Protection	mg/g tissue	(%) Liver Protection
Normal (vehicle treated) (Group I)	4.98 ± 0.22		2.2 ± 0.07		3.8 ± 0.16		9.84 ± 0.46		60.0 ± 2.54		1.3 ± 0.05		6.8 ± 0.31		9.4 ± 0.41	
30% alcohol + CCl ₄ (Group II)	2.21 ± 0.10 ^a		6.8 ± 0.48 ^a		2.1 ± 0.12 ^a		3.55 ± 0.15 ^a		34.6 ± 1.60 ^a		0.83 ± 0.10 ^a		14.3 ± 1.12 ^a		16.1 ± 0.92 ^a	
30% alcohol + CCl ₄ + <i>B. monniera</i> extract (100 mg/kg b wt) (Group III)	3.57 ± 0.35 ^{**}	49.10	4.6 ± 0.24 ^{**}	47.82	2.7 ± 0.14 ^{**}	35.29	5.82 ± 0.12 ^{***}	36.41	46.5 ± 2.42 ^{**}	46.85	1.08 ± 0.16 ^{ns}	53.19	10.8 ± 0.76 [*]	46.67	12.8 ± 0.86 [*]	49.25
30% alcohol + CCl ₄ + <i>B. monniera</i> extract (200 mg/kg b wt) (Group IV)	4.16 ± 0.12 ^{***}	70.40	3.4 ± 0.17 ^{***}	73.91	3.5 ± 0.18 ^{***}	82.35	8.33 ± 0.17 ^{***}	75.99	56.8 ± 3.10 ^{***}	87.40	1.22 ± 0.09 [*]	82.99	8.1 ± 0.45 ^{***}	82.67	10.9 ± 0.72 ^{***}	77.61

Levels of significance:

Data are mean ± SEM (n=6)

a = $P \leq 0.001$ Gp. II compared with control (Group I).*** = $P \leq 0.001$; ** = $P \leq 0.01$; * = $P \leq 0.05$; ns=non-significant Group III & IV compared with Group II.

3.2 Determination of tissue profile

As shown in Table-2, 30% alcohol plus CCl₄ (Group II) caused a significant ($P \leq 0.001$) decrease in the levels of cytochrome *P*-450, GSH, SOD, catalase and ascorbic acid contents alongwith concomitant significant ($P \leq 0.001$) elevation in the level of lipid peroxidation as compared to normal controls (Group I), whereas simultaneously, oral administration of *Bacopa monniera* extract at the doses of 100 mg and 200 mg/kg b wt/day to rats (Group III and Group IV), remarkably antagonized alterations in the antioxidant defense system like cytochrome *P*-450: 49.10% ($P \leq 0.01$) & 70.40% ($P \leq 0.001$); lipid peroxidation: 47.82% ($P \leq 0.01$) & 73.91% ($P \leq 0.001$); GSH: 35.29% ($P \leq 0.01$) & 82.35% ($P \leq 0.001$); SOD: 36.41% ($P \leq 0.001$) & 75.99% ($P \leq 0.001$); catalase: 46.85% ($P \leq 0.01$) & 87.40% ($P \leq 0.001$); and ascorbic acid contents: 53.19% (ns) & 82.99% ($P \leq 0.05$), in a dose dependent manner following 21 days of *Bacopa monniera* extract treatment, respectively.

Apart from this, the rats subjected to 30% alcohol plus CCl₄ in the Group II, resulted hepatocellular damage as evident from a significant ($P \leq 0.001$) hyperlipidemic elevation as the total

cholesterol and triglycerides levels in comparison to normal controls (Group I). Simultaneously, oral treatment of *Bacopa monniera* extract at the doses of 100 mg and 200 mg/kg b. wt./day for 21 days, was dose-dependently restored the levels of total cholesterol: 46.67% ($P \leq 0.01$) and 82.67 ($P \leq 0.001$) and triglycerides: 49.25% ($P \leq 0.01$) & 77.61% ($P \leq 0.001$) in the experimental rats (Group III and IV).

3.3 Determination of tissue histopathology

The liver histopathology of 30% alcohol plus CCl₄-treated rats when compared to normal hepatic architecture (Figure 4) revealed intense centrilobular necrosis, steatosis and often swelling of hepatic cytoplasm (Figure 5). The oral treatment of *Bacopa monniera* extract (100 mg and 200 mg/kg body weight) along with intoxication of 30% alcohol plus CCl₄ showed signs of protection against 30% alcohol plus CCl₄ to a considerable extent as evident from the formation of normal hepatic cords, absence of necrosis and vacuoles in the hepatic architecture of the group III & IV (Figure 6 and 7), respectively.

Explanation of Photomicrographs

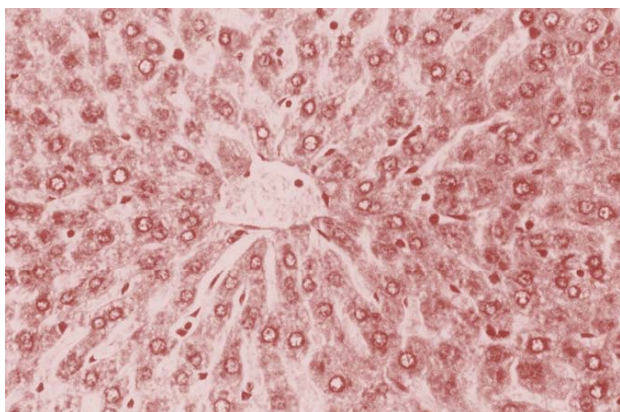


Fig 4: Photomicrograph of control rat liver section showing well brought central vein, hepatic cell with preserved cytoplasm and prominent nucleus at H & E X100

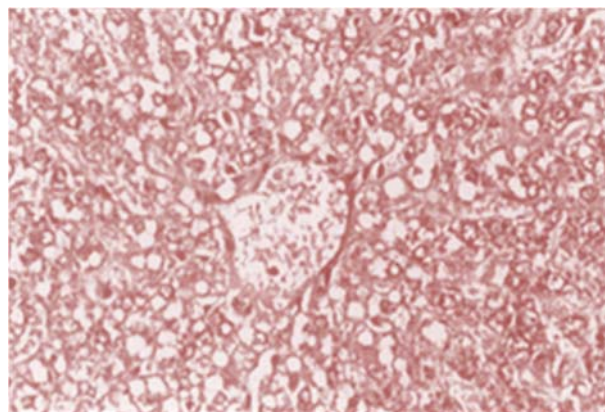


Fig 5: Photomicrograph of rat liver section (30% alcohol plus CCl₄) showing marked steatosis of the hepatocytes with ballooning degeneration & distended portal vein, mild periportal fibrosis and necrosis at H & E X100

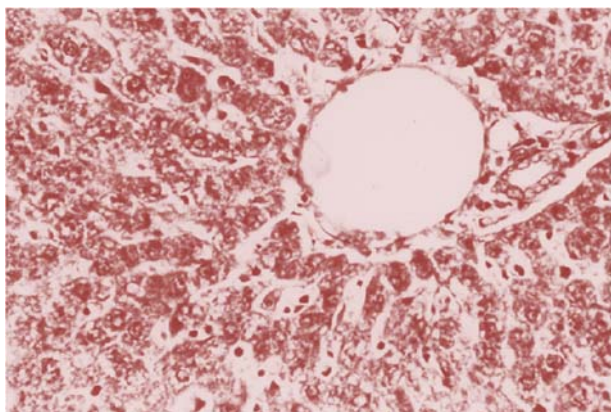


Fig 6: Photomicrograph of rat liver section (30% alcohol plus CCl₄) + *B. monniera* extract (100 mg/kg b wt) showing moderately regeneration in hepatocellular architecture at H & E X100

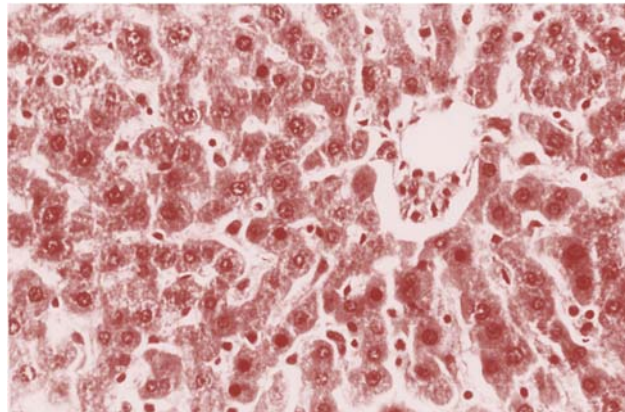


Fig 7: Photomicrograph of rat liver section (30% alcohol plus CCl₄) + *B. monniera* extract (200 mg/kg b wt) showing considerable reduction in necrosis and fatty changes with pyknotic nuclei and cytoplasmic clearing at H & E X100

4. Discussion

Liver cells appear to participate in a variety of enzymatic metabolic activities, scavenge the free radicals which produced oxidative stress and then inhibit the lipid peroxidation [1, 7, 8].

Now a days, carbon tetrachloride (CCl₄) and alcohol are widely used as experimental model hepatotoxicants in the various drug discovery practices against hepatotoxicity [1, 4, 7, 8, 34]. In our present investigation, rats treated with 30% alcohol plus CCl₄, transaminases activities (AST, ALT) were increased in plasma by the release of enzymes from damaged liver parenchymal cells [7, 8, 35]. Enzymes as AST and ALT are the most frequently utilized indicators of hepatic injury and represent markers of hepatocellular necrosis [36, 37]. Oral administration of *B. monniere* extract was reduced the levels of AST, ALT induced by 30% alcohol plus CCl₄. These reduced levels indicate the protective nature of *B. monniere* extract against hepatic oxidative stress might be due to the presence of triterpenoid saponins, alkaloids and flavonoids which have been reported in *Bacopa monniere* extract [38] and/or the isolated purified compounds- betulinic acid, wogonin and oroxindin from the ethanol extract of *Bacopa monniere*.

The CCl₄ induction/excess alcohol consumption has been linked to leakage of the cytoplasmic membrane bound liver enzyme γ -GTP into blood due to the enhanced generation of oxyfree radicals during its oxidation in the liver. This result leads to the elevated level of γ -GTP in serum and its measurement has been claimed to be an extremely sensitive test elevated in Group II [1, 39]. Oral administration of *B. monniere* extract could resume the normal level of γ -GTP through revealed the membrane stabilizing activity might be due to the presence of triterpenoid saponins, alkaloids and flavonoids which have been reported in *Bacopa monniere* extract [38] and/or the isolated purified compounds- betulinic acid, wogonin and oroxindin from the ethanol extract of *Bacopa monniere*.

Alkaline phosphatase (ALP) reflects the pathological alteration in biliary flow [1, 40]. Elevated levels of this enzyme in serum after 30% alcohol plus CCl₄ treatment associated with high level of serum bilirubin content [1, 41]. Oral administration of *B. monniere* extract brought about the suppression of the increased ALP as well as depletion of raised bilirubin suggested that *B. monniere* able to stabilize the biliary dysfunction in liver and indicate the improvement of functional status of the hepatic cells.

The significant lowered level of total protein and albumin recorded in the serum of 30% alcohol plus CCl₄ exposure in the Group II causes considerable hepatic injury through the initiation of peroxidation of lipids and inhibits the protein synthesis due to trichloromethyl free radical covalent bindings produced by CCl₄ along with the potentially generated a number of dangerous by-products such as acetaldehyde that contribute to the production of highly reactive free radicals by alcohol induction [42]. The orally treatment with *B. monniere* extract stabilized the serum total protein and albumin levels. The stabilization of proteins might be considered as an indication of enhanced protein synthesis in the hepatocytes due to inhibition of peroxidation of lipids and scavenge the production of highly reactive free radicals [43].

Metabolically, CCl₄ and alcohol are bio transformed by the cytochrome-P-450 system to produce oxidative stress through the generation of reactive oxygen species (ROS), which in turn, covalently binds to cell-membranes and organelles to initiate the peroxidation of lipids, disturb Ca²⁺ homeostasis

and finally result in cell death [44] as also observed in the Group. II. The metabolic activation of CCl₄ and alcohol believed to be mediated through cytochrome P 450 2E1 which is the central pathway to generate the oxidative stress in hepatocytes [43, 45]. Therefore, the suppression of cytochrome-P-450 can result in a reduction of the reactive metabolites level, and correspondingly, less tissue injury.

The reduced level of cytochrome-P-450 in the CCl₄ and alcohol treated Group II was also compensated by *B. monniere* extract supplementation through maintenance of its normal level. Therefore, the role of *B. monniere* extract in the protection of CCl₄ and alcohol-mediated loss in cytochrome-P-450 content might be considered as an indication for the improvement of protein synthesis in the hepatic cells of treated rats [46] due to the presence of flavonoids, saponins, tannins and phenols in *B. monniere* extract which used in the present study and also may explain the effectiveness of the use of *B. monniere* extract [10, 47].

The production of toxic species—trichloromethyl free radical (CCl₃•) or/and trichloromethyl peroxy radical (CCl₃OO•) by the oxidation of CCl₄, are covalently binds to cell-membranes /organelles to elicit lipid peroxidation and also the oxidation of alcohol into acetaldehyde causes the peroxidation of lipids and stimulates hepatic collagen synthesis, thereby promoting fibrosis and cirrhosis of hepatocytes [42, 43]. Therefore, the measurement of lipid peroxidation is a convenient method to monitor the oxidative hepatic cell damage. The inhibition of lipid peroxidation in hepatic cell membranes has been observed by the treatment of *B. monniere* extract might be due to its antioxidant and free radical scavenging activities through reestablishment of biomembranes of hepatic parenchymal cells [38, 47].

The present study denotes a significant reduction in the level of hepatic-GSH, SOD, catalase and ascorbic acid due to production of hepatic oxidative stress by 30% alcohol plus CCl₄ in the Group II. This could be due to an enhanced utilization of antioxidant system during the detoxification of free radicals generated by peroxidatively hepatocytes injury. GSH is a central non enzymatic component of hepatocellular defensive mechanisms in effecting detoxification of reactive metabolites from the hepatic cells through the protection of membrane protein thiols of hepatocytes from deleterious effects of reactive oxygen metabolites such as hydrogen peroxide and superoxide radicals. Although, the succeeding retrieval in animals treated with *B. monniere* extract might be due to de-novo GSH synthesis or GSH regeneration (GSSG to GSH), dose-dependently [7, 8].

Hepatic SOD and catalase are the first line of defensive antioxidative enzymes which are easily inactivated by lipid peroxides or reactive oxygen species produced by 30% alcohol plus CCl₄ induction in the animals of Group II. SOD is one of the principal steps of the defensive mechanism in the antioxidant defense system against oxidative stress by catalyzing the dismutation of superoxide radicals (O₂⁻) into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂). H₂O₂ is neutralized by the action of catalase [48]. A significant reduction in the activities of hepatic SOD and catalase during alcohol plus CCl₄ intoxication to rats might be due to the enhanced superoxide radical formation leading to oxidative stress in the hepatic cells. However, the dose-dependently significant improved levels of SOD and catalase by the oral treatment of *B. monniere* extract to rats act as strong free radical quencher and protect the hepatic cells. Therefore SOD and catalase are essential for the endogenous antioxidative

defense system to scavenge reactive oxygen species and maintain the cellular redox balance [7, 49].

Ascorbic acid is the principal antioxidant in extracellular fluids and traps peroxy radicals before they can initiate peroxidation of lipids [43, 50]. Therefore, the depleted level of ascorbic acid, thought to be utilized for the restoration of cytoplasmic membranes in the hepatic tissue. Oral administration of *B. monniera* extract to rats showed significant elevation in the depleted level of ascorbic acid due to its antioxidant potential by scavenging the free radicals generated in 30% alcohol plus CCl₄ treated Group II.

It is well recognized that the CCl₄ plus alcohol induction to rats, increased the synthesis of polyunsaturated fatty acids and triglycerides from acetate. This could be due to the transport of acetate into the liver cells, resulting in increased acetate availability which also enhanced the cholesterol synthesis in the hepatocytes. Therefore, the peripheral fat metabolism resulting enhance the hepatic triglycerides synthesis and total cholesterol levels [13]. The *B. monniera* extract supplementation in our study was potentially effective in reduction of excess hepatic total cholesterol accumulation and triglycerides synthesis through its antioxidants capability because the antioxidants act as inhibitors of lipid peroxidation by neutralizing the radicals of polyunsaturated fatty acids and by interrupting the chain reactions [43]. The antioxidant and hypolipidemic capability of *B. monniera* extract might be due to the presence of flavonoids and phenolic compounds which attributes to its rationality of possessing antioxidant and hypolipidemic activity [13, 38]. Although, the polyphenolic compounds are very important plant constituents because of their free radical scavenging ability due to their hydroxyl group's contribution directly to antioxidant and hypolipidemic action [1, 43].

Histopathological explanations suggested that the reactive oxygen metabolites and peroxidative degradation of lipids may play a role as various pathological lesions in the hepatocytes i.e. marked steatosis, cell necrosis, fatty degeneration and peripheral fibrosis etc. with the loss of normal hepatic architecture in the CCl₄ plus alcohol treated Group II. Due to the peroxidative damage of hepatic cells by CCl₄ plus alcohol treatment, the reactive oxidants or free radicals bind covalently to macromolecules of the lipid membranes of adipose tissues and cause peroxidative damage. As a result, fats from the adipose tissues are translocated and accumulated in the hepatic cells and cause the loss of normal hepatic architecture [7, 8]. The degenerative changes were revealed to be minimal or absent with the oral treatment of *B. monniera* extract, dose-dependently. This might be due to the reduction of fat metabolism and re-establishment of antioxidant defense mechanism in the hepatic cells through the antioxidative and hypolipidemic nature of *B. monniera* extract.

5. Conclusion

The protective potential of *Bacopa monniera* extract might be attributed to the presence of triterpenoid saponins, alkaloids, phenolics and flavonoids in the extract and/or isolated pure compounds- betulinic acid, wogonin, and oroxindin by quenching lipid peroxidation, preventing hypercholesterolemia and restoration of antioxidant mechanism against oxidative stress due to their hydroxyl group's contribution directly to antioxidant and hypolipidemic action in the hepatic cells. However, further studies pertaining to possible mechanism of action of isolated pure compounds are required, which are underway in our laboratory.

6. Conflict of interest

The authors declare no conflict of interest.

7. Acknowledgment

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