

PROTECTIVE ROLE OF *BETA VULGARIS* L. LEAVES EXTRACT AND FRACTIONS ON ETHANOL-MEDIATED HEPATIC TOXICITY

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Abstract: The present study was undertaken to investigate the hepatoprotective action of *Beta vulgaris* Linn. (BV, Chenopodiaceae) leaves against ethanol-mediated hepatotoxicity. Rat hepatocyte culture and rats were used as *in vitro* and *in vivo* screening models, respectively. In the *in vitro* studies, different extracts (i.e., petroleum ether, ethanol and aqueous) and fractions derived from ethanol extract (i.e., chloroform, ethyl acetate and n-butanol) of BV leaves were screened. *In vivo* hepatoprotective activity was assessed in rats intoxicated with ethanol. Levels of serum markers enzymes together with antioxidants were measured to evaluate the extent of hepatic protection. Silymarin was taken as reference drug. In the *in vitro* studies, n-butanol fraction of BV leaves (BVBF) was found to be more potent than others. Moreover, in the *in vivo* evaluation, BVBF at doses of 50, 100 and 200 mg/kg showed marked protective action against ethanol-induced hepatic toxicity as evident by restoration of biochemical changes caused by ethanol. The present study concluded that BVBF possess potent hepatoprotective effect against ethanol-induced hepatic toxicity and it may have a great potential role in the management of alcoholic liver disease.

Keywords: *Beta vulgaris*, ethanol, hepatoprotective, antioxidant, Chenopodiaceae

Alcoholism is one of the world's leading health problems as the significant number of peoples are affected by several fatal diseases caused by chronic alcohol consumption (1). Herbal therapy is one of the best ways to minimize these disease conditions. Several plant products have been demonstrated to possess hepatoprotective activity against ethanol-mediated hepatotoxicity (2–4). However, the search for more effective hepatoprotective agents continues to be an area of active research.

Beta vulgaris Linn. (BV, Chenopodiaceae), popularly known as “chukandar” or “beet root”, is an erect annual herb with tuberous root stocks. It is native to Mediterranean region and widely cultivated in America, Europe and throughout India (5). Several parts of this plant are used in traditional Indian medicine for numerous therapeutic properties. Roots are expectorant, diuretic and used as a cure for mental troubles and liver diseases. Leaves are tonic, diuretic and useful in alleviating inflammation, paralysis and diseases of spleen and liver (6, 7).

The presence of phytochemicals such as betalains i.e., betacyanins (red-violet pigments) and betaxanthines (yellow pigments), flavonoids,

polyphenols, vitamins and minerals has been shown in leaves (8). Due to high nutritional value, leaves are widely consumed as vegetables worldwide. Literature review reports that very little work has been done to explore its biological activities. Moreover, no scientific report is available regarding hepatoprotective potential of BV leaves, to the best of our knowledge. Therefore, to validate the traditional use, the present study was undertaken to evaluate the hepatoprotective activity of BV leaves using ethanol-induced hepatotoxicity model.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol, ethylene glycol tetraacetic acid (EGTA), hydroxyethyl piperazine ethane sulfonic acid (HEPES), William's E medium, collagenase, 2,2-diphenylpicryl hydrazyl (DPPH), thiobarbituric acid (TBA) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and estimation kits used were of analytical grade and purchased from commercial sources.

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Experimental animals

Wistar albino rats (200–250 g) of either sex were used for the studies (379/01/ab/CPCSEA). The animals were maintained under standard laboratory conditions of temperature ($25 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) with 12 h light-dark cycle.

Plant material

The fresh leaves of BV were procured from market of Sagar, India and authenticated in the Botany Department of Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India (Herb. No. Bot/Her/892). The fresh leaves were washed with tap water, shade dried and powdered.

Extraction and fractionation

The powdered leaf material (500 g) was successively extracted with petroleum ether (60–80°C) and 95% ethanol using Soxhlet extractor. The marc left after the ethanol extraction was macerated with distilled water for 24 h. Solvents were distilled off under reduced pressure below 45°C to afford petroleum ether extract (BVPEE, yield 5.8% w/w), ethanol extract (BVEE, 11.12% w/w) and aqueous extract (BVAE, 14.3% w/w).

The ethanol extract (BVEE) (30 g) was suspended in water (300 mL) and fractionated successively with chloroform (4×300 mL), ethyl acetate (4×300 mL) and n-butanol (4×300 mL) to afford chloroform fraction (BVCF, 3.1 g), ethyl acetate fraction (BVEAF, 6.3 g) and n-butanol fraction (BVBF, 9.4 g), respectively.

Preliminary phytochemical screening

Preliminary phytochemical analysis was performed to identify the nature of phytoconstituents in different extracts and fractions (9).

In vitro hepatoprotective evaluation

Hepatocyte isolation and culture

Rat hepatocytes were isolated by two step collagenase perfusion technique (10). Briefly, the rats were anesthetized by pentobarbital sodium (50 mg/kg, *i.p.*). After opening the abdomen, livers were perfused *via* the portal vein with Ca^{2+} -free phosphate buffer (pH 7.4), containing 135 mM NaCl, 15 mM NaHCO_3 , 5 mM glucose, 5.9 mM KCl, 0.74 mM KH_2PO_4 and 0.1 mM EGTA, at a flow rate of 25 mL/min to remove blood. After 10 min, the liver was reperused for another 10 min with the same phosphate buffer containing 50 mg collagenase, 3 mM CaCl_2 and 22 mg pyruvate, at a flow rate of 35 mL/min. To produce a single-cell suspension of hepatocytes, the collagenase-digested liver was

removed, passed through a nylon mesh (mesh size, 0.3 mm), washed and centrifuged at 500 rpm for 5 min at 4°C.

Hepatocytes were then resuspended and washed twice with washing medium (pH 7.4) containing 131.7 mM NaCl, 0.12 mM CaCl_2 , 0.85 mM MgSO_4 , 5 mM glucose, 5.2 mM KCl, 15 mM HEPES and 6.5 mM NaOH. The cell viability was more than 85% as determined by trypan blue exclusion. After the final washing, the isolated hepatocytes were then suspended in William's E medium (pH 7.4), containing 15 mM HEPES 10% fetal calf serum, 100 mM dexamethasone, 100 IU/mL penicillin G and 0.1 μM insulin and seeded in 24-well culture plates at a density of 2 to 3×10^3 cells/well at 37°C in humidified atmosphere of 5% CO_2 in a CO_2 incubator.

Toxicity induction and drug treatment

After 24 h of culturing, cells were treated with ethanol (75.7 $\mu\text{g/mL}$) (4) with or without plant samples (100 $\mu\text{g/mL}$) or silymarin (4.82 $\mu\text{g/mL}$) and incubated for another 2 h at 37°C in a CO_2 incubator. After 2 h incubation, the leakage of ALT (11) and LDH (12) in culture medium was determined.

Acute oral toxicity studies

Acute toxicity studies were performed following OECD guidelines 423 (13). On the basis of these studies, the oral dose of 50, 100 and 200 mg/kg were selected for *in vivo* experiments.

In vivo hepatoprotective evaluation

Experimental protocol

The experiment was conducted according to method described previously (2). Rats were randomly divided into six groups, each consisting of six rats. Group I (normal control) rats received distilled water (1 mL/kg, *p.o.*) daily for 7 days. Group II (ethanol control) rats received distilled water (1 mL/kg, *p.o.*) daily for 7 days and ethanol (5 g/kg, *p.o.*) once on 7th day. Groups III–V were treated with BVBF at a dose of 50, 100 and 200 (mg/kg, *p.o.*), respectively, for 7 days and received ethanol (5 g/kg, *p.o.*) once on 7th day. Group VI rats were treated with silymarin (50 mg/kg, *p.o.*) daily for 7 days and ethanol (5 g/kg, *p.o.*) on 7th day. After 16 h of ethanol exposure, blood and liver samples were collected and processed for biochemical studies.

Biochemical assays

Activities of lactate dehydrogenase (LDH) (14), aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase

(ALP) were determined in serum to assess the extent of hepatic toxicity. Level of lipid peroxidation (LPO) (expressed in terms of malondialdehyde (MDA)), glutathione (GSH) and superoxide dismutase (SOD) were also determined by the standard methods (11) to assess oxidative stress.

Statistical analysis

The results are expressed as the mean \pm SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph Pad Prism software; $p < 0.05$ was considered to be significant.

RESULTS

Preliminary phytochemical analysis

In our preliminary phytochemical analysis, different extracts and fractions of BV leaves showed the presence of various phytochemicals such as sterols, triterpenoids, phenolics, tannins, flavonoids, alkaloids, glycosides and saponins (Table 1).

In vitro hepatoprotective activity

In vitro hepatoprotective effect of extracts and fractions against ethanol induced toxicity in cultured rat hepatocytes is shown in Table 2. Hepatocyte culture has been used as screening model for evaluating the hepatoprotective activity of herbal drugs (4). In the present study, level of two hepatic marker enzymes i.e., ALT and LDH were monitored to assess ethanol toxicity and protection. Incubation of hepatocytes with ethanol (96 μ L/mL) resulted in significant increase in ALT (3.12 fold) and LDH (2 fold) levels. Treatment with different extracts and fractions exhibited moderate to high hepatoprotective

effect as evident by restoration of ALT (34.19–59.63%) and LDH (22.89–76.4%) levels, while the silymarin showed good protective effect evident by restoring ALT (53.38%) and LDH (82.6%) levels. Among the plant samples, maximum restoration was observed with BVBF, which was selected further for *in vivo* studies.

Acute toxicity studies

In acute oral toxicity studies, the BVBF did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose, considered relatively safe.

In vivo hepatoprotective activity

The effect of BVBF on serum marker enzymes in ethanol-induced hepatotoxicity is shown in Table 3. Elevated AST (2.5 fold), ALT (3 fold), ALP (2.3 fold) and LDH (2.2 fold) levels caused by ethanol administration were significantly ($p < 0.001$) prevented with BVBF pretreatment in a dose dependent manner when compared with ethanol control rats. Enhanced LPO (2.5 fold) and reduced levels of GSH (2.2 fold) and SOD (2.3 fold) were observed in ethanol control group (Table 4), whereas the BVBF treated groups showed marked ($p < 0.001$) rise in GSH and SOD with significant reduction in lipid peroxidation when compared with ethanol control group. The activity of BVBF at a dose of 200 mg/kg was comparable with that of silymarin.

DISCUSSION

Attempts are being made to develop new and effective therapeutic agents from traditional medicines to treat chronic liver diseases caused by alco-

Table 1. Preliminary phytochemical screening of different extracts and fractions of *Beta vulgaris* L. (BV) leaves.

Nature of phytoconstituents	Chemical test	BVP EE	BV EE	BV AE	BV CF	BV EAF	BV BF
Steroid and triterpenoids	Liebermann-Burchard Test	++	+	–	+	–	++
Phenolic and tannins	5% Alco. FeCl ₃ solution	–	++	+	–	++	+++
Flavonoids	Shinoda test (Mg metal and HCl)	–	++	+	+	++	++
Saponin	Foam test	–	+	++	+	–	+++
Alkaloid	Dragendorff and Mayer's reagent	–	+	–	+	–	–
Anthraquinone glycoside	Borntrager test	–	+	+	–	–	+
Carbohydrate	Molisch reagent and Fehling solution	–	+	++	–	+	+
Protein	Ninhydrin reagent	–	+	++	–	–	+

– absent; + trace; ++ moderate; +++ abundance. BVPEE, petroleum ether extract; BV EE, ethanol extract; BV AE, aqueous extract; BV CF, chloroform fraction; BV EAF, ethyl acetate fraction; BV BF, n-butanol fraction.

Table 2. Effect of different extracts and fractions of *Beta vulgaris* (BV) leaves and silymarin (SIL) on ethanol-induced toxicity in primary culture of rat hepatocytes.

Treatment	% Restoration	
	ALT	LDH O.D./min/3 mL medium
Normal	100.0	100.0
Ethanol control	0.0	0.0
BVPEE (100 µg/mL)	52.14	22.89
BVEE (100 µg/mL)	47.28	23.37
BVAE (100 µg/mL)	34.19	54.0
BVCF (100 µg/mL)	50.05	35.29
BVEAF (100 µg/mL)	40.87	42.73
BVBF (100 µg/mL)	59.63	76.39
SIL (10 µM)	53.38	82.6

Normal values of ALT and LDH were 20.85 ± 2.95 IU/L and 35.97 ± 3.55 IU/L, respectively. In ethanol control group the values were 65.25 ± 1.47 and 74.91 ± 6.20 IU/L, respectively. Each value represent the mean \pm SEM (n = 4). The % restoration was calculated as $100 \times (\text{value of ethanol control} - \text{value of sample}) / (\text{value of ethanol control} - \text{value of normal control})$. ALT, alanine transaminase; LDH, lactate dehydrogenase; BVPEE, petroleum ether extract; BVEE, ethanol extract; BVAE, aqueous extract; BVCF, chloroform fraction; BVEAF, ethyl acetate fraction; BVBF, n-butanol fraction.

Table 3. Effect of n-butanol fraction of *Beta vulgaris* (L.) leaves (BVBF) and silymarin (SIL) on AST, ALT, ALP and LDH in ethanol-induced hepatotoxicity in rats.

Biochemical parameters	Group I Normal	Group II Ethanol (5 g/kg)	Group III BVBF (50 mg/kg) + ethanol	Group IV BVBF (100 mg/kg) + ethanol	Group V BVBF (200 mg/kg) + ethanol	Group VI SIL (50 mg/kg) + ethanol
AST (IU/L)	38.25 ± 4.32	$96.95 \pm 2.06^*$	85.75 ± 1.27	$64.36 \pm 2.16^{\Delta}$	$58.40 \pm 2.91^*$	$47.12 \pm 2.55^*$
ALT (IU/L)	29.30 ± 3.58	$87.71 \pm 2.68^*$	$75.19 \pm 3.61^{\S}$	$60.27 \pm 1.29^*$	$48.03 \pm 2.06^*$	$55.29 \pm 3.36^*$
ALP (IU/L)	45.49 ± 6.07	$107.39 \pm 3.1^{\#}$	$89.06 \pm 2.14^{\S}$	$71.59 \pm 5.0^*$	$60.66 \pm 0.98^*$	$61.70 \pm 3.51^*$
LDH (IU/L)	115.45 ± 17.44	$262.6 \pm 8.28^*$	227.54 ± 10.26	$193.48 \pm 3.63^{\Delta}$	$174.11 \pm 3.24^*$	$153.46 \pm 4.61^*$

Each value represents the mean \pm SEM (n = 6). *p < 0.001 when compared with the normal control group (vehicle only). $^{\Delta}$ p < 0.01, and $^{\#}$ p < 0.05, respectively, when compared with the group treated with ethanol alone (one-way ANOVA followed by Tukey's multiple comparison test). AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

Table 4. Effect of n-butanol fraction of *Beta vulgaris* L. leaves (BVBF) and silymarin (SIL) on hepatic MDA, GSH, and SOD in ethanol-induced hepatotoxicity in rats.

Biochemical parameters	Group I Normal	Group II Ethanol (5 g/kg)	Group III BVBF (50 mg/kg) + ethanol	Group IV BVBF (100 mg/kg) + ethanol	Group V BVBF (200 mg/kg) + ethanol	Group VI SIL (50 mg/kg) + ethanol
MDA(nM/mg protein)	0.44 ± 0.04	$1.12 \pm 0.03^{\#}$	1.01 ± 0.02	$0.80 \pm 0.04^*$	$0.73 \pm 0.03^*$	$0.67 \pm 0.06^*$
GSH (µM/mg protein)	9.78 ± 0.58	$4.26 \pm 0.26^{\#}$	5.14 ± 0.06	$5.89 \pm 0.15^{\Delta}$	$6.34 \pm 0.19^*$	$6.85 \pm 0.09^*$
SOD (U/mg)	37.15 ± 1.64	$15.76 \pm 1.63^{\#}$	19.49 ± 1.21	$25.01 \pm 1.44^{\Delta}$	$31.29 \pm 1.84^*$	$28.94 \pm 0.87^*$

Each value represents the mean \pm SEM (n = 6). *p < 0.001 when compared with the normal control group (vehicle only). $^{\Delta}$ p < 0.01, and $^{\#}$ p < 0.05, respectively, when compared with the group treated with ethanol alone (one-way ANOVA followed by Tukey's multiple comparison test). MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase.

hol abuse. In the present study, we have attempted to explore the hepatoprotective effects of *Beta vulgaris* leaves, a traditionally used herb, in ethanol-induced liver damage, *in vitro* and *in vivo*.

It is proposed that ethanol mediated toxicity is mainly dependent on its metabolic pathway. The main pathway involves hepatic alcohol dehydrogenase (ADH), a cytosolic enzyme that catalyzes the conversion of ethanol into acetaldehyde, which in turn is transformed to acetate through acetaldehyde dehydrogenase (ALDH). Another adaptive pathway involves an ethanol inducible isoform of cytochrome P450 known as CYP2E1 (15). Excessive ethanol intake induces the mass production of free radicals in the body, which are associated with alcoholic liver disease (16).

In the assessment of ethanol-mediated hepatotoxicity, the determination of serum marker enzyme such as AST, ALT, ALP and LDH is largely used (2). In the present study, a significant rise in the level of AST, ALT, ALP and LDH in ethanol control rats was observed, indicated the increased permeability of hepatocytes and considerable cellular damage. Pretreatment with BVBF significantly decreased these enzyme activities, indicating that BVBF could maintain the functional integrity of hepatocyte membrane, thus protecting the hepatocytes against ethanol toxicity. In rat hepatocyte culture, BVBF was also found to be effective in decreasing the leakage of ALT and LDH triggered by ethanol, indicating *in vitro* hepatoprotective activity.

It has been shown that ethanol exposure promotes LPO and decrease the hepatic level of enzymatic and non-enzymatic antioxidants, *in vitro* and *in vivo* (17). MDA is one of the products of LPO; its elevated level could reflect the extent of lipid peroxidative damage in hepatocytes. In this study, a significant increase in hepatic MDA and decrease in GSH was simultaneously observed in ethanol control rats, suggesting that ethanol induced LPO might contribute to decreased GSH content as shown in earlier reports (18).

Pretreatment with BVBF significantly decreased the MDA, and increased GSH to be nearly normal, suggesting that the antioxidant effect of BVBF played an important protective role against ethanol mediated toxicity. SOD, an antioxidant enzyme, was also found to be decreased in ethanol control rats, while pretreatment with BVBF restored its activity, which may also contribute to the decreased LPO. The present findings corroborate with previous report in which BV leaf was shown to improve antioxidant status in the mice fed with high fat diet (19).

Previous phytochemical studies have shown the presence of flavonoids, phenolics and betalains in BV leaves (8) and the presence of such phytoconstituents have been confirmed in BVBF by preliminary phytochemical and chromatographic studies. These phytochemicals have been reported as potent antioxidant (19) and hepatoprotective compounds (20, 21). Therefore, we may consider that these identified classes of compounds in BVBF might be responsible for the offered hepatoprotection and this protection could be associated with its antioxidant properties.

In conclusion, the hepatoprotective effects of BV leaves have been explored, *in vitro* and *in vivo*, for the first time. Our results demonstrated that BVBF possessed significant protection against ethanol mediated hepatotoxicity, which may be partly due to its antioxidant properties through scavenging free radicals to inhibit lipid peroxidation and increasing antioxidant defenses. The phytochemical analysis revealed the betalains, flavonoids, and phenolics in BVBF, which might contribute to its stronger biological activities.

Acknowledgments

One of the authors Nilesh Kumar Jain is grateful to All India Council for Technical Education (AICTE), New Delhi for providing National Doctoral Fellowship (NDF). Sincere thanks go to Dr. N. Gopalan and Director, Defence Research and Development Establishment, Gwalior (MP), for granting permission to carrying out the studies.

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Received: 11. 07. 2011