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A COMPARATIVE STUDY ON EFFECT OF *BOERHAVIA DIFFUSA EXTRACT* ALONE AND IN COMBINATION WITH POMEGRANATE EXTRACT AGAINST ANILINE-INDUCED SPLEEN TOXICITY IN EXPERIMENTAL ANIMALS

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ABSTRACT

Background: Aniline, an industrial aromatic amine, induces oxidative stress and splenic damage through excessive free radical generation. Boerhavia diffusa (Punarnava) possesses potent antioxidant properties, while Punica granatum (pomegranate) is rich in natural polyphenols with strong free radical scavenging activity. This study evaluated the protective effect of Boerhavia diffusa extract alone and in combination with pomegranate extract against aniline-induced spleen toxicity in experimental rats.

Methods: Thirty-six male Wistar rats were divided into six groups (n=6): normal control, negative control (aniline 100 ppm), standard (citric acid 25 mg/kg), Boerhavia diffusa (100 mg/kg), Boerhavia diffusa (200 mg/kg), and combination of Boerhavia diffusa (200 mg/kg) with pomegranate extract (100 mg/kg). Physical, hematological, biochemical, antioxidant, membrane-bound ATPase, and histopathological parameters were evaluated. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test.

Results: Aniline administration significantly increased oxidative stress markers (lipid peroxidation, nitric oxide), organ weight, and histopathological damage while decreasing antioxidant enzyme activities (superoxide dismutase, catalase, reduced glutathione), ATPase activities, and hemoglobin levels. Boerhavia diffusa treatment significantly ameliorated these alterations in a dose-dependent manner. The combination therapy exhibited superior protective effects by restoring antioxidant status, improving biochemical and hematological parameters, and preserving normal splenic architecture.

Conclusion: Boerhavia diffusa possesses significant spleen-protective activity against aniline-induced toxicity, and its combination with pomegranate extract provides enhanced protection through synergistic antioxidant and cytoprotective effects. This herbal combination may serve as a promising natural therapeutic approach for preventing chemically induced splenic damage.

KEYWORDS: Boerhavia diffusa, Punica granatum, Aniline, Spleen toxicity, Oxidative stress, Antioxidant, Histopathology, Experimental rats

1. INTRODUCTION

Toxicology is the scientific discipline that studies the adverse effects of chemical, physical, and biological agents on living organisms. Aniline is an important aromatic amine widely used as an intermediate in the chemical industry for the synthesis of dyes, pharmaceuticals, pesticides, rubber-processing chemicals, and numerous industrial products. Due to its extensive industrial applications and widespread production, aniline has become a significant environmental and occupational contaminant [1,2].

Aniline belongs to the class of primary aromatic amines and consists of an amino group (-NH_2) attached directly to a benzene ring. The molecular formula of aniline is C_6H_7N with a molecular weight of approximately 93.13 g/mol. Aniline can enter the body through inhalation, dermal absorption, and ingestion, with dermal absorption being particularly significant due to its lipophilic nature. Once absorbed, aniline is distributed throughout the body, with high concentrations found in the liver, spleen, kidneys, and blood. Hepatic cytochrome P450 enzymes convert aniline into several metabolites, including phenylhydroxylamine and nitrosobenzene, which generate reactive oxygen species (ROS) contributing to oxidative stress and cellular injury [3].

Oxidative stress is one of the principal mechanisms responsible for aniline-induced toxicity. During metabolism, aniline generates reactive intermediates capable of producing excessive reactive oxygen species, leading to lipid peroxidation, protein oxidation, DNA damage, and cellular dysfunction [4]. The spleen is highly sensitive to

aniline toxicity due to its involvement in red blood cell turnover, immune regulation, and blood filtration. Aniline-induced hemolysis and methemoglobinemia increase the workload of the spleen, leading to splenomegaly, hemosiderin accumulation, fibrosis, and tissue damage [5,6].

Boerhavia diffusa L., commonly known as Punarnava, is a perennial creeping herb belonging to the family Nyctaginaceae, widely recognized in traditional Ayurvedic medicine for its diverse therapeutic properties. The plant contains a wide range of bioactive phytochemicals, including alkaloids, flavonoids, lignans, rotenoids, glycosides, steroids, and phenolic compounds. Boeravinones, punarnavine, and various flavonoids exhibit potent antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, immunomodulatory, and anticancer activities [7-9].

Punica granatum L., commonly known as pomegranate, belongs to the family Lythraceae and is one of the oldest cultivated fruit-bearing plants. Pomegranate contains numerous biologically active compounds, including punicalagin, ellagic acid, anthocyanins, flavonoids, and tannins. These compounds exhibit strong free radical scavenging activity, anti-inflammatory properties, and protective effects against oxidative stress-induced tissue damage [10-12].

Despite extensive studies on aniline toxicity, relatively few investigations have focused specifically on the protective effects of Boerhavia diffusa and pomegranate against aniline-induced splenic injury. Furthermore, there is a lack of scientific evidence regarding the potential benefits of combining these two medicinal plants as a polyherbal intervention against aniline-induced oxidative stress and splenic damage. Therefore, this study was designed to evaluate and compare the protective effects of Boerhavia diffusa and Punica granatum, individually and in combination, against aniline-induced splenic toxicity in experimental rats.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Aniline hydrochloride was procured from Sigma-Aldrich Chemical, Mumbai. Citric acid, 5,5'-Dithiobis-(2-nitrobenzoic acid) [DTNB], sodium sulphite, N-(1-Naphthyl)ethylenediamine dihydrochloride, thiobarbituric acid, trichloroacetic acid, Tris hydrochloride, chloroform, dibasic sodium phosphate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Griess reagent, 2,2'-dipyridyl, adenosine triphosphate standard, ammonium molybdate, and ANSA (1-Amino-2-naphthol-4-sulphonic acid) were obtained from Loba Chem. Pvt. Ltd., Mumbai, and Himedia Lab. Pvt. Ltd., Mumbai.

2.2 Plant Material and Extraction

Boerhavia diffusa and pomegranate extracts were prepared in-house following standard extraction procedures. The plant materials were authenticated by a qualified botanist, dried, powdered, and subjected to extraction using appropriate solvents. The extracts were standardized for phytochemical constituents and stored under suitable conditions until use.

2.3 Experimental Animals

Healthy adult male Wistar albino rats weighing 200-250 g were used in the present study. The animals were procured from Laxmi Biopharma Pvt. Ltd. and housed in clean polypropylene cages containing sterile paddy husk as bedding material. They were maintained under standard laboratory conditions at a temperature of 23±2°C, relative humidity of 55±10%, and a 12-hour light/dark cycle throughout the experimental period. The rats were provided with a standard laboratory pellet diet and filtered drinking water ad libitum. Prior to the initiation of the study, all animals were acclimatized to the laboratory environment for one week. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC), and all experimental procedures were conducted in accordance with CPCSEA guidelines for the care and use of laboratory animals (2274/PO/Re/S/23/CPCSEA).

2.4 Experimental Design

Thirty-six rats were randomly divided into six groups, each consisting of six animals (n=6):

- **Group I (Normal Control):** Received vehicle only
- **Group II (Negative Control):** Received aniline (100 ppm, p.o.) for 4 weeks
- **Group III (Standard Treatment):** Received aniline + citric acid (25 mg/kg, p.o.) for 4 weeks
- **Group IV:** Received aniline + Boerhavia diffusa extract (100 mg/kg, p.o.) for 4 weeks
- **Group V:** Received aniline + Boerhavia diffusa extract (200 mg/kg, p.o.) for 4 weeks
- **Group VI (Combination):** Received aniline + Boerhavia diffusa extract (200 mg/kg, p.o.) + pomegranate extract (100 mg/kg, p.o.) for 4 weeks

Aniline solution of 100 ppm was prepared by accurately weighing 10 mg of aniline and dissolving it in 100 mL of freshly prepared distilled water. The solution was prepared freshly as required and administered orally.

2.5 Sample Collection and Tissue Processing

At the end of the experimental period, blood samples were collected from the retro-orbital plexus using capillary glass tubes. The animals were sacrificed by cervical decapitation, and the spleen was immediately excised and washed thoroughly with ice-cold Tris-HCl buffered saline (pH 7.4). The excised spleen tissue was homogenized

in chilled Tris-HCl buffer (10 mM, pH 7.4) to obtain a 10% (w/v) tissue homogenate. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 0°C, and the resulting supernatant was collected for the estimation of lipid peroxidation (LPO), nitric oxide (NO), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). The sediment was utilized for the determination of membrane-bound ATPase enzymes (Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase). Protein content was estimated by the Lowry method [13].

2.6 Biochemical and Antioxidant Parameters

2.6.1 Lipid Peroxidation (LPO)

Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBARS) according to the method described by Slater and Sawyer [14]. Malondialdehyde (MDA), one of the major end products of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a pink-colored chromogen measured spectrophotometrically at 532 nm. Results were expressed as nmol MDA/mg protein.

2.6.2 Reduced Glutathione (GSH)

Reduced glutathione levels were estimated according to the method described by Sedlak and Lindsay [15]. The method is based on the reaction of GSH with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) to form a yellow-colored chromogen measured at 412 nm. Results were expressed as µmol GSH/mg protein.

2.6.3 Nitric Oxide (NO)

Nitric oxide levels were estimated indirectly by measuring nitrite levels using the Griess reaction as described by Guevara et al. [16]. Nitrite reacts with sulphanilamide under acidic conditions to form a diazonium salt, which subsequently couples with N-(1-naphthyl) ethylenediamine dihydrochloride to produce a stable reddish-pink azo dye measured at 540 nm. Results were expressed as µmol nitrite/mg protein.

2.6.4 Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined according to the method described by Misra and Fridovich [17]. In an alkaline medium, epinephrine undergoes auto-oxidation to form adrenochrome, measured at 480 nm. SOD inhibits this auto-oxidation by scavenging superoxide radicals. Results were expressed as U/mg protein.

2.6.5 Catalase (CAT)

Catalase activity was estimated according to the method described by Luck [18]. Catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen, resulting in a decrease in absorbance measured at 240 nm. Results were expressed as U/mg protein.

2.7 Membrane-Bound ATPase Assays

The activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase in spleen tissue were determined by measuring the amount of inorganic phosphate (Pi) liberated from the hydrolysis of adenosine triphosphate (ATP) [19-21]. Results were expressed as µmol Pi released/mg protein/hour.

2.8 Hematological Parameters

2.8.1 Hemoglobin (Hb) Estimation

Hemoglobin concentration was estimated by Sahli's acid hematin method using a Sahli hemoglobinometer. Results were expressed as g/dL.

2.8.2 Total Erythrocyte Count (RBC Count)

Total erythrocyte count was determined using an Improved Neubauer Hemacytometer. Blood was diluted with RBC diluting fluid (sodium citrate 3.0 g, formalin 1.0 mL, distilled water up to 100 mL) at a dilution of 1:200. Results were expressed as $\times 10^6$ cells/µL.

2.8.3 Total Leukocyte Count (WBC Count)

Total leukocyte count was determined using an Improved Neubauer Hemacytometer. Blood was diluted with WBC diluting fluid (glacial acetic acid 2 mL, distilled water up to 100 mL, aqueous methylene blue solution 10 drops), which lyses erythrocytes while preserving leukocyte nuclei. Results were expressed as $\times 10^3$ cells/µL.

2.9 Biochemical Parameters

2.9.1 Total Protein

Total protein concentration was estimated using a commercial diagnostic kit based on the Biuret method. Results were expressed as g/dL.

2.9.2 Total Iron

Serum iron concentration was estimated according to the method described by Ramsay [22]. Iron bound to transferrin is released under acidic conditions and reduced from ferric (Fe^{3+}) to ferrous (Fe^{2+}) state, reacting with 2,2'-dipyridyl to form a colored complex measured at 520 nm. Results were expressed as µg/dL.

2.10 Histopathological Examination

Spleen tissues were fixed in 10% neutral buffered formalin, processed by routine paraffin embedding technique, and sections of approximately 5 µm thickness were prepared using a microtome. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope at 400× magnification for evaluation of histopathological alterations.

2.11 Statistical Analysis

The experimental data were expressed as Mean ± SEM for six animals per group (n=6). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical significance was considered at $p < 0.05$. The levels of significance were represented as ns = non-significant, ###$p < 0.001$ for comparisons versus the control group; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ for comparisons versus the negative control group; and \$\$\$$p < 0.001$, \$\$$p < 0.01$, \$$p < 0.05$ for comparison between Boerhavia diffusa extract 200 mg/kg and combination groups. All statistical analyses were performed using Excel and Real Statistics.

3. RESULTS

3.1 Effect on Organ Weight, Water Intake, and Feed Intake

Aniline administration significantly increased organ weight (2.140 ± 0.034 g) compared to the normal control group (0.870 ± 0.021 g, $p < 0.001$), along with a marked reduction in water and feed intake across all four weeks. Treatment with Boerhavia diffusa extract at doses of 100 mg/kg and 200 mg/kg significantly reduced organ weight and enhanced water and feed intake, indicating
a dose-dependent protective action. The combination-treated group exhibited the greatest improvement among all treatment groups, with organ weight (1.090 ± 0.019 g) approaching normal values and substantially higher water and feed consumption (Table 1).

Table 1: Effect on Organ Weight, Water Intake, and Feed Intake

Parameters	G-I (Control)	G-II (Aniline Control)	G-III (Citric Acid)	G-IV (BD 100)	G-V (BD 200)	G-VI (BD + POM)
Organ Weight (g)	0.870 ± 0.021	2.140 ± 0.034###	1.280 ± 0.019***	1.610 ± 0.025***	1.420 ± 0.021***	1.090 ± 0.019***\$\$\$
Water Intake Wk 01 (mL/day)	70.200 ± 1.782	39.100 ± 1.384###	52.400 ± 1.291***	45.300 ± 1.050*	50.800 ± 1.062***	58.600 ± 1.217***\$\$\$

Parameters	G-I (Control)	G-II (Aniline Control)	G-III (Citric Acid)	G-IV (BD 100)	G-V (BD 200)	G-VI (BD + POM)
Organ Weight (g)	0.870 ± 0.021	2.140 ± 0.034###	1.280 ± 0.019***	1.610 ± 0.025***	1.420 ± 0.021***	1.090 ± 0.019***\$\$\$
Water Intake Wk 01 (mL/day)	70.200 ± 1.782	39.100 ± 1.384###	52.400 ± 1.291***	45.300 ± 1.050*	50.800 ± 1.062***	58.600 ± 1.217***\$\$\$

Water Intake Wk 02 (mL/day)	71.400 ± 1.728	37.800 ± 1.363###	59.200 ± 1.132***	50.100 ± 0.994***	56.985 ± 1.053***	63.700 ± 1.115***\$\$\$
Water Intake Wk 03 (mL/day)	72.100 ± 1.739	43.600 ± 1.365###	66.340 ± 1.074***	55.200 ± 0.998***	61.400 ± 1.065***	68.500 ± 1.114***\$\$\$
Water Intake Wk 04 (mL/day)	73.000 ± 1.711	45.100 ± 1.303###	67.800 ± 1.102***	59.300 ± 0.931***	63.200 ± 1.051***	70.400 ± 1.096***\$\$\$
Feed Intake Wk 01 (g/day)	20.150 ± 0.349	11.020 ± 0.270###	15.880 ± 0.322***	13.460 ± 0.251***	14.920 ± 0.266***	17.050 ± 0.295***\$\$\$
Feed Intake Wk 02 (g/day)	21.080 ± 0.323	10.880 ± 0.264###	18.240 ± 0.294***	14.100 ± 0.242***	15.600 ± 0.260***	18.730 ± 0.278***\$\$\$
Feed Intake Wk 03 (g/day)	20.440 ± 0.338	11.300 ± 0.255###	17.960 ± 0.313***	14.880 ± 0.231***	16.340 ± 0.253***	19.020 ± 0.278***\$\$\$
Feed Intake Wk 04 (g/day)	21.260 ± 0.347	11.150 ± 0.266###	18.410 ± 0.290***	15.370 ± 0.235***	16.880 ± 0.252***	19.480 ± 0.268***\$\$\$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001, **p<0.01, *p<0.05 vs Negative Control; $$$p<0.001, $$p<0.01, $p<0.05 vs BD 200. BD = Boerhavia diffusa, POM = Pomegranate.

3.2 Effect on Antioxidant Parameters

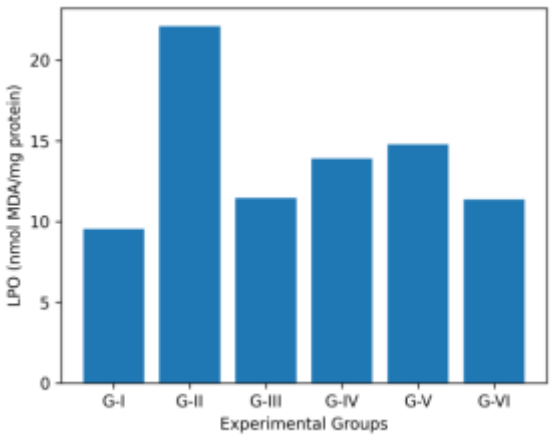
3.2.1 Lipid Peroxidation (LPO)

Aniline administration significantly increased splenic lipid peroxidation levels in the negative control group (22.124 ± 0.703 nmol MDA/mg protein) compared to the control group (9.564 ± 0.260 nmol MDA/mg protein, $p < 0.001$), indicating severe oxidative stress. Treatment with Boerhavia diffusa extract at both 100 mg/kg (13.909 ± 0.374 nmol MDA/mg protein) and 200 mg/kg (14.801 ± 0.441 nmol MDA/mg protein) significantly attenuated the aniline-induced elevation in LPO levels ($p < 0.001$). The combination treatment (11.390 ± 0.318 nmol MDA/mg protein) produced a further significant reduction when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 2, Figure 1).

Table 2: Lipid Peroxidation (LPO) Levels

Group	Treatment	LPO (nmol MDA/mg protein)
G-I	Control	9.564 ± 0.260
G-II	Negative Control	22.124 ± 0.703###
G-III	Citric Acid	11.474 ± 0.307***
G-IV	BD (100 mg/kg)	13.909 ± 0.374***
G-V	BD (200 mg/kg)	14.801 ± 0.441***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	11.390 ± 0.318***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.



Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on lipid peroxidation levels in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200. BD = Boerhavia diffusa, POM = Pomegranate.

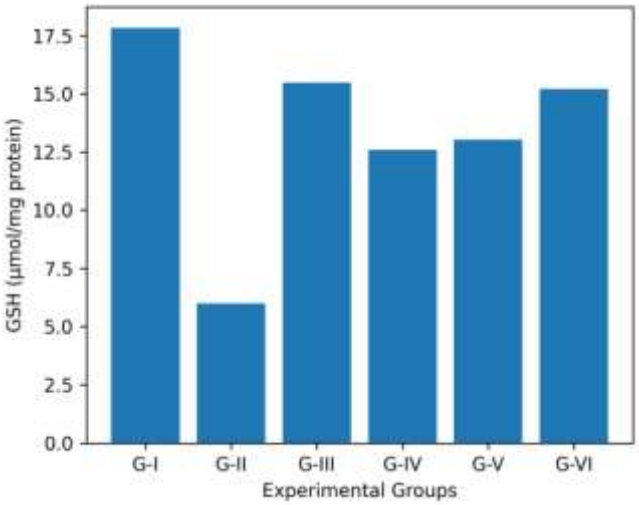
3.2.2 Reduced Glutathione (GSH)

Aniline administration significantly decreased splenic GSH levels in the negative control group (6.001 ± 0.194 µmol/mg protein) compared to the control group (17.826 ± 0.218 µmol/mg protein, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (12.591 ± 0.220 µmol/mg protein) and 200 mg/kg (13.036 ± 0.244 µmol/mg protein) significantly elevated GSH levels compared to the negative control group ($p < 0.001$). The combination treatment (15.209 ± 0.219 µmol/mg protein) showed a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 3, Figure 2).

Table 3: Effect on Reduced Glutathione (GSH) Levels

Group	Treatment	GSH (µmol/mg protein)
G-I	Control	17.826 ± 0.218
G-II	Negative Control	6.001 ± 0.194###
G-III	Citric Acid	15.468 ± 0.231***
G-IV	BD (100 mg/kg)	12.591 ± 0.220***
G-V	BD (200 mg/kg)	13.036 ± 0.244***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	15.209 ± 0.219***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on reduced glutathione levels in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.2.3 Nitric Oxide (NO)

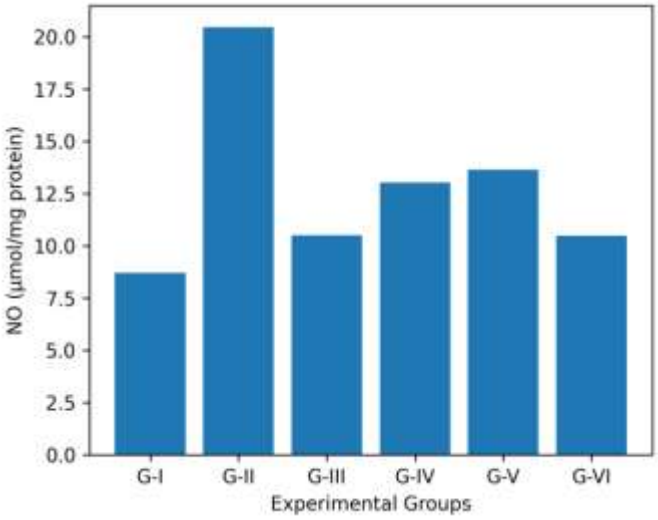
Aniline administration significantly increased splenic NO levels in the negative control group (20.460 ± 0.354 µmol/mg protein) compared to the control group (8.712 ± 0.162 µmol/mg protein, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (13.013 ± 0.201 µmol/mg protein) and 200 mg/kg (13.626 ± 0.164 µmol/mg protein) significantly lowered the elevated NO levels ($p < 0.001$). The combination treatment (10.484 ± 0.183 µmol/mg protein) produced a further significant reduction when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 4, Figure 3).

Table 4: Effect on Nitric Oxide (NO) Levels

Group	Treatment	NO (µmol/mg protein)
G-I	Control	8.712 ± 0.162
G-II	Negative Control	20.460 ± 0.354###
G-III	Citric Acid	10.504 ± 0.176***
G-IV	BD (100 mg/kg)	13.013 ± 0.201***

Group	Treatment	NO (µmol/mg protein)
G-V	BD (200 mg/kg)	13.626 ± 0.164***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	10.484 ± 0.183***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on nitric oxide levels in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

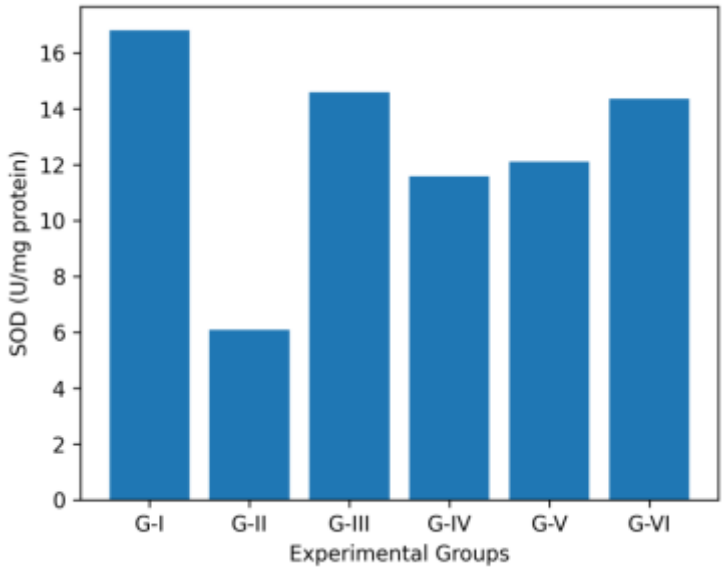
3.2.4 Superoxide Dismutase (SOD)

Aniline administration significantly decreased splenic SOD activity in the negative control group (6.086 ± 0.191 U/mg protein) compared to the control group (16.824 ± 0.214 U/mg protein, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (11.594 ± 0.218 U/mg protein) and 200 mg/kg (12.107 ± 0.214 U/mg protein) significantly increased SOD activity ($p < 0.001$). The combination treatment (14.367 ± 0.226 U/mg protein) showed a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 5, Figure 4).

Table 5: Effect on Superoxide Dismutase (SOD) Activity

Group	Treatment	SOD (U/mg protein)
G-I	Control	16.824 ± 0.214
G-II	Negative Control	6.086 ± 0.191###
G-III	Citric Acid	14.594 ± 0.223***
G-IV	BD (100 mg/kg)	11.594 ± 0.218***
G-V	BD (200 mg/kg)	12.107 ± 0.214***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	14.367 ± 0.226***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.01 vs BD 200.



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on superoxide dismutase activity in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

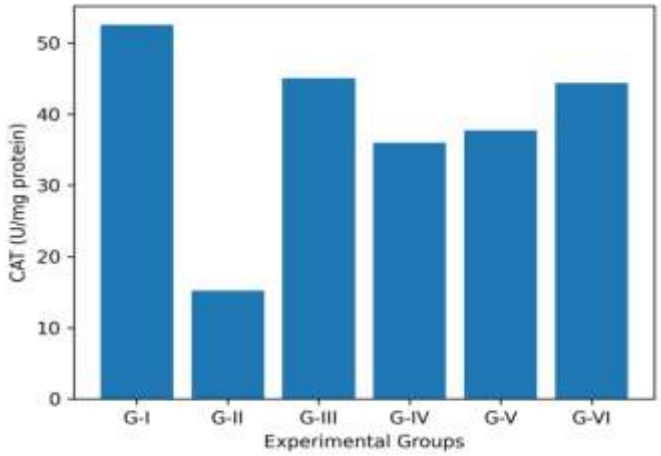
3.2.5 Catalase (CAT)

Aniline administration significantly decreased splenic catalase activity in the negative control group (15.232 ± 0.422 U/mg protein) compared to the control group (52.572 ± 0.514 U/mg protein, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (36.009 ± 0.486 U/mg protein) and 200 mg/kg (37.730 ± 0.514 U/mg protein) significantly increased CAT activity ($p < 0.001$). The combination treatment (44.403 ± 0.501 U/mg protein) produced a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 6, Figure 5).

Table 6: Catalase (CAT) Activity

Group	Treatment	CAT (U/mg protein)
G-I	Control	52.572 ± 0.514
G-II	Negative Control	15.232 ± 0.422###
G-III	Citric Acid	45.056 ± 0.497***
G-IV	BD (100 mg/kg)	36.009 ± 0.486***
G-V	BD (200 mg/kg)	37.730 ± 0.514***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	44.403 ± 0.501***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on catalase activity in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.3 Effect on Membrane-Bound ATPases

3.3.1 Na^+/K^+-ATPase

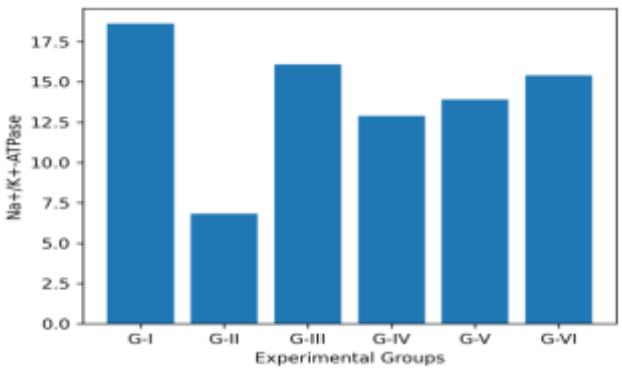
Aniline administration significantly decreased Na^+/K^+-ATPase activity in the negative control group (6.820 ± 0.194 µmol Pi/mg protein/hour) compared to the control group (18.624 ± 0.228 µmol Pi/mg protein/hour, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (12.884 ± 0.221 µmol Pi/mg protein/hour) and 200 mg/kg (13.901 ± 0.213 µmol Pi/mg protein/hour) significantly increased enzyme activity ($p < 0.001$). The combination treatment (15.405 ± 0.224 µmol Pi/mg protein/hour) showed a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.01$) (Table 7, Figure 6).

Tale 7: Effect on Na^+/K^+-ATPase Activity

Group	Treatment	Na^+/K^+-ATPase (µmol Pi/mg protein/hour)
G-I	Control	18.624 ± 0.228
G-II	Negative Control	6.820 ± 0.194###
G-III	Citric Acid	16.074 ± 0.216***
G-IV	BD (100 mg/kg)	12.884 ± 0.221***
G-V	BD (200 mg/kg)	13.901 ± 0.213***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	15.405 ± 0.224***$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$p<0.01 vs BD 200.

Effect of Treatments on Na^+/K^+-ATPase Activity



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on Na^+/K^+-ATPase activity in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$p<0.01 vs BD 200.

3.3.2 Ca^{2+}-ATPase

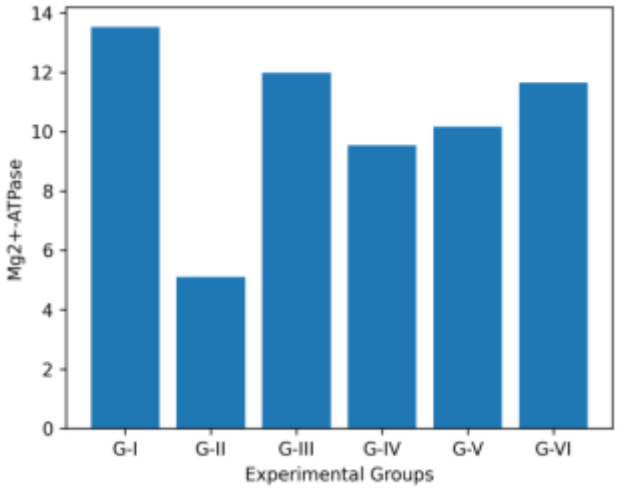
Aniline administration significantly decreased Ca^{2+}-ATPase activity in the negative control group (5.788 ± 0.184 µmol Pi/mg protein/hour) compared to the control group (15.824 ± 0.206 µmol Pi/mg protein/hour, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (10.889 ± 0.201 µmol Pi/mg protein/hour) and 200 mg/kg (11.586 ± 0.194 µmol Pi/mg protein/hour) significantly increased enzyme activity ($p < 0.001$). The combination treatment (13.069 ± 0.203 µmol Pi/mg protein/hour) showed a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.01$) (Table 8, Figure 7).

Table 8: Effect on Ca^{2+}-ATPase Activity

Group	Treatment	Ca^{2+}-ATPase (µmol Pi/mg protein/hour)
G-I	Control	15.824 ± 0.206
G-II	Negative Control	5.788 ± 0.184###
G-III	Citric Acid	13.469 ± 0.198***
G-IV	BD (100 mg/kg)	10.889 ± 0.201***
G-V	BD (200 mg/kg)	11.586 ± 0.194***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	13.069 ± 0.203***$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$p<0.01 vs BD 200.

: Effect of Treatments on Mg^{2+}-ATPase Activity



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on Mg^{2+}-ATPase activity in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.3.3 Mg^{2+}-ATPase

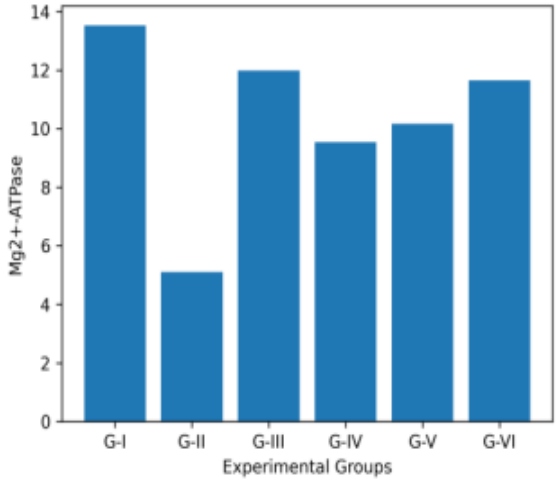
Aniline administration significantly decreased Mg^{2+}-ATPase activity in the negative control group (5.108 ± 0.221 µmol Pi/mg protein/hour) compared to the control group (13.517 ± 0.273 µmol Pi/mg protein/hour, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (9.539 ± 0.236 µmol Pi/mg protein/hour) and 200 mg/kg (10.165 ± 0.229 µmol Pi/mg protein/hour) significantly increased enzyme activity ($p < 0.001$). The combination treatment (11.647 ± 0.241 µmol Pi/mg protein/hour) produced a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 9, Figure 8).

Table 9: Effect on Mg^{2+}-ATPase Activity

Group	Treatment	Mg^{2+}-ATPase (µmol Pi/mg protein/hour)
G-I	Control	13.517 ± 0.273
G-II	Negative Control	5.108 ± 0.221###
G-III	Citric Acid	11.976 ± 0.248***
G-IV	BD (100 mg/kg)	9.539 ± 0.236***
G-V	BD (200 mg/kg)	10.165 ± 0.229***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	11.647 ± 0.241***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

Effect of Treatments on Mg^{2+}-ATPase Activity



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on Mg^{2+}-ATPase activity in spleen tissue. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.4 Effect on Hematological Parameters

3.4.1 Hemoglobin (Hb)

Aniline administration significantly reduced hemoglobin levels in the negative control group (7.480 ± 0.177 g/dL) compared to the control group (14.612 ± 0.168 g/dL, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (10.701 ± 0.166 g/dL) and 200 mg/kg (11.383 ± 0.181 g/dL) significantly improved Hb levels ($p < 0.001$). The combination treatment (12.767 ± 0.174 g/dL) showed a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.01$) (Table 10, Figure 9).

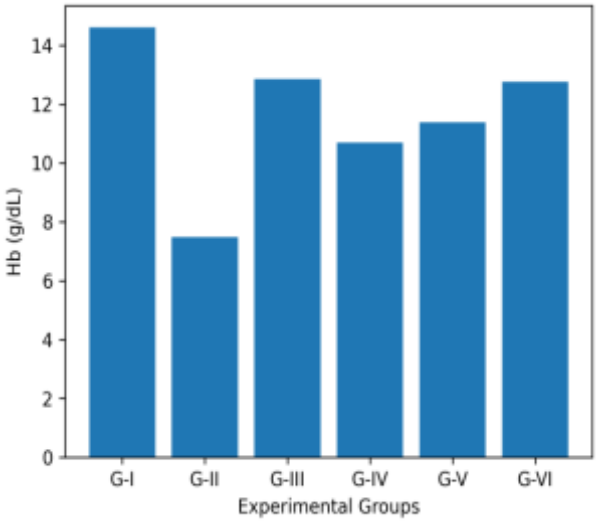
Table 10: Effect on Hemoglobin (Hb) Levels

Group	Treatment	Hb (g/dL)
G-I	Control	14.612 ± 0.168
G-II	Negative Control	7.480 ± 0.177###
G-III	Citric Acid	12.848 ± 0.171***
G-IV	BD (100 mg/kg)	10.701 ± 0.166***

Group	Treatment	Hb (g/dL)
G-V	BD (200 mg/kg)	11.383 ± 0.181***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	12.767 ± 0.174***$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$p<0.01 vs BD 200.

Effect of Treatments on Hemoglobin (Hb) Levels



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on hemoglobin levels. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$p<0.01 vs BD 200.

3.4.2 Total Red Blood Cell Count (RBC)

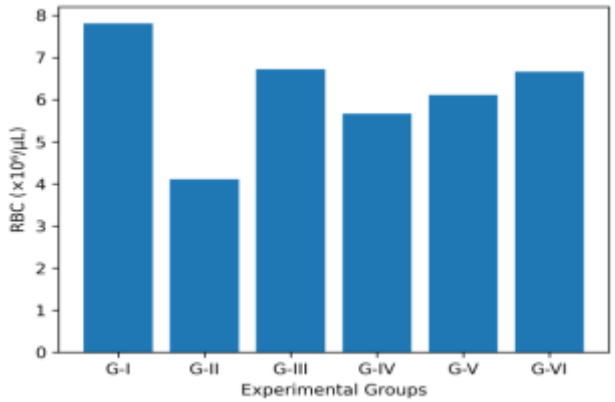
Aniline administration significantly decreased RBC count in the negative control group (4.110 ± 0.109 ×10⁶ cells/µL) compared to the control group (7.812 ± 0.124 ×10⁶ cells/µL, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (5.666 ± 0.113 ×10⁶ cells/µL) and 200 mg/kg (6.108 ± 0.121 ×10⁶ cells/µL) significantly restored RBC levels ($p < 0.001$). The combination treatment (6.662 ± 0.116 ×10⁶ cells/µL) was effective, but no significant difference was observed between the combination-treated group and the Boerhavia diffusa extract (200 mg/kg)-treated group ($p > 0.05$) (Table 11, Figure 10).

Table 11: Effect on Total Red Blood Cell (RBC) Count

Group	Treatment	Total RBC (×10⁶ cells/µL)
G-I	Control	7.812 ± 0.124
G-II	Negative Control	4.110 ± 0.109###
G-III	Citric Acid	6.724 ± 0.118***
G-IV	BD (100 mg/kg)	5.666 ± 0.113***
G-V	BD (200 mg/kg)	6.108 ± 0.121***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	6.662 ± 0.116***ns

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; ns = non-significant vs BD 200.

Effect of Treatments on Total RBC Count



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on total red blood cell count. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; ns = non-significant vs BD 200.

3.4.3 Total White Blood Cell Count (WBC)

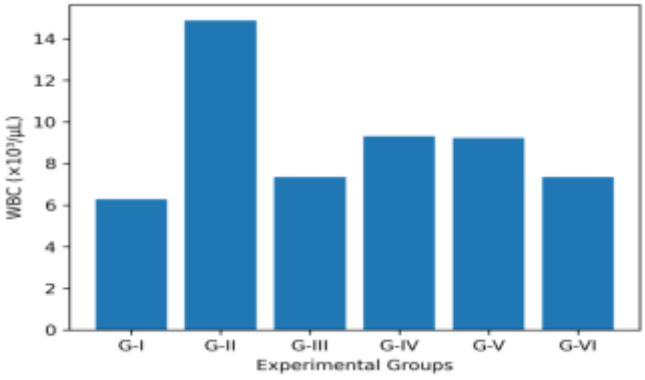
Aniline administration significantly increased WBC count in the negative control group (14.882 ± 0.185 ×10³ cells/µL) compared to the control group (6.284 ± 0.152 ×10³ cells/µL, p < 0.001). Treatment with Boerhavia diffusa extract at both 100 mg/kg (9.317 ± 0.171 ×10³ cells/µL) and 200 mg/kg (9.237 ± 0.164 ×10³ cells/µL) significantly decreased the elevated WBC count (p < 0.001). The combination treatment (7.345 ± 0.159 ×10³ cells/µL) produced a further significant reduction when compared with Boerhavia diffusa extract (200 mg/kg) alone (p < 0.001) (Table 12, Figure 11).

Table 12: Effect on Total White Blood Cell (WBC) Count

Group	Treatment	Total WBC (×10³ cells/µL)
G-I	Control	6.284 ± 0.152
G-II	Negative Control	14.882 ± 0.185###
G-III	Citric Acid	7.348 ± 0.168***
G-IV	BD (100 mg/kg)	9.317 ± 0.171***
G-V	BD (200 mg/kg)	9.237 ± 0.164***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	7.345 ± 0.159***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

Effect of Treatments on Total WBC Count



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on total white blood cell count. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.5 Effect on Biochemical Parameters

3.5.1 Total Protein

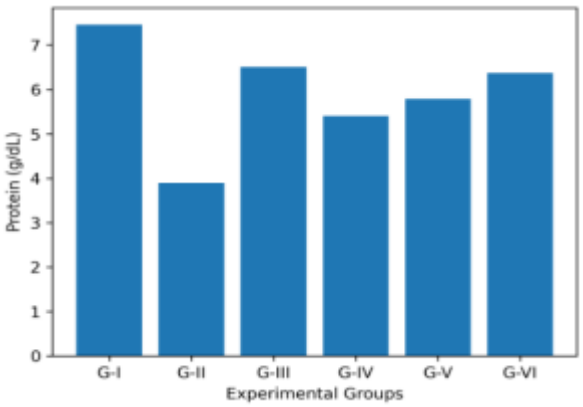
Aniline administration significantly decreased total protein levels in the negative control group (3.894 ± 0.087 g/dL) compared to the control group (7.462 ± 0.098 g/dL, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (5.409 ± 0.091 g/dL) and 200 mg/kg (5.788 ± 0.095 g/dL) significantly increased total protein levels ($p < 0.001$). The combination treatment (6.376 ± 0.093 g/dL) produced a further significant increase when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.05$) (Table 13, Figure 12).

Table 13: Effect on Total Protein Levels

Group	Treatment	Total Protein (g/dL)
G-I	Control	7.462 ± 0.098
G-II	Negative Control	3.894 ± 0.087###
G-III	Citric Acid	6.512 ± 0.094***
G-IV	BD (100 mg/kg)	5.409 ± 0.091***
G-V	BD (200 mg/kg)	5.788 ± 0.095***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	6.376 ± 0.093***$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $p<0.05 vs BD 200.

Effect of Treatments on Total Protein Levels



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on total protein levels. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $p<0.05 vs BD 200.

3.5.2 Total Iron

Aniline administration significantly decreased total iron levels in the negative control group (36.075 ± 1.058 µg/dL) compared to the control group (118.462 ± 1.124 µg/dL, $p < 0.001$). Treatment with Boerhavia diffusa extract at both 100 mg/kg (79.817 ± 1.096 µg/dL) and 200 mg/kg (88.136 ± 1.072 µg/dL) significantly increased total iron levels ($p < 0.001$). The combination treatment (100.968 ± 1.118 µg/dL) produced a further significant improvement when compared with Boerhavia diffusa extract (200 mg/kg) alone ($p < 0.001$) (Table 14, Figure 13).

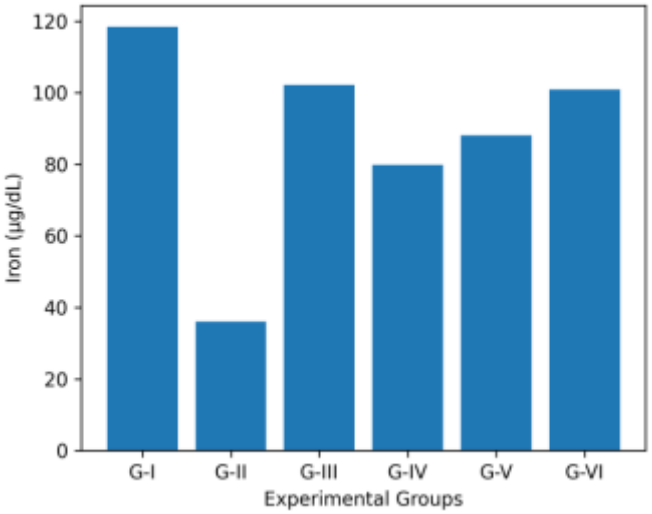
Table 14: Effect on Total Iron Levels

Group	Treatment	Total Iron (µg/dL)
G-I	Control	118.462 ± 1.124
G-II	Negative Control	36.075 ± 1.058###

Group	Treatment	Total Iron (µg/dL)
G-III	Citric Acid	102.153 ± 1.087***
G-IV	BD (100 mg/kg)	79.817 ± 1.096***
G-V	BD (200 mg/kg)	88.136 ± 1.072***
G-VI	BD (200 mg/kg) + POM (100 mg/kg)	100.968 ± 1.118***$$$

Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; ***p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

Effect of Treatments on Total Iron Levels



*Caption: Bar diagram showing the effect of Boerhavia diffusa extract alone and in combination with pomegranate extract on total iron levels. Values are expressed as Mean ± SEM (n=6). ###p<0.001 vs Control; **p<0.001 vs Negative Control; $$$p<0.001 vs BD 200.

3.6 Histopathological Assessment

Histopathological examination of spleen tissue revealed marked differences among the experimental groups. Spleen tissue sections from the normal control group showed normal histological architecture with well-organized lymphoid follicles and clearly defined red and white pulp regions (Grade 0). In contrast, the aniline-treated group exhibited severe histopathological alterations characterized by congestion, fibrinoid necrosis of follicular arteries, follicular hemorrhage, severe atrophy and necrosis of lymphoid cells, reticular cell hyperplasia, numerous apoptotic cells, and extensive deposition of eosinophilic hyaline material and fibrin threads within the splenic parenchyma (Grade 4).

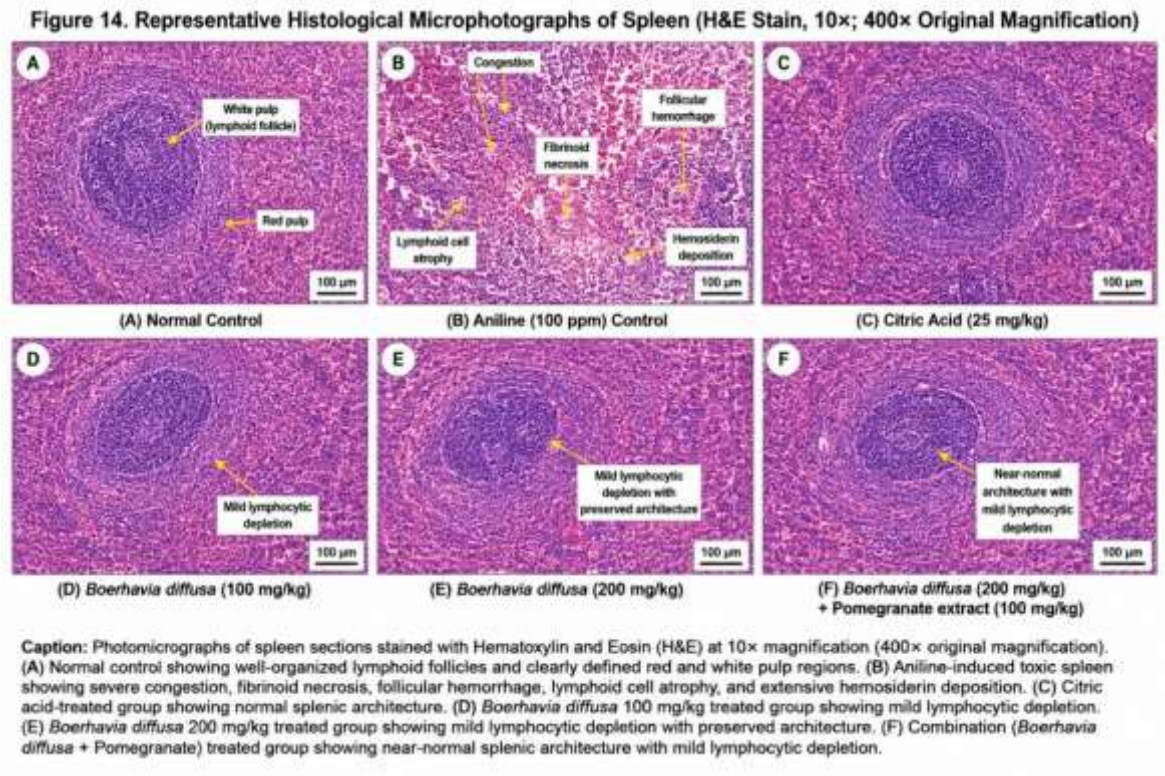
The standard treatment group (citric acid) showed normal histology with intact lymphoid follicles and preserved splenic architecture (Grade 0). Treatment with Boerhavia diffusa extract 100 mg/kg showed appreciable protection with mild to moderate depletion of lymphocytes within lymphoid follicles (Grade 1-2). The Boerhavia diffusa extract 200 mg/kg group demonstrated moderate splenoprotective activity with mild to moderate lymphocyte depletion (Grade 1-2). Notably, the combination-treated group exhibited improved histological preservation with only mild lymphocytic depletion and near-normal splenic architecture (Grade 1) (Table 15, Figure 14).

Table 15: Histopathology Assessment of Spleen

Group	Treatment	Histopathology Assessment
G-I	Control	No Abnormality Detected
G-II	Negative Control	Congestion and fibrinoid necrosis of follicular arteries associated with follicular hemorrhage; severe atrophy and necrosis of lymphoid cells with reticular cell hyperplasia; severe lesion

Group	Treatment	Histopathology Assessment
G-III	Citric Acid	No Abnormality Detected
G-IV	BD (100 mg/kg)	Mild depletion of lymphocytes within lymphoid follicles; architecture largely preserved
G-V	BD (200 mg/kg)	Mild depletion of lymphocytes within lymphoid follicles; moderate preservation of splenic architecture
G-VI	BD + POM	Mild lymphocytic depletion within follicles; near-normal splenic architecture

Caption: Photomicrographs of spleen sections stained with Hematoxylin and Eosin (H&E) at 10× magnification (400× original magnification). (A) Normal control showing well-organized lymphoid follicles and clearly defined red and white pulp regions. (B) Aniline-induced toxic spleen showing severe congestion, fibrinoid necrosis, follicular hemorrhage, lymphoid cell atrophy, and extensive hemosiderin deposition. (C) Citric acid-treated group showing normal splenic architecture. (D) Boerhavia diffusa 100 mg/kg treated group showing mild lymphocytic depletion. (E) Boerhavia diffusa 200 mg/kg treated group showing mild lymphocytic depletion with preserved architecture. (F) Combination (Boerhavia diffusa + Pomegranate) treated group showing near-normal splenic architecture with mild lymphocytic depletion.



4. DISCUSSION

The present study was undertaken to evaluate the splenoprotective potential of Boerhavia diffusa extract alone and in combination with pomegranate extract against aniline-induced spleen toxicity in experimental rats. The findings demonstrated that aniline administration produced significant systemic toxicity and splenic damage, as evidenced by increased organ weight, reduced feed and water intake, profound oxidative stress, hematological disturbances, membrane enzyme inhibition, and severe histopathological alterations.

4.1 Oxidative Stress and Antioxidant Defense

Oxidative stress is one of the principal mechanisms responsible for aniline-induced toxicity [3,4]. During metabolism, aniline generates reactive intermediates capable of producing excessive reactive oxygen species, leading to lipid peroxidation, protein oxidation, DNA damage, and cellular dysfunction [23]. In the present study, aniline administration significantly increased lipid peroxidation (LPO) and nitric oxide (NO) levels while depleting endogenous antioxidant defenses including reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). These findings are consistent with previous reports demonstrating that aniline exposure induces oxidative stress in splenic tissue [24,25].

Lipid peroxidation is a free radical-mediated process involving the oxidative degradation of membrane lipids, resulting in cellular damage and dysfunction [26]. The elevated MDA levels observed in the negative control group indicate extensive oxidative damage to splenic cell membranes. This is in agreement with Khan et al. [27], who demonstrated increased lipid peroxidation products in aniline-induced splenic toxicity. The significant reduction in LPO levels following treatment with Boerhavia diffusa extract, particularly in the combination group, suggests that the phytoconstituents present in the extracts possess potent free radical scavenging activities capable of protecting spleen tissue from oxidative damage.

Reduced glutathione is one of the most abundant intracellular antioxidants and plays a crucial role in maintaining cellular redox homeostasis [28]. The significant depletion of GSH levels in aniline-treated animals reflects increased utilization of antioxidant defenses and impaired protection against oxidative injury. Restoration of GSH levels by Boerhavia diffusa and pomegranate extracts indicates enhancement of the endogenous antioxidant defense system. The superior effect observed with combination therapy suggests a synergistic interaction between the bioactive constituents of the two plant extracts.

Superoxide dismutase and catalase are key enzymatic antioxidants that protect cells against oxidative damage by catalyzing the dismutation of superoxide radicals and decomposition of hydrogen peroxide, respectively [29]. The significant decrease in SOD and CAT activities following aniline exposure indicates severe impairment of the endogenous antioxidant defense mechanism. Treatment with Boerhavia diffusa extract significantly restored these enzyme activities in a dose-dependent manner, while the combination therapy produced the greatest improvement. These findings are consistent with previous studies demonstrating the antioxidant potential of Boerhavia diffusa [30,31] and pomegranate [32,33].

4.2 Nitrosative Stress

Nitric oxide is an important biological mediator involved in various physiological and pathological processes. Under oxidative conditions, nitric oxide is rapidly converted into stable end products, namely nitrite and nitrate [34]. The significantly elevated NO levels observed in aniline-treated animals indicate enhanced nitrosative stress and inflammatory responses. Increased NO production can lead to the formation of peroxynitrite, a potent oxidant that contributes to cellular damage [35]. The significant reduction in NO levels following treatment with Boerhavia diffusa and pomegranate extracts suggests attenuation of nitrosative stress and inflammation, with combination therapy showing superior effects.

4.3 Hematological Parameters Aniline exposure resulted in significant hematological disturbances, including marked reduction in hemoglobin concentration, red blood cell count, and serum iron levels, along with a significant elevation in total white blood cell count. These findings are consistent with the known hematotoxicity of aniline, which oxidizes hemoglobin to methemoglobin, reducing the oxygen-carrying capacity of blood [36,37]. The elevated WBC count reflects an inflammatory response associated with toxic injury [38].

Administration of Boerhavia diffusa extract significantly improved these parameters when compared with the aniline-treated group. The 200 mg/kg dose was more effective than the 100 mg/kg dose, demonstrating dose-dependent protection. Furthermore, the combination treatment produced greater restoration of hemoglobin, WBC count, total protein, and iron parameters than the individual extract treatments. These findings suggest that the extracts not only protect against oxidative stress but also contribute to normalization of hematological function and metabolic homeostasis under toxic conditions.

4.4 Membrane-Bound ATPases

Membrane-bound ATPases play a crucial role in maintaining cellular ion homeostasis, membrane integrity, and energy metabolism [39]. The activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase in spleen tissue were significantly reduced following aniline administration, indicating severe membrane damage and disruption of normal cellular function. Na^+/K^+-ATPase is responsible for maintaining intracellular sodium and potassium ion gradients, while Ca^{2+}-ATPase regulates intracellular calcium homeostasis, and Mg^{2+}-ATPase is involved in ATP hydrolysis and cellular energy metabolism [40-42].

The inhibition of these enzymes by aniline can be attributed to oxidative damage to membrane lipids and proteins, as well as the generation of reactive metabolites [43]. Treatment with Boerhavia diffusa extract significantly restored ATPase activities in a dose-dependent manner, while the combination of Boerhavia diffusa and pomegranate extract produced the greatest improvement. Restoration of these enzyme activities indicates preservation of membrane structure and function, protection against oxidative membrane injury, and maintenance of cellular homeostasis. These findings are consistent with previous studies demonstrating the membrane-stabilizing effects of Boerhavia diffusa [44] and pomegranate [45].

4.5 Histopathological Findings

Histopathological examination of spleen tissue confirmed the biochemical and hematological findings. Aniline-treated animals exhibited severe pathological alterations, including congestion, fibrinoid necrosis of follicular arteries, follicular hemorrhage, severe atrophy and necrosis of lymphoid cells, reticular cell hyperplasia, apoptotic cell formation, and extensive deposition of eosinophilic hyaline material and fibrin threads within the splenic

parenchyma. These changes are characteristic of aniline-induced splenic toxicity and are consistent with previous reports [46,47].
The standard treatment group (citric acid) showed complete preservation of splenic architecture with no detectable abnormalities. Treatment with Boerhavia diffusa extract at both dose levels reduced the severity of histological damage and preserved splenic structure to a considerable extent. Notably, the combination-treated group displayed near-normal histological architecture with only mild lymphocytic depletion, indicating superior protection against aniline-induced tissue injury. These observations strongly correlate with the improvements observed in antioxidant, biochemical, and hematological parameters.

4.6 Mechanism of Protective Action

The protective effects of Boerhavia diffusa and pomegranate extracts against aniline-induced splenic toxicity can be attributed to multiple mechanisms:
1. Antioxidant Activity: Both plants contain bioactive phytochemicals with potent free radical scavenging properties. Boerhavia diffusa contains flavonoids (quercetin, kaempferol), rotenoids (boeravinones), alkaloids (punarnavine), and phenolic compounds [7,48], while pomegranate is rich in punicalagin, ellagic acid, anthocyanins, and flavonoids [10,49]. These compounds neutralize reactive oxygen species, inhibit lipid peroxidation, and protect cellular structures from oxidative injury.
2. Anti-inflammatory Activity: Both plants suppress the production of inflammatory mediators and cytokines [50,51]. The significant reduction in NO levels and WBC count observed in this study indicates attenuation of inflammatory responses.
3. Membrane Stabilization: The restoration of membrane-bound ATPase activities suggests preservation of membrane integrity and function. This may be attributed to the membrane-stabilizing properties of the phytoconstituents present in both extracts.
4. Enhancement of Endogenous Antioxidant Defenses: Treatment with the extracts enhanced the activity of endogenous antioxidant enzymes (SOD, CAT) and restored GSH levels, strengthening cellular antioxidant defense mechanisms.
5. Synergistic Interaction: The enhanced efficacy observed with combination therapy suggests a synergistic interaction between the bioactive constituents of the two plant extracts. This may involve complementary mechanisms of action and multiple targeting of biochemical pathways.

4.7 Clinical Significance and Future Perspectives

The findings of this study have significant clinical implications. Industrial and environmental exposure to aniline continues to increase due to its widespread use in dye, rubber, pharmaceutical, and chemical industries [52]. Such exposure poses significant health risks, particularly through oxidative stress-mediated organ toxicity. The development of safe, cost-effective, and naturally derived therapeutic agents capable of mitigating chemical-induced tissue damage is of great importance.
Boerhavia diffusa and pomegranate are widely available, have been used in traditional medicine for centuries, and possess well-established safety profiles [53,54]. The present study demonstrates that these plants, particularly in combination, offer significant protection against aniline-induced splenic toxicity. These findings suggest that the herbal combination may serve as a promising natural therapeutic approach for preventing chemically induced splenic damage.
Future studies should focus on:
1. Isolation and characterization of the active constituents responsible for the protective effects
2. Elucidation of the molecular mechanisms involved in the synergistic interaction
3. Evaluation of the protective effects in other target organs (liver, kidneys, blood)
4. Clinical studies to establish efficacy and safety in humans
5. Development of standardized formulations for therapeutic use

5. CONCLUSION

The present study demonstrates that Boerhavia diffusa extract possesses significant splenoprotective activity against aniline-induced toxicity in experimental rats. Treatment with the extract restored antioxidant defenses, improved hematological and biochemical parameters, stabilized membrane-bound ATPases, and preserved normal histological architecture. The combination of Boerhavia diffusa with pomegranate extract produced superior protective effects across most of the evaluated parameters, indicating enhanced therapeutic efficacy through synergistic antioxidant and cytoprotective mechanisms.
The beneficial effects may be attributed to the antioxidant, anti-inflammatory, and membrane-stabilizing phytoconstituents present in both plant extracts. Therefore, Boerhavia diffusa alone and particularly in combination with pomegranate extract may serve as promising natural therapeutic agents for the prevention and management of chemically induced spleen toxicity.

6. CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest regarding the publication of this manuscript. No financial or personal relationships with other people or organizations that could inappropriately influence the work have been reported. All authors have read and approved the final manuscript.

7. ETHICAL APPROVAL STATEMENT

All experimental procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Royal College of Pharmaceutical Education and Research, Malegaon (Approval No: 2274/PO/Re/S/23/CPCSEA). All efforts were made to minimize animal suffering and to reduce the number of animals used in the study.

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