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Investigation of the Antioxidant Signalling Mechanisms of *Rosmarinus officinalis* L. in an Aniline-induced Splenic Toxicity Rat Model

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Abstract

Background: Aniline is an industrial chemical whose reactive metabolites induce oxidative stress and structural damage in splenic tissue, making aniline-induced splenic toxicity in rats a well-established experimental model for evaluating protective interventions. *Rosmarinus officinalis* L. (rosemary) possesses potent antioxidant and anti-inflammatory phytochemicals that may mitigate chemically induced tissue injury. **Aim:** To investigate the antioxidant-mediated splenoprotective effects of Ethanolic Rosemary Extract (ERE) against aniline-induced splenic toxicity in male Wistar rats. **Methods:** Male Wistar rats (200-250 g) were randomly divided into five groups (n = 6) and treated for 30 days. Splenic toxicity was induced by oral administration of aniline hydrochloride (50 mg/kg/day). Protective treatment groups received ERE at doses of 100 or 200 mg/kg/day orally, while citric acid (100 mg/kg/day, p.o.) served as the reference standard. Physiological parameters, oxidative stress biomarkers, antioxidant enzyme activities (GSH and CAT), ATPase activity, serum biochemical indices, histopathological changes, and hemosiderin deposition were assessed. Statistical analysis was performed using one-way ANOVA, with $p<0.05$ considered statistically significant. **Results:** Aniline administration produced splenomegaly, excessive iron accumulation, elevated lipid peroxidation, impaired antioxidant defences, reduced ATPase activity, and pronounced histopathological alterations including fibrosis and disruption of normal splenic architecture. Treatment with ERE significantly ameliorated these toxic effects in a dose-dependent manner. Rosemary extract restored antioxidant enzyme levels, reduced lipid peroxidation and nitric oxide production, improved ATPase activity, decreased hemosiderin deposition, normalised haematological and biochemical parameters, and preserved splenic histoarchitecture. The higher dose (200 mg/kg) demonstrated the greatest protective efficacy. **Conclusion:** ERE effectively protects against aniline-induced splenic toxicity by attenuating oxidative stress, restoring antioxidant defences, reducing iron accumulation, and preserving tissue architecture, suggesting its potential as a natural splenoprotective agent.

Major Findings: ERE significantly ameliorated oxidative stress, restored antioxidant enzyme activity, improved haematological indices, reduced splenic iron overload, and preserved splenic histoarchitecture, with the 200 mg/kg dose producing the greatest protective effect.

Keywords: Antioxidants, Fibrosis, Histopathology, *Rosmarinus officinalis,* Splenomegaly

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Abbreviations: AH: Aniline Hydrochloride; ERE: Ethanolic Rosemary Extract; CA: Citric Acid; PHA: Phenylhydroxylamine; NB: Nitrosobenzene; TCA: Trichloroacetic Acid; TBA: Thiobarbituric Acid; LPO: Lipid Peroxidation; MDA: Malondialdehyde (a marker of lipid peroxidation); GSH: Reduced Glutathione; SOD: Superoxide Dismutase; GPx: Glutathione Peroxidase; Nrf2: Nuclear Factor Erythroid 2-related Factor 2 (antioxidant pathway).

1. Introduction

The spleen is a critical immune organ responsible for hematopoiesis, filtration of blood, and immune regulation[1]. In rats, long-term aniline exposure results in splenic toxicity, where fibrosis appears as an early preneoplastic lesion that may progress to sarcoma. However, little is known about the molecular processes underlying aniline-induced fibrogenesis[2]. The antioxidant, anti-inflammatory, hepatoprotective and neuroprotective effects of rosemary (*R. officinalis* L.) and its active constituents, such as carnosic and rosmarinic acid, have been extensively studied in a variety of experimental models[3].

Nevertheless, no research has been published that explicitly investigates the protective effects of ERE on spleen tissue exposed to chemically induced splenic toxicity, such as that caused by long-term aniline exposure. Therefore, it is unknown whether ERE can prevent or ameliorate spleen-related alterations. Assessing ERE in a model of aniline-induced splenic toxicity thus represents a novel and significant contribution to phytotherapeutic and toxicological research[4]. It is unclear how aniline causes specific toxicity to the spleen. Aniline exposure causes lipid peroxidation and protein oxidation in the spleen, according to earlier research.

The current investigation sought to ascertain the spleen's release of free iron and oxidative damage[5]. Aniline shows high reactivity and functions as a precursor for several intermediates; it is an important industrial chemical[6]. It is widely utilised in the production of pigments and dyes, chemicals for rubber processing, polyurethane foams made from (methylene diphenyl di-isocyanine), medications including paracetamol, and a variety of pesticides, herbicides, explosives, and photographic reagents[7]. Since too much iron is extremely harmful to the spleen, iron overload is widely recognised as a health issue in developed nations[8]. Phenylhydroxylamine and nitrosobenzene, these reactive metabolites are produced by long-term aniline exposure, which undergo redox cycling and produce an excess of ROS, which encourage oxidative damage to membrane lipids, proteins, and nucleic acids. An increase in ROS destabilises iron-regulatory pathways. When combined, these processes cause splenic fibrosis, extracellular matrix buildup, and chronic inflammation of the spleen[9-12].

Although the antioxidant and anti-inflammatory properties of *R. officinalis* have been extensively reported in hepatic and neurological disorders[13-15], its protective role against aniline-induced splenic toxicity has not been investigated[16-18]. Therefore, the present study aimed to evaluate the splenoprotective effects of ERE by assessing oxidative stress markers, antioxidant defence systems, iron accumulation, ATPase activity, haematological parameters, and histopathological alterations in an experimental rat model.

2. Materials and Methods

2.1 Material

A standardised ethanolic extract of *R. officinalis* L. (Batch No. SSB/REE/081124) was purchased from Shree Sai Biotech, Indore, India. Phytochemical standardisation confirmed that the extract contained 4.5-6 % (w/w) rosmarinic acid, as verified by the manufacturer's certificate of analysis. AH was obtained from Sigma-Aldrich (Mumbai, India). All other chemicals and reagents used in biochemical and enzymatic assays were of analytical grade and procured from reliable commercial suppliers. Protein estimation was performed using commercial diagnostic kits. The standard laboratory diet was obtained from NutriVita Lab, Pune, India. All reagents and solutions were freshly prepared in accordance with standard laboratory procedures.

2.2 Methods

2.2.1 Study Design and Experimental Animals

Five groups of six healthy adult male Wistar rats weighing between 200 and 250 g were housed in individual polypropylene cages with paddy husk bedding. The animals were acclimated before the experiment began. Throughout the investigation, the

rats were kept at a temperature of 23 ± 2°C, a relative humidity of 55 ± 5%, and a 12-h light/dark cycle. The rats had free access to water and a standard laboratory diet.

2.2.2 Experimental Grouping

Thirty rats were randomly allocated into five experimental groups (n = 6 per group).

Group 1 was the Normal Control (NC): Rats received normal saline as a vehicle.

Group 2 was the Disease Control (DC): Rats received AH (50 mg/kg/day, p.o.).

Group 3 was the Standard Treatment (STD): Rats received AH along with (CA; 100 mg/kg/day, p.o.).

Group 4 received AH along with (ERE; 100 mg/kg/day, p.o.).

Group 5 received AH along with (ERE; 200 mg/kg/day, p.o.).

Dosages and treatment durations of AH, CA, and ERE were based on established protocols and literature. Rats in the DC group received AH to induce splenic toxicity. The STD group received AH along with CA as a standard control. Treatment groups were administered AH together with ERE at either a low or a high dose to evaluate dose-dependent protective effects. To allow sufficient time for structural and biochemical changes in splenic tissue and to comprehensively assess the protective efficacy of ERE, all interventions were administered for 30 consecutive days.

2.2.3 Physical Parameters

Body weight was recorded before treatment initiation, weekly during the study period, and at the end of the experiment. Every day, the animals' general physical state, behaviour, and any indications of toxicity were monitored. Absolute and relative spleen weights were evaluated at the conclusion of the experiment to evaluate treatment-related changes.

2.2.4 Induction of Splenic Toxicity

Rats were orally administered (AH; 50 mg/kg/day, p.o.) for 30 consecutive days to induce splenic toxicity. Aniline undergoes splenic metabolism to produce reactive oxidative metabolites, primarily Phenylhydroxylamine (PHA) and Nitrosobenzene (NB), which promote redox cycling and oxidative damage to erythrocytes. PHA rapidly enters erythrocytes, interacts with oxyhemoglobin, and contributes to methemoglobin formation. Damaged erythrocytes are sequestered by splenic macrophages, resulting in splenomegaly and hyperplasia[19,20].

2.2.5 Necropsy and Collection of Spleen

Animals were euthanised under appropriate anaesthesia before tissue collection, and the spleen was carefully removed and cleaned with cold saline. For histopathology, spleens were fixed in 10% neutral-buffered formalin for 24 h, processed, embedded in paraffin, sectioned at 4-5 µm, and stained with Haematoxylin and Eosin (H and E). Special stains such as Masson's trichrome or Prussian blue were used. Histological assessment focused on white and red pulp architecture, lymphoid cell density, and vascular alterations. For biochemical investigations, a part of the spleen was snap-frozen at -80°C immediately after collection[21]. Every procedure complied with CPCSEA regulations and was authorised by IAEC[22].

2.2.6 Quantification of Oxidative Stress Markers

2.2.6.1 Determination of Lipid Peroxidation (LPO)

Two millilitres of the spleen tissue homogenate (supernatant) was combined with 2.0 ml of freshly made 10% w/v Trichloroacetic Acid (TCA) and maintained in an ice bath for 15 minutes to precipitate proteins. After that, the mixture was centrifuged for ten minutes at 2,000 rpm. Two millilitres of the resulting clear supernatant was combined with 2.0 ml of freshly made Thiobarbituric Acid (TBA) reagent, and the mixture was heated in a boiling water bath for 10 minutes. The tubes were quickly chilled in an ice bath after being heated. Using spectrophotometry, the pink chromogen produced when TBA and Malondialdehyde (MDA) reacted was quantified at 532 nm in comparison to a reagent blank. The calibration curve was obtained by processing standard MDA (0-23 nM), which was made from 1, 1, 3, 3-tetraethoxypropane. The findings were reported as nM MDA per mg of tissue[23].

2.2.6.2 Determination of Reduced Glutathione (GSH)

The samples were centrifuged, and the supernatant was collected following the deproteinisation of the spleen homogenate using 20% TCA. Two millilitres of DTNB reagent and phosphate buffer were added to

0.25 millilitres of the supernatant. Spectrophotometric measurements of the developed yellow colour's intensity were made at 412 nm. A calibration curve was created using standard glutathione solutions (10-50 µg). Protein levels of reduced Glutathione (GSH) in spleen tissue were measured in nmol/mg[24].

2.2.6.3 Determination of Nitric Oxide (NO)

Following centrifugation and deproteinisation of the spleen homogenate with 20% sulphosalicylic acid, 1 mL of the supernatant was mixed with 1 mL of freshly prepared Griess reagent and incubated at 37°C for 15 min. The absorbance of the resulting crimson azo compound was measured spectrophotometrically at 540 nm. Sodium nitrite standards (0-100 µM) were used to construct a calibration curve. Nitrite concentration, reflecting nitric oxide levels, was expressed as nmol nitrite/mg protein[25].

2.2.6.4 Determination of Membrane-Bound Na^+/K^+-ATPase

Spleen homogenate was used to assess Na+/K+-ATPase activity. Solutions of MgSO4 (5 mM), KCl (5 mM), NaCl (60 mM), EDTA (0.1 mM), and ATP (40 mM) were produced along with Tris-HCl buffer (92 mM, pH 7.5). 0.2 mL of homogenate, 1 mL of Tris-HCl, and 0.2 mL of each reagent were combined in a test tube, incubated for 15 minutes at 36°C, and the reaction was terminated with 1 mL of 10% TCA. Following centrifugation, the amount of Inorganic Phosphorus (Pi) in the supernatant was measured. The unit of measurement for enzyme activity was µM Pi emitted per mg protein per minute[26].

2.2.6.5 Tissue homogenisation

The spleens of the sacrificed rats were carefully removed[27]. After blotting to remove blood, the tissues were rinsed with ice-cold Tris-hydrochloric buffered saline (pH 7.4) and weighed using an electronic scale (WENSAR, Model PGB-200). The spleens were then finely chopped with a sterile scalpel and placed in cold 0.25 M sucrose solution. The tissue slices were homogenised in cold 10 mM Tris-HCl buffer (pH 7.4) to prepare a 10% (w/v) homogenate. The homogenate was centrifuged at 10,000 rpm for 15 min at 0°C using a Remi C-24 high-speed refrigerated centrifuge. The supernatant was used for estimation of antioxidant indices including LPO, NO, SOD, GSH, and CAT, while the pellet was used for membrane-bound ATPase activity estimation[28]. Protein concentration of the homogenate was determined to normalise the biochemical data.

2.2.6.6 Histopathological Analysis

Spleens were dissected post-decapitation and fixed in 10% formalin. Five-micrometer paraffin sections were stained with hematoxylin and eosin (H and E) and examined under a light microscope (400×) for morphological changes. Representative photomicrographs were captured at Biotox Laboratory, Nashik, with standardised symbols and abbreviations used to annotate histological alterations. Splenic histology was evaluated semi-quantitatively using a 0-4 scale (0 = normal/nil, 1 = mild, 2 = minimal, 3 = moderate, 4 = severe), with independent scoring for White Pulp Area (WPA), Red Pulp Area (RPA), Hemosiderin-Laden Macrophages (HLM), Splenic Sinus Congestion (SSC), Follicular Tissue Thickness (FTT), and Peripheral Cell Volume (PCV)[29,30].

2.2.7 Statistical Work

A statistical study was carried out, with one-way Analysis of Variance (ANOVA), followed by Bonferroni's multiple comparison test for all parameters performed in GraphPad Prism software, version 8.2, USA. Results are shown as mean ± SD. The findings are displayed as mean ± SD. $p<0.05$ was the threshold for statistical significance, and $p<0.01$ was deemed highly significant[31,32].

3. Results

3.1 Physiological Parameters (Body Weight and Spleen Weight)

AH exposure in the DC group significantly reduced body weight (213.6 ± 7.25 g, ###$p<0.001$) and increased spleen weight (2.079 ± 0.27 g, ###$p<0.001$) compared with NC, indicating systemic toxicity and splenomegaly. STD and ERE treatments restored body weight and normalised spleen weight in a dose-dependent manner. ERE-2 (200 mg/kg/day, p.o) was more effective (263.0 ± 5.90 g; 0.870 ± 0.13 g) than ERE-1 (100 mg/ kg/day, p.o.), as shown in Table 1. Water and Feed Intake: DC rats consumed significantly less water (44.25 ± 1.92 ml)

Table 1. Effect of ERE- 1, 2 and CA on body weight, spleen weight, water intake and feed consumption

Group (g)	Parameters			
	Water intake (ml)	Feed intake (g)	Body weight (g)	Organ weight (g)
NC	72.5±4.93	21.5±0.5477	276.8±6.89	0.840±0.04
DC	44.25±1.917	12.75±0.2739	213.6±7.249 ###	2.079±0.27###
STD	67.5±2.739	18.25±0.2739	251.0±10.19 ***	0.903±0.20***
ERE-1	58±2.191	14.40±0.1807	228.8±7.88 *	0.945±0.16***,a
ERE-2	62±0	15.5±0.5477	263.0±5.90 ***, aa, bb	0.870±0.13***,aa,bb

Values are expressed as mean ± SD; n = 6. One-way ANOVA followed by Bonferroni's multiple comparison test. Significance: ###p<0.001 vs. NC (normal control); *p<0.05, **p<0.01, ***p<0.001 vs. DC (disease control); ap<0.05, aap<0.01, aaap<0.001 vs. STD; bp<0.05, bbp<0.01, bbbp<0.001 vs. ERE-1.

Table 2. Effect of ERE and standard treatment on splenic oxidative stress markers and antioxidant enzymes in aniline-induced splenic toxicity

Group	Parameters				
	LPO (nmol /mg protein)	GSH (mg/g protein)	NO (µmol / mg protein)	SOD (U/mg protein)	CAT (nmol /mg protein)
NC	6.113±1.40	8.013±0.82	5.272±1.43	9.817±1.499	15.36±3.286
DC	23.89±8.67###	1.622±0.45###	24.27±1.97###	22.19±2.616###	5.232±1.224###
STD	13.22±1.91***	4.985±0.70***	13.25±2.59***	14.86±2.585***	8.895±1.33**
ERE-1	14.10±0.92***	2.488±0.22***,a	17.53±2.30***	12.8±0.7301***,a	7.848±0.9623*
ERE-2	13.45±1.85***,aa, bb	3.584±0.36***,aa,bb	14.24±1.35***,aa,bb	11.18±2.178***,aa,bb	11.04±1.123***,bb

Values are expressed as mean ± SD; n = 6. One-way ANOVA followed by Bonferroni's multiple comparison test. Significance: ###p<0.001 vs. NC (normal control); *p<0.05, **p<0.01, ***p<0.001 vs. DC (disease control); ap<0.05, aap<0.01, aaap<0.001 vs. STD; bp<0.05, bbp<0.01, bbbp<0.001 vs. ERE-1.

and feed (12.75 ± 0.27 g) than NC (72.5 ± 4.93 ml; 21.5 ± 0.55 g), indicating systemic stress. ERE-2 restored intake more effectively (62 ± 0 ml; 15.5 ± 0.55 g) than ERE-1, reflecting dose-dependent recovery.

3.2 Assessment of Oxidative Stress and Antioxidant Status

In DC rats exhibited pronounced oxidative stress, as indicated by a significant increase in LPO (23.89 ± 8.67 nmol/mg protein, ###p<0.001) and marked depletion of GSH (1.622 ± 0.45 mg/g protein, ###p<0.001) compared with NC. NO levels were also significantly elevated in DC rats (24.27 ± 1.97 µmol/mg protein, ###p<0.001), while antioxidant enzyme activities were altered, with increased SOD (22.19 ± 2.62 U/mg protein) and decreased CAT (5.23 ± 1.22 nmol/mg protein). Treatment with STD, ERE-1, and ERE-2 significantly ameliorated these changes, with ERE-2 showing superior efficacy. ERE-2 markedly reduced LPO (13.45 ± 1.85 nmol/mg protein, aap<0.01, bbp<0.01) and NO (14.24 ± 1.35 µmol/mg protein, aap<0.01, bbp<0.01), while restoring GSH (3.584 ± 0.36 mg/g protein, aap<0.01, bbp<0.01), SOD (11.18 ± 2.18 U/mg protein), and CAT (11.04 ± 1.12 nmol/mg protein) toward normal levels. The results of the assessment of oxidative stress and antioxidant status are shown in Table 2.

3.3 Hematological Parameters

In DC rats, significant hematological alterations were observed. Hemoglobin (Hb) and RBC counts were markedly decreased (7.03 ± 0.68 g/dL and 5.57 ± 0.54 $\times 10^6$/cmm, ###p<0.001), WBC counts were elevated (13.6 ± 0.95 $\times 10^3$/cmm, ###p<0.001), and platelet counts were reduced (454.1 ± 127.7 $\times 10^3$/cmm, ###p<0.001) compared with NC. Treatment with STD, ERE-1, and ERE-2 significantly ameliorated these changes. ERE-2 showed superior efficacy, restoring Hb (10.42 ± 1.83 g/dL, aap<0.01, bbp<0.01) and RBC (7.45 ± 0.86 $\times 10^6$/cmm, aap<0.01, bbp<0.01), normalising WBC (10.87 ± 0.85 $\times 10^3$/cmm, bbp<0.001), and increasing platelets (750.3 ± 48.78 $\times 10^3$/cmm, **p<0.01; bbp<0.01) toward

Table 3. Effect of ERE- 1, 2 and CA on hemopoietic parameters in AH-induced splenic toxicity in rats

Group	Parameter			
	Hb (g/dL)	RBC (x106/cmm)	WBC (x103/cmm)	Platelets (x103/cmm)
NC	13.6±1.244	8.290±0.3381	10.17±0.6121	843.3±50.11
DC	7.033±0.6831###	5.565±0.5415###	13.6±0.9493###	454.1±127.7###
STD	11.12±1.098***	7.633±0.7187***	11.1±1.311**	783±59.02***
ERE-1	7.583±0.7305*,a	6.802±1.121**	11.82±0.828*	561.8±126.8**
ERE-2	10.42±1.837***, aa, bb	7.450±0.8625***,a,bb	10.87±0.8548***,bb	750.3±48.78***,a,bb

Values are expressed as mean ± SD; n = 6. One-way ANOVA followed by Bonferroni's multiple comparison test. Significance: ###p<0.001 vs. NC (normal control); *p<0.05, **p<0.01, ***p<0.001 vs. DC (disease control); ap<0.05, aap<0.01, aaap<0.001 vs. STD; bp<0.05, bbp<0.01, bbbp<0.001 vs. ERE-1.

Table 4. Effect of ERE- 1, 2 and CA on ATPase in AH induced splenic toxicity in rats

Group	ATPase (nmol Pi/mg protein/min)		
	Na+/K+ ATPase	Ca+ ATPase	Mg+ ATPase
NC	13.42±0.56	14.34±1.31	19.44±0.5057
DC	9.661±1.36###	9.195±0.4572###	11.91±0.8570###
STD	13.73±0.65***	12.23±1.822**	17.28±1.668***
ERE-1	11.02±1.412**	9.209±1.67	13.79±2.573***
ERE-2	13.35±0.3238***,aa,bb	11.13±1.23**	15.53±1.224***

Values are expressed as mean ± SD; n = 6. One-way ANOVA followed by Bonferroni's multiple comparison test. Significance: ###p<0.001 vs. NC (normal control); *p<0.05, **p<0.01, ***p<0.001 vs. DC (disease control); ap<0.05, aap<0.01, aaap<0.001 vs. STD; bp<0.05, bbp<0.01, bbbp<0.001 vs. ERE-1.

Table 5. Effect of ERE- 1, 2 and CA on iron and total protein in AH induced splenic toxicity in rats

Group	Parameters	
	Iron (ug/dL)	Protein (mg/dL)
NC	247.2±35.90	7.87±0.999
DC	781.0±53.82###	5.50±0.488
STD	444.2±76.27***	7.85±0.616***
ERE-1	477.2±125.0***	7.29±1.082**,a
ERE-2	418.5±92.17***	7.74±0.2268***,aa,bb

Values are expressed as mean ± SD; n = 6. One-way ANOVA followed by Bonferroni's multiple comparison test. Significance: ###p<0.001 vs. NC (normal control); *p<0.05, **p<0.01, ***p<0.001 vs. DC (disease control); ap<0.05, aap<0.01, aaap<0.001 vs. STD; bp<0.05, bbp<0.01, bbbp<0.001 vs. ERE-1.

normal levels. The results of hematological parameters are shown in Table 3.

3.4 Membrane-bound ATPase Activities

DC rats exhibited significant reductions in Na^+/K^+-, Ca^{2+}-, and Mg^{2+}-ATPase activities (###p<0.001 vs NC). STD and ERE treatments restored enzyme activities, with ERE-2 showing superior efficacy: significantly increasing Na^+/K^+-ATPase (aap<0.01 vs STD; bbp<0.01 vs ERE-1), Ca^{2+}-ATPase (**p<0.01 vs DC), and Mg^{2+}-ATPase (***p<0.001 vs DC). Both ERE doses significantly improved all ATPase activities compared to DC. The results of membrane-bound ATPase activities are shown in Table 4.

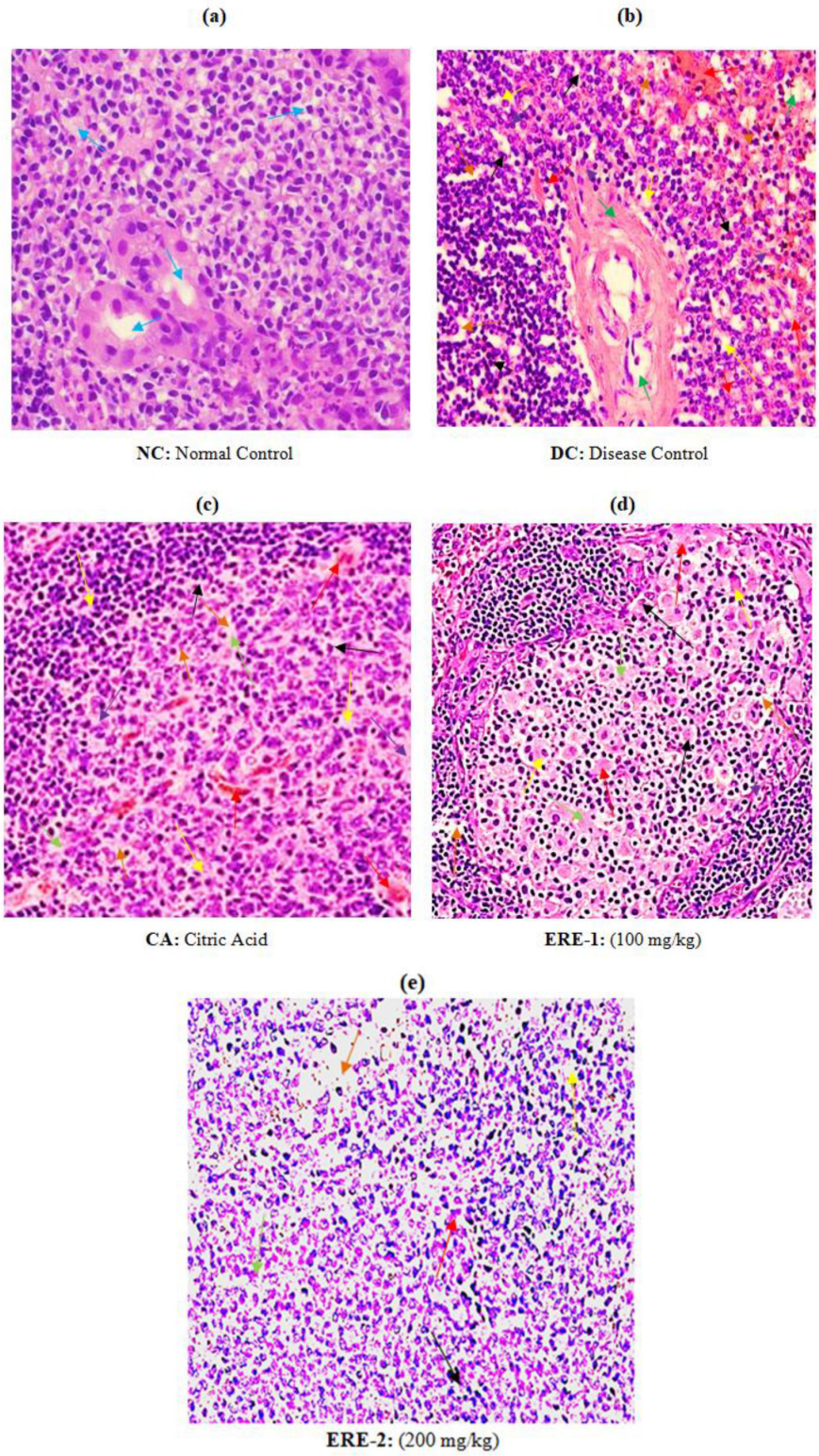
Table 6. Semi-quantitative histopathological scores of splenic tissues in different treatment groups

Animal groups	WPA	RPA	HLM	SSC	FTT	PCV
NC	0	0	0	0	0	0
DC	4	3	4	4	4	4
CA	3	3	2	3	2	2
ERE-1	2	2	2	2	1	2
ERE-2	1	1	1	1	0	1

Scoring Criteria: 0, nil or normal; 1, mild; 2, minimal; 3, moderate; 4, severe.

3.5 Splenic Iron and Total Protein Levels

Table 5 shows that splenic iron levels were significantly elevated in the DC group (781.0 ± 53.82 µg/dL)



Colored Arrows Indicate Histopathological Changes: orange - White Pulp Atrophy (WPA); red - Red Pulp Degeneration (RPA); green - Hemosiderin-Laden Macrophages (HLM); yellow - Splenic Sinusoidal Congestion (SSC); purple - Fibrous Trabecular Thickening (FTT); black - Parenchymatous Cell Vacuolisation (PCV).

Figure 1. Photomicrographs of splenic tissue sections showing the effect of ERE-1, 2, and CA on AH induced splenic toxicity in rats (n = 6).

compared with the NC group (247.2 ± 35.90 µg/dL; ###p<0.001), while total protein levels were significantly decreased in DC (5.50 ± 0.488 mg/dL) compared with NC (7.87 ± 0.999 mg/dL; ###p<0.001). Treatment with STD significantly reduced iron levels (444.2 ± 76.27 µg/dL; ***p<0.001 vs DC) and restored protein levels (7.85 ± 0.616 mg/dL; ***p<0.001 vs DC). Both doses of ERE also significantly decreased iron and increased protein compared with DC, with ERE-1 at 477.2 ± 125.0 µg/dL and 7.29 ± 1.082 mg/dL (**p<0.01 vs DC, ap<0.05 vs STD) and ERE-2 at 418.5 ± 92.17 µg/dL and 7.74 ± 0.2268 mg/dL (***p<0.001 vs DC, aap<0.01 vs STD, bbp<0.01 vs ERE-1). Iron levels in ERE-2 were lower and protein levels higher than in ERE-1, approaching NC values, indicating a dose-dependent effect.

3.6 Histopathology of Spleen

Histopathological results of splenic tissues from all experimental groups, as illustrated in Figure 1 (a-e) and summarised in Table 6, clearly demonstrate the protective effect of ERE against AH-induced splenic toxicity[33,34]. The NC group served as the baseline reference and exhibited intact splenic architecture with well-defined red and white pulp regions, indicating normal histological features. In contrast, the Disease Control group (DC) showed severe splenic damage characterised by pronounced fibrous trabecular thickening, parenchymatous cell vacuolisation, marked splenic sinusoidal congestion, extensive infiltration of hemosiderin-laden macrophages, along with significant white pulp atrophy and red pulp degeneration, confirming the successful induction of splenic toxicity[35,36].

Treatment with the CA resulted in partial restoration of splenic architecture, as evidenced by moderate reductions in white pulp atrophy (score 3), red pulp degeneration (score 3), splenic sinusoidal congestion (score 3), fibrous trabecular thickening (score 2), parenchymatous cell vacuolisation (score 2), and hemosiderin-laden macrophage accumulation (score 2) compared with the disease control group. Administration of ERE at a low dose (ERE-1, 100 mg/ kg/day, p.o) produced noticeable protective effects, showing further attenuation of histopathological alterations, including reduced white pulp atrophy and red pulp degeneration (score 2), decreased splenic sinusoidal congestion (score 2), reduced fibrous trabecular thickening (score 2), diminished parenchymatous cell vacuolisation (score 2), and fewer hemosiderin-laden macrophages (score 2). Notably, the high-dose ERE-2 group (200 mg/kg/day, p.o) exhibited marked preservation of splenic architecture, characterised by minimal white pulp atrophy and red pulp degeneration (score 1), mild splenic sinusoidal congestion (score 1), minimal hemosiderin-laden macrophage infiltration (score 1), absence of fibrous trabecular thickening (score 0), and only mild parenchymatous cell vacuolisation (score 1), closely resembling the normal control group.

The semi-quantitative histopathological scoring further corroborated these observations, demonstrating dose-dependent reductions in white pulp atrophy, red pulp degeneration, hemosiderin-laden macrophages, splenic sinusoidal congestion, fibrous trabecular thickening, and parenchymatous cell vacuolisation in ERE-treated groups compared with the disease control group, with the most pronounced protective effect observed in the ERE-2 group. Collectively, these findings indicate that ERE confers significant, dose-dependent protection against AH-induced splenic injury and effectively preserves splenic structural integrity. Representative photomicrographs were captured at a scale bar of 50 µm.

4. Discussion

AH exposure induced pronounced splenic toxicity, evidenced by reduced body weight, increased spleen weight, and decreased feed and water intake, indicating systemic stress and metabolic disruption. Hematological analysis revealed decreased haemoglobin and RBC counts, elevated WBCs, and reduced platelets, reflecting impaired hematopoiesis and immune imbalance[37].

Oxidative stress was a central mechanism, as DC rats showed elevated LPO and NO, depleted GSH, and altered SOD and CAT activities, indicating redox imbalance under excessive ROS[38]. These changes were accompanied by increased splenic iron, reduced total protein, and decreased membrane-bound ATPase activities (Na^+/K^+, Ca^{2+}, and Mg^{2+}- ATPases), suggesting iron-mediated oxidative damage, impaired protein synthesis, and disrupted membrane integrity[38-40].

Histopathology confirmed severe splenic injury, including trabecular thickening, parenchymal vacuolisation, sinusoidal congestion, macrophage

infiltration, and pulp degeneration[33-36, 41]. Treatment with STD and ERE, particularly ERE-2 (200 mg/kg/day, p.o), restored body and spleen weights, normalised hematological parameters (indicating protection of splenic hematopoietic function), reduced oxidative stress, and preserved ATPase activities[42-46]. These findings demonstrate that ERE exerts dose-dependent splenoprotective effects through attenuation of oxidative stress, restoration of endogenous antioxidant defences, reduction of iron accumulation, stabilisation of membrane-associated ATPase activity, and preservation of splenic histoarchitecture.

5. Conclusion

This study demonstrates that AH induces significant splenic toxicity through oxidative stress, iron dysregulation, hematological disturbances, and histopathological damage. The injury is primarily mediated by excessive ROS generation and iron-dependent cytotoxicity. Administration of ERE, particularly at 200 mg/kg/day (ERE-2), provided marked dose-dependent protection. ERE-2 restored body and spleen weights, normalised hematological parameters, reduced lipid peroxidation and nitric oxide levels, enhanced antioxidant defences (GSH, SOD, CAT), preserved membrane integrity, and improved splenic histology. These protective effects are likely mediated through antioxidant mechanisms, including Nrf2 pathway activation and phenolic constituents of rosemary. Overall, ERE demonstrates significant potential as a natural therapeutic candidate for mitigating oxidative stress-mediated splenic injury and warrants further mechanistic and clinical investigation.

6. Ethical Approval

The research was conducted after obtaining approval from the Institutional Animal Ethics Committee (IAEC), Biotox Laboratory, Nashik (Approval No.: IAEC/05/L2025/RP-32a). All animal care and experimental procedures were performed in accordance with the guidelines prescribed by the Committee for Control and Supervision of Experiments on Animals (CPCSEA).

7. References

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Author Contribution

Chandrashekhar Patil: Conceptualisation, investigation, and original draft preparation; Durgesh Pagar and Kajal Pansare: Investigation and data collection; Ganesh Sonawane: Data analysis, writing, and editing; Deepak Somavanshi: Phytochemical and pharmacological investigations; Durgesh Gautam and Sujit Jadhav: Review, editing, and supervision; Sandip Ahire: Investigation and manuscript review. All authors have read and agreed to the published version of the manuscript.