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PROTECTIVE ROLE OF WITHANIA SOMNIFERA ETHANOLIC ROOT EXTRACT AGAINST POLYURETHANE MICROPLASTIC-INDUCED HAEMATOTOXICITY AND INTESTINAL DAMAGE IN RABBIT (ORYCTOLAGUS CUNICULUS)

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ABSTRACT
The systemic toxicity of synthetic polymer microplastics (MPs) is an emerging and growing concern for mammalian health, but how MPs cause systemic toxicity in mammals and how to effectively mitigate their effects are not fully understood. This present investigation aimed to study the sub-chronic toxicological effects of oral administration of polyurethane (PU) and phytoprotective effects of Withania somnifera (Ashwagandha) ethanolic root extract (EREWS) in the rabbit model. Twenty rabbits were randomly distributed to 5 experimental groups (n = 5), and treated daily for 60 days, with the following treatments: Group A (vehicle control); Groups B (PU at 300 mg/kg B.W); Groups C and D (PU with low- (75 mg/kg B.W.) or high-dose (150 mg/kg B.W.EREWS). Microplastic caused significant systemic pathogenesis ($p < 0.05$): a decrease in erythrocytic indices (RBC count, haemoglobin, packed cell volume) and platelet counts, and an increase in total white blood cell counts and mean corpuscular volume, all suggestive of microcytic anaemia, haematopoietic suppression, and systemic inflammation. Histopathology of the intestine showed marked disruption of the mucosa with villous blunting and fusion, lifting and sloughing of the epithelium, inflammatory infiltration of the lamina propria and disorganisation of the crypt architecture. Of particular interest was the greater effect of PE on erythrocyte degradation and leukocytosis at the same dose compared to PU. All haematological and histopathological aberrations were significantly improved in a dose dependent manner when EREWS was co-administered. High-dose EREWS (150 mg/kg B.W.) was significantly more effective in restoring erythrocytic indices and platelet counts to physiological levels and in maintaining intestinal mucosal architecture by regeneration of villous height, restoration of epithelial integrity and attenuation of inflammatory infiltration. These results show that the root extract of W. somnifera has a strong protective effect against haematotoxicity and enterotoxicity induced by MP, suggesting that it could be used as a natural countermeasure against environmental exposure to xenobiotics.

Keywords: Microplastics; Withania somnifera; Phytoprotection; Haematotoxicity; Intestinal histopathology Polyurethane

INTRODUCTION
Global plastic waste production has been increasing at an exponential rate (currently at more than 380 million metric tonnes per year), with waste management systems not keeping up with the amount of plastic waste generated, leading to the widespread presence of plastic waste in terrestrial, freshwater, and marine environments (Deng et al., 2022; Saeedi, 2024). Of the plastic produced, around 85% ends up in the environment, where it is continuously broken down by physicochemical and biological processes into microplastics (MPs) (1 μm – 5 mm) and nanoplastics (<1 μm) (Huang et al., 2021; Liaqat et al., 2024).

MPs are produced from primary sources such as engineered microspheres in personal care products, industrial pellets, synthetic textile fibres, and tyre wear particles, as well as secondary fragmentation of macro-debris such as agricultural mulch films, packaging materials and fishing gear (Ling et al., 2020). MPs are also more likely to be ecotoxicologically relevant because of their ability to bind chemical contaminants, such as persistent organic pollutants, endocrine disrupting compounds and heavy metals, and to enhance their transport (Amelia et al., 2021). Critically, MPs are more persistent in aquatic and terrestrial food webs than conventional wastewater treatment facilities can remove.

Of all the synthetic polymers most significant to the environment, polyethylene (PE) and polyurethane (PU) are two that deserve special attention. PE is the largest thermoplastic produced worldwide, and is widely used in food contact packaging, agricultural mulch films and consumer products (Ghatge et al., 2020). PU is a thermoset elastomer synthesized by diisocyanate–polyol condensation reactions, and is widely used in automotive interior, thermal insulation foams and building materials (Othman et al., 2021; Levchik and Weil, 2005). Although MPs are commonly used in industry, the specific haematotoxic and gastroenteric effects of chronic oral exposure to PE and PU MPs in mammalian models have not been fully characterized when compared to polystyrene (Deng et al., 2017; Espinosa et al., 2019).

Oral exposure to humans is the main source of exposure to MPs through contaminated food and water. When ingested, MPs can pass through the intestinal epithelial barrier, enter into visceral organs, and cause oxidative stress, disruption of the intestinal barrier, and systemic inflammatory responses (Jin et al., 2018; Lu et al., 2018; Qiao et al., 2019). Haematological changes in MP exposed organisms such as erythropenia, anaemia, thrombocytopenia and leukocytosis are a consequence of bone marrow suppression, oxidative damage to the erythrocyte membrane and innate immune activation (Hamed et al., 2019).

The new paradigm of phytotherapy against environmental xenobiotics has attracted much scientific interest. Withania somnifera (L.) Dunal or Solanaceae is one of the most widely studied medicinal plants in Ayurvedic pharmacopoeia, commonly referred to as Ashwagandha or Indian ginseng. The roots are rich in a variety of bioactive secondary metabolites, such as withanolides (steroidal lactones), withanine and somniferine (alkaloids), polyphenols and saponins, which contribute to its antioxidant, anti-inflammatory, immunomodulatory, and adaptogenic properties (Mikulska et al., 2023; Singh et al., 2011; Mirjalili et al., 2009). W. somnifera extract has been shown to modulate the hypothalamic–pituitary–adrenal (HPA) axis, reduce cortisol hypersecretion and normalize haematopoietic function in chronic stress (Gupta et al., 2021; Mukherjee et al., 2020).

There is, however, no empirical evidence on the effectiveness of W. somnifera for MP-specific toxicity, especially in mammalian models exposed to PE and PU, in the current literature. For this reason, the present study aimed to (i) characterize the haematotoxic and intestinal histopathological effects of chronic oral exposure to PE and PU MPs in a rabbit model; (ii) characterize the phytochemical profile of EREWS using GC-MS; and (iii) assess the dose-dependent phytoprotective potential of EREWS against systemic toxicity induced by MPs.

MATERIALS AND METHODS

Ethical approval and experimental animals

The experimental procedures were approved by the Institutional Animal Ethics Committee of Institute of Zoology, Bahauddin Zakariya University, Multan, Pakistan, with Ref. No. Zool 126 dated 27-11-2024, according to internationally accepted guidelines for the care and use of laboratory animals. A total of 20 rabbits (Oryctolagus cuniculus) were purchased from a certified local supplier (Multan) at age of 5–6 weeks (initial body weight 750–800 g). Animals were maintained in separate polypropylene cages under controlled environmental conditions (12 h light/dark cycle; 27 ± 1°C; 55 ± 5% relative humidity) with free access to standard commercial pelleted chow and filtered potable water. Experimental allocation was preceded by a seven day quarantine and acclimatisation.

The design of an experiment and the selection of treatment regimens.

After acclimatisation, rabbits were randomly grouped into 4 experimental groups (n = 5 rabbits per group) and treated orally each day for 60 days with the following treatment groups:

Group A (Vehicle Control): Standard basal diet and distilled water, no administration of microplastic or extract.

Group B (PU): Polyurethane microplastics in drinking water at 300mg/kg B.W./day.

Group C (PU + EREWS 75): PU microplastics (300 mg/kg B.W.) co-administered with EREWS (75 mg/kg B.W.).

Group D (PU + EREWS 150): PU microplastics (300 mg/kg B.W.) were co-administered with EREWS (150 mg/kg B.W.).

Microplastic suspensions were freshly prepared every day using distilled water by ultrasonication, and given via oral gavage. EREWS was delivered orally using gavage.

PREPARATION OF WITHANIA SOMNIFERA ETHANOLIC ROOT EXTRACT (EREWS)

Roots of W. somnifera were collected from its natural habitat in montane area of Soon Valley, District Khushab, Punjab, Pakistan (GPS co-ordinates marked). A voucher specimen (Voucher accession No. 288-BOT-21) was deposited with a senior taxonomist of the Department of Botany, GC University Faisalabad for botanical identification. Harvested roots were washed thoroughly under running tap water, cut into uniform pieces (about 2 cm) and dried in the shade for 14 days with the periodic turning to ensure uniform drying. Dried material was powdered using a stainless-steel electric blender and passed through a 40-mesh sieve to obtain a homogeneous powder.

The crude extraction was done by maceration of 750 g of root powder in 1,000 mL of 95% (v/v) analytical grade ethanol for 24 h in a Soxhlet extraction apparatus at reflux temperature. The extract was filtered on Whatman No. 1 filter paper under vacuum and the filtrate was concentrated to a viscous residue by rotary evaporation (40°C, 120 rpm). The crude extract was lyophilised to give a dry powder with a defined yield (% w/w). The standardised colorimetric and precipitation methods were used to confirm the presence of alkaloids, flavonoids, tannins, cardiac glycosides, carbohydrates, saponins, vitamins and individual phenolic compounds in the plant extracts through qualitative phytochemical screening using the methodlogy of Ali et al 2020.

After 60 days of experimentation, the rabbits were anaesthetised by inhaling diethyl ether and blood samples (3 mL) were taken aseptically from the marginal ear vein into EDTA coated vacutainer tubes (1.8 mg EDTA/mL blood). Haematological parameters were determined within 2 hours after collection, using a fully automated five-part differential haematology analyser (Sysmex XN-550 or equivalent). The following parameters were evaluated: red blood cell (RBC; ×106/µL), haemoglobin concentration (Hb; g/dL), packed cell volume/haematocrit (PCV/HCT; %), mean corpuscular volume (MCV; fL), mean corpuscular haemoglobin (MCH; pg), mean corpuscular haemoglobin concentration (MCHC; g/dL), platelet count (PLT; ×103/µL), total white blood cell count (WBC; ×103/µL) and differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils, basophils; %).

HISTOPATHOLOGICAL EXAMINATION

Animals were scarified for blood collection by cervical dislocation under anaesthetic. Midline laparotomy was performed and representative sections of small intestine (duodenum, jejunum, and ileum) were cut, washed in ice-cold phosphate-buffered saline (PBS, pH 7.4), and then fixed in 10% neutral-buffered formalin (NBF) for 48 h. Fixed tissues were dehydrated using a graded ethanol series (70–100%), cleared in xylene, infiltrated with molten paraffin wax and oriented blocks were used to allow both cross-sectional and longitudinal sectioning.

The serial sectioning was performed with a calibrated rotary microtome (Leica RM2235) and the sections were placed on poly-L-lysine coated glass slides. The sections were stained with Ehrlich's haematoxylin (10 min) and counterstained with aqueous eosin (3 min) according to standard H&E procedure, cleared in xylene and mounted in Canada balsam. Histological assessment was conducted by a board-certified veterinary pathologist blinded to treatment group by using a light microscope (Olympus BX 53) at ×40 and ×100 magnification, with calibration. The following morphological endpoints were evaluated: villous height, crypt depth, villous to crypt ratio, epithelial continuity (0 = normal to 3 = severe injury), goblet cell density, lamina propria cellularity and degree of inflammatory infiltration (0 = normal to 3 = severe injury).

STATISTICAL ANALYSIS

The mean ± SEM (standard error of the mean) is used to report all continuous data. One-way analysis of variance (ANOVA) was used to determine inter-group differences and Duncan's Multiple Range Test (DMRT) was conducted for post-hoc pairwise comparisons, with statistical significance set at $p < 0.05$. Levene's test was used to verify homogeneity of variance. IBM SPSS Statistics Version 26.0 (IBM Corp., Armonk, NY, USA) was used for all analyses.

RESULTS

QUALITATIVE PHYTOCHEMICAL SCREENING OF Erewspreliminary qualitative phytochemical analysis confirmed the presence of eight major secondary metabolite classes in the ethanolic root extract of W. somnifera (Table 1). All screened phytochemical classes were detected, consistent with the established pharmacognostic profile of Ashwagandha root preparations and corroborating the extract's broad bioactive potential.

Table 1. Qualitative Phytochemical Profile of EREWS

Phytochemicals	Detection Status
Alkaloids	**Present**
Flavonoids	**Present**
Tannins	**Present**
Cardiac Glycosides	**Present**
Carbohydrates	**Present**
Saponins	**Present**
Vitamins	**Present**
Individual Phenolic Compounds	**Present**

EREWS = Ethanolic Root Extract of Withania somnifera; + = Detected.

Study of Behavioral Parameters

Different behavioral parameters were studied during the whole experiment in all treated groups; detail is given below in Table 2.

Table 2: Behavioral changes observed during the experiment

Behavioral Parameter	A (Control)	B (PU 300)	C (PU + EREWS 75)	D (PU + EREWS 150)
Locomotor Activity	+	−	±	+
Grooming Behavior	+	−	±	+
Feeding Behavior	+	−	±	+
Water Intake	+	−	±	+
Response to External Stimuli	+	−	±	+
Aggression/Irritability	+	+	±	+
Anxiety-Like Behavior	+	+	±	+
Tremors/Shaking	+	+	±	−
Convulsions/Seizures	+	+	±	−
Posture	+	−	±	+
Coordination and Balance	+	−	±	+
Respiratory Pattern	+	−	±	+
Social Interaction	+	−	±	+
Mortality/Survival	−	−	−	−

Where as, + = Normal / Present, **−** = Abnormal / Absent / Observed (for negative parameters like mortality = no deaths) and **±** = Partially affected / Partially recovered

HAEMATOLOGICAL PROFILE

Microplastic monotherapy produced statistically significant and clinically meaningful haematotoxicity across all erythrocytic parameters (Table 3; $p < 0.05$). PU exposure (Group B) reduced RBC count to 5.06 ± 0.39 $\times 10^6/\mu L$ compared to 8.15 ± 0.55 in controls (Group A), accompanied by a reduction in PCV from 42.04 ± 0.32% to 37.96 ± 0.33% and an increase in MCV from 40.87 ± 0.65 fL to 47.92 ± 0.32 fL, indicative of a macrocytic compensatory response and erythropoietic stress. Platelet counts declined significantly from 309.36 ± 0.45 to 287.92 ± 0.36 $\times 10^3/\mu L$, suggesting microplastic-mediated thrombopoietic suppression or enhanced

platelet consumption. Concomitantly, WBC counts increased significantly from 11.13 ± 0.68 to 11.72 ± 0.68 $\times 10^3/\mu L$, consistent with a systemic inflammatory leukocytosis.

EREWS co-administration produced dose-dependent haematoprotection. Low-dose EREWS (Group C; 75 mg/kg) partially restored RBC counts (7.04 ± 0.23 $\times 10^6/\mu L$), PCV (40.28 ± 0.54%), and PLT (303.40 ± 0.60 $\times 10^3/\mu L$). High-dose EREWS (Group D; 150 mg/kg) achieved near-complete restoration, with RBC at 7.68 ± 0.47, PCV at 43.02 ± 0.28%, and PLT at 304.18 ± 0.46 $\times 10^3/\mu L$, values statistically comparable to the vehicle control ($p > 0.05$). WBC counts were normalised to 11.40 ± 0.57 in Group D, not significantly different from Group A ($p > 0.05$). The ameliorative efficacy was significantly greater at the 150 mg/kg dose relative to the 75 mg/kg dose ($p < 0.05$), demonstrating a clear dose–response relationship.

Table 3. Haematological Profile of Experimental Rabbits (Mean ± SEM) Across Treatment Groups

Group	RBC ($\times 10^6/\mu L$)	WBC ($\times 10^3/\mu L$)	PLT ($\times 10^3/\mu L$)	Hb (g/dL)	PCV (%)	MCV (fL)
A — Control	8.15 ± 0.55^a	11.13 ± 0.68^d	309.36 ± 0.45^a	11.19 ± 0.68^b	42.04 ± 0.32^b	40.87 ± 0.65^f
B — PU 300 mg/kg	5.06 ± 0.39^e	11.72 ± 0.68^c	287.92 ± 0.36^f	11.13 ± 0.51^b	37.96 ± 0.33^e	47.92 ± 0.32^b
C — PU + EREWS 75	7.04 ± 0.23^c	11.98 ± 0.30^b	303.40 ± 0.60^c	11.92 ± 0.47^a	40.28 ± 0.54^d	45.31 ± 0.51^e
D — PU + EREWS 150	7.68 ± 0.47^b	11.40 ± 0.57^d	304.18 ± 0.46^b	10.85 ± 0.49^b	43.02 ± 0.28^a	46.36 ± 0.47^d

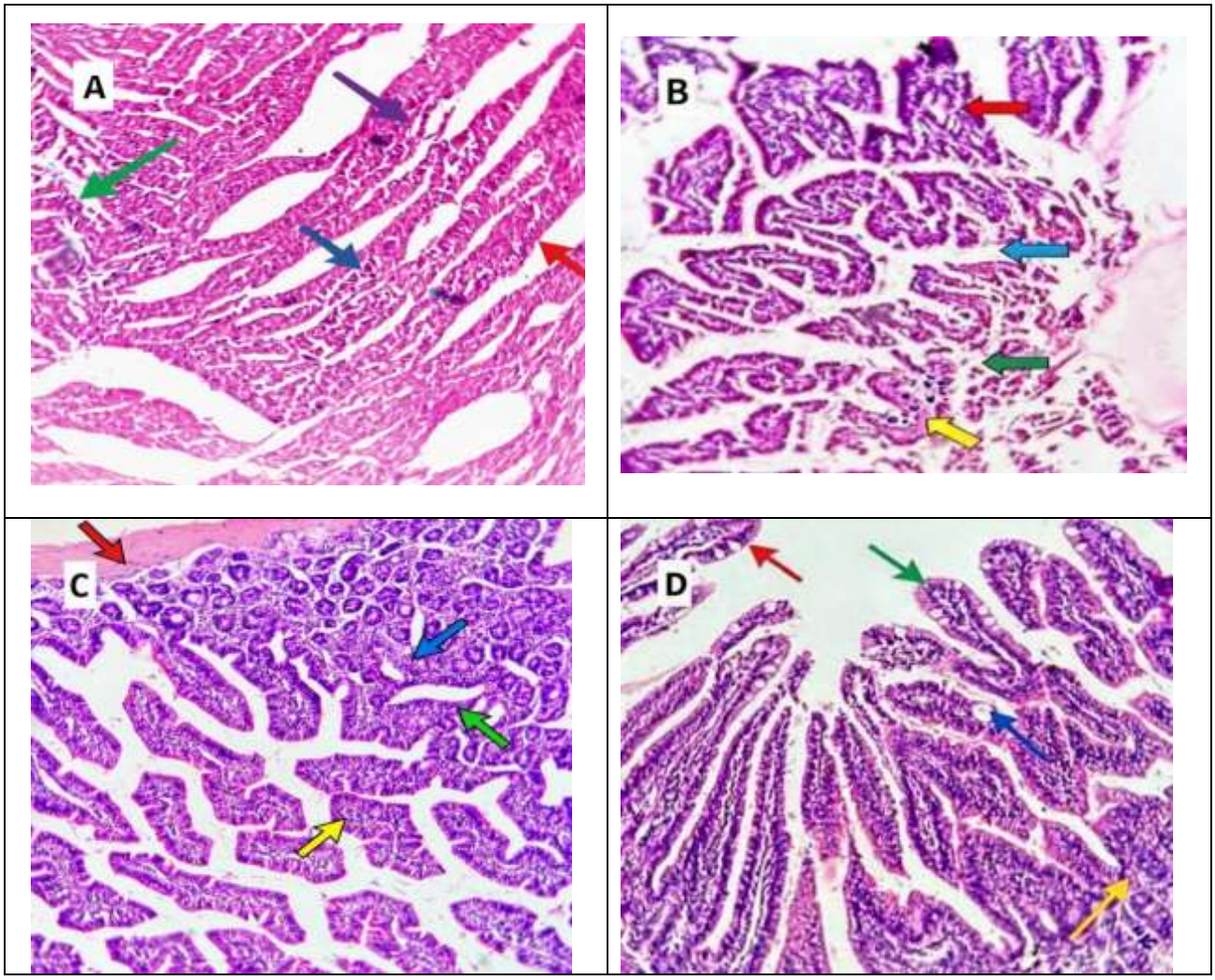
Different superscript letters within each column denote statistically significant differences (ANOVA + DMRT; $p < 0.05$).

INTESTINAL HISTOPATHOLOGICAL FINDINGS

Microscopic examination of intestinal sections revealed pronounced polymer-specific enterotoxicity in MP-exposed groups relative to healthy control architecture (Group A: tall, uniform finger-like villi with intact brush border, abundant goblet cells, organised lamina propria, and well-defined crypts of Lieberkühn). Group B (PU 300 mg/kg) exhibited severe villous blunting and fusion, extensive inflammatory cell infiltration of the lamina propria, multifocal mucosal degeneration, and epithelial lifting. Group C (PU + EREWS 75) co-administered at the low dose displayed moderate protection, with partial recovery of villous height and attenuated inflammatory infiltration.

The intestinal histopathology sections from A to D sequentially in Fig:1, indicate the severe mucosal and structural damage caused by the isolated microplastics and the significant protective and regenerative efficacy of the Withania somnifera ethanolic root extract. Group A (Control) shows healthy, normal intestinal architecture with tall, finger-like villi lined by intact enterocytes (green arrow), well distributed goblet cells (purple arrow), an intact lamina propria core (blue arrow) and structured crypts of Lieberkühn (red arrow). On the other hand, exposure to microplastics alone causes severe mucosal damage and inflammation in the following three groups: Group B (Polyurethane) shows prominent epithelial shedding and sloughing at the villi tips (red and blue arrows), architectural disorganisation (green arrow) and severe submucosal haemorrhage or vascular congestion (orange arrow); Group C (Polyurethane + Extract) has significant recovery of villi height and mucosal architecture (blue and green arrows) and also a well-defined muscularis layer (red arrow) and healthy crypt zones (yellow arrow) Importantly, the co-administration of the extract of Withania somnifera successfully attenuates this microplastic-induced enterotoxicity and promotes cellular repair in the protected groups: Group D (Polyurethane + Extract) has well-preserved, elongated villi projections with a distinct, healthy brush border (green arrow), normally aligned columnar enterocytes (red and blue arrows), and minimal inflammatory presence (yellow arrow); all indicating the robust protective shield of the extract against intestinal tissue injury. Group D (PU + EREWS 150) demonstrated near-complete histological restoration, with

regenerated mucosal epithelium, a stabilised lamina propria matrix, normal crypt architecture, and minimal residual inflammatory infiltrate — findings quantitatively confirmed by the semi-quantitative injury scoring.



DISCUSSION

The present study provides extensive experimental evidence that sub-chronic oral exposure of mammalian model to microplastic of polyurethane and occupationally relevant doses (300 mg/kg B.W./day) causes significant haematotoxicity and gastrointestinal histopathological damage, which could be significantly reduced by co-administration of W. somnifera ethanolic root extract in a dose dependent manner.

The haematotoxic profile is characterized by erythropenia, decreased haemoglobin and PCV, macrocytosis, thrombocytopenia and leukocytosis, and is consistent with oxidative peroxidation of the erythrocyte membrane, decreased iron-utilisation in haem biosynthesis, and bone marrow stromal toxicity by MP. The production of ROS in the Fenton reaction on the MP surface affects membrane phospholipids and sulphydryl groups of spectrin and band-3 proteins, reducing the deformability of erythrocytes and inducing premature haemolysis (Hamed et al., 2019; Espinosa et al., 2019). An increase in MCV in MP-exposed groups is probably a macrocytic erythropoietic compensatory response to peripheral red cell loss, similar to what was previously reported in MP-exposed European sea bass and rodent models (Espinosa et al., 2019; Deng et al., 2017). The data from the differential count suggests that leukocytosis is mostly neutrophilic, reflecting the activation of pattern-

recognition receptor (PRR)-mediated innate immune signalling, perhaps through the activation of the Toll-like receptor (TLR)-4 by lipopolysaccharide (LPS) on the MP surface or polymer degradation products.
The histopathological changes: villous atrophy, epithelial sloughing, lamina propria oedema and crypt degeneration all suggest a breakdown in the intestinal barrier function. The combined effect of physical damage and disruption of the villous epithelium by angular MP particles, ROS-mediated downregulation of tight junction proteins (occludin, claudin-1), and activation of the NLRP3 inflammasome, are responsible for the observed mucosal disruption (Jin et al., 2018; Lu et al., 2018). Villous atrophy decreases absorptive surface area, leading to malabsorption syndromes and crypt destruction decreases the ability of the mucosa to regenerate itself. These results corroborate similar findings in zebrafish (Jin et al., 2018), mice (Lu et al., 2018), and aquatic invertebrates, further enhancing the translational relevance.
Haematoprotective and gastroprotective activity of EREWS is dose-dependent, which is similar to the haematinic and gastro-protective activity of W. somnifera in experimental animals reported earlier (Pandey et al., 2014; Mishra et al., 2000). The superiority of the 150 mg/kg dose over the 75 mg/kg dose may be due to saturation of bioavailability constraints at higher doses leading to higher tissue level antioxidant and anti-inflammatory activity, or a threshold effect on HPA axis modulation and cortisol normalisation (Gupta et al., 2021). Importantly, intestinal histological recovery at the high dose was near-complete, and this may have been achieved through stimulation of intestinal stem cell proliferation in the crypt compartment (Mishra et al., 2000) as well as through passive cytoprotection. The mechanistic hypotheses should be explored in future studies using molecular endpoints such as tight junction protein immunohistochemistry, oxidative stress biomarker panels (malondialdehyde, glutathione) and pro-inflammatory cytokine profiling (IL-1β, TNF-α, IL-6).
The present results have important implications for the development of accessible, low-cost phytotherapeutic strategies to reduce the negative health impacts of MP exposure in populations living in highly polluted areas. W. somnifera is well established as a plant used in South Asia, the Middle East and Africa, commercially available as standardised extracts and with a long safety profile in humans at conventional therapeutic doses (Mikulska et al., 2023). Direct extrapolation of the present findings to human exposure scenarios should be done with caution, because there are differences in exposure routes, particle size distributions, polymer surface chemistries, and species-specific pharmacokinetics.

CONCLUSION

In the current study, chronic oral administration of microplastics (PU and PE) at 300 mg/kg B.W. for 60 days causes significant haematotoxicity (erythropenia, anaemia, thrombocytopenia, and inflammatory leukocytosis) in a rabbit experimental model and severe histopathological damage of the gut (villous atrophy, epithelial sloughing, lamina propria inflammatory infiltration). The haematological and histopathological changes induced by microplastics were successfully and dose-dependently reversed by Ethanolic root extract of Withania somnifera with a higher therapeutic dose of 150mg/kg B.W., thereby bringing the erythrocytic and platelet indices back to physiological levels and maintaining the architecture of the intestinal mucosa.
Overall, these results provide scientific evidence to support the use of W. somnifera root extract as a phytotherapeutic agent with potential for environmental microplastic contamination, offering a scientifically sound and pharmacologically rational approach to address the escalating public health risk posed by environmental microplastic contamination. Using molecular mechanistic strategies such as quantification of oxidative stress biomarkers, expression analysis of tight junction proteins, and characterization of pro-inflammatory cytokines, future studies should determine the exact cellular and molecular mechanisms responsible for EREWS-mediated cytoprotection.

AUTHOR CONTRIBUTIONS

Design: [Tahira Ruby and Sadaqat ali]. Conceptualization, study design, Animal experiments and sample collection. The authors' own analysis of phytochemicals analysis. Histopathological evaluation and scoring: [Kashif ali]. Statistical analysis: [Sadaqat Ali]. Manuscript preparation and original draft writing: [Tahira Ruby and Kashif ali]. Critical revision and final approval: All authors have read and approved the published version of the manuscript.

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CONFLICTS OF INTEREST
The authors have no conflict of interest.

DATA AVAILABILITY
The raw data that support the conclusions of this article are available upon reasonable request from the corresponding author.

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