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RESEARCH ARTICLE

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Hematological Parameters and Splenic Histopathology in Offspring of Wistar Rats Exposed to Prenatal Stress and *Moringa oleifera*

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ABSTRACT

This study investigates the effects of prenatal exposure to Moringa oleifera leaf extract (MoLE) and chronic unpredictable stress (CUS) on the immune development and splenic histopathology of offspring in rat model. Twenty-five pregnant Albino-Wistar rats were divided into five groups: a control group, two MoLE-treated groups (5 mg/kg and 10 mg/kg), and two CUS-exposed MoLE-treated groups (5 mg/kg and 10 mg/kg). MoLE was administered via gavage from gestation day 8 to 21. Hematological parameters and splenic histopathology were assessed in offspring at puberty. Results showed that MoLE exposure affected blood cell counts and splenic histology. Specifically, MoLE treatment resulted in decreased monocyte and granulocyte counts, while lymphocyte levels remained unchanged. The high-dose MoLE group exhibited increased mean platelet volume (MPV) and platelet distribution width (PDW). Severe histopathological changes, including fibrosis and hemorrhage, were observed in the CUS-exposed groups. These findings suggest that prenatal MoLE and CUS exposure influence immune development.

Keywords

Prenatal Stress, Hematotoxicity, Immunity, Blood, Moringa oleifera

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Number of References:: 24
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Abbreviations

AE-FUNAI: Alex Ekwueme Federal University Ndufu-Alike
ANOVA: Analysis of Variance
CUS: Chronic Unpredictable Stress
CV: Coefficient of Variation
EDTA: Ethylenediaminetetraacetic Acid
GC–MS: Gas Chromatography-Mass Spectrometry

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Introduction

Chronic unpredictable stress (CUS) during pregnancy represents a significant public health concern due to its potential to disrupt fetal development, and impacting both physiological and behavioral outcomes in offspring [1]. Prenatal stress, especially in the form of CUS, has been associated with alterations in hematological profiles, such as changes in white blood cell (WBC) counts and platelet function, which could have lasting effects on immune development and blood clotting processes [1-3]. These hematological alterations may increase offspring susceptibility to infections and other health complications [4]. Thus, identifying potential interventions to mitigate these adverse effects is crucial for promoting healthy offspring development.

Moringa oleifera, commonly referred to as the "drumstick tree," is renowned for its health-promoting properties. This drought-resistant tree is cultivated in tropical and subtropical regions and is particularly valued for its leaves, which are rich in essential nutrients, vitamins, minerals, and bioactive compounds, including flavonoids, glucosinolates, and phenolic acids [5, 6]. These compounds are believed to contribute to the plant's antioxidant, anti-inflammatory, and immunomodulatory effects [7].

Although the therapeutic potential of MoLE, has been increasingly documented, its effects during sensitive developmental periods, particularly during pregnancy, remain insufficiently characterized. Furthermore, the combined influence of MoLE and CUS exposure on offspring development, especially regarding hematological parameters and splenic health, has not been fully elucidated. This study aims to bridge this gap by examining the effects of MoLE on hematological markers and splenic histopathology in offspring exposed to prenatal CUS, thus contributing valuable insights into maternal health and offspring development.

Abbreviations-Cont'd

GD: Gestational Day
Gran: Granulocytes
H&E: Hematoxylin and Eosin
HPA: Hypothalamic–Pituitary–Adrenal (Axis)
i.p.: Intraperitoneal
Lym: Lymphocytes
MID: Monocytes/Intermediate Cells
MoLE: Moringa oleifera Leaf Extract
MPV: Mean Platelet Volume
PCT: Plateletcrit
PDW: Platelet Distribution Width
PDW-CV: Platelet Distribution Width Coefficient of Variation
PDW-SD: Platelet Distribution Width Standard Deviation
P-LCR: Platelet Large Cell Ratio
PLT: Platelet Count
SD: Standard Deviation; SEM: Standard Error of the Mean
BC: White Blood Cell.

Results

The majour compounds identified in the GC-MS analysis of MOLE are known for their antioxidant and anti-inflammatory activities[8](Table 1).

White Blood Cell and Differential Counts

Prenatal exposure to Moringa oleifera leaf extract (MoLE) and/or chronic unpredictable stress (CUS) resulted in significant alteration in leukocyte profiles in the offspring (Table 2). Total white blood cell (WBC) counts were significantly elevated in the High dose MoLE group ($17.42 \pm 1.78 \times 10^9$/L) and the CUS + High dose MoLE group ($15.32 \pm 3.39 \times 10^9$/L) when compared with the Control group ($5.40 \pm 0.22 \times 10^9$/L, $p < 0.05$). Lymphocyte counts exhibited a similar pattern, with significant increases observed in the High-dose MoLE and CUS + High dose MoLE groups ($p < 0.05$).

Granulocyte counts were significantly higher in the High dose MoLE ($3.45 \pm 0.15 \times 10^9$/L) and the CUS + High dose MoLE groups ($2.83 \pm 0.72 \times 10^9$/L) compared to Control ($0.46 \pm 0.15 \times 10^9$/L, $p < 0.05$). In contrast, no significant changes were observed in monocyte counts across groups ($p > 0.05$). These findings suggest a stress induced inflammatory response that was partially modulated by MoLE supplementation in a dose dependent manner.

Platelet Counts and Indices

Platelet profiles also significantly affected by prenatal treatments (Table 2). Offspring from High dose MoLE and both CUS exposed groups exhibited a sharp reduction in platelet counts compared to Control, showing a decrease of over 70% ($p < 0.05$). In contrast, CUS + Low-dose MoLE group exhibited a significant increase in platelet count ($1445.0 \pm 101.9 \times 10^9$/L, $p < 0.05$) compared to Control.

Analysis of platelet indices revealed that mean platelet volume (MPV) and platelet distribution width (PDW-SD and PDW-CV) were significantly elevated only in the High-dose MoLE group ($p < 0.05$). No significant differences in MPV or PDW values were observed in the CUS + Low-dose MoLE group compared with Control, indicating preservation of platelet morphology under low-dose supplementation.

Histopathological Findings

Representative photomicrographs of spleen sections are shown in Figure 1 (Panels A–E). Spleens from Control animals exhibited normal histological architecture, characterized by well-demarcated white pulp and red pulp regions (Figure 1A). Low-dose

Table 1.
GC-MS analysis of MOLE; An exempt from "GC–MS analysis of *Moringa oleifera* leaf extract and effects of administration on histology of reproductive organs and liver of female rats exposed to chronic unpredictable stress" by Chukwu et al., 2024 [10]

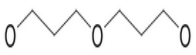
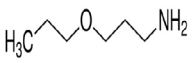
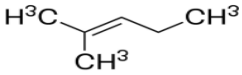
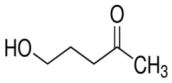
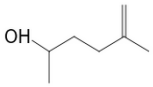
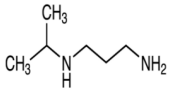
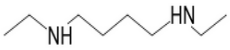
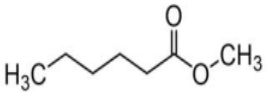
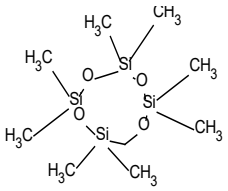
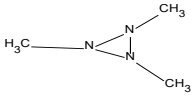
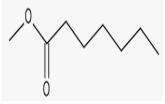
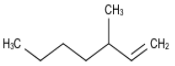
S / No	Name of Compound	Mol. formular	Mol. Wt(g)	RT (min)	% TIC	Structure	Activity
1	1-Propanol, 3,3'-oxybis-	$C_6H_{14}O_3$	134.	3.045	1.521		Humectants (Food additive/ Moisturizer)
2	1-Propanamine, 3-propoxy-	$C_5H_{12}NO$	117	3.327	0.803		Textile resins, Drugs, Pesticides
3	2-Pentene, 2-methyl-	C_6H_{12}	84	3.778	0.593		Photochemical and ozonolysis studies
4	Pyridine	C_5H_5N	79	4.285	4.763		Drugs, Vitamins, Food flavorings, Pesticides,
5	2-Pentanone, 5-hydroxy-	$C_5H_{10}O_2$	102	4.820	0.630		Anti-malarial drugs, Vitamin B1
6	5-Hexen-2-ol, 5-methyl-	$C_7H_{14}O$	114	5.102	0.887		Natural substances and Extractives
7	1,3-Propanediamine, N-(1-methylethyl)-	$C_6H_{16}N_2$	116	5.440	0.546		Useful research chemical compound
8	1,4-Butanediamine, N,N'-diethyl-	$C_8H_{20}N_2$	144	5.553	0.183		Unidentified
9	Hexanoic acid, methyl ester	$C_7H_{14}O_2$	130	5.722	0.843		Flavouring agents
10	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	296	6.031	0.468		Pharmaceuticals, Polymers, Hair/Skin care products, Antiperspirants and Deodorants, Lubricants, Sealants, Adhesives, Waxes and Coating.
11	1,2,3-Trimethyldiaziridine	$C_4H_{10}N_2$	86	6.285	0.515		Unidentified
12	2-Hexyn-1-ol	$C_6H_{10}O$	98	6.426	0.212		Flavour and fragrance
13	Heptanoic acid, methyl ester	$C_8H_{16}O_2$	144	6.595	1.204		Human Metabolite, Flavouring agents, Fragrance,
14	1-Heptene, 3-methyl-	C_8H_{16}	112	6.905	0.608		Hydrocarbon
15	1-Fluorononane	$C_9H_{19}F$	146	7.158	0.459		Unidentified

Table 1 Cont'd

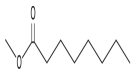
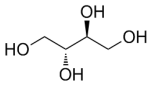
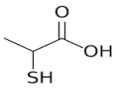
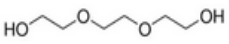
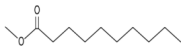
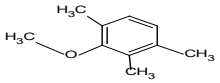
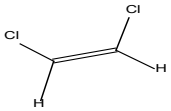
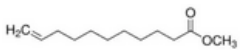
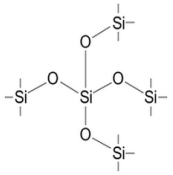
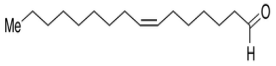
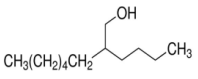
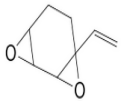
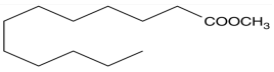
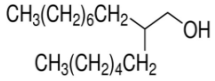
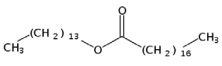
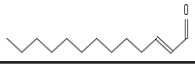
S / No	Name of Compound	Mol. formular	Mol. Wt(g)	RT (min)	% TIC	Structure	Activity
16	Octanoic acid, methyl ester	$C_9H_{18}O_2$	158	7.440	2.299		Metabolite
17	Erythritol	$C_4H_{10}O_4$	122	7.834	6.842		Food additive and Sugar substitutes
18	2-Mercaptopropanoic acid	$C_3H_6O_2S$	106	8.313	2.240		Flavour and Fragrance agents
19	Triethylene glycol	$C_6H_{14}O_4$	150	9.243	1.079		Pesticides, Fragrance, Humectant, Disinfectant, Plasticizer for vinyl polymers
20	Decanoic acid, methyl ester	$C_{11}H_{22}O_2$	186	9.440	1.750		Biodiesel surrogate
21	Benzene,2-methoxy-1,3,4-trimethyl	$C_{10}H_{14}O$	150	10.849	2.298		unidentified
22	Ethylene, 1,2-dichloro-, (Z)-	$C_2H_2Cl_2$	97	10.894	0.200		Pharmacology, Refrigerant, Degreaser, Adhesives, Lacquers, oils, and Resins
23	10-Undecenoic acid, methyl ester	$C_{12}H_{22}O_2$	198	11.102	0.275		Flavouring agents
24	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl) oxy]-	$C_{12}H_{36}O_4Si_5$	385	11.581	0.360		Paints, Coatings, and Cosmetics, including some Personal care products
25	7-Hexadecenal, (Z)-	$C_{16}H_{30}O$	238	12.539	0.016		Derivative of essential oils with potential antibacterial activities.
26	1-Octanol, 2-butyl-	$C_{12}H_{26}O$	186	12.623	0.015		Human metabolite, Humectant
27	3,8-Dioxatricyclo[5.1.0.0(2,4)] octane, 4-ethenyl-	$C_8H_{10}O_2$	138	12.905	0.003		Undefined
28	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	214	13.356	6.008		Therapeutic uses, Flavouring agents
29	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	242	14.342	0.497		Fungicidal properties, Inhibitor (Candida glabrata), suggesting useful for treating Skin cancer
30	Myristyl stearate	$C_{32}H_{64}O_2$	481	14.623	0.181		Skin conditioning and Deodorant
31	2-Tridecenal, (E)-	$C_{13}H_{24}O$	196	14.877	0.219		Flavour and Fragrance agents

Table 1 Cont'd

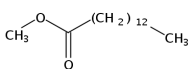
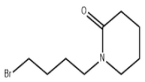
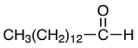
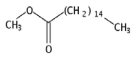
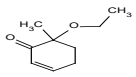
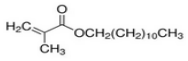
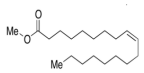
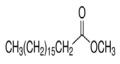
S / No	Name of Compound	Mol. formular	Mol. Wt(g)	RT (min)	% TIC	Structure	Activity
32	Cyclotetradecane	$C_{14}H_{28}$	196	15.468	1.427		Plant metabolite and a human metabolite
33	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	15.891	4.899		Plant metabolite, Flavouring agent and a Fragrance.
34	2-Piperidinone, N-[4-bromo-n-butyl]-	$C_9H_{16}BrNO$	234	16.426	1.766		Antimicrobial
35	Tetradecanal	$C_{14}H_{28}O$	212	16.736	1.853		Human metabolite, Flavouring agent and Fragrance
36	Ethanol, 2-(octadecyloxy)-	$C_{20}H_{42}O_2$	314	16.877	1.420		Surfactant
37	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	17.384	26.182		Metabolite
38	6-Ethoxy-6-methyl-2-cyclohexenone	$C_9H_{14}O_2$	154	17.778	3.234		Flavouring agent
39	n-Dodecyl methacrylate	$C_{16}H_{30}O_2$	254	18.004	3.814		Drug (Clinical trials), Metabolites, Fragrance, Pesticides etc.
40	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	18.567	4.770		Flavouring agents and Fragrance
41	Methyl stearate	$C_{19}H_{38}O_2$	298	18.736	12.113		Metabolites and Flavouring agents

Table 2.
Hematological Parameters (White Blood Cell, Differential Count, and Platelet Indices) of Offspring of Dams Exposed to CUS and MoLE

Variable (Units)	Control	Low-dose MoLE	High-dose MoLE	CUS + Low-dose MoLE	CUS + High-dose MoLE
Total & Dif-ferential WBC					
WBC ($\times 10^9$/L)	5.40 ± 0.22^a	10.87 ± 1.64^b	17.42 ± 1.78^c	7.32 ± 2.15^{ab}	15.32 ± 3.39^c
Lymphocytes ($\times 10^9$/L)	4.43 ± 0.07^a	7.93 ± 0.77^b	13.03 ± 1.54^c	6.90 ± 2.07^{ab}	11.86 ± 2.49^c
Monocytes ($\times 10^9$/L)	0.51 ± 0.10^a	0.54 ± 0.19^a	0.95 ± 0.11^{ab}	0.30 ± 0.06^a	0.63 ± 0.18^{ab}
Granulocytes ($\times 10^9$/L)	0.46 ± 0.15^a	2.40 ± 0.71^b	3.45 ± 0.15^c	0.12 ± 0.03^a	2.83 ± 0.72^c
Platelet Indices					
PLT ($\times 10^9$/L)	1087.3 ± 115.9^a	355.7 ± 51.0^b	312.3 ± 40.1^b	1445.0 ± 101.9^c	316.0 ± 17.2^b
MPV (fL)	7.07 ± 0.03^a	7.27 ± 0.12^{ab}	7.77 ± 0.23^c	6.83 ± 0.09^a	7.60 ± 0.15^{bc}
PDW-CV (%)	13.97 ± 0.13^a	14.00 ± 0.25^a	15.07 ± 0.12^b	13.83 ± 0.09^a	14.10 ± 0.17^{ab}
PDW-SD (fL)	9.77 ± 0.19^a	10.53 ± 0.45^{ab}	12.30 ± 0.44^c	9.43 ± 0.03^a	11.50 ± 0.42^{bc}
PCT (mL/L)	7.71 ± 0.86^a	2.59 ± 0.35^b	2.44 ± 0.37^b	5.59 ± 2.80^{ab}	2.41 ± 0.13^b
P-LCR (%)	14.60 ± 0.26^a	15.33 ± 0.94^a	18.77 ± 1.27^b	12.60 ± 0.65^a	16.77 ± 0.98^b

Values are expressed as Mean ± SEM. Within each row, means that do not share the same superscript letter differ significantly ($p < 0.05$). Superscripts with the same letter indicate no statistically significant difference between the corresponding groups.

MoLE-treated offspring displayed mild splenic tissue degeneration accompanied by moderate inflammatory infiltration and focal ground-glass changes (Figure 1B). In contrast, High-dose MoLE offspring showed mild to moderate fibrotic changes with occasional hemorrhage (Figure 1C). The most severe histopathological changes were observed in the CUS + Low-dose MoLE group, characterized by marked tissue degeneration, extensive fibrosis, and hemorrhagic red pulp (Figure 1D). The CUS + High-dose MoLE group exhibited moderate tissue degeneration with focal fibrosis but less extensive damage compared to the CUS + Low-dose MoLE group (Figure 1E), suggesting a partial protective effect of higher MoLE supplementation under stress exposure. Semi-quantitative lesion scoring (Table 3), confirmed these histological observations: fibrosis and hemorrhage were significantly more severe in the CUS + Low-dose MoLE group compared to Control ($p < 0.05$). In contrast, co-treatment with High-dose MoLE attenuated lesion severity under CUS conditions ($p < 0.05$).

Figure 1. Representative photomicrographs of spleen sections (H&E staining) showing:

(B) Low-dose MoLE: Mild degeneration with inflammatory infiltration;

(C) High-dose MoLE: Mild fibrosis and hemorrhage;

(D) CUS + Low-dose MoLE: Severe degeneration, fibrosis, and hemorrhagic red pulp;

(E) CUS + High-dose MoLE: Moderate degeneration with focal fibrosis.

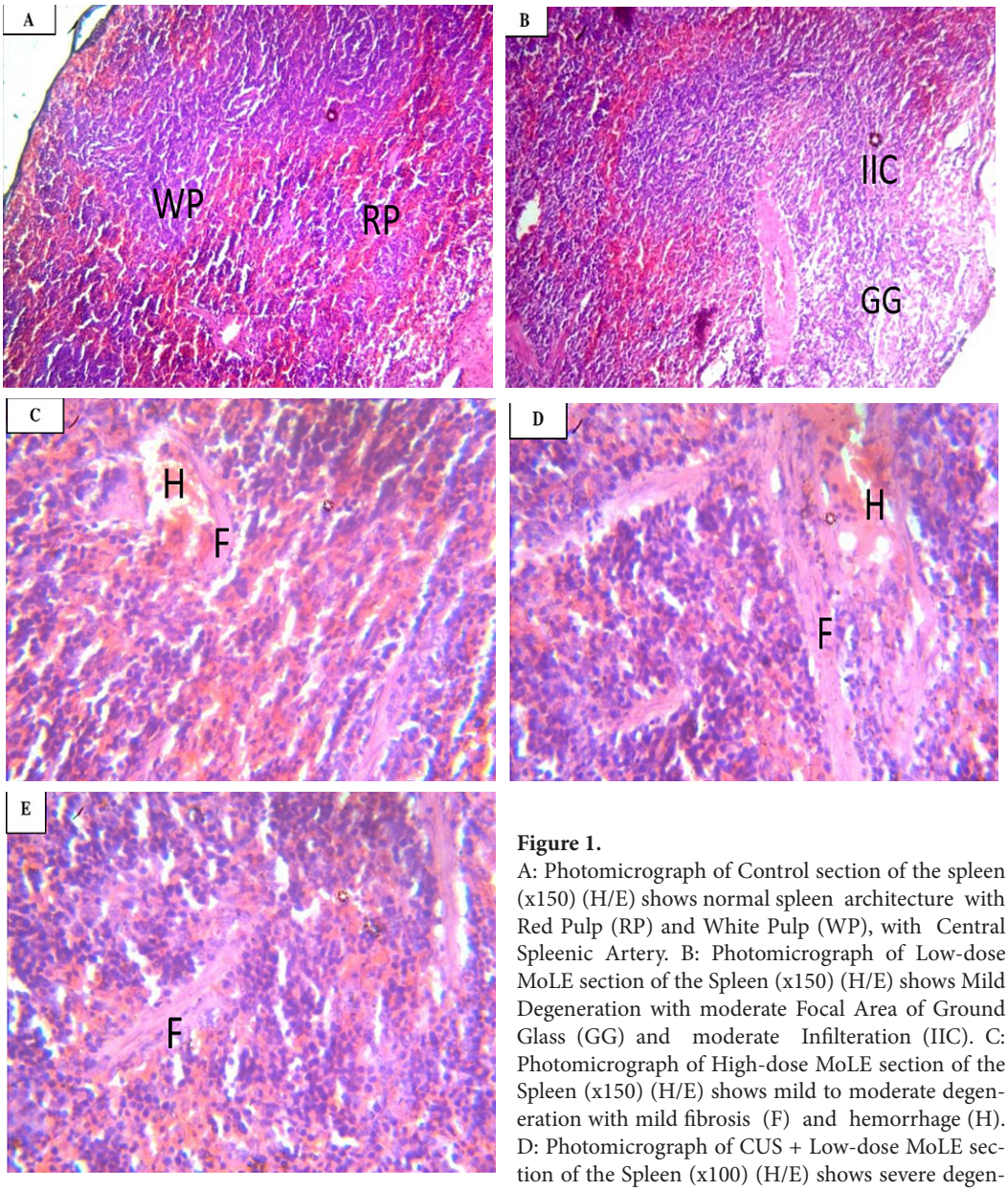


Figure 1.
A: Photomicrograph of Control section of the spleen (x150) (H/E) shows normal spleen architecture with Red Pulp (RP) and White Pulp (WP), with Central Spleenic Artery. B: Photomicrograph of Low-dose MoLE section of the Spleen (x150) (H/E) shows Mild Degeneration with moderate Focal Area of Ground Glass (GG) and moderate Infilteration (IIC). C: Photomicrograph of High-dose MoLE section of the Spleen (x150) (H/E) shows mild to moderate degeneration with mild fibrosis (F) and hemorrhage (H). D: Photomicrograph of CUS + Low-dose MoLE section of the Spleen (x100) (H/E) shows severe degeneration of the Spleenic Tissue with severe Fibrosis (F) and Hemorrhagic (H) Red Pulp. E: Photomicrograph of CUS + High-dose MoLE section of the Spleen (x100) (H/E) shows moderate Degeneration with mild Focal Area of Fibrosis (F).

Table 3.
Semi-quantitative Scoring of Splenic Lesions in Offspring

Group	Fibrosis (0–3)	Hemorrhage (0–3)	Inflammatory Infiltrate (0–3)	Ground Glass Areas (0–3)	Total Lesion Score (0-12)
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[a]
Low-dose MoLE	1.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	5.00 ± 0.00[b]
High-dose MoLE	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.00[b]
CUS + Low-dose MoLE	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.00	0.00 ± 0.00	8.00 ± 0.00[c]
CUS + High-dose MoLE	2.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.00[b]

Values are mean ± SEM (n = 3 animals per group). 0 = absent, 1 = mild, 2 = moderate, 3 = severe. Superscripts within the Total Lesion Score column indicate statistically distinct groups ($p < 0.05$). Groups sharing the same superscript are not significantly different from each other. [a]Control, [b]Low-dose MoLE, High-dose MoLE, and CUS + High-dose MoLE (not significantly different from one another), [c]CUS + Low-dose MoLE (significantly higher than all other groups)

Materials & Methods

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During manuscript preparation, a generative AI tool (ChatGPT, OpenAI) was used only as a supportive language aid to improve sentence structure, grammar, and clarity in selected sections of the manuscript. The tool was not used to generate scientific hypotheses, experimental design, data, statistical analyses, figures, or interpretations. All content was critically reviewed, edited, and finalized by the authors, who take full responsibility for the originality, accuracy, and integrity of the work.

Ethical Approval

All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Faculty of Basic Medical Sciences Research Ethics Committee, Alex Ekwueme Federal University Ndufu-Alike, Ebonyi State, Nigeria (Approval Code: FBMS/EC/AE/1983).

Plant Collection, Identification, and Extraction

Fresh Moringa oleifera leaves were collected in the early morning from a cultivated garden in Abakaliki, Ebonyi State. Botanical authentication was confirmed at the Herbarium Unit, Department of Biology, Alex Ekwueme Federal University Ndufu-Alike (AE-FUNAI). Leaves were thoroughly washed, air-dried at room temperature for seven days, and pulverized into a coarse powder using an electric blender (Model MS-233, China). Extraction was performed using methanol according to standardized procedures [5, 6]. The filtrate was concentrated at 40°C under reduced pressure to yield a dark-green paste, which was stored at 4°C until use.

Phytochemical characterization of the extract was performed using gas chromatography–mass spectrometry (GC–MS) as previously described by Chukwu et al. [8]. Analysis was performed using an Agilent GC–MS system equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm). The oven temperature was programmed from 60°C (2 min hold) to 280°C at 10°C/min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Major compounds identified are known for antioxidant and anti-inflammatory activity [8] (Table 1).

Experimental Animals and Housing Conditions

Twenty-five mature, nulliparous, virgin female Albino-Wistar rats (weighing 150–180 g) were obtained from the Animal House, AE-FUNAI. Animals were housed in well-ventilated polypropylene cages under standard laboratory conditions, including 12h light/dark cycle, temperature of 23±2°C, and humidity of 50-60%. Rats had free access to standard rat chow (Vital feed®, Nigeria) and tap water. Animals were acclimatized for two weeks before mating.

Estrous Cycle Monitoring and Mating

Estrous cycle monitoring was performed by vaginal cytology using light microscopy following established protocols [9]. Females with two consecutive regular four-day cycles were considered for mating. During the proestrus phase, identified by the predominance of nucleated epithelial cells and absence of cornified cells, females were co-housed overnight with proven male breeders (1:2 ratio). The presence of spermatozoa in morning smears confirmed successful mating and was designated as gestational day (GD) 1 [10].

Experimental Design and MoLE Administration

Pregnant rats were randomly assigned (n=5 per group) to the following treatment groups:

- Control: Standard diet and water ad libitum.
- Low-dose MoLE: MoLE 5 mg/kg/day via oral gavage from GD 8–21.
- High-dose MoLE: MoLE 10 mg/kg/day via oral gavage from GD 8–21.
- CUS + Low-dose MoLE: CUS exposure plus 5 mg/kg/day MoLE from GD 8–21.
- CUS + High-dose MoLE: CUS exposure plus 10 mg/kg/day MoLE from GD 8–21.

Doses were freshly prepared in distilled water daily and administered in volumes not exceeding 1 mL/100 g body weight to avoid gastric discomfort [8]. The gestational window GD 8-21 was selected to reflect the late second to third trimester in human pregnancy, a critical period for immune and hematopoietic development [11, 12].

Chronic Unpredictable Stress (CUS) Protocol

Animals in CUS groups were subjected to a validated CUS protocol [13] consisting of the following randomly applied stressors:

- Wet bedding (300 mL water mixed with sawdust).
- Cage tilting at 45° for 6 hours.
- Overnight food deprivation.
- Psychological stress by exposure to a caged cat.
- Sleep deprivation using a pedestal in shallow water.
- Restraint stress in 50 mL plastic tubes for 2-hour intervals.
- Continuous overnight light exposure.
- Social isolation for 6-12 hours.

Stressors were rotated daily in an unpredictable order from GD 8–21 to mimic human psychosocial stress [13. 14].

Sample Collection and Litter Effect Consideration

At the onset of puberty (postnatal day 21 onward), five offspring per treatment group were sampled for hematological analysis, with at least one pup selected from each dam (total 5 dams per group). To minimize litter effect bias and maintain balanced sex representation, pups were randomly selected across litters, ensuring that no more than one male and one female pup per dam contributed to the dataset. This approach ensured equal representation of each dam while avoiding over-representation of littermates.

Although individual pup measurements were recorded, the dam (litter) was treated as the experimental unit (n = 5 dams per group) for all statistical analyses, in accordance with internationally accepted developmental toxicology guidelines [12, 16], thereby preventing pseudo-replication. Approximately 2 mL of blood was collected via retro-orbital venous puncture under light isoflurane anesthesia into EDTA-coated tubes and processed within 3 hours at 4°C to preserve cell morphology and ensure accurate hematological profiling.

Hematological Analysis

A calibrated automated hematology analyzer (Mindray BC-2800, Shenzhen, China) was used to determine:

- Total WBC counts ($\times 10^9$/L).
- Differential counts: Lymphocytes (Lym), monocytes (MID), and granulocytes (Gran), reported as absolute counts and percentages.
- Platelet parameters: Platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW-CV, PDW-SD), plateletcrit (PCT), and platelet-large cell ratio (P-LCR) [16, 17].

Histological Studies

Following blood collection, animals were humanely euthanized using sodium pentobarbital (150 mg/kg, i.p.). Spleens were excised, trimmed of fat, and fixed in 10% neutral-buffered formalin for 24-48 hours. Standard tissue processing, paraffin embedding, and hematoxylin-eosin (H&E) staining were performed [18]. Sections were examined under a light microscope (×100–150 magnification). Histopathological changes, including fibrosis, hemorrhage, inflammatory infiltration, ground-glass appearance; were semi-quantitatively graded on a scale of 0-3 by two independent blinded observers. Any scoring discrepancies were resolved by consensus [19].

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). The dam was considered as the experimental unit (n = 5 per group), to avoid pseudo-replication. When more than one pup per dam was sampled, values were averaged to obtain a single representative data point per dam. Hematological parameters were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Non-parametric data from semi-quantitative splenic lesion scoring were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparisons. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism (Version 9.0, GraphPad Software, San Diego, CA, USA).

Authors' Contributions

O.O.C., A.C.U.E, conceived, planned and carried out the experiments, O.O.C., S.N.I.and N.G.K carried out data collection and analysis. O.O.C. wrote the first draft of the manuscript, and all authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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