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Prenatal Neem Leaves Extract Alters Immunological Functions of Male Wistar Albino Rats Offsprings

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Abstract | The neem plant, Azadirachta indica, is one of Asia's and Africa's most important medicinal plants. This research intended to explore the immunological potential of dietary neem leaves extract prenatal exposure on male Wistar albino rats' offspring. In this experiment, 18 adult female and 6 male Wistar rats were used. All experimental animals were kept in the same environment which was suitable, away from stresses and of adequate hygiene was provided. They were alienated into two groups. Control and treated with neem leaves extract groups; they were allowed to mate. Neem extract was administered from gestation day 1 (GD1) till full term. The weight of birth was recorded. At postnatal day 40, the body weight was recorded, hematological analysis, peripheral lymphocyte transformation, lymphocytes' comet assay, splenic immunohistochemistry for interleukin-6 (IL-6), nuclear factor kappa B (NFKB) and tumor necrosis factor-alpha (TNF-α) and histopathology for spleen and thymus were performed. Oral administration of neem extract to pregnant female rats caused significant ($P<0.05$) declines in birth and final body weights of male offspring and their thymus absolute weight ($P<0.05$). It also caused significant neutrophil (%) promotion ($P<0.05$) and lymphocyte (%) reduction ($P<0.05$), reduced serum TAC levels statistically ($P<0.05$), and affected DNA and lymphoid tissues evidenced by increased ($P<0.05$) immunohistochemical expression of IL-6, NFKB and TNF-α in addition to induced spleen and thymus histopathological alterations. The study concluded that methanolic neem leaves extract has a cytotoxic effect that affects the histo-architecture of lymphoid organs, promoted inflammatory markers and the mononuclear cells' DNA damage that seemed to be as a result of neem extract oxidative stress. This effect could be implemented on both human and animals with future prospective studies suggestion to estimate the dose dependent effect of prenatal neem extract exposure.

Keywords: Hematology, Immunity, Neam, Prenatal, Lymphoid depletion

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INTRODUCTION

Pregnancsy is a sensitive time for the embryo and fetus development; exposure to certain compounds throughout their stages can lead to immunomodulation (Hertz-Picciotto *et al*., 2008), which includes immunodeficiency and increased susceptibility to both viral and chronic diseases. Alternatively, the immune system may become more immunoreacted, which might result in autoimmune diseases, hypsersensitivity, inflammation, or allergies and the development of tumor cells. In addition, these compounds may cause natural hormone elimination, which is important for regulating homeostasis in the development processes (Hertz-Picciotto *et al*., 2008).

Herbal treatments made from medicinal plants are utilized traditionally in many parts of the world (Brahmachari, 2004), according to estimates from the World Health Organization (WHO), up to 80% of individuals worldwide obtain their prime medical care from traditional herbal remedies (Eid *et al*., 2017). *Azadirachta indica* (neem) is one of the utmost adaptable medicinal plants with a wide range of biological activity. Neem trees possess a diverse spectrum of pharmacological activities, including antifungal, antibacterial, antiulcer, repellent, antifeedant, inhibitor, pesticide and sterilant effects in all plant parts (Eid *et al*., 2017). Besides treating a range of conditions, including jaundice, ulcers in the stomach, and parasitic infections like malaria, chicken pox, and leprosy. In addition to being used as a diuretic and for diabetes, infusions and teas made from leaves have been used to treat various skin conditions, intestinal complaints, dental issues, headaches, stimulating appetite, and heartburn. It is common knowledge that head lice can be treated using aqueous seed extracts. Neem oil has strong antibacterial properties; India has long employed neem-based products derived from *Azadirachta indica* for pest-control in gardening and agriculture (Eid *et al*., 2017). Neem leaf have immuno-stimulatory activity for instance, Beuth *et al*. (2006) reported that neem leaves increased the weight of the spleen and enhanced the peritoneal macrophage activity, beside activated marker CD-44 expression Beuth *et al*. (2006).

Neem plant was reported to have a wide range of safety margin where Raizada *et al*. (2001) demonstrated that no acute toxicity symptoms for *Azadirachta* were observed in male and female rats. They added that 500, 1000 and 1500 mg/kg/day *Azadirachta* for successive 90 days to both male and female rats did not produce any mortality, signs of toxicity, changes in tissue weight, serum parameters, hematology and pathology except aggressiveness symptoms in rats, which was time and dose dependent. Deng *et al*. (2013) showed that neem oil administration to mice at dose the dose of 1600 mg/kg/day for 4 weeks produced retrogressive changes in testes, liver and kidney histoarchitecture with reduction of food consumption at 3rd and 4th weeks of treatment. They added that acute exposure to high dose 45 mg/ kg induced slow movement, hypesthesia and convulsions with gut swelling and fluid accumulation at necropsy. Gbotolorun *et al*. (2004) observed that diarrhea was the common side effect in female Sprague Dawley rats administered neem seeds methanolic extract. They reported no deaths and no teratogenic effect However, Partial ovulation blockage and estrous pattern perturbations were noted. Mbaya *et al*. (2010) had found anorexia, malaise, dehydration, respiratory depression, coma and death in male and female Wistar rats given i.p. 100, 200, 400, 800, 1600 and 3200 mg/kg of *A. indica* stem bark ethanolic extract for 24 h. They found also histopathological changes in the trachea, lungs, bronchioles, bronchi, and kidney. They observed that the later effects were dose dependent. Another study conducted by Ashafa *et al*. (2012) revealed that neem ethanolic extract at 50, 100, 200 and 300 mg/kg resulted in weight of the kidney, liver, lungs and heart of male Wistar rats besides significant reduction in leukocytes count.

Most herbal treatment can be used without prescription and without specific dose, more studies are required to detect the influences of this herbal treatment on human health and especially during pregnancy to detect the effect of the exposure of this treatment on the immune system of the offspring. Therefore, this study aimed to explore the maternal exposure of neem leaf extracts in diet on some immunological parameters of rats' offspring via estimation of the hematological analysis, peripheral lymphocyte transformation, lymphocytes' comet assay, splenic immunohistochemistry for interleukin-6 (IL-6), nuclear factor kappa B (NFKB), and tumor necrosis factor-alpha (TNF-α) and histopathology for spleen and thymus.

MATERIALS AND METHODS

Plant extract

According to Mamoon-ur-Rashid *et al*. (2011), the leaves of the *Azadirachta indica* tree were dried, then ground and homogenized with distilled water in an electric blender. Triple-folded gauze was used to filtrate the homogenate. Before use, a rotary vacuum evaporator was used to evaporate the solvent, and 70% dilation of the extracts was prepared.

Experimental animals

This study cast off 18 mature regular cyclic female rats (5.5–6 months; 181–200g) and 6 males (6-6.5 months; 190-218g) of the Wistar strain. The rats were acquired from Laboratory Animal House, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. They were kept in polyethylene cages, 3 animals/ cage at room temperature

(24°C ± 1°C) and natural daylight cycles. Water and feed were given ad libitum. Suitable environment away from noises, cold, or any overstresses and adequate hygiene were provided for the rats during the experiment. Rats were kept for 1 week to adapt. The experimental procedures were adhered to the ethical rules for the utilization of animals in laboratory settings at the Faculty of Veterinary Medicine, Suez Canal University, Egypt (SCU-VET- REC 2024041).

Design of the experiment

Eighteen pregnant females were split into two groups; the control group (n = 9) that given a basal diet misted with 150 mL distilled water/kg diet, and the neem extract group (n = 9) that fed a basal diet misted with 10 mL neem leaves extract mixed with 140 mL distilled water with a dose 10 mL neem leaves extract /kg diet as mentioned by Al-Awadhi *et al*. (2024). A total of 200 g diet was offered / cage. The experimental diet was offered daily, starting from GD1 till birth. The sample size was calculated according to Charan and Kantharia (2013).

Sampling

At the end of 40 days after birth and weaning, the male offspring were weighed and under tetrahydrofuran inhalation blood was drawn from retro-orbital venous vessels and put in ethylenediaminetetraacetic acid, lithium heparin, and plain tubes. The sere were separated by allowing the plain tubes to clot and then centrifugated at 3000 rpm. Sera were kept at -70 °C. The thymus and spleen were dissected.

Feed intake and weight gain of dams and relative lymphoid organ weights

The feed intake for each pregnant female and maternal weight gain were recorded all over the gestation period. The remaining food was subtracted from the offered food, and the obtained value was divided by the number of rats per cage. Body weights of male pups were recorded at postnatal day 1 (PND1) and at PND40. The dissected thymus and spleen were weighed, and their relative weights were obtained as follows (organ weight/body weight X100).

Hematology

Blood specimens in EDTA were imperiled to manual routine hematological examination of red and white cell count (RBCs and WBCs), hemoglobin (Doudi and Setorki), hematocrit, blood indices, and differential white cell count according to Washington and Van Hoosier (2012).

Total antioxidant capacity (TAC)

The serum TAC of both groups was assessed by means of a calorimetric kit (Biodiagnostic, Egypt). All analysis steps were performed following the manufacturer's protocol.

Lymphocyte transformation assay

Blood samples collected in lithium heparinized tubes were promptly placed on cold packs and immediately transported to the laboratory for lymphocyte transformation assay. In summary, the isolated buffy coat was rinsed with RPMI-1640 medium (Cat. No. R8758, Sigma-Aldrich, Egypt), and the resulting sedimented lymphocytes were suspended in 1 ml of RPMI-1640 medium enriched fetal calf serum (FCS) 10% (Cat. No. F2442, Sigma-Aldrich, Egypt) following the protocols of Boyum (1968) and Burrells and Well (1977). The quantification of viable lymphocytes per milliliter of RPMI medium was assessed using the method mentioned by Hudson and Hay (1980). The lymphocytes transformation assay was conducted using MTT staining techniques, as mentioned by Abdelrazek *et al*. (2019). All samples were run in triplicate, and the average of the three measurements were obtained. Negative control wells were established and contained RPMI-1640 medium + 10% FCS only.

Comet assay

The steps of the test were done according to that mentioned by Abdelrazek *et al*. (2015). All the steps are done dimly to diminish DNA damage artifacts. A positive control of H_2O_2 treated cells to give standard DNA breaks was implemented and examined as well as negative control of untreated cells. Analysis was done at a 100× magnification power by using a light microscope (Olympus, China). A minimum of 100 cells were scanned and examined per slide.

Histopathology

The thymus and spleen were dissected and put in 10% formalin solution for 24 hours. Afterward, after treatment, they were immersed in paraffin wax with a series of steps, including dehydration, clearing with xylene, infiltration, and embedding in paraffin wax. After that, they were cut into sections using a microtome. Then, the slices were deparaffinized using xylene, followed by alcohol and water, before being stained with hematoxylin and eosin (H and E), as mentioned by Bancroft and Gamble (2008). The stained sections were mounted in D.P.X. and allowed to dry for image capture and interpretation. Five tissue sections per organ were examined by the same person who was blinded to treatment.

A semiquantitative lesion scoring was used to assess the severity of histopathological changes in the spleens and thymus of the control and neem-treated group. Six slides from six rats in the experimental group were examined to grade histopathological changes in the mentioned tissues at 100x magnification using a light microscope. Histopathological lesions were scored based on the following criteria: mild (+) for < 25% area, moderate (++) for 25–50% area and severe (+++) for > 50% area (Sayed and Younes, 2017).

Immunohistochemistry

The spleen specimens were managed using an automated tissue processor, entrenched in paraffin blocks, and then cut into 5 µm thick sections with a rotary microtome. The tissue slices were then mounted on glass slides that were positively charged. The paraffin sections underwent de-paraffinization by passing through xylene, graded ethyl alcohol concentrations, and Q water. The tissue slices were boiled in citrate buffer for 5 minutes was used to prepare them for proper retrieval of the antigen. HRP/DAB kit (Abcam, United Kingdome) was used for immunohistochemistry. The primary antibodies of the examined cytokines' were applied in the following dilutions: interleukin 6 (IL-6) 1:400, tumor necrosis factor-alpha (TNF-α) 1:100, and nuclear factor kappa B (NFKB) 1:300. The slices of tissue were treated with primary antibodies, then incubated with horseradish peroxidase (HRP) conjugate, followed by DAB substrate. Finally, the tissue slices were counterstained with Mayer Hematoxylin for examination by light microscope. Three slides/ animal were examined. Eight fields per slide were eamined by the same person who was blinded to treatment.

Statistical analysis

Shapiro-Wilk test for univariate normality was implemented to test data normality. The present work results were examined by student t-test. Then the data was demonstrated as a mean ± standard error (mean±SE), p ≤ 0.05 is considered statistically significant. Correlations between tested parameters were performed. All of the analyses were carried out using the R programming language (Team, 2021).

RESULTS AND DISCUSSION

Feed intake and weight gain of dams and relative lymphoid organ weights

Table (1) declared that there were statistical ($p<0.05$) declines in birth and final body weights of male offspring in the neem leaves extract group in comparison to the control group. However, maternal feed intake and maternal weight gain showed no statistical variation between both groups (control and neem leaves extract group). In comparison to the control, the thymus absolute weight showed a statistical ($p = 0.0341$) decline in the males administered neem leaves extract during prenatal period. Relative thymus weight, as well as absolute and relative spleen weights, were non-statistically altered.

Table (1): Influence of Neem leaves extract on male rats' body weights and relative spleen and thymus weights.

Groups / Parameters	Control (0 % neem leaves extract/kg diet)	Neem extract (10 mL neem leaves extract / kg diet)	P-value
Maternal feed intake (g/day)	25.60 ± 0.70	25.40 ± 0.56	= 0.8344
Maternal weight gain during gestation (g/day)	9.08±1.35	7.67±1.20	=0.439
Birth body weight (g)	4.20 ± 0.05	3.50 ± 0.09 *	<0.0001
Final body weight (g)	49.00 ± 1.02	44.50 ± 1.48 *	= 0.0165
Thymus absolute weight (g)	0.06± 0.01	0.05 ± 0.01 *	= 0.0341
Thymus relative weight	0.125 ± 0.01	0.09 ± 0.02	= 0.1379
Spleen absolute weight (g)	0.12 ± 0.01	0.11 ± 0.01	= 0.4037
Spleen relative weight	0.24 ± 0.01	0.23 ± 0.02	= 0.7297

Values were expressed as mean ± SE (n = 20 /group). According to the statistical analysis T-test, the mean with the * sign in the same row is significantly different at p ≤ 0.05. Relative weight = (Organ weight/body weight) x 100.

Table (2): Hematological profiles and blood indices of male albino rats treated with neem leaves extract.

Groups / Parameters	Control (0 % neem leaves extract/ kg diet)	Neem extract (10 mL neem leaves extract / kg diet)	P-value
Hb (g/dL)	8.63 ± 0.33	9.43 ± 0.24	= 0.0812
RBCs count (10^6/ µL)	7.92 ± 0.68	9.31 ± 0.36	= 0.1009
PCV (%)	26.00 ± 0.99	28.60 ± 0.69	= 0.0562
TLC count (10^3/µL)	12326 ± 1220	12675± 1495	= 0.8601
Neutrophils (%)	18.00 ± 0.45	23.30 ± 0.56 *	<0.0001
Eosinophils (%)	2.00 ± 0.63	2.67 ± 0.62	= 0.4671
Basophils (%)	0.500 ± 0.224	0.33 ± 0.21	= 0.5995
Lymphocytes (%)	74.80 ± 1.19	68.70 ± 0.56 *	= 0.0009
Monocytes (%)	4.67 ± 0.62	5.00 ± 0.26	= 0.6279

Values were expressed as mean ± SE (n = 9 samples /group). According to the statistical analysis T-test, the mean with the * sign in the same row is significantly different at p ≤ 0.05. Hb: hemoglobin concentration, RBCs: red blood cell, PCV: Packed cell volume. TLC: total leukocyte count.

Hematology

The red blood corpuscle count (p= 0.1009), packed cell volume (PCV) at p= 0.0562, hemoglobin (Hb) at p= 0.0812 and total leucocyte count (TLC) at p= 0.8601 were non-significantly altered between both experimental groups. The neutrophils % revealed a significant (p<0.0001) promotion in the neem leaves extract group. Meanwhile, lymphocytes % showed a significant (p= 0.0009) reduction in the males administered neem leaves extract at prenatal period compared to the control group. Eosinophils (p= 0.4671), monocytes (p= 0.6279), and basophils (p= 0.5995) percentages exhibited non-significant alterations between the two groups (Table 2).

Total antioxidant Capacity (TAC)

Exposure to neem leaves extract during gestation significantly (p= 0.0001) reduced serum TAC in male offspring in comparison to the control rats (Figure 1).

Lymphocyte transformation assay and comet assay

The lymphocyte transformation denoted by optical density was shown in Table 3. The neem leaves extract group revealed significantly (p<0.0001) reduced LTT values as matched to the control group. The prenatal neem leaves extract exposure produced a statistical intensification in the percentage of DNA damage (p<0.0001), tail length (p= 0.0081), DNA percentage in the tail (p= 0.0131), and tail moment (p<0.0001) than control (Table 3, Figure 2).

Histopathology

The histopathological findings of stained tissues from the spleen and thymus of both the control and neem-treated groups were depicted in Figures (3) and (4). Table (4) presented the severity of histopathological alterations observed in the examined tissues.

The control spleen exhibited a normal architecture with well-differentiated hematogenous red pulp and white pulp (Figure 3A). During the prenatal period, the H and E-stained sections of neem-extract-treated male rats showed degeneration of white pulp; the follicles that formed the white pulp seemed atrophied (atrophied follicles) and were not bounded by a marginal zone. In addition, the germinal center appeared absent in these follicles (Figure 3B).

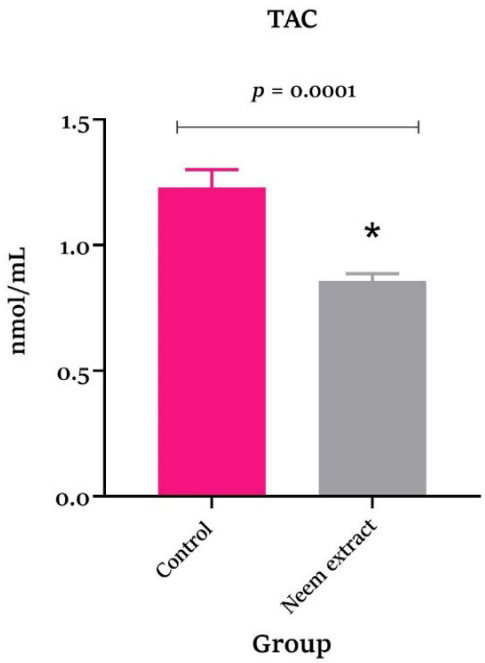

Figure (1): Effect of neem methanolic extract on serum TAC levels. Values were expressed as mean ± SE at $p \leq 0.05$ (n= 10 samples/group). According to the statistical analysis T-test, a column with the * sign is significantly different at $p \leq 0.05$.

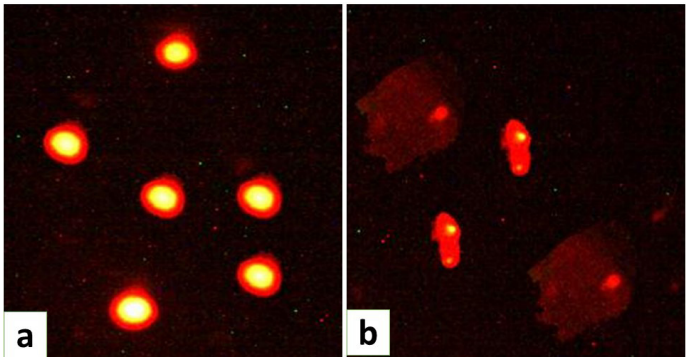

Figure (2): Comet assay images of blood mononuclear cells in control male offspring group (a) and neem methanolic extract prenatally treated (b) male rat offspring.

Table (3): LTT and comet assay data in the blood of male albino rats treated with neem leaves extract.

Groups / Parameters	Control (0 % neem leaves extract/kg diet)	Neem extract (10 mL neem leaves extract / kg diet)	P-value
LTT	0.500 ± 0.01	0.400 ± 0.01*	<0.0001
Percentage of damage	7.02 ± 0.10	12.20 ± 0.43*	<0.0001
Tail length	6.72 ± 0.53	8.49 ± 0.29*	= 0.0081
Percentage of DNA in tail	12.80 ± 0.81	15.30 ± 0.47*	= 0.0131
Tail moment	0.78 ± 0.02	1.37 ± 0.04*	<0.0001

Values were expressed as mean ± SE (n = 20 samples /group). Based on the T-test statistical analysis, the mean with the asterisk (*) in the same row is significantly different at p ≤ 0.05. LTT: Lymphocyte transformation assay.

Table (4): Scoring the detectable lesions in the spleen and thymus tissues of male Albino rats treated with neem leaves extract.

Lesion type	Experimental group	
	Control (0 % neem leaves extract/kg diet)	Neem extract (10 mL neem leaves extract/kg diet)
Spleen		
Atrophied follicles	-	+++
Depletion of white blood cells	-	+++
Depletion of Mz	-	++
Dilated veins	-	++
Dilated sinusoids	-	+
Thickening of endothelium	-	+++
Fibrosis	-	+++
Thymus		
Depletion of thymocytes	-	+++
Reticular cell proliferation	-	+
Apoptotic debris	-	++

Absent (–) (no lesions); mild (+) < 25% section area; moderate (++) 25–50% section area; severe (+++) > 50% section area.

Other follicles appeared shrunken. White blood cells, including lymphocyte cells, were depleted from the red pulp, and depletion of splenic parenchyma was frequently seen (Figure 3B, D). Large dilated splenic sinusoids and veins with signs of thickening endothelium and fibrosis and disturbed trabeculae were also seen (Figure 3C, D).

The normal thymus in the control group, as shown in Figure 4A, was composed of a darkly stained cortex with densely packed small and immature lymphocytes. The medulla had a paler staining and less dense cellular content than the cortex, and it contained more mature lymphocytes and reticular cells. The neem extract-treated thymus during the prenatal period exhibited a severe reduction in the lymphocyte (thymocyte) population in both the cortex and medulla, with significantly moderate macrophages engulfing apoptotic debris. Additionally, a mild increase in reticular cell proliferation was observed (Figure 4B).

Immunohistochemistry

As shown in Figure 5, sections of the spleen treated prenatally with neem leaves extract and immunohistochemically stained displayed increased expression of IL-6 (Figure 5B), TNF-α (Figure 5D), and NFKB (Figure 5F) in positive immune cells. These cells were primarily situated in the red pulp, with some dispersed in the white pulp and around splenic sinusoids and veins. There were statistically significant intensification in the immune stained area percentage of NFKB, Il-6 and TNF- α on neem methanolic extract male offspring than control ones (Figure 5G).

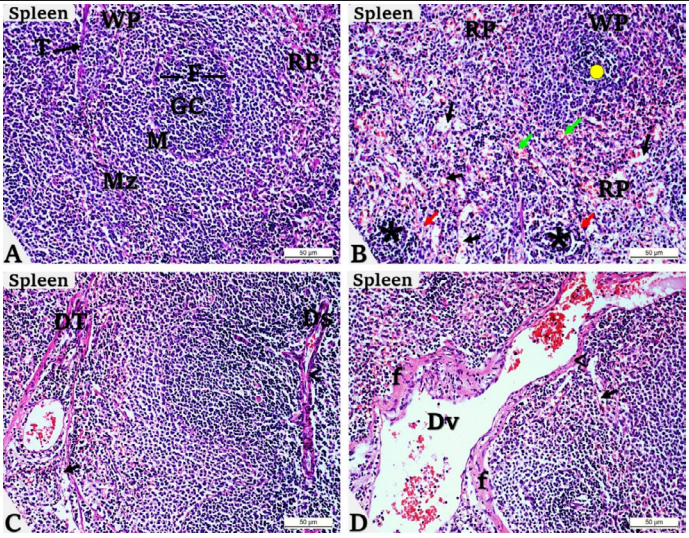

Figure (3): Splenic tissue sections (H and E x 200) from male rats' offspring following prenatal exposure to neem leaves extract. (A) control spleen exhibited a normal architecture with well-differentiated red pulp (RP) and white pulp (Wp). The red pulp (Rp) mainly consists of red blood cells, with some white blood cells, including lymphocytes. Lymphoid follicles (LF) of white pulp (WP) include distinct compartments such as the germinal center (GC), mantle (M), and marginal zone (MZ). Trabeculae (T) were also observed. (B-D) treated splenic tissues illustrated that all the white and red pulp compartments were similarly affected and changed in size and cellularity. Decreased Wp in size (•), atrophied Wp (*), depletion of Mz (red arrows), depletion of white blood cells of the red pulp (green arrows), depletion of splenic parenchyma (black arrows), and disturbed trabeculae (DT). In addition to dilated splenic sinusoids (Ds), dilated splenic veins (Dv), thickening of endothelium (<), and fibrosis (f) were also seen.

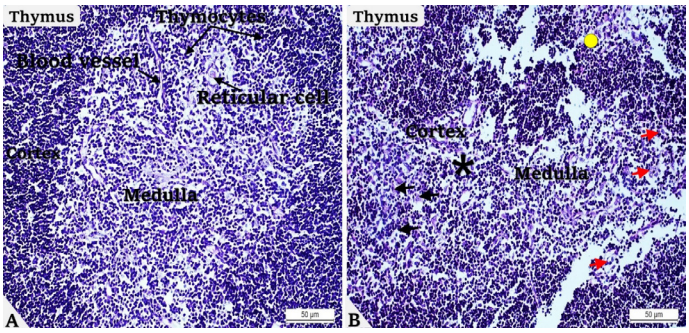

Figure (4): Thymus tissue sections (H and E x 200) from male rats' offspring following prenatal exposure to neem leaves extract. (A) control thymus exhibited a normal cortex and medulla with regular cellularity of thymocytes or lymphocytes and reticular cells. (B) treated thymus tissues showed a decrease in the lymphocyte (thymocyte) population in both cortex (•) and medulla (*) with macrophages phagocytized apoptotic debris (red arrows). In addition, a proliferation of reticular cells (black arrows) was also seen.

Table (5): correlation between tested parameters of control and neem extract group.

Treatment	Variables	Body weight		DNA damage		IL-6		TNF- α		NFKB	
		r	P	r	P	r	P	r	P	r	P
Control	Body weight	1.000	-	0.285	0.363		0.656	0.369	0.889	- 0.419	0.105
	DNA damage			1.000	-	0.287	0.366	0.025	0.939	- 0.018	0.957
	IL-6					1.000	-	- 0.377	0.136	0.496	0.043
	TNF-a							1.000	-	- 0.052	0.843
	NFKB									1.000	-
Neem - extract	Body weight	1.000		- 0.125	0.699	- 0.082	0.484	0.182	0.484	- 0.132	0.613
	DNA damage			1.000	-	- 0.184	0.567	0.591	0.043	0.424	0.170
	IL-6					1.000	-	0.231	0.373	0.097	0.712
	TNF- α							1.000	-	- 0.187	0.473
	NFKB									1.000	-

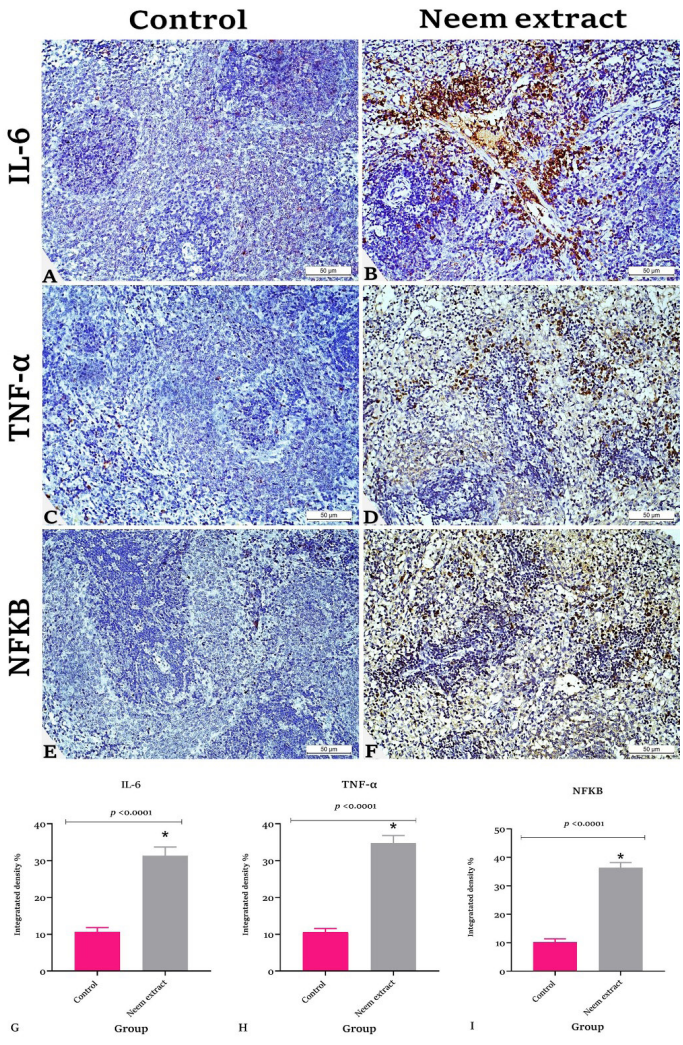

Figure (5): Immunohistochemical staining of IL-6 (A and B), TNF-α (C and D), and NFKB (E and F) on splenic tissue sections of male albino rats after 40 days of trial. (x 200). Compared to the control spleen sections (A, C, and E), the Neem-treated spleen exhibited an increased expression of IL-6 (B), TNF-α (D), and NFKB (F) positive immune cells predominantly in the red pulp of the spleen. (G), (H) and (I) graphs showed the integrated intensity percentage analysis of immunostaining for IL-6, TNF-a, and NFKB, respectively; according to the statistical analysis T-test, a column with the * sign is significantly different at $p \leq 0.05$.

Correlation of parameters

In the control group there was a statistical (P<0.043) positive correlation between NFKB and IL-6. In neem extract group, TNF- α revealed a statistical (P<0.043) positive correlation with DNA damage. There were non-significant correlations between the other parameters to each other (Table 5).

The research into new medications has been primarily focused on plant-based medications, with neem, *Azadirachta indica,* being extensively studied for its various medicinal properties and effectiveness (Alzohairy, 2016; Islas *et al.*, 2020; Hemdan *et al.*, 2023). However, animal toxicity studies are often used to assess potential health risks from plant extract effects on humans (Braga *et al.*, 2021). Rat model has a similar immune similarity to human where it has been widely used for several research to understand immune response of developmental immunotoxicology (Skaggs *et al.*, 2019), T cells in autoimmune diseases (Wildner, 2019), human skin immune response to infection (Agarwal *et al.*, 2020). Neem extracts have been investigated previously for their antioxidant activity (Veerendrakumar *et al.*, 2023). On the other hand, it has been identified and recognized that neem leaves contains a specific glycoprotein group that is thought to have an immune-modulatory effect when tested on mammals and may be able to limit tumor development by altering both systemic and local immunity (Kundu *et al.*, 2015).

Despite the growing usage of neem and its products for therapeutic and other purposes, there is limited information available regarding their toxicity in comparison to their application level. This highlights the need for further

research to approve the safety of neem extracts and compounds for numerous applications. The current study aimed to explore the influence of the prenatal exposure to neem leaves extract on the hematological and some immune parameters of male rat offspring.

Hematological parameters are crucial for diagnosis the functional and structural status of animals' exposure to a material. Whereas, hematological parameters are extremely subtle to physiological, environmental changes and health conditions (Kwawukume, 2013). The current data showed that treatment with a 10 mL neem leaves extract/kg diet produced non-significant alterations in the TLC, RBCs, Hb, and PCV. This finding contradicts the previous study, which stated that neem extract increased total TLC, RBCs, and Hb after administering toxic substances to animal hematology (Kaur *et al*., 2019). Meanwhile, Ikwuka *et al*. (2020) reported that there is no significant difference in PCV. In addition, Chibuike *et al*. (2020) detected that neem extract had non-significant variation in the values of TLC, PCV, RBCs, and Hb in Wistar rats, which is consistent with the current study's findings.

Evaluating the differential count of WBCs is vital for detecting the specific effects of a given substance influence on the immune system of the body. A rise in any type of WBCs can indicate an immunological reaction to an infection or allergy (Chibuike *et al*., 2020). In the neem leaves extract treated rats, there was an increase in neutrophils count compared to the control group. Similar results were also observed by Ashafa *et al*. (2012). The present study demonstrated that neutrophils and lymphocytes were affected. Whereas there were nonaffected percentages of eosinophils, monocytes, and basophils between the control and neem-treated groups, as previously confirmed by Chibuike *et al*. (2020). Physiologically, neutrophils assist in healing damaged tissues and resolving infections. Their levels naturally increase in response to injuries, infections and other categories of stress (Ogbuewu *et al*., 2010). This might confirm the stressful influence of methanolic neem leaves extract, which led to the increase in neutrophil percentage in this study. Also, lymphocyte percentage in the neem-treated group was statistically lesser than the control one; this finding was supported by the histological lesion of lymphocyte depletion in both spleen and lymphoid-examined tissues in this study. However, it has been reported that neem leaves extract increased lymphocytes count without exhibiting any cytotoxic effects in the body (Parshad *et al*., 1994).

One of the primary active components in neem extract is quercetin, which induces the formation of reactive oxygen species (ROS) in somatic cells, probably through two distinct mechanisms (Subapriya and Nagini, 2005). One possible explanation is that quercetin transforms into radicals in the form of quercetin-O after scavenging the peroxyl radical (Lee *et al*., 2010). Another mechanism involves the inhibition of antioxidant molecules such as glutathione and thioredoxin by quercetin (Pelicano *et al*., 2004; Jeong *et al*., 2009). Therefore, these explanations supported the current study's finding that neem leaves extract decreased the levels of TAC in the blood of male offspring.

As the spleen witnesses many lymphocytes passage daily extra than other lymphoid tissue, it types the spleen the utmost crucial secondary lymphoid organ in the lymphoid system (Bajénoff *et al*., 2008). Examining the spleen tissues of male rats' offspring after exposure to neem leaves before birth showed that both the white and red pulp areas were affected. There were changes in their size and cellularity, and the main lesion observed was a decline in the percentage of lymphocytes. In addition, the thymus also displayed a depletion of lymphocytes in the neem-treated group that could attributed to the observed decrease in thymus weight.

These alterations might result in the decreased levels of TAC detected in this study as an indication of increased ROS. Another previous study reported that exposure to ethanolic neem extract orally induced spleen alterations in male albino rats (Chibuike *et al*., 2020). Moreover, LTT is significantly deteriorated in neem leaves extract group due to increased oxidative load. LTT is a crucial pointer for lymphocytes blastogenesis throughout the adaptive immunity emergence toward attacking pathogen (Miszczyk *et al*., 2014). Therefore, neem extract seems to diminish number and function of lymphocytes.

In the current study, administering neem orally increased immunohistochemical expression of IL-6, NFKB and TNF-α, in positive immune cells in spleen sections. Inversely, Ruslie and Darmadi (2020) found that administering neem leaves extract decreased IL-6 and TNF-α expressions in rats induced with dextran sodium sulfate-induced colitis. Our observation contrasted with the findings of several studies reporting that the extract of *A. indica* leaves has an inhibiting inflammatory effect (Schumacher *et al*., 2011; Lee *et al*., 2017; Sarkar *et al*., 2021). The depletion of antioxidant system by neem extract prenatal exposure was prone by the reduced TAC. The resulted oxidative stress is capable of causing DNA breaks that was reflected by the increased DNA damage %, tail length, moment and % of DNA in tail of peripheral blood mononuclear cells. This reflected impaired immunological function.

Furthermore, oxidative stress can promote NFKB expression (Lingappan, 2018) which is responsible for various aspects of adaptive and innate immunological functions and assists as a fundamental mediator regarding the inflammatory responses (Liu *et al*., 2017). well-recognized role of NFKB

is guideline of inflammation responses. Whereas, NFKB intercedes initiation of several innate immune cells especially neutrophils, pro-inflammatory genes, regulates the differentiation, activation and influences function of T inflammatory cells (Tak and Firestein, 2001; Lawrence, 2009). Among pro-inflammatory genes NFKB induced array; IL-6 and TNF-α from M1 cells are existed (Wang *et al*., 2014) where NFKB activation binds to DNA promotor of the 2 genes initiating their expression (Collart *et al*., 1990; Galien *et al*., 1996). The expression of the later cytokines was significantly promoted in neem leaves extract group as a consequence for NFKB promotion in this group. This denoted the existence of an active inflammatory state in neem extract exposed male offspring where IL-6 and TNF-α encourage infiltration of leukocytes, inflammation and is considered as a motivator of other inflammatory cytokines that aggravate inflammation and may predispose autoimmune response (Jeong *et al*., 2002; Tanaka *et al*., 2014). The promotion of inflammatory cytokines especially TNF-α aggravates apoptosis (Rath and Aggarwal, 1999) as noted by the increased DNA damage in comet in neem extract group. This was confirmed by the existence of statistically significant positive correlation between TNF-α and DNA damage percentage.

CONCLUSIONS AND RECOMMENDATIONS

Orally administering pregnant female albino rats to methanolic neem leaves extract (70%) at a dosage of 10 mL extract/kg diet has a cytotoxic effect on offspring by decreasing birth body weight, inducing inflammation and antioxidant inhibition, and causing DNA damage and lymphoid tissue histopathological alterations. These results suggest that neem may have a potential cytotoxic effect in prenatal use as a therapeutic agent or contraceptive, as documented several times previously. Oxidative stress induction seems to be the definite mechanism behind neem prenatal immune-toxic effect that triggers DNA fragmentation of blood mononuclear cells as well as cytokines expression in splenic tissue. Administration of neem during pregnancy should be avoided in animals and human. This study has several limitations; the study lacks dose dependent effect to determine the safety margin for using neem extract without side effects that would be relevant to human exposure limit. This point should be addressed in future research. Also, lack of time interval for immunological evaluation that would confirm whether this effect is permanent or could be reversed after certain time that should be addressed in future research that will correspond to human cases. Identifying the key signaling pathways involved in the inflammatory response and exploring how neem extract modulates these pathways are deficient in the present study. Therefore, potential targets could include cytokine receptors, transcription factors involved in inflammation, or enzymes related to DNA repair mechanisms should be addressed in future studies.

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NOVELTY STATEMENT

This research article provides a new scope on prenatal neem exposure immunological disturbances in malerat offspring.

AUTHOR'S CONTRIBUTION

EMA, HMAA: Conceptualization. HMAA, HMA, NAA, AMZ, SES, NAE: Metshodology. EMA, HMAA: Formal analysis. HMA, NAA, AMZ, SES, NAE: Investigation. HMAA, EMA: Resources. HMA, NAA, AMZ, SES, NAE: Writing- original draft preparation. HMAA, EMA, NAE: Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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The authors have declared no conflict of interest.

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