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RESEARCH ARTICLE

Protective mechanisms of *Azadirachta indica* against titanium dioxide nanoparticle-induced reproductive toxicity through antioxidant and endocrine axis regulation

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Abstract

Aim: This study investigated the protective effects of *Azadirachta indica* (Neem) against Titanium Dioxide Nanoparticles (TiO_2-NPs)-induced reproductive toxicity in male Sprague Dawley rats.

Materials and Methods: *A. indica* leaves were extracted in methanol and the Lethal Concentration (LC_{50}) was determined ('2155.21 mg/kg' BW). Rats were divided into six groups with eight rats in each group (n=8), including a control group, saline group, positive group (TiO_2-NPs, '120 mg/kg' BW) and treatment groups receiving additional low ('100 mg/kg'), medium ('150 mg/kg'), or high ('300 mg/kg') doses of neem extract for '90 days'.

Results: TiO_2-NPs significantly reduced body weight, testicular somatic index and hormone levels and induced significant oxidative stress, while the plant extract dose-dependently attenuated these effects, with 300 mg/kg showing the strongest protective response and restoring antioxidant status toward control levels. While high-dose *A. indica* extract (300 mg/kg) markedly improved all parameters. TiO_2-NPs caused severe testicular damage, while high-dose mitigated structural abnormalities and restored tissue architecture.

Conclusion: High-dose *A. indica* extract effectively countered TiO_2-NPs-induced reproductive toxicity, highlighting its potential as a natural therapeutic agent for nanoparticle-related oxidative damage.

Keywords: *Azadirachta indica*, TiO_2 NPs, Hormonal parameter, Histology, Male reproductive organ (Testis)

Introduction

Nanotechnology plays a crucial role in contemporary science and significantly contributes to the synthesis and enhancement of nanoparticles (Yang, et al. 2021). Nanoparticles are present in our environment in both natural and synthetic forms. Naturally occurring nanoparticles emerge from sources like volcanic eruptions, Windblown particles, and sea spray. These fine particles are often dispersed through natural processes like wind and ocean currents. On the other hand, many nanoparticles are engineered for specific purposes. These artificial particles are developed in laboratories and industries for use in technology, medicine, and manufacturing (Chintagunta, et al. 2021). The use of nanoparticles in consumer products from textiles to cosmetics to paints offers convenience and

efficiency, but it also introduces significant environmental and health concerns (Hong, et al. 2018).

Among the many nanoparticles utilized today, titanium dioxide stands out. It is a transition metal with a color ranging from white to silver and holds great importance due to its broad applications in products like tiles, filtration systems for air and water and self-cleaning windows (Ziental, et al. 2020, Bakare, et al. 2016). Materials integrated into common accessories can enter the human body with regular use. Once inside, they are quickly distributed across many organs and tissues, traveling through the circulatory system. This fast dispersion enables the substances to reach cellular targets efficiently. The effects on cells can be profound, as these materials may disrupt or modify normal cell activity. Li, et al. 2016 examined this process, noting the swift distribution and cellular impact. Their findings highlight potential health risks associated with these materials.

Reproduction ensures continuity of life by transmitting genetic traits from parents to offspring. Male reproductive organs have specific functions: sperm production, maintenance, and transport. Titanium dioxide, prevalent in many modern products, may enter the body and accumulate in these organs, affecting critical cells like Sertoli cells and those responsible for sperm development. This leads to a decline in sperm production (Suthar, et al. 2023).

The modern resurgence of herbal medicine highlights its enduring value. As global populations seek integrative healthcare options, plant-based treatments offer promising avenues for developing new, effective, and safer pharmacological agents (Pathan, et al. 2025).

Neem, a revered medicinal plant in traditional systems like Ayurveda, holds potent antibacterial and antifungal properties, making it a natural remedy for treating skin diseases such as eczema, psoriasis, acne, and infections, with its leaves, oil, and extracts cleansing the skin and preventing microbial growth without harsh chemical side effects (Jamshidi, et al. 2025). Neem's antiviral and antiparasitic attributes contribute significantly in treating ailments like malaria, chickenpox, and intestinal worms, as compounds like azadirachtin and nimbin found in neem interfere with pathogen development and strengthen the body's resistance to infections. (Khan, et al. 2024) Its strong anti-inflammatory and immune-boosting abilities support the treatment of chronic illnesses like arthritis and joint pain, where neem helps reduce swelling and pain, enhances blood circulation, and offers a natural alternative to synthetic medications for managing autoimmune and inflammatory conditions (Aini and Andri, 2025). Secondary metabolites in neem plants, including alkaloids, flavonoids, saponins, tannins and total phenolic compounds, play a significant role against diseases and environmental stress. Alkaloids exhibit antimicrobial activity that helps suppress the growth of harmful pathogens. Flavonoids and total phenols function as powerful antioxidants, reducing oxidative damage and enhancing defense responses. Saponins and tannins contribute to disease resistance by disrupting pathogen development and strengthening natural protective barriers. Collectively, these bioactive compounds make neem a valuable natural source of remedial agents for disease management while improving plant resilience under adverse conditions (Mshelmbula, et al. 2025). Lastly, neem supports blood purification and liver detoxification, which indirectly benefits conditions like diabetes and cardiovascular disease by regulating blood sugar levels, improving metabolic activity, and aiding in the removal of toxins, thus enhancing overall systemic health naturally (Pathak and Avijit, 2024).

Materials and Methods

Ethics and patient admission information

The all procedure of animal study was carried out in compliance with the Ethics Review Ethics approval number, Ref. No. GCUF/ERC/3122, dated 14-05-2024.

Patient approval has not been obtained as it is performed on animals.

Plant sample collection and extraction methodology

Leaves of *Azadirachta indica* (Neem) were sourced and thoroughly washed to eliminate contaminants. The leaves were sun-dried and ground into a fine powder using a Sigma grinder. For extraction, 500 g of powdered leaves were soaked in 1.5 L methanol at ambient temperature for seven days with daily stirring. The methanolic extract was filtered by muslin cloth and concentrated *via* rotary evaporation at 40°C. The residue was reconstituted in 0.9% NaCl, ultra-sonicated for 20 minutes at 40°C, and vortexed for 1 minute before oral administration (Alzohairy, et al. 2016).

Determination of LC_{50} of plant extract

Male Sprague Dawley rats were exposed to *A. indica* at doses of '1000-5000 mg/kg' BW in six replicates, with mortality recorded every 12 hours over '96 hours'. The LC_{50} was determined following standard (Nagano, et al. 2021) protocols, without feeding the rats during the test.

Quantitative analysis of plant extracts

Plants secondary metabolites contents were quantified by following the method of (Saleem, et al. 2021).

Alkaloids: 1 g of each plant sample along with 200 ml CH_3COOH and C_2H_5OH (20%) were mixed in a breaker and filtered after 4

hours. It was then heated in water bath (Memmert) at 90°C until solution remained 1/4th of original solution. Precipitates were filtered with NH_4OH, oven dried and weighted by using weight balance (Shimedzu).

Following formula was used to calculate alkaloids: Alkaloids%=Weight of precipitate/Weight of original sample × 100

Flavonoids: 5 g plant sample (*A. indica*) was extracted with 80% methanol and then filtered with Whatman filter paper. Filtrate was heated into water bath (Memmert) for 30 min, evaporated in incubator (JSR-100T) at 30°C and weighed in mg/100 g.

Saponins: Plant sample (10 g) and 200 ml ethanol (20%) were taken in a Flask (Pyrex), mixed and placed on shaker (KS-130B, Basic) for 30min. After that time it was then heated into water bath (Memmert) at 50°C for 3 hours. Extraction was completed with 200 ml ethanol (20%) after filtration. The mixture was again evaporated into water bath (90°C) until 30 ml volume was attained. 20 ml diethyl ether and 30 ml filtrate were shaken together vigorously and only bottom ether layer was used for n-butanol extraction. 50 ml n-butanol was added in funnel along with bottom layer of ether and again shaked very well on shaker. Further, upper layer of n-butanol extract in funnel was washed two times with 5% Sodium Chloride (NaCl) buffer (10 ml). The remaining mixture was evaporated to dry into water bath and oven dried it at 40°C. Percentage of saponins was evaluated using the formula;

Saponins%=Weight of residues/Weight of plant material used × 100

Tannins: Plant sample (1 g) along with double distilled water (10 ml) were kept in 250 ml flask and placed on shaker (KS-130B, basic) at 1000 rpm for one hour. Afterwards, filtration was completed in 50 ml flask to make 50 ml volume. 5 ml of filtrate, 2 ml of diluted (10 fold) iron chloride (0.1 M) in hydrochloric acid (0.1M) and $K_4[Fe(CN)_6]•3H_2O$ (0.008M) were mixed and then placed at 37°C. Absorbance were noted at 395 nm within 10 minutes in mg/100 ml.

Total phenols: Plant sample (2 g) and 50 ml ether was taken in flask and boiled for 15 minutes. 5 ml of boiling solution and 10 ml double distill water was added in a flask and stirred gently. 5 ml NH_4OH and 2 ml $CH_3(CH_2)CH_2OH$ was also added with constant stirring, It was kept at room temperature for 30 minutes for color observation and absorbance was noted at 505 nm and expressed as mg/100 g of sample.

Dosage and administration of drugs

Forty-eight healthy male Sprague Dawley rats weighing '200-220 g' were obtained and kept in stainless steel cages under controlled environmental conditions ('20-23°C', '12h' light/dark cycle, ~'5 %' humidity) for a two-week acclimation period. The rats were divided into six groups, each comprising eight animals (n=8). Group I served as the negative Control (C) and was given only distilled water. Group II, the placebo group (S), received saline solution ('0.9% 'saline). Group III, TiO_2NPs treated group@ 120 mg/kg' BW (represented by G*) was exposed to TiO_2 nanoparticles (120 mg/kg' BW) *via* intraperitoneal injection. Groups IV-VI were treated with the same TiO_2NPs dose plus low (G1='100 mg/kg'), medium (G2='150 mg/kg'), or high (G3='300 mg/kg) doses of *A. indica*, administered orally for 90 consecutive days.

Sample collection

Following the 91-day experimental period, the rats were subjected to an overnight fast, anesthetized with ketamine hydrochloride (30 mg/kg body weight), and subsequently euthanized for sample collection. Blood samples were collected in gel-based coagulation tubes for testosterone analysis (Jannyketchuly, et al. 2016) while testes were excised, weighed using a Shimadzu Sartorius balance, and processed for further evaluation. One testis portion was frozen at '-80°C' for sperm and cell viability studies, and another was preserved in a fixative mixture for histopathological examination, following Institutional Animal Ethics Committee (IAEC) approved animal care guidelines.

Body and somatic index

Body weight was measured weekly using a weighing balance (Shemazu 230), while testis weight was recorded post-sacrifice of the rats.

Hormonal parameter

Testosterone level: Testosterone levels were measured using a commercial kit, each for 25 µL of samples (blood), standards and controls were added to coated 96-well plates. Then '100 µL' of conjugate reagent was added, shaken for '30 min' and incubated at room temperature for '1 h'. After washing, '100 µL' of 3,3',5,5'-Tetramethylbenzidine (TMB reagent) was added and shaken for '1 min' before adding '100 µL' stop solution. Absorbance was measured at '450 nm' and testosterone concentrations were calculated using a standard curve (Akdogan, et al. 2004).

Luteinizing hormone: Blood samples were obtained from the experimental animals immediately after sacrifice. Following clot formation, serum was isolated by centrifugation and preserved at -80°C until biochemical analysis. Serum levels of Luteinizing Hormone (LH) were quantified using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cat. No. CK-E90904) in accordance with the manufacturer's instructions. Absorbance values for the standards and serum samples were recorded at a wavelength of 450

nm using a Bio-Rad ELISA microplate reader.

Oxidative stress markers

Oxidative stress was analyzed through Malondialdehyde (MDA), lipid peroxidation, Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Reduced Glutathione (GSH) levels were measured using standard established protocols (Demirci and Sahin, 2019).

Homogenate preparation: A 0.25 g sample of liver tissue was homogenized in 2.5 mL of 0.1 M Tris-HCl buffer (pH 7.4) using a bullet blender under chilled conditions (4°C) to preserve enzymatic activity. The homogenate was subsequently centrifuged at 10,000 rpm for 20 minutes at 4°C to separate the cellular debris. The resulting supernatant was carefully collected, stored at -20°C, and later utilized for the assessment of oxidative stress biomarkers.

Estimation of Malondialdehyde (MDA): Malondialdehyde (MDA), a secondary by-product of Lipid Peroxidation (LPO), was quantified following the method of Ohkawa, et al. 1979. Briefly, the reaction mixture consisted of 0.2 ml tissue homogenate, 0.2 ml of 8.1% Sodium Dodecyl Sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% Thiobarbituric Acid (TBA), with distilled water added to obtain a final volume of 4 ml. The mixture was incubated at 95°C for 60 minutes in a water bath to allow color development. After cooling, 1 ml distilled water and 5 ml of n-butanol: Pyridine (15:1, v/v) were added, followed by vigorous mixing and centrifugation at 4000 rpm for 10 minutes. The absorbance of the upper organic layer was recorded at 532 nm using a spectrophotometer, and MDA levels were expressed as nmol per gram of tissue.

Estimation of lipid peroxidation: A 0.5 mL aliquot of freshly prepared tissue homogenate was combined with 0.9 mL of Ferrous Oxidation-Xylenol Orange (FOX) reagent. The reagent consisted of butylated hydroxytoluene (88 mg), xylenol orange (7.6 mg), and ammonium iron sulfate (9.8 mg), dissolved in a methanol and sulfuric acid mixture at a ratio of 90:10 (v/v). Following thorough mixing, the samples were incubated at 37°C for 30 minutes to allow color formation. The absorbance of the developed color was then measured at 560 nm using a spectrophotometer. Lipid peroxidation (LPO) levels were calculated and expressed as mM per gram of tissue.

Superoxide Dismutase (SOD): A 10% (w/v) liver homogenate was prepared in ice-cold 50 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 × g for 15 minutes at 4°C, after which the supernatant was collected. The reaction mixture consisted of phosphate buffer (pH 7.8), 0.1 mM Ethylenediaminetetraacetic Acid (EDTA), 13 mM methionine, 75 µM NBT, and 2 µM riboflavin. An appropriate volume of liver supernatant was added to this mixture and exposed to fluorescent light for 10-15 minutes to initiate the reaction. Absorbance was measured at 560 nm, and one unit of SOD activity was defined as the amount of enzyme required to produce 50% inhibition of Nitroblue Tetrazolium (NBT) reduction.

Estimation of Catalase (CAT): Catalase (CAT) activity in liver tissue homogenate is commonly determined spectrophotometrically by monitoring the decomposition of hydrogen peroxide (H_2O_2). Fresh liver tissue is homogenized in ice-cold phosphate buffer (50 mM, pH 7.0) and centrifuged to obtain a clear supernatant. The reaction mixture typically contains phosphate buffer, a defined concentration of H_2O_2 (e.g., 10-30 mM), and an appropriate volume of tissue homogenate, and the decrease in absorbance is recorded at 240 nm for 1-3 minutes using a Ultraviolet (UV)-visible spectrophotometer. Catalase activity is calculated from the rate of H_2O_2 degradation using its molar extinction coefficient and expressed as units per milligram of protein.

Estimation of Glutathione Peroxidase (GPx): Glutathione Peroxidase (GPx) activity in liver homogenate was determined using a coupled assay method. Briefly, liver tissue was homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000 × g for 15 min at 4°C to obtain the supernatant. The reaction mixture contained phosphate buffer, reduced Glutathione (GSH), reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH), glutathione reductase, and hydrogen peroxide as substrate, and the decrease in absorbance was monitored at 340 nm. GPx activity was calculated from the rate of NADPH oxidation and expressed as units per mg protein.

Estimation of Reduced Glutathione (GSH) levels: Reduced Glutathione (GSH) levels in liver tissue homogenates were quantified using the classical Ellman's method. Briefly, liver samples were homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000 × g for 15 minutes at 4°C to obtain the supernatant. An aliquot of the supernatant was reacted with 5,5'-Dithiobis (2-Nitrobenzoic Acid) (DTNB), and the formation of the yellow chromophore (TNB) was measured spectrophotometrically at 412 nm. GSH concentration was calculated from a standard curve prepared using reduced glutathione and expressed as µmol GSH per gram of tissue.

Histology

A small portion of gonadal tissue from both control and treated groups was collected and fixed using Sera's solution (formaldehyde, ethanol, and glacial acetic acid). The samples were then dehydrated through a graded alcohol series, followed by clearing with cedar wood oil at room temperature. Embedding was carried out in multiple steps involving benzol and paraplast at '50°C'. Once embedded, the tissue was sectioned into 5 µm slices using a microtome (Histo-line MR 2258). To assess tissue morphology and histopathological changes, the prepared sections were stained with Hematoxylin and Eosin (H and E) and subsequently examined micro-

scopically (Irais, et al. 2021).

Statistical analysis

The Data in this study was expressed as mean ± Standard Error (SE). Statistical significance was determined using Minitab software (minitab15). Body weights, organ weights, Hormonal parameter and oxidative stress Biomarker were analyzed through one way ANOVA by using general linear model to found out the effect of various treatment of *A. indica* on different parameter at ($p<0.05$) compared using the Duncan test. Histological changes in testis were assessed through visual inspection of photographic images from control and treated groups.

Results

LC_{50} of plant extract

As presented in Fig. 1, the LC_{50} value of *A. indica* for Sprague Dawley rats over '96 h' was found to be '2155.21 mg/kg' body weight.

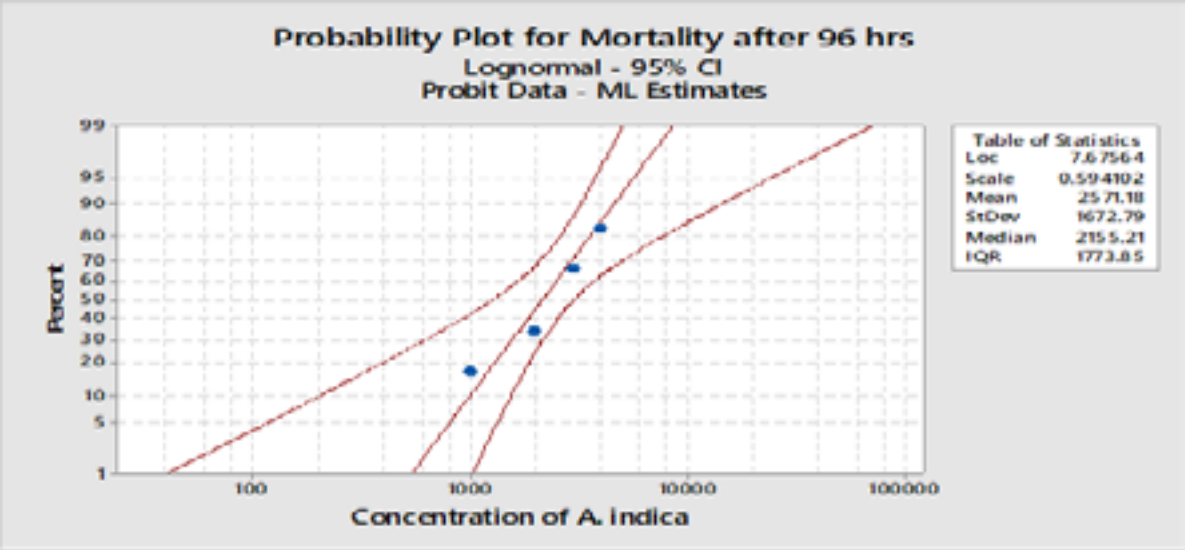

Figure 1. Mortality response of Sprague-Dawley rats to acute 96-hour exposure to *Azadirachta indica*.

Quantitative analysis of plant extracts

The phytochemical analysis of *A. indica* revealed the presence of several important secondary metabolites with varying concentrations (See in Tab. 1). Among the analyzed compounds, alkaloids were detected at the highest concentration (9.42%), indicating that they are the predominant secondary metabolite in the plant. Flavonoids were recorded at 3.24 mg/100 g, followed by tannins at 2.88 mg/100 mL and total phenols at 2.36 mg/100 g, demonstrating moderate levels of these bioactive constituents. In contrast, saponins were present at the lowest concentration (1.55%), suggesting that they are comparatively less abundant than the other metabolites. Overall, the findings indicate that *A. indica* is particularly rich in alkaloids while also containing appreciable amounts of flavonoids, tannins, total phenols, and saponins, all of which may contribute to its biological and therapeutic properties.

Table 1. Quantitative analysis of secondary metabolites in *Azadirachta indica*.

Secondary Metabolites	Research stage/vegetation period
Alkaloids (%)	9.42 ± 0.06^{A}
Flavonoids (mg/100g)	3.24 ± 0.05^{A}
Saponins ((%)	1.54 ± 0.08^{A}
Tannins (mg/100ml)	2.88 ± 0.13^{B}
Total phenols (mg/100g)	2.36 ± 0.04^{A}

Note: Mean values having different alphabets (A and B) in rows are significantly different ($p<0.05$).

Body and somatic index

The G* group (Positive control group) showed a marked drop in body weight compared to untreated and plant-treated rats. High-dose *A. indica* ('300 mg/kg') effectively countered TiO_2-NPs toxicity, more than the low ('100 mg/kg') and medium ('150 mg/kg') doses. In low-dose groups, weight rose in month one then declined, while medium-dose rats showed early loss followed by later gain (see Tab. 2).

The testicular somatic index was significantly lower ($p<0.05$) in the positive control group, whereas high-dose *A. indica* significantly restored it compared with the positive control and low-dose groups (see Tab. 2).

Table 2. Mean body weight and testicular somatic index (g; Mean ± SD) of Sprague-Dawley rats following 90-day *Azadirachta indica* exposure

Weight Results	Groups						P-value
	Control	Saline	Positive	*Azadirachta indica*			
Parameters	C	S	G*	G1	G2	G3	
Start of experiment AW(g) ± SD	207 ± 7.37[a]	209 ± 6.98[a]	206 ± 1.53[a]	207 ± 1.60[a]	206 ± 5.27[a]	205 ± 6.37[a]	P=0.35
End of experiment AW(g) ± SD	422 ± 2.00[a]	420 ± 1.89[a]	253 ± 0.70[b]	251 ± 0.83[c]	371 ± 2.37[d]	392 ± 1.92[d]	P=0.00
OW (g) ± SD (left testis)	1.77 ± 0.02[a]	1.75 ± 0.01[a]	0.97 ± 0.01[d]	1.01 ± 0.04[d]	1.18 ± 0.01[c]	1.32 ± 0.02[b]	P=0.00
OW (g) ± SD (right testis)	1.72 ± 0.012[a]	1.70 ± 0.01[a]	0.92 ± 0.016[d]	0.93 ± 0.01[d]	1.04 ± 0.01[c]	1.34 ± 0.03[b]	P=0.00

Note: AW: Animal Weight, OW: Organ Weight, SD: Standard Deviation, a: Similar to each other, different from b and c, b: Different from a, similar within itself, c: Different from both a and b, ab: Similar to both a and b.d; Different from a,b and c

Hormonal parameter

Tab. 3 illustrates a marked reduction ($p<0.05$) in testosterone and luteinizing hormone levels from the positive control group (G*) when compared to the non-treated and other treated groups. However, administration of a high dose of *A. indica* (G3) led to a significant rise ($p<0.05$) in both testosterone and luteinizing hormone levels in the gonads of these rat relative to the positive control group. Low and medium doses caused limited changes, whereas G3 exhibited the most significant hormonal restoration.

Table 3. Hormonal profile of Sprague-Dawley rats: Serum testosterone and luteinizing hormone responses to 90-day *Azadirachta indica* exposure.

Weight Results	Groups						P-value
	Control	Saline	Positive	*Azadirachta indica*			
Parameters	C	S	G*	G1	G2	G3	
Testosterone level (ug/dl) ± SD	1.88 ± 0.05[a]	1.86 ± 0.04[a]	0.64 ± 0.06[d]	0.64 ± 0.06[d]	0.97 ± 0.05[c]	1.32 ± 0.060[b]	P=0.000
Luteinizing hormone (ng/ml) ± SD	1.42 ± 0.01[a]	1.40 ± 0.01[a]	0.92 ± 0.01[d]	0.93 ± 0.01[d]	1.08 ± 0.01[c]	1.39 ± 0.02[b]	P=0.000

Note: SD: Standard Deviation, a: Similar to each other, different from b and c, b: Different from a, similar within itself, c: Different from both a and b, ab: Similar to both a and b.d; Different from a,b and c

Oxidative stress markers

The results showed that TiO_2-NP exposure significantly increased MDA and LPO levels, indicating enhanced oxidative stress, while antioxidant markers (SOD, CAT, GPx, and GSH) were also significantly elevated compared with the control group ($p<0.05$). Plant extract treatment at 100, 150, and 300 mg/kg body weight significantly reduced these TiO_2-NP-induced alterations in a dose-dependent manner. The magnitude of reduction was greater at higher extract doses, with the 300 mg/kg dose producing the most pronounced protective effect. At this dose, most oxidative stress parameters were restored to values close to those of the control group. Overall, the plant extract effectively attenuated TiO_2-NP-induced oxidative stress and improved the antioxidant status of the treated groups (Tab. 4).

Table 4. Oxidative stress markers of Sprague-Dawley rats in responses to 90-day *Azadirachta indica* exposure.

Oxidative stress markers						
Parameter	**C**	**S**	**G***	**G1**	**G2**	**G3**
MDA (nM/g)	136.47 ± 0.3^c	135.21 ± 0.4^c	300.34 ± 0.6^a	252.05 ± 0.7^b	185.14 ± 0.8^c	139.08 ± 0.4^c
LPO(m/100g)	17.06 ± 0.4^c	17.21 ± 0.6^c	31.11 ± 0.5^a	26.04 ± 0.5^b	22.24 ± 0.4^c	18.21 ± 0.7^c
SOD(U/mg)	345.40 ±1.3^c	346.85 ± 0.6^c	643.14 ± 1.8^a	585.43 ± 1.4^b	433.87 ± 1.2^c	351.25 ± 0.8^c
CAT (U/ml)	341.23 ± 0.5^c	342.16 ± 0.6^c	438.09 ± 0.3^a	401.74 ± 0.7^b	378.47 ± 0.4^c	348.21 ± 0.2^c
GPx (U/mg)	41.08 ± 0.17^c	41.03 ± 0.14^c	58.09 ± 0.03^a	53.21 ± 0.09^b	49.13 ± 0.06^c	42.21 ± 0.04^c
GSH(uM/g)	273 ± 0.3^c	273 ± 0.8^c	336 ± 0.5^a	299 ± 1.02^b	287 ± 0.8^c	276 ± 0.9^c

Note: MDA: Malondialdehyde, LPO: Lipid Peroxiation, SOD: Superoxide Dismutase, CA: Catalase, GPx: Glutathione Peroxidase, GSH: Glutathione, SD: Standard Deviation
Mean values having different alphabets in rows are significantly different ($p<0.05$).

Histology

The histopathological assessment of testicular tissue is summarized in Tab. 5. The control and saline-treated groups exhibited normal testicular architecture, characterized by intact seminiferous tubules, well-organized seminiferous epithelium, normal Sertoli cells, and active spermatogenesis. No signs of seminiferous tubule destruction, degeneration, necrosis, vacuolation, torsion, or basement membrane damage were observed in these groups. In contrast, the positive control group treated with TiO_2 nanoparticles (TiO_2-NPs) showed severe histopathological alterations (+++). These changes included marked destruction and degeneration of seminiferous tubules, extensive Sertoli cell damage, vacuolar degeneration of the seminiferous epithelium, sloughing of germinal epithelium, basement membrane disruption, testicular atrophy, arrested or impaired spermatogenesis, and pronounced necrosis. These findings confirm the toxic effect of TiO_2-NPs on testicular tissue. Administration of *A. indica* extract at a low dose resulted in considerable histological damage, with moderate to severe alterations (++ to +++) comparable to those observed in the positive control group. However, treatment with the medium dose of *A. indica* extract significantly reduced the severity of these lesions, showing only mild histopathological changes (+). Notably, animals treated with the high dose of *A. indica* extract (300 mg/kg) demonstrated near-normal testicular architecture, with preservation of seminiferous tubules, intact basement membranes, normal Sertoli cells, and restoration of spermatogenesis. No significant pathological alterations were observed in this group, similar to the control and saline groups. Overall, these findings indicate that *A. indica* extract exerts a dose-dependent protective and remedial effect against TiO_2-NP-induced testicular toxicity, with the 300 mg/kg dose providing maximum protection and restoring normal testicular histology.

Table 5. Histological scoring of tissue alterations in control, saline, positive control (TiO_2 NPs), and *A. indica*-treated groups (100, 150, and 300 mg/kg).

Parameter/ effects	**Groups**					
	C	**S**	**G***	**G1**	**G2**	**G3**
Seminiferous tubule destruction	-	-	+++	++	-	-
Sertoli cell destruction	-	-	+++	++	-	-

Torsion	-	-	+++	+	-	-
Vacuolar degeneration of seminiferous epithelium	-	-	+++	++	-	-
Seminiferous tubule degeneration	-	-	+++	++	-	-
Testicular atrophy	-	-	+++	+	-	-
Arrested/impaired spermatogenesis	-	-	+++	++	-	-
Necrosis	-	-	+++	+	-	-
Seminiferous epithelium sloughing	-	-	+++	++	-	-
Destruction of basement membrane	-	-	+++	++	-	-
Vacuolation	-	-	+++	++	+	-

Note: -(No abnormality), +(Mild), ++(Moderate), +++(severe)

Fig. 2 shows histology of control (A-D) shows normal Seminiferous Tubules (ST) and Sertoli Cells (SC). TiO_2-NPs @120 mg/kg (E-J) caused severe changes: Torsion (T), Vacuolar Degeneration of Seminiferous epithelium (VDS), Seminiferous Tubule Degeneration (STD), Testicular Atrophy (TA), Arrested/Impaired Spermatogenesis (AS) and Necrosis (N) shown in Fig. 3. *A. indica* @100 mg/kg (K&L) showed Seminiferous Epithelium Sloughing (SES) and Destruction of Basement Membrane (DBM); @150 mg/kg (M&N), animals treated with the high dose of *A. indica* extract (300 mg/kg) demonstrated near-normal testicular architecture, with preservation of seminiferous tubules, intact basement membranes, normal Sertoli cells, and restoration of spermatogenesis (O-P) shown in Fig. 4.

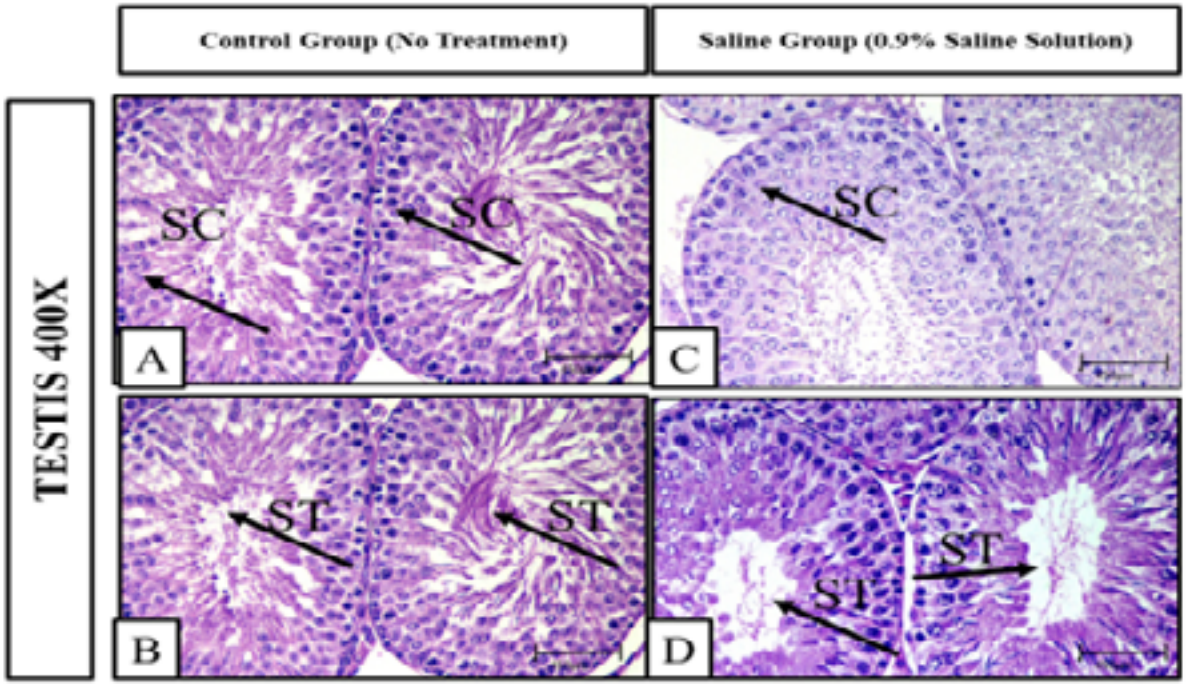

Figure 2. Photomicrograph (H and E; X400) of the testis tissue in control (non-treated) group (A and B) and saline group (0.9% saline solution) showing normal Sertoli cell and seminiferous tubules in Sprague Dawley rats.

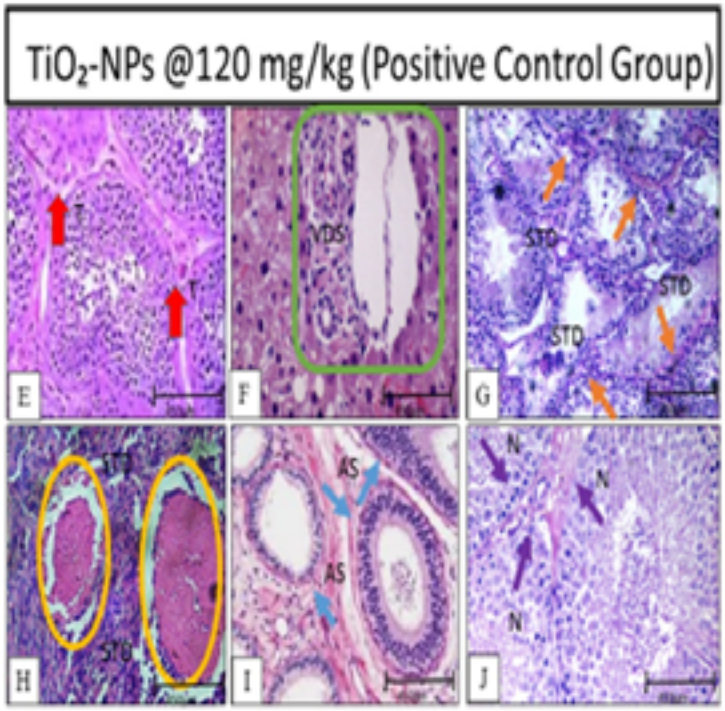

Figure 3. Photomicrograph (H and E; X400) of the testis tissues in Sprague Dawley rats with intraperitoneal injection of TiO_2-NPs @ 120 mg/kg BW (E-J).

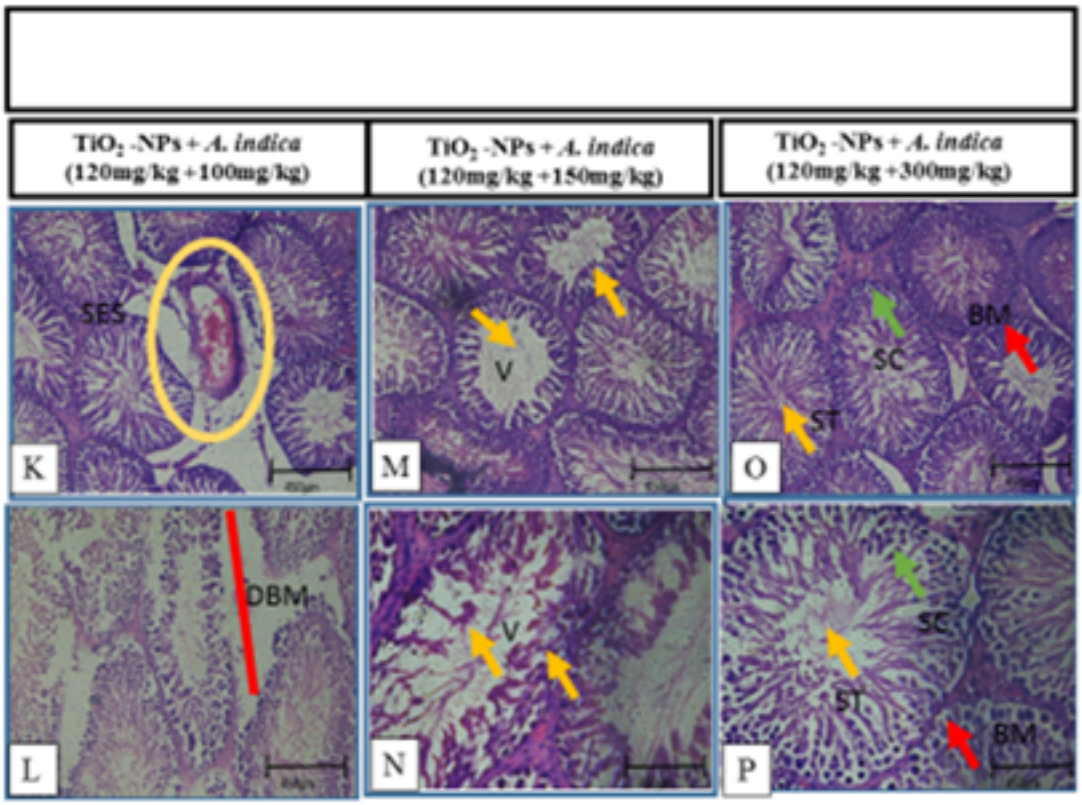

Figure 4. Photomicrograph (H& E; X400) of the testicular tissues of Sprague Dawley rats orally treated with different doses of *A. indica* along with intraperitoneal injection of TiO_2-NPs @ 120mg/kg BW.

Discussion

The current study entitled 'mitigating effects of Neem (*A. indica*) on reproductive damage caused by TiO_2NPs in Sprague Dawley rats' was carried out. The benefits of titanium dioxide are diverse in application but they can pose environmental and health risks

due to their potential toxicity. Plants can help reduce these adverse effects by absorbing or breaking down nanoparticles through phytoremediation. The neem plant plays a significant role in reducing the toxicity of (TiO_2 NPs) through its natural antioxidant and bioactive compounds (Seifu, et al. 2012). Neem extracts, rich in flavonoids, terpenoids, and polyphenols, can effectively stabilize and cap the nanoparticles, minimizing their harmful effects. These phytochemicals help in reducing oxidative stress caused by TiO_2 NPs in biological systems (Fernandes, et al. 2012). Additionally, neem-based synthesis of TiO_2 NPs produces more biocompatible particles compared to chemically synthesized ones. The plant's anti-inflammatory and antimicrobial properties also contribute to lowering the toxic impact of the nanoparticles (Naik, et al. 2014). Neem extracts also improve the dispersion and stability of TiO_2 NPs in solutions. Overall, neem serves as a natural agent to mitigate nanoparticle toxicity in biomedical and environmental applications.

The present study demonstrated that *A. indica* contains a diverse range of secondary metabolites, with alkaloids (9.42%) being the most abundant constituent, followed by flavonoids (3.24 mg/100 g), tannins (2.88 mg/100 mL), total phenols (2.36 mg/100 g), and saponins (1.55%). These findings are generally consistent with previous investigations on *A. indica*, which have reported the occurrence of the same major phytochemical groups in varying concentrations depending on plant age, geographical origin, environmental conditions, and extraction methods. (Ogbonna, et al. 2016) similarly quantified alkaloids, flavonoids, tannins, phenols, and saponins in neem leaves and concluded that these compounds are responsible for the plant's pesticidal and medicinal potential. Likewise, Nwali, et al. 2018 reported that neem leaves possess appreciable levels of alkaloids, flavonoids, saponins, and tannins, emphasizing that differences in phytochemical abundance among plant parts reflect their biological functions. In agreement with our observations, (Shrestha, et al. 2021) confirmed that *A. indica* leaves contain significant quantities of alkaloids, flavonoids, and other secondary metabolites, although the relative concentrations varied among different plant organs. More recently, (Olayemi, et al. 2026) also identified alkaloids, flavonoids, saponins, tannins, and polyphenols as dominant constituents of neem leaves and highlighted their contribution to antioxidant, antimicrobial, and anti-inflammatory activities. Similarly, (Ousman, et al. 2025) detected the same phytochemical classes in methanolic neem leaf extracts and associated their presence with broad-spectrum antibacterial activity, further supporting the biological significance of these metabolites. The relatively high abundance of alkaloids observed in the present study may explain the strong defensive capacity of *A. indica* against microbial pathogens, whereas flavonoids and total phenols contribute substantially to antioxidant activity by scavenging reactive oxygen species. Tannins are known to inhibit the growth of microorganisms through protein precipitation, while saponins enhance plant defense by disrupting the cell membranes of invading pathogens. Collectively, the phytochemical profile obtained in this investigation confirms that *A. indica* is a rich source of biologically active secondary metabolites, supporting its traditional use as a natural antimicrobial, antioxidant, and disease-management agent.

The present study determined the '96-h' median Lethal Concentration (LC_{50}) of *A. indica* in Sprague Dawley rats to be '2155.21 mg/kg' BW, indicating relatively low acute toxicity. This finding aligns with the work of (Dad and Yousuf, 2011), who reported that neem extracts exhibited low toxicity in mammals. Similarly, (Krishnan and Wong, 2015) observed no significant toxic effects of neem extract up to '2000 mg/kg' in rodents. Our LC_{50} value is consistent with that of (Maranho, et al. 2014) who concluded that *A. indica* has a wide safety margin in acute exposure studies. In contrast, some studies such as (Azmi, et al. 1998) have noted possible toxicity at higher doses, emphasizing the importance of dose regulation. The mild toxicity observed in our study supports its traditional medicinal use with caution. Overall, the data reinforce the safety of *A. indica* at controlled doses in animal models.

The current study observed a marked decline in body weight and testicular somatic index in the TiO_2-NP-treated group, which was notably improved at higher doses of *A. indica*. This enhancement was reflected in better body weight and increased somatic indices of both brain and testis. Similar trends have been documented, where oral administration of *A. indica* at various dosages led to recovery of body weight in rodent models (Janika, et al. 2018, Adeyi, et al. 2015, Abdelnour, et al. 2021) These results align with previous investigations demonstrating the protective and restorative potential of *A. indica* against chemically induced weight loss (Adki, et al. 2014). However, one study reported no significant impact on body weight or testicular index at lower doses of the extract (Aigbiremolen and Odigie, 2018).

The present findings demonstrate that *A. indica* extract effectively attenuated TiO_2-NP-induced oxidative stress, as evidenced by the dose-dependent reduction in MDA and LPO and restoration of antioxidant defenses. These results are consistent with the findings of (Ghosh, et al. 2016) who reported that *A. indica* leaf and bark extracts reduced MDA and restored SOD, CAT, and GPx activities in diabetic rats. Similarly, (Dinda, et al. 2020). observed that neem treatment enhanced SOD, CAT, and GSH while reducing lipid peroxidation, supporting its strong antioxidant potential. The protective effects observed in the present study are also in agreement with (Guchhait, et al. 2022) showing that *A. indica* attenuates cisplatin-induced renal and hepatic oxidative damage by restoring GSH and antioxidant enzymes and reducing MDA levels. Furthermore, study of (Hussain, et al. 2024) involving arsenic- and lead-induced toxicity demonstrated that *A. indica* significantly increased endogenous antioxidant defenses while suppressing oxidative-stress markers. This antioxidant activity may be attributed to the diverse bioactive constituents of neem, including flavonoids, polyphenols, quercetin, nimbin, nimbidin, and other limonoids, which can contribute to free-radical scavenging and preservation of cellular redox balance. Collectively, these findings strengthen the evidence that *A. indica* is a potent natural antioxidant capable of maintaining cellular integrity and protecting tissues against nanoparticle-induced oxidative injury.

Testosterone plays a crucial role in male fertility by supporting sperm production. Luteinizing Hormone (LH) stimulates the testes to produce testosterone, making it essential for normal reproductive function. Balanced levels of both hormones are vital for optimal reproductive potential. The significant reduction in testosterone and luteinizing hormone levels observed in the positive con-

trol group aligns with previous reports by (Azekhumen, et al. 2017), who documented similar hormonal declines in toxicant-exposed rodents. Conversely, the notable restoration of these hormone levels following high-dose *A. indica* administration corresponds with findings by (Ekaluo, et al. 2010, Al-awadhi, et al. 2024) who highlighted the plant's endocrine-modulating properties. Similarly, (Patel, et al. 2021, Irais, et al. 2021) observed dose-dependent improvements in reproductive hormones with *A. indica* extracts. Moreover, the mild effects at lower doses in our study reflect the dose-responsive trends reported by (Dhondiram, et al. 2021, Parkunan, et al. 2024). Together, these parallels strengthen the evidence for *A. indica*'s protective role against gonadal toxicity.

In the current investigation, exposure to TiO_2-NPs resulted in a range of histopathological alterations in testicular tissue of male Sprague Dawley rats showed pathological changes such as marked destruction and degeneration of seminiferous tubules, extensive Sertoli cell damage, vacuolar degeneration of the seminiferous epithelium, sloughing of germinal epithelium, basement membrane disruption, testicular atrophy, arrested or impaired spermatogenesis, and pronounced necrosis. However, administration of high-dose of *A. indica* exhibited a protective influence, mitigating the histological damages triggered by TiO_2-NPs. These observations are consistent with the findings of (Al-Khamas, et al. 2017, Omar, et al. 2015) who reported that *A. indica* conferred a protective role against acrylamide-induced testicular injuries characterized by spermatogenic cell degeneration, formation of spermatid giant cells, vascular congestion in interstitial areas, and extensive necrosis among spermatogenic elements in male Sprague Dawley rats. Furthermore, the current findings align with those of (Silva, et al. 2019) who demonstrated the remedial potential of *A. indica* in alleviating histological disruptions caused by aluminum phosphide in the testes of male quails.

Conclusion

Titanium dioxide nanoparticles (TiO_2-NPs) significantly impair male reproductive health, reducing body weight, testicular somatic index, hormone levels, and cell viability in rats. High-dose *A. indica* (300 mg/kg) effectively mitigated these toxic effects, showing the greatest improvement across all parameters. Histopathological analysis confirmed the protective role of neem extract against TiO_2-induced testicular damage. Therefore, *A. indica* offers a promising natural remedy against nanoparticle-induced reproductive toxicity.

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Data Availability Statement

Data will be made available on request from the Corresponding author.

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