Research Article

Moringa oleifera Leaf Extract Modulates Oxidative Stress and Hormonal Imbalance To Alleviate Di-(2-ethylhexyl) Phthalate-Induced Testicular Injury in Rats

Putri Ayu Ika Setiyowati[1,2,7]*, Yuningtyaswari Yuningtyaswari[3], Risya Secha Primindari[4], Lilis Maghfuroh[5], Nastiti Intan Permata Sari[6], Nur Fahim Oktavia[7], Vuanghao Lim[8], Alfiah Hayati[9]

[1]Research Fellow at Universitas Muhammadiyah Yogyakarta, Yogyakarta-55183, Indonesia; [2]Doctoral Program of Mathematics and Natural Science, Airlangga University, Surabaya-60115, Indonesia; [3]Study Program of Medical Science, Faculty of Health and Medical Science, Universitas Muhammadiyah Yogyakarta, Yogyakarta-55183, Indonesia; [4]Study Program of Midwifery, Faculty of Health Science, Universitas Muhammadiyah Lamongan, Lamongan-62215, Indonesia; [5]Study Program of Nursing, Faculty of Health Science, Universitas Muhammadiyah Lamongan, Lamongan-62215, Indonesia; [6]Center for Biomedical Research, Research Organization for Health, National Research and Innovation Agency (BRIN), Cibinong Science Centre, Bogor-16911, Indonesia; [7]Study Program of Biology, Faculty of Science Technology and Education, Universitas Muhammadiyah Lamongan, Lamongan-62215 Indonesia; [8]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bandar Putra Bertam-13200, Malaysia; [9]Departement of Biology, Faculty of Science and Technology, Airlangga University, Surabaya-60115, Indonesia.

Abstract | Di-(2-ethylhexyl) phthalate (DEHP) is a widely used plasticizer known for its endocrine-disrupting effects, particularly on the male reproductive system. Exposure to DEHP induces oxidative stress, reduces testosterone levels, impairs sperm quality, and disrupts testicular histoarchitecture. This study aimed to evaluate the protective role of *Moringa oleifera* leaf extract (MOLE) against DEHP-induced testicular damage in rats. Thirty-five male rats were randomly divided into five groups: Vehicle (DMSO 5%), DEHP only (200 mg/kg bw), DEHP + quercetin (20 mg/kg bw), and two treatment groups receiving DEHP plus MOLE at 200 or 400 mg/kg bw for 55 consecutive days. Antioxidant enzyme activities (SOD, CAT), lipid peroxidation (MDA), serum testosterone levels, sperm quality (motility, viability, morphology, and concentration), and testicular histopathology (spermatogonia, primary spermatocytes, and spermatids) were evaluated. DEHP significantly ($p < 0.01$) increased MDA levels and decreased SOD, CAT, testosterone, and all sperm quality parameters, along with a marked depletion of spermatogenic cells. MOLE treatment, particularly at 400 mg/kg, effectively ($p < 0.01$ or $p < 0.0001$) restored antioxidant status, hormone levels, and germ cell populations in a dose-dependent manner. The findings indicate that MOLE ameliorates DEHP-induced reproductive damage through antioxidant enhancement, hormonal restoration, and preservation of spermatogenic cells. This study supports the potential of *Moringa oleifera* as a natural therapeutic agent against phthalate-induced male reproductive toxicity.

Keywords | Di-(2-ethylhexyl) phthalate, *Moringa oleifera*, Oxidative stress, Sperm quality, Testicular histopathology, Endocrine disruptor

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***Correspondence** | Putri Ayu Ika Setiyowati, Research Fellow at Universitas Muhammadiyah Yogyakarta, Yogyakarta-55183, Indonesia; **Email:** putri.ayu@umy.ac.id

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INTRODUCTION

Phthalates are synthetic chemicals widely used as plasticizers to increase the flexibility and durability of polyvinyl chloride (PVC) materials. Among them, Di-(2-ethylhexyl) phthalate (DEHP) is one of the most prevalent and concerning due to its widespread application in medical devices, food packaging, cosmetics, and toys (Mariana *et al*., 2023; Gorini *et al*., 2025). Human exposure to DEHP occurs through ingestion, inhalation, dermal contact, or parenteral administration, and has been detected in urine, semen, and blood plasma, suggesting systemic bioavailability (Wang *et al*., 2019; Li *et al*., 2022). Accumulating evidence has raised significant concern regarding the reproductive toxicity of DEHP, particularly its adverse effects on male fertility (Li *et al*., 2024).

Experimental and epidemiological studies indicate that DEHP disrupts endocrine function by impairing steroidogenesis, reducing testosterone biosynthesis, and damaging Leydig and Sertoli cells (Yang *et al*., 2025; Hlisníková *et al*., 2020). These disruptions result in diminished serum testosterone levels, impaired spermatogenesis, and poor sperm quality, including reductions in motility, morphology, viability, and concentration (Oyovwi *et al*., 2025). Additionally, DEHP induces oxidative stress by generating reactive oxygen species (ROS), which surpass the capacity of endogenous antioxidants such as superoxide dismutase (SOD) and catalase (CAT) (Chang *et al*., 2022; Brahmana *et al*., 2024). This redox imbalance leads to lipid peroxidation, protein oxidation, DNA fragmentation, and ultimately, testicular cell apoptosis (Meida *et al*., 2023). The increased malondialdehyde (MDA) levels observed following DEHP exposure reflect enhanced lipid peroxidation and serve as a critical biomarker of oxidative damage in testicular tissue (Taufani *et al*., 2023; Setiyowati *et al*., 2022).

Oxidative stress not only compromises sperm integrity but also alters the histological organization of the seminiferous tubules. Several studies have reported that DEHP exposure reduces the number of germ cells, including spermatogonia, primary spermatocytes, and spermatids, disrupting the tightly regulated process of spermatogenesis (Han *et al*., 2025). These structural impairments in the testicular architecture translate into functional reproductive deficits and long-term fertility risks (Yuri *et al*., 2023). Considering the complex pathophysiological pathways involved in DEHP-induced testicular injury, therapeutic strategies must aim at mitigating oxidative stress, preserving hormonal balance, and supporting germ cell regeneration (Lin *et al*., 2023).

Natural antioxidants derived from medicinal plants are increasingly being explored as alternative therapeutic interventions against reproductive toxicity induced by environmental toxicants. *Moringa oleifera*, a plant extensively used in traditional medicine, has garnered attention due to its remarkable antioxidant, anti-inflammatory, and androgenic properties (Pareek *et al*., 2023). The leaves of *M. oleifera* are rich in polyphenolic compounds, particularly flavonoids, phenolic acids, gamma tocopherol, and other bioactives known to scavenge free radicals and enhance antioxidant defense systems (Harimurti *et al*., 2024; Kashyap *et al*., 2022). Preclinical studies have demonstrated that *M. oleifera* leaf extract (MOLE) confers protective effects against testicular damage in various toxicant-induced models by restoring antioxidant enzyme activity, preserving testosterone levels, and improving sperm parameters (Wurlina *et al*., 2025).

Although several studies have reported the protective effects of MOLE, many have primarily emphasized either biochemical or hormonal markers, with relatively few integrating these endpoints with detailed assessments of spermatogenic cell populations and sperm quality indices. Moreover, evidence on the dose-dependent effects of MOLE in DEHP-induced models that simultaneously evaluate antioxidant enzyme activity, testosterone levels, comprehensive sperm parameters (motility, viability, morphology, and concentration), and quantitative histopathological outcomes remains limited. The lack of comprehensive data integrating these parameters represents a significant gap in understanding the full therapeutic potential of MOLE.

Therefore, the present study aims to evaluate the protective effects of *Moringa oleifera* leaf extract on male reproductive toxicity induced by DEHP in a murine model. Specifically, we investigated the impact of MOLE on antioxidant enzyme activity (SOD, CAT), lipid peroxidation (MDA), serum testosterone levels, sperm quality (motility, viability, morphology, concentration), and testicular histopathology, including the quantification of spermatogonia, primary spermatocytes, and spermatids. This approach is expected to provide additional insight into the multilevel protective mechanisms of MOLE in a dose-responsive manner and contribute to the growing evidence supporting the use of plant-based interventions in mitigating reproductive toxicity caused by environmental endocrine disruptors.

MATERIALS AND METHODS

Materials and equipment

The primary test material used in this study was dried *Moringa oleifera* leaf powder, obtained from PT. Karya Herbal Nasional (Gresik, Indonesia), and stored in sealed, opaque containers at room temperature to maintain phytochemical stability. Di-(2-ethylhexyl) phthalate (DEHP, ≥99% purity) and quercetin (≥95%

purity) were obtained from Sigma-Aldrich (USA), while dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). Phosphate-buffered saline (PBS, pH 7.4), 10% neutral-buffered formaline (NBF), eosin Y, and nigrosin powder were also procured from Sigma-Aldrich and used in histological and sperm quality assessments. Enzyme-linked immunosorbent assay (ELISA) kits for the quantification of SOD, CAT, MDA, and testosterone were purchased from Redbiotech (Shanghai, China), and all analyses were performed according to the manufacturer's instructions. Standard laboratory equipment included oral gavage needles, micropipettes (Eppendorf, Germany), microtubes (Biologix, USA), a light microscope (Olympus CX23, Japan), a UV-VIS spectrophotometer (DLAB 1100, China), a microplate reader (Thermo Multiskan GO, USA), and a refrigerated centrifuge (Thermo Fisher Scientific, USA). A Neubauer hemocytometer was used for sperm concentration analysis. For tissue lysis, a probe-type ultrasonic homogenizer (Sonics Vibra-Cell™, USA) was employed to ensure efficient disruption of testicular samples prior to centrifugation and subsequent biochemical assays.

Preparation of *Moringa oleifera* leaf extract

One hundred grams of dried *Moringa oleifera* leaf powder was used for extraction. The powder was macerated in 1 L of 96% ethanol (1:10 w/v) for 72 hours at room temperature (~25 °C) with occasional shaking to enhance solute diffusion. After maceration, the mixture was filtered using Whatman No. 1 filter paper to separate the ethanol extract from plant residue. The filtrate was then concentrated using a rotary evaporator (Heidolph Laborota 4000, Germany) under reduced pressure at 40 °C to obtain a viscous crude extract. The extract was stored in amber-colored glass bottles at 4 °C to protect it from light and oxidative degradation until further use (Syahputra *et al*., 2021).

Animal and experimental design

This study was approved by the Animal Ethics Committee of Committee of the Faculty of Dental Medicine, Universitas Airlangga (Approval No. 0736/HRECC. FODM/VII/2025). Thirty-five healthy male Wistar rats (*Rattus norvegicus*), aged 6–8 weeks and weighing approximately 200±20 g, were obtained from the Faculty of Veterinary Medicine, Universitas Airlangga. The animals were housed in polypropylene cages under standard laboratory conditions (22–25 °C, 50–60% relative humidity, and a 12 h light/dark cycle), with ad libitum access to standard pellet diet and drinking water. After a 7-day acclimatization period, rats were randomly assigned into five experimental groups (n = 7 per group). The control group (C) received 5% dimethyl sulfoxide (DMSO) as vehicle. The negative control group (CN) was administered di-(2-ethylhexyl) phthalate (DEHP) at a dose of 200 mg/kg body weight (bw). The positive control group (CP) received DEHP (200 mg/kg bw) in combination with quercetin (20 mg/kg bw). The treatment groups received DEHP (200 mg/kg bw) co-administered with either MOLE at 200 mg/kg bw (T1) and 400 mg/kg bw (T2). All treatments were prepared in 5% DMSO and delivered orally by gavage once daily for 55 consecutive days. At the end of the treatment period, animals were fasted overnight and anesthetized using ketamine-xylazine (80:10 mg/kg bw, intraperitoneally). Blood samples were collected via cardiac puncture and stored appropriately for biochemical analysis. Testes and epididymides were carefully excised, cleaned of surrounding fat and connective tissue, weighed, and subsequently processed for histological, biochemical, and sperm quality assessments. The DEHP dose (200 mg/kg bw) was selected based on previous studies reporting its ability to induce testicular damage and impair spermatogenesis in rodents without causing systemic toxicity (Hosseinzadeh *et al*., 2021). MOLE doses (200 and 400 mg/kg bw) were chosen from prior *in vivo* findings showing antioxidant and anti-inflammatory effects at non-toxic levels (Elnaby *et al*., 2022).

Oxidative stress assay

Testicular tissue samples (100 mg) were lysed in 0.9 mL of cold PBS (pH 7.4; Gibco, USA) at a 1:9 (w/v) ratio. Tissue lysis was performed using a probe sonicator (Qsonica Q125, USA) in three 10-second bursts with cooling intervals on ice to avoid heat denaturation. The homogenates were centrifuged at 12,000 rpm for 15 minutes at 4 °C (Eppendorf 5424R, Germany), and the resulting supernatants were collected for biochemical assays. Antioxidant endogenous (SOD, CAT), and lipid peroxidation (MDA) concentrations were quantified using commercial ELISA kits (Redbiotech, China; Cat. No. RB-SOD-E01, RB-CAT-E01, and RB-MDA-E01) following the manufacturer's protocols. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Multiskan FC, USA). Quantification of each parameter was performed based on the standard curves provided within each ELISA kit, and results were expressed in units specified by the manufacturer (ng/mL).

Testosterone measurement

Serum testosterone levels were measured using a rat-specific enzyme-linked immunosorbent assay (ELISA) kit (Redbiotech, China; Cat. No. E0460Ra), according to the manufacturer's protocol. Whole blood was collected and centrifuged at 3,000 rpm for 15 minutes at 4 °C to obtain serum, which was stored at −20 °C until analysis. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Multiskan FC, USA). Testosterone concentrations were calculated from the standard curve provided in the kit. and results were expressed in units

specified by the manufacturer (ng/mL).

Sperm quality assessment

Spermatozoa were collected from the cauda epididymis of each animal immediately after euthanasia. The cauda was dissected and minced in 1 mL of PBS (pH 7.4; Gibco, USA) at 37 °C, and the suspension was incubated for 10 minutes in a water bath to allow spermatozoa to swim out. The resulting suspension was used to evaluate sperm motility, viability, morphology, and concentration. Motility was assessed by placing a drop of sperm suspension on a clean glass slide, covering it with a coverslip, and observing under a light microscope (Olympus CX23, Japan) at 100× magnification. A total of 100 spermatozoa per sample were randomly examined and evaluated for motility, viability, morphology, and concentration. Motility was assessed under light microscopy (100×) and categorized into motile (showing progressive forward movement) and immotile (non-progressive or no movement). Only the number of motile spermatozoa per 100 cells was recorded and reported as the representative outcome. Viability was evaluated using eosin-nigrosin staining. Equal volumes of sperm suspension and eosin-nigrosin dye (Sigma-Aldrich, USA) were mixed and smeared onto clean glass slides. After air-drying, 100 sperm cells were observed under a light microscope (400×), and only the number of viable (unstained) spermatozoa was quantified and expressed as the viability percentage. For morphology, smears were stained with eosin and examined at 400× magnification. Sperm cells (n = 100) were classified based on head, midpiece, and tail integrity. Only the proportion of spermatozoa with normal morphology was considered for analysis. Sperm concentration was determined using a Neubauer hemocytometer after 1:20 dilution in formalin-buffered saline (5% formalin in 0.9% NaCl). Spermatozoa within the central grid were counted at 400× magnification, and the results were expressed as $\times 10^6$ sperm/mL (Setiyowati *et al.*, 2022; Syaputra *et al.*, 2023).

Histological analysis of testis

Testes were excised and immediately fixed in 10% NBF for 24–48 hours. Fixed tissues were dehydrated in a graded ethanol series (70% to absolute), cleared in xylene, and embedded in paraffin wax using an automated tissue processor (Leica TP1020, Germany). Serial sections (5 µm thick) were cut using a rotary microtome (Leica RM2125, Germany) and mounted on glass slides. The slides were stained with hematoxylin and eosin (H & E) following standard protocols and observed under a light microscope (Olympus CX23, Japan) at 400× magnification (Yuningtyaswari and Dwi, 2016). For each animal, ten seminiferous tubules were randomly selected, and the total number of spermatogonia, primary spermatocytes, and spermatids across the selected tubules was counted and recorded as total cell number (cells). Histological evaluation was performed in a blinded manner by two independent observers to ensure objectivity (Hakemi *et al.*, 2019).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA). Data were first tested for normality using the Shapiro–Wilk test. For normally distributed (parametric) data, comparisons among groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Results were expressed as mean ± standard deviation (SD), and statistical significance was set at $p < 0.05$. Each treatment group consisted of seven animals (n= 7). All tests were two-tailed.

RESULTS AND DISCUSSION

Oxidative stress

Exposure to DEHP significantly altered oxidative stress markers in testicular tissue. In the DEHP group (CN), SOD and CAT activities were markedly suppressed ($p < 0.0001$), indicating impaired antioxidant defense. MOLE administration restored these activities in a dose-dependent manner. The 200 mg/kg group (T1: 5.440 ± 0.517 ng/mL) showed a moderate increase in SOD ($p < 0.01$), while the 400 mg/kg group (T2: 6.600 ± 0.561 ng/mL) reached levels comparable to the quercetin group (CP: 6.760 ± 0.770) ($p < 0.0001$). CAT activity improved significantly in the T2 group (2.720 ± 0.465 ng/mL) ($p < 0.0001$), whereas the increase in T1 (2.171 ± 0.308 ng/mL) was not statistically significant (Figure 1a-b).

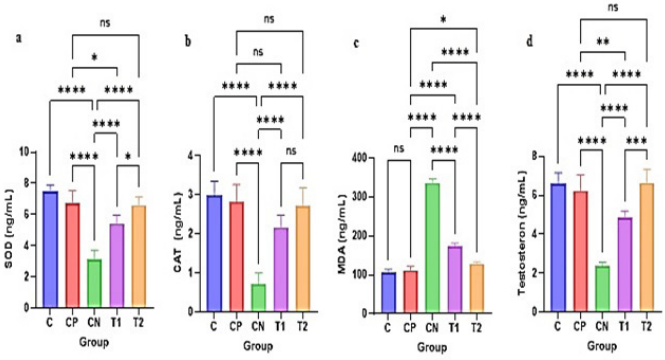

Figure 1: The effects of *Moringa oleifera* leaf extract on testicular oxidative stress markers and serum testosterone levels in rats exposed to DEHP. (a) Superoxide dismutase (SOD) activity; (b) Catalase (CAT) activity; (c) Malondialdehyde (MDA) levels; and (d) Serum testosterone levels. Data are presented as mean ± SD. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns = not significant.

MDA levels, a marker of lipid peroxidation, were significantly elevated in the DEHP group ($p < 0.0001$). Both MOLE doses reduced MDA concentrations, with the T2 group (128.4 ± 5.271 ng/mL) showing the most substantial decrease ($p < 0.0001$ when compared to CN (336.2 ± 10.290 ng/mL), aligning closely with the quercetin group (CP: 111.8 ± 9.542 ng/mL) (Figure 1c). These findings confirm that MOLE alleviates DEHP-induced oxidative stress by enhancing antioxidant enzyme activity and reducing lipid peroxidation.

Oxidative stress is another critical mechanism underlying DEHP-induced testicular toxicity. Elevated MDA levels and suppressed activities of SOD and CAT observed in the negative control group support the hypothesis that DEHP may enhance the generation of ROS, surpassing the capacity of endogenous antioxidants. This imbalance has been previously documented in rat models by Chang *et al.* (2022) and Zheng *et al.* (2024), who demonstrated that DEHP increases lipid peroxidation and oxidative stress in testicular tissue. Excessive oxidative stress not only impairs the structural integrity of the seminiferous epithelium but also disrupts mitochondrial function in spermatozoa (Hussain *et al.*, 2024).

Importantly, MOLE supplementation, particularly at 400 mg/kg bw, reversed these biochemical and functional alterations in a dose dependent manner. The observed improvements in SOD and CAT activities suggest that MOLE enhances the antioxidant defense system, thereby reducing oxidative burden. These effects are likely attributed to the high content of polyphenolic compounds in *M. oleifera* leaves, including quercetin, kaempferol, and chlorogenic acid, which are well known for their free radical scavenging properties (Arshad *et al.*, 2025; Kurniawan *et al.*, 2024). Similarly, Mardatillah *et al.* (2022) reported that MOLE 500 mg/kg/day restored antioxidant enzyme activity in gentamicin-induced testicular injury, supporting its systemic antioxidant effects. The α-tocopherol in MOLE may also inhibit ROS activated cell death signalling pathway such as p53 and Bax/Bcl-2 (Tokoro *et al.*, 2021).

The reduction in MDA levels observed in MOLE treated groups indicates mitigation of lipid peroxidation, thereby preserving sperm plasma membrane integrity, a critical determinant of motility and fertilization capacity (Pomi *et al.*, 2025; Rao *et al.*, 2021). Another research reported that MOLE reduced testicular MDA content in rats exposed to cadmium, a heavy metal known to induce oxidative testicular damage (Ragab *et al.*, 2024). This consistency suggests that MOLE's antioxidant properties are effective against various toxicants, including phthalates.

Serum testosterone levels

DEHP exposure (CN: 2.384 ± 0.194 ng/mL) led to a significant decline in serum testosterone levels ($p < 0.0001$ compared to control group (6.600 ± 0.547 ng/mL)), reflecting its endocrine-disrupting effect. MOLE treatment reversed this reduction in a dose-dependent manner. The T1 group (4.876 ± 0.317 ng/mL) showed a moderate but significant increase ($p < 0.05$ compared to CN), while T2 (6.634 ± 0.707 ng/mL) exhibited testosterone levels comparable to CP (6.228 ± 0.805 ng/mL) ($p < 0.0001$) (Figure 1d).

DEHP has been extensively documented as an endocrine-disrupting chemical (EDC) that targets the male reproductive system through multiple mechanisms (Dutta *et al.*, 2023). Upon metabolism, DEHP is converted to mono-(2-ethylhexyl) phthalate (MEHP), which acts directly on testicular cells, particularly Leydig and Sertoli cells, impairing steroidogenic enzyme expression and inducing oxidative stress (Awny *et al.*, 2021). The reduction in serum testosterone observed in the DEHP group corroborates previous findings that reported MEHP disrupts the hypothalamic pituitary gonadal (HPG) axis and downregulates key enzymes involved in testosterone biosynthesis (Odetayo *et al.*, 2024). This hormonal imbalance likely contributed to the marked decline in sperm quality, as testosterone plays a central role in spermatogenesis and sperm maturation (Xie *et al.*, 2024).

Another key finding of this study is the improvement of testosterone levels in MOLE-treated rats. Although we did not directly measure the expression of steroidogenic enzymes, the elevation of testosterone may reflect improved testicular function secondary to reduced oxidative damage. In this context, MOLE's effect on testosterone may be indirectly mediated through its ability to counteract oxidative insults to Leydig cells. The recovery of antioxidant status may underlie the normalization of testosterone levels and support the maintenance of germ cell lineage and seminiferous tubule architecture, as demonstrated in this study.

Sperm quality

Sperm analysis revealed that DEHP exposure severely impaired sperm motility, viability, morphology, and concentration ($p < 0.0001$ compared to control group (C)), confirming reproductive toxicity. MOLE treatment improved all parameters in a dose-dependent manner. In terms of motility, T1 showed significant restoration (52. 80 ± 3.701 cells; $p < 0.05$), and T2 values (70.0 ± 1.581 cells) were comparable to those in the CP group (72.20 ± 1.483 cells). Viability also improved moderately in T1 (55.80 ± 3.194 cells; $p < 0.05$), with T2 showing a more pronounced increase (71.20 ± 1.924 cells; $p < 0.001$). Morphological normalities, which were significantly decreased by DEHP (30.40 ± 3.050 cells; $p < 0.0001$), were improved by MOLE

treatment, particularly in the T2 group (75.60 ± 2.408 cells; $p < 0.001$), where values approached normal. Sperm concentration increased significantly in both T1 and T2 (148.8 ± 4.658 million/mL; $p < 0.05$ and 179.6 ± 5.941 million/mL; $p < 0.01$, respectively), suggesting enhanced spermatogenesis (Figure 2a-d).

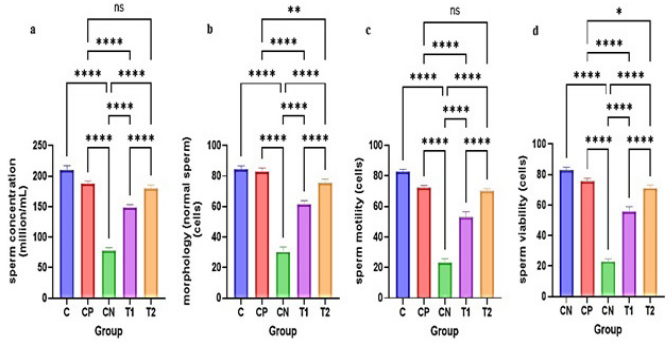

Figure 2: The effects of *Moringa oleifera* leaf extract on sperm quality in rats exposed to DEHP. (a) sperm concentration; (b) morphology (normal sperm); (c) sperm motility; and (d) sperm viability. Data are presented as mean ± SD. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns = not significant.

The current study provides compelling evidence that DEHP exposure induces marked deterioration in sperm quality, as demonstrated by a significant reduction in progressive motility, viable spermatozoa, morphologically normal sperm, and overall sperm concentration. These findings align with existing evidence showing that phthalates impair spermatogenesis by reducing testosterone synthesis and increasing oxidative stress through ROS generation, lipid peroxidation, and mitochondrial dysfunction in testicular cells (Oyovwi *et al*., 2025; Hosseinzadeh *et al*., 2021).

The recovery of sperm motility and viability suggests that mitochondrial function and membrane integrity were maintained. The increase in viable spermatozoa in the T1 and T2 groups supports the hypothesis that MOLE attenuates membrane damage caused by lipid peroxidation. Moreover, the reduction in abnormal sperm morphology and increase in sperm concentration imply that MOLE supports normal spermatogenesis and proper maturation of germ cells.

This observation aligns with the findings of Abou-Zeid *et al*. (2021), who reported improved spermatogenic output in rats treated with antioxidant-rich plant extracts Our study extends these findings by demonstrating that the sperm-protective effects of MOLE are not only observable at the morphological level but also quantitatively validated by improvements in concentration, supporting its therapeutic potential against DEHP-induced reproductive dysfunction. Our data extend these findings by integrating sperm functional assays with biochemical and histological markers.

Histological analysis of testis

H&E staining revealed marked testicular damage in the DEHP group, including germinal epithelium disorganization and germ cell loss. Quantitative histology confirmed significant reductions in spermatogonia, primary spermatocytes, and spermatids ($p < 0.0001$ compared to control group (C)). MOLE treatment mitigated these effects. The T1 group showed partial restoration of tubular architecture and germ cell numbers ($p < 0.05$ compared to CN), while T2 displayed near-normal histology with germ cell counts significantly improved ($p < 0.001$ compared to CN), approaching those of the quercetin group (Figure 3a-c). Figure 4 presents representative testicular histology across treatment groups. The control (C) shows normal seminiferous tubules with abundant germ cells. The positive control (CP) maintains normal tubular structure and cell density. The negative control (CN) reveals disrupted epithelium, reduced spermatogenic cells, and vacuolization. T1 (MOLE 200 mg/kg) shows partial recovery, while T2 (MOLE 400 mg/kg) displays restored testicular architecture and germ cell populations. Together, these results indicate that MOLE offers dose-dependent protection against DEHP-induced testicular damage, likely via antioxidant enhancement and hormonal support.

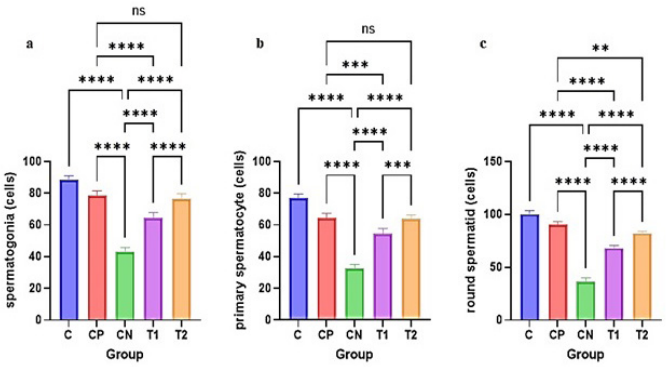

Figure 3: The effects of *Moringa oleifera* leaf extract on spermatogenic cells in rats exposed to DEHP. (a) spermatogonia; (b) primary spermatocyte; and (c) round spermatid. Data are presented as mean ± SD. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns = not significant.

Histological examination of the testis revealed that DEHP exposure markedly reduced the number of spermatogonia, primary spermatocytes, and spermatids, indicating disrupted spermatogenesis and correlated with decrease of sperm quality. This is consistent with (Gouri *et al*., 2022), who reported germ cell loss and seminiferous tubule

atrophy following DEHP exposure. The reduction in germ cells observed in this study may be attributed to oxidative damage and impaired testosterone signalling, both of which are essential for maintaining the spermatogenic cycle. The dose-dependent restoration of germ cell populations in MOLE-treated groups suggests that the extract may promote germ cell survival and proliferation, possibly by attenuating ROS induced DNA fragmentation and cellular apoptosis.

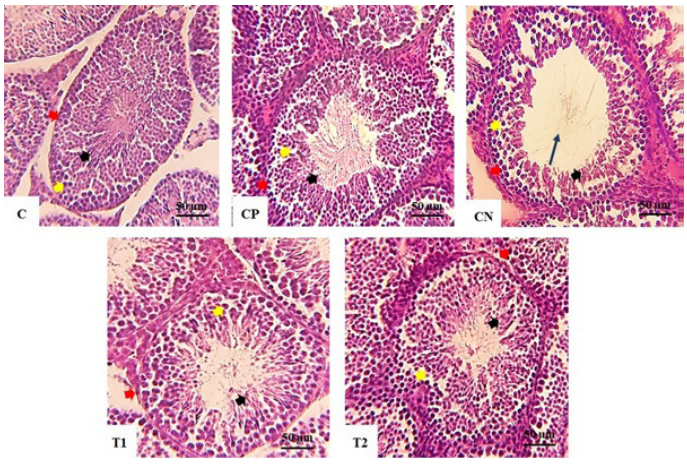

Figure 4: The representative photomicrographs of testicular histology (H & E staining, 400× magnification) from different treatment groups. Scale bar = 50 µm. (C) Control group (DMSO 5%) showing normal seminiferous tubules with intact germinal epithelium and abundant spermatogonia (red arrow), primary spermatocytes (yellow arrow), and spermatids (black arrow); (CP) Positive control group (DEHP 200 mg/kg bw + quercetin 20 mg/kg bw) displaying preserved tubular architecture and high density of spermatogenic cells similar to normal; (CN) Negative control group (DEHP 200 mg/kg bw) showing disorganized seminiferous epithelium, marked reduction of spermatogenic cells, and visible cytoplasmic vacuolization within the germinal layer (blue arrow); (T1) DEHP 200 mg/kg bw + MOLE 200 mg/kg bw group exhibiting partial improvement in spermatogenic cell populations and tubular structure with reduced vacuolization; (T2) DEHP 200 mg/kg bw + MOLE 400 mg/kg bw group demonstrating restored seminiferous organization and nearly normal numbers of spermatogonia, primary spermatocytes, and spermatids.

Interestingly, the positive control group receiving quercetin also demonstrated significant improvements across all parameters, but the efficacy of MOLE at 200 mg/kg bw was comparable, indicating that whole-plant extracts may offer synergistic benefits due to the presence of multiple bioactive compounds. This aligns with the concept of phytochemical synergy, wherein the combined effects of plant constituents may outperform isolated compounds in complex biological systems (Noor *et al*., 2022; Syahputra *et al*., 2021).

It is noteworthy that the current study employed two doses of MOLE (200 and 400 mg/kg bw) to explore its dose-response relationship. The greater efficacy observed at 400 mg/kg is in agreement with previous studies using similar or higher doses of *M. oleifera* in models of reproductive toxicity (Pop *et al*., 2022). Translating this to humans yields an human equivalent dose (HED) of approximately 64.9 mg/kg (≈4.5 g/day for a 70 kg adult) using standard body surface area scaling (Jacob *et al*., 2022). Given the limited data on long term safety at this intake in humans, we recommend further chronic toxicity and pharmacokinetic studies prior to direct clinical translation. For initial human supplementation studies, we suggest starting with lower doses (e.g., 1–3 g/day) and implementing careful clinical monitoring.

Despite the promising findings, several limitations must be acknowledged. The present study did not include molecular assays to assess gene or protein expression of key regulators in steroidogenesis, oxidative pathways, or apoptosis (e.g., StAR, 3β-HSD, Bax/Bcl-2). In addition, Sertoli cell function was not directly evaluated, and thus conclusions regarding the spermatogenic niche remain speculative. Future studies incorporating these endpoints will be essential to further delineate the mechanistic pathways by which MOLE confers its protective effects.

CONCLUSION

In conclusion, this study demonstrates that *Moringa oleifera* leaf extract mitigates DEHP-induced testicular injury through a combination of antioxidant restoration, hormonal modulation, and structural preservation of the seminiferous epithelium. These findings support the growing evidence for plant-derived antioxidants as potential therapeutic agents in the management of male reproductive toxicity. The dose-dependent benefits observed highlight the therapeutic relevance of MOLE and warrant further investigation in preclinical and clinical settings.

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NOVELTY STATEMENT

The novelty of this study lies in the dose-dependent evaluation of Moringa oleifera leaf extract on DEHP-induced testicular toxicity through integrated biochemical, hormonal, sperm quality, and histopathological analyses. This approach reveals new insights into MOLE's antioxidant,

endocrine, germ cell protective mechanisms and its potential as a phytotherapeutic agent.

AUTHOR'S CONTRIBUTION

PAIS: Concepts or ideas, design, literature search, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing, manuscript review; Y: Design, experimental studies, manuscript preparation, manuscript review; RSP: Experimental studies, data acquisition; LM: Experimental studies, literature search, manuscript preparation; NIPS: Literature search, manuscript preparation, manuscript review, manuscript editing; NFO: statistical analysis, data acquisition, manuscript preparation; VL: manuscript review, manuscript revision; supervision; AH: manuscript review, manuscript revision, concepts, supervision.

Generative AI and AI-assisted technology statement

No generative AI or AI-assisted technologies were used in the preparation or writing of this manuscript

Conflict of interest

The authors have declared no conflict of interest.

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