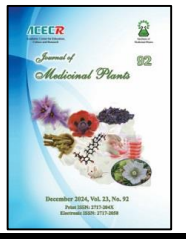
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Research Article

Effect of *Malva neglecta* Wallr. extract on infertility caused by $CdCl_2$ in Wistar male rats

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ABSTRACT

Background: *Malva neglecta* is a commonly used medicinal plant known for its content of tannins, hydroxycinnamic acids, alkaloids, flavonoids, saponins, anthocyanins, and flavonols. **Objective:** The objective of this research was to assess the effects of *M. neglecta* extract on male rat infertility. **Methods:** Forty adult male rats were divided into eight groups. The *M. neglecta* extract was administered daily for 28 days. At the conclusion of the treatment period, all animals were anesthetized with ether. Following euthanasia, dissection was performed, and sperm morphology, viability, and motility were assessed in the epididymis. The right testis was subjected to histological analysis after tissue processing and H/E staining, with cell counts conducted under a light microscope. The left testis was examined for superoxide dismutase (SOD) and malondialdehyde (MDA) enzyme levels. **Results:** Sperm analysis showed a significant improvement in viability, motility, and concentration in the infertile groups treated with *M. neglecta* extract at doses of 100, 200, and 400 mg/kg. SOD enzyme levels were notably higher in the experimental groups receiving the extract, surpassing those of the healthy control group. Furthermore, increasing the extract dosage led to a rise in SOD production within the infertile groups. MDA levels, an indicator of lipid peroxidation, were lower in the experimental groups compared to the control group, with the MDA concentration nearly identical to that of the healthy controls. This suggests that *M. neglecta* extract significantly reduced lipid peroxidation. **Conclusion**: The findings indicate that *M. neglecta* extract has a notable positive effect on spermatogenesis, sperm viability, motility, and concentration, particularly under oxidative stress conditions.

Abbreviations: MDA, Malon Di Aldehode; SOD, Superoxide Dismutase

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1. Introduction

Infertility is defined as the inability to conceive after one year of unprotected intercourse, prompting many couples to seek treatments. Infertility affects both men and women, with male infertility contributing to over 50 % of childless couples' cases [1]. According to the World Health Organization, male infertility accounts for over 33 % of infertility cases, while 50 % are attributed to female infertility. Around 10 % of cases involve both partners, and the remaining cases are categorized as unexplained [2]. While a clear cause is not always identified in nearly half of infertile men, male infertility can result from a variety of factors, including physical conditions, hormonal imbalances, sexually transmitted infections, genetic factors, environmental influences, and lifestyle choices [3].

The testicles, essential male reproductive organs, are protected by the tunica albuginea, a capsule within the scrotum [4]. The testis is divided into two distinct regions: the tubular section, which contains seminiferous tubules, and the interstitial spaces between the tubules, involved in blood circulation and protective responses [5]. Numerous medicinal plants are now recognized for their potential to enhance male fertility, offering an affordable and effective alternative to synthetic treatments [6].

M. neglecta Wallr, a plant with a range of pharmacologically active compounds such as saponins, alkaloids, hydroxycinnamic acids, flavonols, flavonoids, anthocyanins, tannins, proanthocyanidins, proteins, oils, sugars, and organic acids, exhibits a variety of beneficial effects. These include anti-inflammatory, anti-ulcer, antimicrobial, antioxidant, and anticholinesterase activities [7].

In addition to these properties, *M. neglecta* contains isoflavonoids and phytoestrogens, with its extract having been used for various therapeutic purposes [8]. Notably, the phytoestrogens in *M. neglecta* are known to alleviate pregnancy-related symptoms and support infertility recovery in women. Clinical benefits have also been reported for postmenopausal women [9]. However, there is limited research exploring the potential benefits of *M. neglecta* extract for male infertility. This study analyzed the effects of *M. neglecta* on infertile male rats induced by cadmium chloride.

2. Material and Methods

2.1. Preparing M. neglecta hydroalcoholic extract

M. neglecta flowers were collected during the winter season in Khuzestan province and delivered to the herbarium laboratory of Islamic Azad University (The Branch for Sciences and Research) for identification, where they were assigned the herbarium code PMP-1918.

In order to replicate the traditional soaking extraction method, the dried plant material was first ground into a powder, weighed (477 g), and then mixed with 80% methanol. The mixture was stirred thoroughly to ensure the plant powder was completely saturated with the solvent, thereby optimizing the extraction process. After 72 hours, the purple solution was filtered through paper to eliminate plant particles. The resulting filtered solution was then returned to the percolator, where methanol was removed by evaporation under low pressure at 40 ºC using a rotary evaporator. The methanol solvent was concentrated and recycled for reuse. The extraction process was repeated with fresh 80 % methanol until the solution became colorless. After the final concentration, the extract was placed in a crystallizer and left under a fume hood to evaporate any remaining solvent, resulting in the complete plant extract.

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2.2. Experimental animals

This study utilized 40 male *Wistar* rats with an average weight of 200 ± 20 g. The rats were maintained in a controlled environment under a 12-hour natural dark/light cycle and housed in suitable cages. They were given a one-week acclimatization period under standard laboratory conditions, with a regulated temperature of 25±2°C. All procedures adhered to the ethical guidelines established by the research ethics committee. The study protocol received approval from the Research Ethics Committee of the Islamic Azad University, Science and Research Branch (approval number: IR.IAU.SRB.REC.1400.054).

2.3. Groups

The experiment rats were divided into eight groups, including two controlled groups (a fertile control group, GI, and an infertile control group, GII), and six experimental groups, with each group comprising five male rats. The GII group (infertile ones) was given 3 mg/kg of $CdCl_2$ daily in the course of 28 days [10].

The GIII, GIV, and GV received *M. neglecta* extract for 28 consecutive days but with different dosages. GIII received 100 mg/kg, GIV received 200 mg/kg, and GV received 400 mg/kg, in order to consider the impact of different dosages. On the other hand, the other three rat groups (GVI, GVII and GVIII) were administered both $CdCl_2$ and *M. neglecta* extract on the same timeline and the same dosages, in order to measure the difference in materials (Table 1).

Cadmium is known to cause significant damage to the testicles, as it adversely affects Sertoli cells (SCs), spermatogenic tubules, and the testicular blood barrier, leading to sperm loss. This reduction in sperm count and infertility can be observed through microscopic examination [11].

2.4. Administration of $CdCl_2$

Pure cadmium chloride (CdCl2) powder was acquired, and deionized water was used as the solvent to prepare a solution of soluble cadmium chloride. The resulting solution was then administered intraperitoneally as a single dose of 3 mg/kg to the experimental groups [12].

2.5. Testis extraction and Histological Study

On the 28th day, all rats were weighed and anesthetized using an ether solution. The testicles and epididymides were collected from the euthanized rats and weighed. For exactly 4 hours, the testicular tissues were preserved in Bouin's solution, dehydrated using a graded series of ethanol, cleared in xylene, and embedded in paraffin. Thin sections, 6 µm thick, were prepared using a microtome and stained with hematoxylin-eosin for microscopic evaluation. The maturation and quality of the seminiferous tubules were evaluated using Johnson's score [13], which rates the tubules on a scale from 1 to 10 based on specific characteristics, as outlined in Table 2.

2.6. Sperm Count and Motility

Sperm quality was evaluated by examining parameters like motility, viability, morphology, and sperm count. Using a scalpel, the caudal segment of the epididymis from the left testis was meticulously dissected and transferred into a Petri dish with Hank's buffer solution. The suspension was kept at 37 ºC for a minimum of 10 minutes to allow for sperm dispersion. The assessment involved counting motile (both rapid and slow) and non-motile sperm under a light microscope, with the findings reported as a percentage of the total sperm count [14].

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Table 1. Treatment method in 28 days

Groups / Drugs	(GI)	(G II)	(GIII)	(GIV)	(GV)	(GVI)	(GVII)	(GVIII)
CdCl2 (Interperitoneal)	-	3mg/kg	-	-	-	3 mg/kg	3 mg/kg	3 mg/kg
M. neglecta Extract (Gavage)	-	-	100 mg/kg	200 Mg/kg	400 Mg/kg	100 mg/kg	200 Mg/kg	400 Mg/kg

Table 2. The Johnson's score to quality the seminiferous tubules

Level	Description
10	Complete spermatogenesis and perfect tubules with the presence of a large number of spermatozoa were located on the around, and a regular lumen was seen
9	There were a large number of mature spermatids, but the epithelium was degraded and the rounded and regular lumen was not seen
8	Less than five spermatozoa were seen in each tube, and a few mature spermatids were seen
7	No adult spermatozoa and spermatid were seen, but a large number of primary spermatids was seen
6	No spermatozoa or spermatids pulp was seen, but a few primary round spermatids were presented
5	No spermatozoa or spermatids were seen
4	No spermatozoa or spermatid were observed
3	Only spermatogonia was presented
2	There were no germinal cells and only Sertoli cells were recognized
1	The atrophic tubules were defined as seminiferous tubules with no epithelial

2.7. Assessment of the oxidative stress status

In brief, the tissue sample (0.1 g) was homogenized in a KCl solution in the proportions 1 to 9. A homogenized sample was mixed with two volumes of thiobarbituric acid solution containing trichloroacetic acid, thiobarbituric acid, and hydrochloric acid. In order to stabilize the color formation at 1000 g, it was then immersed in boiling water for 15 minutes and cooled. It was centrifuged for 10 minutes, then the top layer was gradually removed, and the optical absorbance of the samples at 535 nm was measured using a spectrophotometer to quantify the quantity of MDA [15]. SOD was measured by adding 45.2 ml of SOD reagent solution to test tubes. Each tube was filled with 50 µl of tissue homogenate sample, 450 µl of distilled water, and 50 µl of xanthin solution oxide. The samples were maintained at 25 degrees for 20 minutes. Pipes were treated with 50 µl of copper chloride (0.8 mM $CuCl_2$). Then by keeping the measurements at a wavelength of 560 nm, the optical absorbance of the samples was measured [16].

2.8. Statistical analysis

The data were analyzed statistically using analysis of variance (ANOVA), followed by the Tukey-Kramer test. A significance level of $P < 0.0001$ was considered indicative of a meaningful difference between the groups.

3. Results

3.1. Histopathological Result

Histopathological analysis revealed a significant difference between the infertile groups treated with *M. neglecta* extracts and the infertile control group, as well as a comparable outcome (based on Johnsen Score) between the fertile experimental groups and the healthy control group ($P < 0.0001$) (Fig. Graph 1). Additionally, the results showed a marked improvement in the infertile experimental groups treated with *M. neglecta* extract at doses of 100, 200, and 400 mg/kg ($P < 0.0001$) (Graph 1).

Histopathological examination showed severe damage to the seminiferous tubules in the infertile control group, with the absence of cell

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clusters (Fig. A2). In the infertile group treated with 100 mg/kg *M. neglecta* extract, a small number of spermatocytes were observed (Fig. A1). However, at the 400 mg/kg dose, there was a significant increase in the population of spermatocytes within the seminiferous tubules (Fig. A3). Along with spermatocytes, spherical spermatids and spermatozoa were also present in this group.

3.2. Stress oxidative Enzymes Result

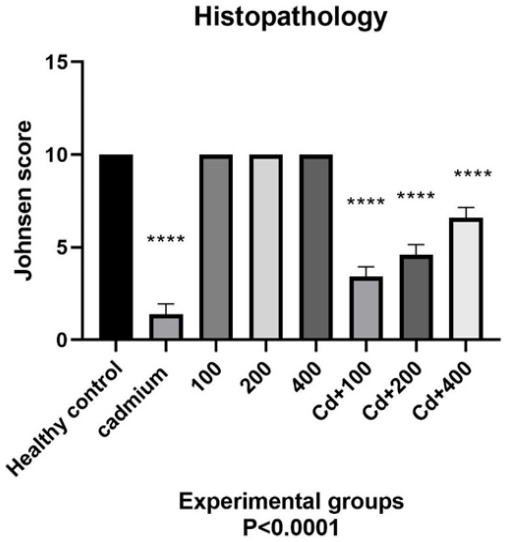
SOD analysis demonstrated that the healthy experimental groups receiving 100-400 mg/kg of M. neglecta extract exhibited the highest levels of enzyme production, surpassing even the healthy control group. Additionally, higher doses of M. neglecta extract had a significant impact on enzyme production ($P < 0.0001$) (Graph 2.A).

At the 400 mg/kg concentration of *M. neglecta* extract, a significant positive effect on MDA enzyme production was observed, when compared to the other concentrations ($P < 0.0001$). (Graph 2.B). Furthermore, the study found that the healthy control group produced similar levels of MDA as the three normal experimental groups.

Sperm Microscopic Factors Test: Sperm viability analysis indicated that healthy experimental groups given a 400 ml/kg dose of *M. neglecta* extract had the greatest viability rate, followed by the healthy control group.

Infertile experimental groups 6, 7, and 8 showed increased viability ($P < 0.0001$) after receiving dosages of 100, 200, and 400 ml/kg of *M. neglecta* extract (Graph3.A). Sperm motility testing revealed substantial differences between *M. neglecta* extract-treated infertile groups and the infertile control group. The results indicated comparable motility between the fertile experimental groups and the healthy control group ($P < 0.0001$) (Graph 3.B).

According to this study the healthy experimental group that was treated with 400 mg/kg of *M. neglecta* extract had a higher sperm count in comparison with the healthy control group. Additionally, this group exhibited a significantly larger sperm population than the infertile control group ($P < 0.0001$) (Graph 3.C).



Graph 1. Histopathology analysis based on the Johnsen score ($P < 0.0001$).

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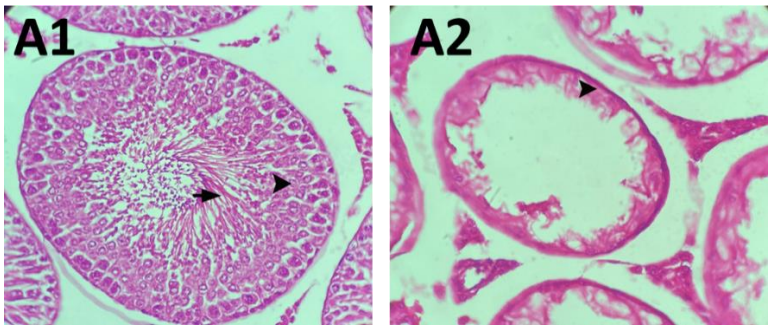


Fig. 1. Testicular seminiferous tubes in healthy control group (A1) and infertile control group (A2)

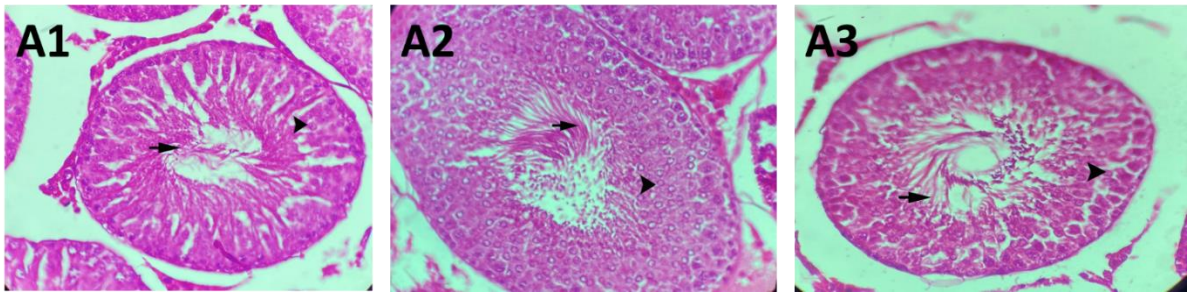


Fig. 2. Seminiferous tubules of the testes in the normal groups that were exclusively treated with *M. neglecta* extract (100mg/kg A1), (200 mg/kg A2), (400 mg/kg A3)

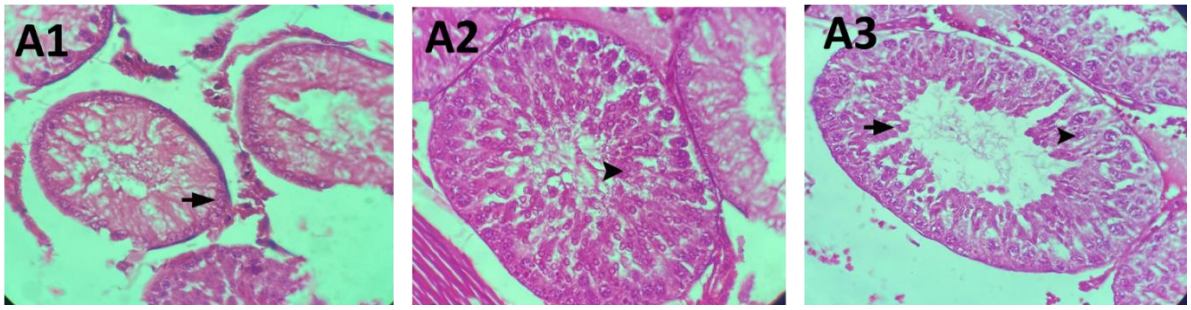
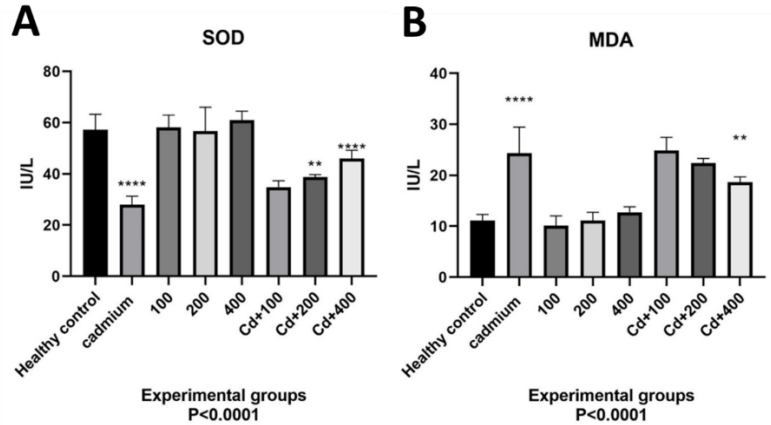


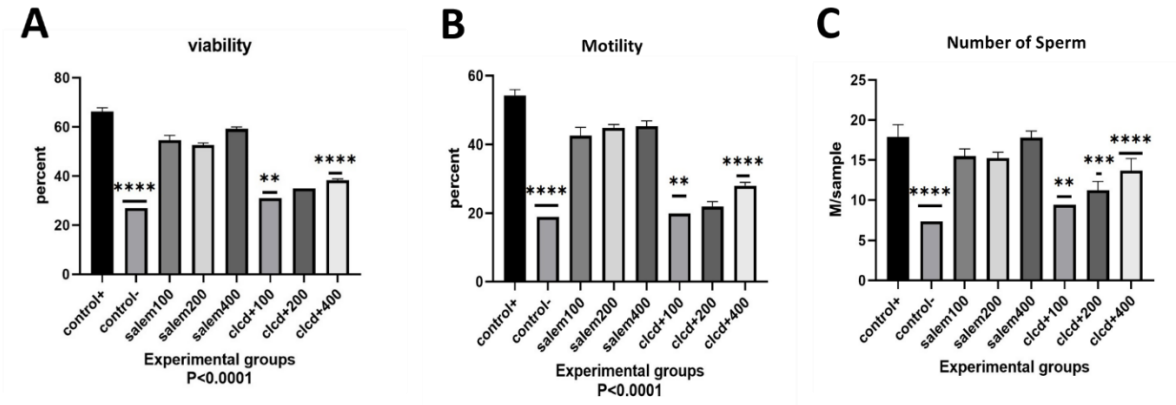
Fig. 3. Seminiferous tubules of the testes in the infertile groups treated with *M. neglecta* extract 100 mg/kg (A1), 200 mg/kg(A2), and 400 mg/kg (A3))



Graph 2. The analysis of oxidative stress in experimental groups, Oxidative stress analysis across experimental groups, including Superoxide Dismutase (SOD) activity (A) and Malondialdehyde (MDA) levels (B) ($P < 0.0001$).

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Graph 3. Sperm quality analyses, Evaluation of the quality of sperms: viability (A), motility (B), and concentration (millions/ml) (C).

4. Discussion

Male infertility is a significant global issue, influenced by a range of factors such as genetics, epigenetics, anatomical abnormalities, biochemical imbalances, hormonal disturbances, infections, immune system issues, lifestyle choices, and environmental exposures [17]. Due to the high cost and potential side effects of conventional pharmaceutical treatments for infertility, many individuals turn to alternative therapies, including herbal medicine.

M. neglecta has recently gained attention as a potential treatment for infertility. However, while most studies on this plant have focused on its effects on female reproductive health, there is limited research examining its impact on male infertility [18].

This study aimed to explore the in vitro effects of *M. neglecta* extract on male infertility induced by cadmium chloride. Histological examinations revealed that cadmium chloride induction resulted in significant damage to the seminiferous tubules [19].

In addition to its effects on fertility, *M. neglecta* has been shown to possess a broad spectrum of biological activities, such as antibacterial, antifungal, antiviral, anticancer, anti-toxic, and antioxidant effects. These beneficial properties are likely attributed to its antioxidant capabilities [20].

Several studies have evaluated the impact of *M. neglecta* on reducing convulsions in Balb/C mice. After intraperitoneal injection of *M. neglecta* extract, the results indicated a reduction in the frequency of convulsions, supporting the plant's antioxidant activity [21].

Furthermore, *M. neglecta* extract has demonstrated positive effects in treating urolithiasis induced by ethylene glycol and ammonium chloride. The results clearly showed that calcium oxalate deposition could be prevented, and interstitial tubular damage could be reduced in a dose-dependent manner. These therapeutic effects are likely linked to the plant's chemical components, including saponins, flavonoids, mucilage, and phenolic compounds.

According to this study, flavonoid compounds have a positive effect on reproductive tissues such as the testis, and it can be concluded that they are effective in reducing CaOx deposits and spermatogenesis in the testis tissue, both in terms of histopathology and sperm analysis [22].

Furthermore, antioxidant and flavonoid compounds found in *M. neglecta* extract have been shown to reduce the toxic effects of carbon tetrachloride in the liver [23], as well as to support the blood-testis barrier and aids in

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protecting and repairing sperm DNA in infertile men by reducing radical damage. In a previous study, examining the methanolic extract of *M. neglecta* extract in the healing of skin wounds in mice, they concluded that the use of *M. neglecta* extract in the form of a topical ointment for fourteen days due to its secondary metabolites and antioxidant properties is very effective in the healing of wounds [24].

Considering the increasing rate of infertility caused by testicular tissue dysfunction and disorders in sperm production or structure due to the increase in the number of oxidant compounds, as well as exposure to toxic compounds such as cadmium and its toxicity for human germ cells, it appears that the use of herbal compounds is critical as a barrier against antioxidant compounds. The results demonstrated the positive and effective impact of *M. neglecta* extract in mitigating the harmful effects of cadmium chloride. [25-27].

The sperm quality assessments, including viability, motility, and concentration, showed that the control group with infertility induced by cadmium chloride had the lowest values across all parameters. In contrast, treatment with *M. neglecta* extract led to a significant improvement in all of these characteristics.

As a result, the group with infertility was given 400 mg/kg *M. neglecta* extract, which showed increased viability, motility, and sperm concentration. The healthy control group exhibited the highest viability among all the groups, with the healthy experimental group following closely behind who were given 400 mg/kg *M. neglecta* extract, indicating the influence of *M. neglecta* antioxidants on sperm production and survival.

In experimental groups 6, 7, and 8, which were treated with 100, 200, and 400 mg/kg of *M. neglecta* extract, the protective effects of the herbal extract resulted in increased sperm percentages in comparison with the infertile group that only received $CdCl_2$. According to the graphs, the healthy experimental group treated with 400 mg/kg of *M. neglecta* extract showed the highest sperm count, surpassing even the healthy control group. This demonstrates the positive influence of *M. neglecta* extract on spermatogenesis.

The highest sperm motility was observed in the healthy control group, followed by the healthy experimental groups treated with 100, 200, and 400 mg/kg *M. neglecta* extract. The infertile control group, which was only administered cadmium chloride, showed the lowest motility [28].

5. Conclusion

The present study found that *M. neglecta* extract stimulated higher enzyme production in the healthy experimental groups than in the control group with no treatment. Additionally, increasing the dose of *M. neglecta* extract led to higher enzyme production in the infertile groups. MDA, a byproduct of lipid peroxidation, was present in the highest concentration in the infertile control group. However, after treatment with *M. neglecta* extract, which contains flavonoids and antioxidants, there was a significant reduction in lipid peroxidation and MDA levels. In contrast, the healthy experimental groups produced less MDA, similar to the levels found in the healthy control group.

The study demonstrated that *M. neglecta* extract has the potential to enhance spermatogenesis, protect testicular tissue from oxidative stress, and improve sperm motility, viability, and quantity under oxidative stress conditions. Future research should explore the effects of cadmium chloride and *M. neglecta* extract on the female reproductive system and

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ovulation processes. Additionally, examining the impact of these substances on sperm fertilization outcomes and calculating the rate of successful fertilizations could provide further insights. Expanding this research to include the reproductive systems of other species, such as cattle and sheep, would also be valuable. Given *M. neglecta*'s antioxidant properties, its potential use in treating mastitis in dairy cows warrants further investigation.

Conflict of interest

The authors declare that no known competing financial or personal interests were involved in conducting this research.

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Author Contributions

F. S.: methodology, conceptualization, validation, formal analysis, investigation, project administration. M. A. E.: editing, quality supervision, project administration. P. M.: conceptualization, writing original draft, methodology, validation, resources, data curation, writing review, and editing supervision.

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