



# Reduction of reproductive toxicity in murine sperm model using *Moringa peregrina* leaves extracts

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## ABSTRACT

Male infertility is a critical health issue associated with different causes, of which is oxidative stress. This study aims to investigate the protective effect of *Moringa peregrina* against male infertility. The antioxidant activity of leaf extracts was measured, and the phytochemical constituents of which phenols and flavonoids were quantified using standard methods. The effect of *M. peregrina* on male fertility profile was evaluated based on biochemical parameters and semen analysis. Serum levels of follicle-stimulating hormone, luteinizing hormone, and total testosterone were measured. Sperm motility and other properties such as morphology, vitality, teratogenicity, and speed tracking were reported. For the aqueous and methanolic extracts, the total phenol contents were  $170.64 \pm 0.94$  and  $185.78 \pm 4.12$  and total flavonoids content were  $60.22 \pm 1.3$  and  $57.03 \pm 2.4$   $\mu\text{g}/\text{mg}$  plant extract  $\pm$  SD, respectively. The obtained results confirmed the potential activity of the 500 mg/kg water extract in ameliorating the harmful effect of metronidazole and enhancing sperm quality. Significant improvement ( $p < 0.05$ ) in the physical characteristics of the semen was observed, while nonsignificant alteration in testosterone serum level and undetectable gonadotrophin levels were reported. *Moringa peregrina* water extract was suggested as a potential remedy for preventing asthenoteratozoospermia and necrozoospermia, with no effects on sex hormones.

## INTRODUCTION

The failure to conceive children after recurrent trials is experienced as a major social and psychological issue by couples worldwide (Cousineau and Domar, 2007). Infertility on account of the malefactor is reported to be responsible for nearly a third of infertility cases. Infertility rates differ concerning regional components, with the highest rate reported in Africa (Agarwal et al., 2015). Despite the limited knowledge of the reasons behind male infertility, declining sperm count has still considered the most

common cause. Other potential risk factors for male infertility were categorized as biological, physiological, genetic, lifestyle, environmental, and sociodemographic reasonings (Okonofua et al., 2022).

Medications' side effects on the endocrine system leading to hormonal disturbances and decreased sperm count could induce infertility even in males with healthy reproductive organs leading to temporary or, in some cases, permanent failure to conceive children (Mishail et al., 2009).

Metronidazole (MTZ) is a prescribed nitroimidazole antimicrobial for treating microbial infections of organs. It is also used to treat trichomoniasis, a sexually transmitted disease caused by a parasite (Margarita et al., 2022). The medicine MTZ is confirmed to have a negative effect on the spermatogenesis process. Male gonadotropins and testosterone levels would decrease after treatment with the drug (Sohrabi et al., 2007).

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Oxidative stress caused by boosting in the reactive oxygen species (ROS) generation or a decreased level of available antioxidants has been considered a causative agent of male infertility. The increase in ROS leads to generating poor sperm quality. Cytoplasmic droplets caused by spermiogenesis abnormalities have been reported to be the primary source of ROS (Gomez *et al.*, 1996; Mannucci *et al.*, 2022). Therefore, the effects exerted by oxidative stress on male reproductive health cannot be neglected.

The reliance on alternative herbal medicines, based on their claimed biological activities or in response to traditional recipes, is considered a common practice among individuals. Recently, published reports have declared the potential activity of herbal remedies against several illnesses, including hyperglycemia, hyperlipidemia, wounds, cytotoxicity, obesity, and dysmenorrhea (Al-Halaseh *et al.*, 2022a, 2022b; Al-Samydai *et al.*, 2022; Awwad *et al.*, 2023; Kattuo *et al.*, 2022).

Regarding infertility, plant extracts have been proven to enhance semen parameters and overall male fertility profile (Park *et al.*, 2017). Several *in vivo* and *in vitro* studies have demonstrated the benefit of herbal remedies in improving fertilization, such as *Ginkgo biloba*, *Glycyrrhiza uralensis*, *Zingiber officinale*, and *Silybum marianum* (Abedi *et al.*, 2016; Ahmed *et al.*, 2016; Khaki *et al.*, 2009; Wang *et al.*, 2016).

*Moringa* is a medicinal plant genus that contains flavonoids, quercetin, saponins, triterpenoids/steroids, cyanogens, alkaloids, vitamins, carotene, proteins, and other phenolics compounds in abundance (El-Haddad *et al.*, 2019; Widiastini *et al.*, 2022). There are 46 identified secondary metabolites in *Moringa* species known for their potent antioxidant properties. Their mechanism of action includes preventing new free radicals, inhibiting chain reactions, protecting cells in the body from free radical attacks, and preventing oxidative damage to most biomolecules (Sreelatha and Padma, 2011). *Moringa oleifera* was discovered to establish and preserve critical sperm functions, avoid excess sperm-produced superoxide, and conserve the acrosome reaction as well as DNA integrity (Moichela *et al.*, 2021).

*Moringa peregrina* is a Jordanian species that has colonized areas of high aridity and edaphic conditions in Jordan's southern regions, especially Wadi Araba (Alrawashdeh *et al.*, 2016). The phenolic and flavonoid content of *M. peregrina* leaf extract can be connected to its antioxidant activity, in addition to terpenes and other compounds found in the plant's aerial section (Dehshahri *et al.*, 2012; Hasan *et al.*, 2022).

In this study, we are considering investigating the male infertility prevention effect of the water extract of *M. peregrina* leaves in an *in vivo* study, considering its well-known antioxidant activity. In addition, this study aims to justify the self-prescription of *Moringa* herbal tea by folk as an infertility prophylactic agent used by men.

## MATERIAL AND METHODS

### Plant material

Fresh *M. peregrina* leaves were collected from South Jordan. The leaves were washed, dried at shades, and then

crushed into reduced size. Identification and authentication of the plant were made, and a voucher sample was deposited in the herbarium. Aqueous and methanolic extracts were prepared separately by maceration, then concentrated under reduced pressure. The aqueous was lyophilized using a Benchtop Manifold Freeze Dryer from Millrock Technology®, UK. On the other hand, the methanol extract was concentrated under reduced pressure using the Rotary-Evaporator machine.

The extraction yield % was estimated using Equation 1:

$$\text{Yield of extract (\%w/w)} = \frac{\text{dried extract weight}}{\text{dried leaves weight}} \times 100\% \quad (1)$$

## Phytochemical analysis

### Antioxidant activity

Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the specification published by Proestos *et al.* (2013) with some modifications. The radical scavenging activity was computed using Equation (2) for each sample after measuring the absorbance at  $\lambda_{\text{max}} = 517 \text{ nm}$ .

$$\% \text{ inhibition} = \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (2)$$

where  $A_{\text{control}}$  is the absorbance of the control sample and  $A_{\text{sample}}$  is the absorbance of the sample.

The effective concentration required to scavenge 50% of the free radicals ( $IC_{50}$ ) was calculated and used to compare the antioxidant activity of each extract to each other and to the ascorbic acid standard solution prepared at various concentrations.

### Determination of total flavonoids and total phenol content

Total flavonoids were determined using a colorimetric approach based on the production of a complex flavonoid–aluminum complex, as reported by Agbo *et al.* (2015). The total flavonoid content was calculated as quercetin equivalent.

The Folin–Ciocalteu technique was used to determine total phenol content following the specification reported by Margraf *et al.* (2015). The total flavonoid content was calculated as gallic acid equivalent.

### Animal model

Male albino mice, with an average weight of  $28 \pm 3.21 \text{ g}$ , were obtained from the Animal House, Faculty of Pharmacy, Mutah University. Male mice were moved from the animal house to the animal laboratory a week before the experiment to be acclimatized.

### Ethical approval for the study

All procedures were performed in accordance with international regulations for the care and use of laboratory animals. Ethical approval on the study was obtained by the Institutional Review Board at Mutah University, Karak, Jordan, Approval no. 6/2021/2022.

### Induction of male infertility

Infertility was gradually induced in male mice after successive daily intraperitoneal doses of MTZ solution at a concentration of 40 mg/kg. The protocol was adopted and modified after several pilot studies based on previous work by Al-Alami *et al.* (2017). The experimental animals were distributed into four groups of eight mice each as follows: the negative control group (no medication was given), the positive control group (given MTZ), the low-dose treatment group (MTZ doses in combination with the herbal aqueous extract (500 mg/kg), and the high-dose treatment group (MTZ doses in combination the herbal aqueous extract at a concentration of (1,000 mg/kg). The medications were given daily via the intraperitoneal route for 2 weeks. The prophylactic herbal treatments were given 8 hours prior to the MTZ doses. No anesthesia was needed during this experiment. The experimental animals were confirmed to develop infertility when semen analysis showed at least one abnormal result, including azoospermia, inactivity, teratozoospermia, and motility.

### Blood samples collection

Blood samples were collected from the heart of the experimental animals under mild anesthesia using diethyl ether. The mouse was constrained on the anatomical plate, a cotton pad filled with the anesthetic agent covering the nostrils. Blood samples were collected in gel tubes and centrifuged for 5 minutes (3,500 rpm). The supernatant was collected and kept at  $-20^{\circ}\text{C}$  for further biochemical analysis.

### Hormonal analysis

Serum analysis for hormonal profile was undertaken in MedLabs® AlKarak branch. The collected serum was analyzed for the concentration of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and total testosterone.

### Semen collection and analysis

Directly after collecting the blood samples, the vasa deferentia and cauda epididymis were dissected using a sterile surgical blade. The sperm was stripped from the deferens and the epididymis was cut in a test tube containing 1 ml of normal saline. The sperm suspension samples were transferred to the laboratory for further analysis. The freshly extracted samples were analyzed via Spermolyzer®, computed-assisted semen analysis. Physical properties, including volume, pH, color, odor, and liquefaction time, were recorded. In addition, concentration, total sperm count, vitality, and morphology were investigated.

Sperms morphology was considered normal according to the morphology index and teratogenicity index compared to the negative control group. These are concluded after examining the head abnormalities, neck and midpiece abnormalities, excess residual cytoplasm, and tail abnormalities. The visualized sperm were compared to schematic drawings of abnormal sperms by expert laboratorians.

### Statistical analysis

Data analysis was performed using Microsoft 13 Excel sheet to calculate the average  $\pm$  SD for the numerical data. *p*-values were computed using data analysis and paired two samples for the mean. Significant values were considered at  $p < 0.05$ .

## RESULTS

### Extraction yield

The extraction yields for the aqueous and methanol extracts of *M. peregrina* leaves were calculated ( $3.65\% \pm 0.002\%$  and  $4.08\% \pm 0.025\%$ ) % w/w dry weight  $\pm$  SD.

### Phytochemical analysis

#### Antioxidant effect (DPPH assay) for *M. peregrina* leaf extracts

Free radical inhibition activity was used to calculate the antioxidant efficiency of serial concentrations of plant extracts and the standard ascorbic acid using the scavenging DPPH assay methods. Figure 1 demonstrates the percentage inhibition of free radical activity for serial concentrations of plant extracts and ascorbic acid.

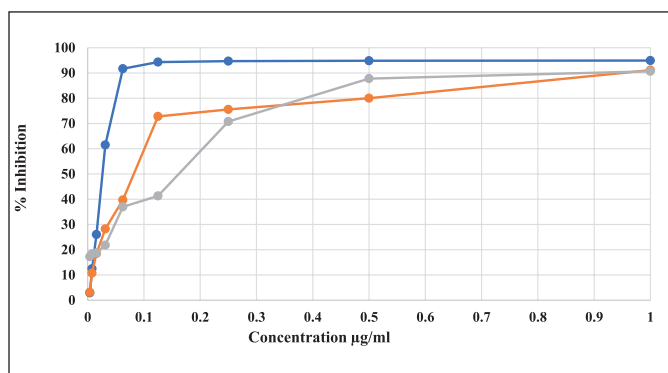
#### Quantification of total flavonoid and total phenolic in *M. peregrina* leaf extracts

According to the findings, comparable levels of total flavonoids and total phenols were found in both the aqueous and methanolic extracts. The average total phenols content for the aqueous and methanolic extracts were found to be  $170.64 \pm 0.94$  and  $185.78 \pm 4.12$   $\mu\text{g}/\text{mg}$  plant extract  $\pm$  SD, respectively. The average total flavonoids content for the aqueous and methanolic extracts were found to be  $60.22 \pm 1.3$  and  $57.03 \pm 2$   $\mu\text{g}/\text{mg}$  plant extract  $\pm$  SD, respectively.

### In vivo animal study

#### Serum sex hormones analysis

The aqueous plant extract showed no effect on the levels of both FSH and LH hormones. The total testosterone levels showed to be lowered using the prepared plant extract with a nonsignificant effect. The concentrations of FSH and LH in all the tested groups were undetectable ( $<0.3$  IU/l). The concentration of total testosterone dropped after inducing toxicity with MTZ to be  $1.249 \pm 0.376$  compared to  $4.563 \pm 3.46$  ng/ml in untreated groups. Treatment with the herbal remedy failed to significantly reverse the effect of MTZ on the total testosterone; the total concentration after treatment with low and high doses was found to be  $0.772 \pm 0.443$  and  $0.27 \pm 0.006$  ng/ml.



**Figure 1.** The % inhibition of free radical activity for serial concentrations of plant extracts and ascorbic acid, using the scavenging DPPH assay methods. The percentage inhibition of ascorbic acid is represented in blue, the aqueous extract in orange, and for the methanol extract in grey color.

### Semen analysis

No variations were detected in the physical properties of the tested semen samples in all experimental groups. The pH was measured to be six in both the negative and positive control groups, while it was measured to be slightly higher (pH = 7) in the groups treated with low- and high-dose herbal extracts. There was no variation in color and odor to be recorded.

Semen analysis for sperm count, motility, and vitality for all experimental animal groups is shown in Table 1. Results showed that sperm count and motility did not improve when using the plant extract as a prophylactic agent in combination with MTZ. On the other hand, sperm vitality showed an improvement when treated with plant extract as a prophylactic agent in combination with MTZ.

The progressive and the nonprogressive motility patterns in the sperms were color-coded by the Spermolyzer® and then analyzed. Images showing different types of motility were taken, and reports were generated by expert laboratorians.

A dramatic drop in the percentage of viable sperms exhibiting progressive speed patterns was recorded after treatment with MTZ. On the other hand, conjugated prophylactic herbal doses enhanced the motility patterns and increased the percentage of progressive motile viable sperms compared to those treated only with MTZ. Overall, herbal treatment improves not only total motility but also the pattern of motility and the spermatozoa speed track. The percentage of sperms that exhibits different speed rates in the semen sample was analyzed, as shown in Figure 2. The graphs indicate an increase in the percentage of sperms that exhibit higher speed after treatment with the herbal remedy (low and high concentration).

According to the vitality parameter, live and dead sperms were color-coded by the Spermolyzer®, and the generated reports were read by expert laboratorians. The percentage of viable sperms was computed via the computer-assisted system. The curled tail sperms were considered to live, while sperms with straight tails were considered dead. Viable sperms have semipermeable cell membranes which allow the passage of surrounding fluid across the membrane. The accumulated fluid should result in swollen spermatozoa. Therefore, swollen cytoplasm causes the tail to curl. On the other side, dead sperms have damaged membranes that allow free passing of the fluid in and out of the cytoplasm with no accumulation; accordingly, the tail remains straight. Sperms vitality was enhanced after treating the experimental animals with the herbal remedy compared to those treated only with MTZ (Table 1).

Regarding teratogenicity, the percentage of normal to terato sperms was reported by the computer-assisted system and expert laboratorians. Sperms were considered normal when no

defect in the head, tail, neck, midpiece, and the excess residual cytoplasm were reported. Teratogenicity is confirmed when the mean of the terato sperms exceeds the reference value of morphological specialties. The scan system reported a tapered and big head as the most observed defect of the examined sperms in the MTZ-treated groups. Expert laboratorians described other defects after closely examining the slides, such as a detached head and abnormal cytoplasmic droplets. The morphology of the scanned sperms showed more abnormalities in the MTZ-treated groups than that of the negative control. A slight reduction in the number of teratogenic sperms after treatment with the herbal remedy was reported by at least two expert laboratorians with no statistical data. Clarifying images are shown in Figure 3.

### DISCUSSION

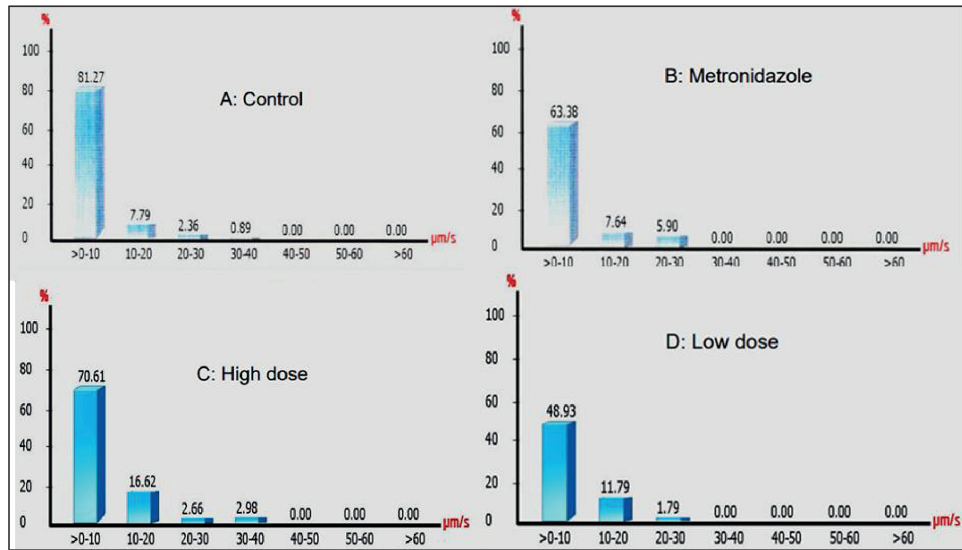
Findings suggest that the *M. peregrina* leaves aqueous extract as a potential remedy for the reduction of reproductive toxicity in the murine sperm model that was induced by MTZ medication. In addition, improvement in the physical characteristics of the semen but not the hormonal values are interesting findings to investigate. In the interpretation of the obtained results, we compared them with the previously published reports. Previous reports support our findings, where low FSH and LH values do not necessarily negatively affect the fertility window (Ma *et al.*, 2022). Some other reports confirm the negative correlations between FSH value and the morphology of the sperm (Meeker *et al.*, 2007). Although the role of testosterone in male puberty and maturation is not doubtful, some controversial reports support our findings and conclude that decreased serum testosterone levels may not account for sperm motility. There is a lack of correlation between testosterone serum level and sperm's physical characteristics, in particular motility.

There was good agreement with the result reported by Al-Owaisi *et al.* (2014), as the antioxidant effects of the aqueous and methanolic extracts had similar IC<sub>50</sub>. The prepared crude extracts showed to contain a variety of plant phytochemical components, revealed by phytochemical analysis, including phenols and flavonoids, among many others. The methanolic extract was found to contain the highest phenolic content. The total flavonoid content of the two extracts was comparable and relatively equal. Therefore, the antioxidant activity of *M. peregrina* leaf extract can be linked to its phenolic and flavonoid content, which agrees with several reports on the Moringaceae family (Dehshahri *et al.*, 2012; Senthilkumar *et al.*, 2018).

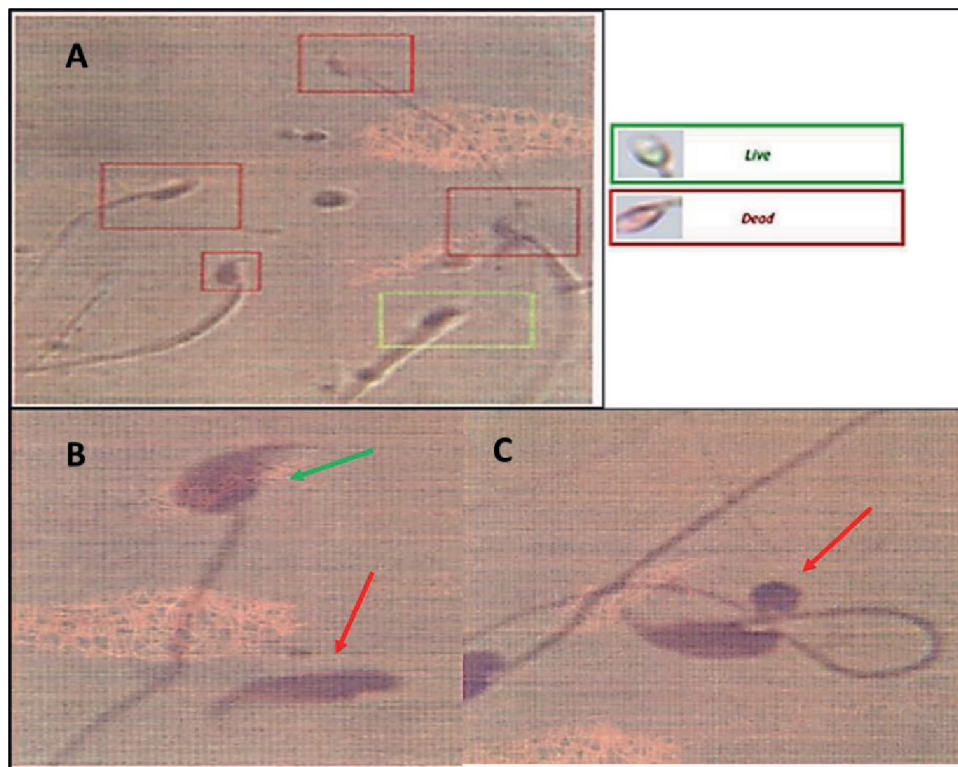
Various phytocompounds have been previously detected in the study plant extract by our research group (Hasan *et al.*, 2022), demonstrating significant pharmacological actions, including protection effects for male fertility. Of special interest in this study are the flavonoid compounds, including spiraeoside, known to act

**Table 1.** Semen analysis for sperm count, motility, and vitality for all experimental animal groups.

| Sample group                       | Semen analysis                |                                     |   |  | Reference value |
|------------------------------------|-------------------------------|-------------------------------------|---|--|-----------------|
|                                    | Negative control (value ± SD) | MTZ (positive control) (value ± SD) | Low-dose (500 mg/kg) extract (value ± SD) | High-dose (1,000 mg/kg) extract (value ± SD) |                 |
| Total sperm count (n = 8)          | 19.54 ± 5.63                  | 8.73 ± 3.35                         | 8.27 ± 2.11                               | 9.64 ± 3.96                                  | >15 million/ml  |
| Total motility (PR and NP) (n = 8) | >40%                          | 18.74 ± 4.05                        | 19.423 ± 5.38                             | 17.85 ± 7.99                                 | >40%            |
| Vitality (n = 8)                   | 34.26 ± 6.52                  | 21.46 ± 1.80                        | 36.52 ± 6.16                              | 24.68 ± 2.06                                 | >58%            |



**Figure 2.** Sperms speed tracking. (A) control; (B) MTZ-treated group; (C) high-dose extract-treated group (1,000 mg/kg); and (D) low-dose extract-treated group (500 mg/kg).



**Figure 3.** Sperms viability and morphology: (A) live sperm is surrounded by a green rectangle, while dead sperm are surrounded by red rectangles. (B) a green arrow points to a normal sperm and a red arrow points to a sperm with a detached head. (C) a red arrow points to cytoplasmic droplet abnormality.

by regulating abnormal sex hormones, in addition to decreasing oxidative stress and improving fertility and apoptosis (Karna *et al.*, 2019). Rutin is also known to possess a protective role in the context of male reproduction, but further studies remain to be elucidated (Kolarevic *et al.*, 2019; Sun *et al.*, 2017). Chlorogenic acid was also found to increase the sex hormone binding globulin,

which plays an important role in male infertile (Esther *et al.*, 2017). Succinic acid was shown to restore reproductive ability in 85.6% of cows (Mikhailova *et al.*, 2021). Benzoic acid was also found to affect male infertility by altering DNA integrity in germ cells (Rabiu *et al.*, 2021). Similarly, 2,5-dihydroxybenzoic acid was found to lower seminal plasma lipid peroxidation levels

but increase sperm nuclear DNA fragmentation (Camargo *et al.*, 2014). In addition, 4-hydroxybenzoic acid was found to play an important role in the treatment of infertility among bovines via mitochondria-directed antioxidant effect when it was studied on bovine sperm function and embryo production (Santos *et al.*, 2022). *p*-Coumaric acid was found to have a protective effect on sperm parameters and apoptosis. It can also protect from ethanol-induced testicular dysfunction and improve fertility (Nishi *et al.*, 2018). Caffeic acid was found to have an inhibitory effect on testicular damage induced by Cd, where this effect is shared by the most possible pathway, scavenging ROS extensively (El-Refaei and Abdallah, 2021).

Therefore, the findings of the present study would be of special interest among researchers in the failure of herbal medicine, infertility, and pharmaceutical nutrition that are seeking the power of natural medicine and the discovery of new treatments, which would be utilized to improve human health.

## CONCLUSION

The proposed activity *M. peregrina* was largely referring to the antioxidant properties of the plant extract, which contains numerous valuable constituents such as polyphenols and flavonoids. Many detected compounds were previously reported to have protective and treatment roles on male infertility. These findings were important when treating male infertility as directed toward semen's physical properties rather than hormonal imbalances. It would also suggest the investigation of the combined effect of the study plant extract with other conventional medications that are widely used to treat male infertility, with the aim of getting the benefit of potentially additive effects. This approach of alternative treatments is well recognized among traditional and herbal medicine healers.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## CONFLICTS OF INTEREST

The authors declare that no conflicts of interest are associated with this work.

## ETHICAL APPROVAL

All procedures were performed in accordance with international regulations for the care and use of laboratory animals. Ethical approval on the study was obtained by the Institutional Review Board at Mutah University, Karak, Jordan, Approval no. 6/2021/2022.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

## PUBLISHER'S NOTE

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