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Protective effects of *Calligonum comosum* extract on histological alterations and apoptosis-related gene expression in vitrified-thawed neonatal mouse testicular tissue

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ABSTRACT

Introduction: In this study, we aimed to evaluate the antioxidant effect of the *Calligonum comosum (C. comosum)* extract on the improvement of spermatogenesis in vitro.

Methods: Neonatal testicular tissues were divided into four groups and cultured for two weeks: freshly cultured tissues (F2W); freshly cultured tissues supplemented with 10 µg/mL *C. comosum* extract (F2W + Ca); frozen–thawed tissues (FT2W); and frozen–thawed tissues supplemented with 10 µg/mL *C. comosum* extract (FT2W + Ca). Then, the expression of apoptosis-related genes (*Bcl2*, *Bax*, *P53*, and *Fas*) and the number of seminiferous tubules were evaluated after culture.

Results: The results of gene expression indicated significant upregulation of the *Bax*, *P53* and *Fas* genes in the FT2W group compare to the F2W group ($P < 0.001$). The C. comosum extract significantly downregulated the expression of *Bax*, *P53* and *Fas* genes in neonatal testicular tissue of the FT2W+ Ca group compared to the FT2W group ($P < 0.001$). Histological results showed that the number of abnormal tubules in neonatal testicular tissue considerably increased in the FT2W group compared to the F2W group ($P < 0.001$). Additionally, the *C. comosum* extract significantly reduced the number of abnormal seminiferous tubules in testicular tissue of the FT2W+ Ca group compared to the FT2W group ($P < 0.001$).

Conclusions: The *C. comosum* extract was associated with reduced histological abnormalities and decreased the expression of apoptosis-related genes in cultured neonatal mouse testicular tissue.

Keywords: Spermatogenesis, *Calligonum* camosum, Testicular tissue, Testosterone.

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Introduction

In male, fertility ability depends on the process of spermatogenesis. The process of spermatogenesis occurs within the seminiferous tubules of the testis. Spermatogenesis is a process that takes place in the seminiferous tubules of the testis (Walker, 2022).Recently, the prevalence of infertility in men has increased due to diseases and environmental factors that negatively affect spermatogenesis (Nazari et al., 2019; Silva et al., 2022).

Cancer can affect spermatogenesis and lead to infertility in men. Chemotherapeutic agents are widely recognized as an important factor of gonadotoxicity that lead to infertility in men(Quinn et al., 2025; Tiwari et al., 2024). Currently, sperm or testicular tissue cryopreservation are methods used for fertility preservation in male cancer patients (Li et al., 2024; Mansi et al.). There are two methods for freezing testicular tissue: slow freezing and vitrification. Two strategies exist for the cryopreservation of testicular tissue, which include vitrification and slow freezing (Mohaqiq et al., 2018). In vitro maturation (IVM) method generated sperm from immature fresh or cryopreserved testicular tissues (Oblette et al., 2017; Yokonishi et al., 2014). The freeze-thaw process of testicular tissue may damage spermatogenesis resulting from the production of reactive oxygen species (ROS) (Fleming & Thomson, 2025) . ROS can induce apoptosis and cause testicular tissue damage (Sanei-Ataabadi et al., 2026). Programmed cell death, commonly referred to as apoptosis, is a essential process essential for maintaining tissue homeostasis.

Disruption in the apoptosis process leads to various disorders including cancer, infertility, autoimmune and neurodegenerative diseases (Mosadegh et al., 2025).The integrity of seminiferous tubules is crucial for adult sperm production, as any damage to structural integrity of seminiferous tubules can lead to reduce fertility or abnormalities in sperm quality (Misiakiewicz-Has et al., 2021). However, testicular tissue cryopreservation has disadvantages, and there is a vital need to establish advanced treatment strategies for men affected by cancer.

Medicinal plants have bioactive compounds that possess antioxidative, anti-inflammatory, anti-apoptotic , antibacterial and antimicrobial properties (El-Saadony et al., 2025). These medicinal species exhibit extensive therapeutic applications in the various disorders such as, oncological, diabetes ,infectious, cardiovascular, inflammatory, and gastrohepatic disorders.

C. comosum (Polygonaceae), is a desert plant that is widely distributed in North Africa, South Europe, and Western Asia (Belaabed et al., 2017). The *C. comosum* has various features, including anti-inflammatory, anti-apoptosis , anti-oxidant, anti-microbial, , and anti-cancer activity (CHOUIKH et al., 2020). This plant is used to treat different diseases such as toothache, hepatotoxicities, polycystic ovaries, male infertility, wound healing, osteoporosis, gum sores, and ulcer (Barati & Movahedin, 2021; Tahmasebi et al., 2018). According to properties of *C. comosum*. in this research, we investigated the effects of this plant on maintaining testicular tissue integrity and reducing apoptosis.

Materials and Methods

Experimental animal

This experimental work was performed on NMRI mice. Six-day-old NMRI neonatal male mice prepared from the Royan Institute of Iran. The sample size for each experimental group was n=5 mice.The mice were housed under a 12-12 hour dark -light- cycle and had free access to food and water. Newborn male offspring were separated and were anesthetized by 1 % xylazine and ketamine and. Then, we removed the male offspring testes of all groups. Then, testicular tissue was sectioned into approximately 1 mm^3 fragments, and randomly distributed among four experimental groups: F2w group: Freshly cultured testicular tissue samples for 2 weeks without treatment; F2W+ *C. comosum* (Ca) group: Freshly cultured testis tissue samples with 10 µg/ml *C. comosum* extract for 2 weeks, FT2W group: Frozen-thawed testicular tissue samples cultured for 2 weeks without treatment and FT2W+ Ca group: Freezing-thawinged testicular tissue samples cultured with dosage of 10 µg/ml *C. comosum* extract for 2 weeks. After 2 weeks of testicular tissue culture in different groups, to investigate the effect of *C. comosum*, the histological changes and gene expression were examined. All experimental procedures were approved by the institutional animal ethics committee of Islamic azad university (Ethical code: IR.I AU.SRB.REC.1348.115).

Freezeing-thawing method of testicular tissue

In this study, we performed neonatal testicular tissue vitrification based on a previously published protocol (Radaelli et al., 2017). Briefly, the neonatal testicular tissues were transferred to a cryovial containing vitrification solution 1, with 7.5% DMSO (Sigma-Aldrich), 7.5% ethylene glycol (EG, Sigma-Aldrich), and 20 % fetal bovine serum (FBS, Life Technologies) for 10 min at 4°C. Then, the pieces were transferred to a vitrification solution 2, which consisted of 15% EG, 20% FBS, 0.5 M sucrose and 15% DMSO, for 2 minutes at 4°C. The testicular tissue fragments were transferred into cryovials containing liquid nitrogen for 24. Then, the cryovials were isolated from the storage tank, and the samples were thawed.

Preparation of C. comosum extract

We isolated the *C. comosum* plant extract according to previously published protocol (Kiani et al., 2019). Briefly, various parts of *C. comosum*, such as root, fruit, stem, and flower were collected from Isfahan province. The parts were cut into smaller pieces and left to dry in the shade under room-temperature conditions for 4 weeks.

Then, the dried pieces powdered and immersed in 70% ethanol (Merck) in a shaker for 3 days at room temperature. Ethanol and water were evaporated to dryness using Rotary Evaporator (60 rpm at 40 °C). Finally, the extract is obtained and stored at 4°C. 20 mg of *C. comosum* extract was dissolved in 40 µl of DMSO. In this study, the selected dose is 10 µg/ml, which was chosen based on the previous studies (Barati et al., 2018; Kumar et al., 2022). In order to prepare this dose, 1 ml of the resulting

solution was dissolved in 50 ml of the base culture medium to prepare the medium, and then filtered through a 0.45 µm millipore filter.

Culture of testicular tissue

Agarose gel was used to culture testicular tissues. We prepared agarose gel according to previously published protocol (Yokonishi et al., 2013). Briefly, to prepare agarose 1.5% w/v agarose solution, agarose (1.5 g; Merck) was dissolved in 100 mL of distilled water. About 2 ml of 1.5% this solution was placed in each well of the 6-well plates for 2 h. After stiffening the gel, the segments of neonatal testicular tissue were placed in 6-well plates containing α-MEM culture medium (Life Technologies) together with 10% knockout serum replacement (KSR, Gibco), 60ng/ml progesterone (Gibco), 30 ng/ml β-estradiol (Gibco), 20 ng/ml EGF (Sigma-Aldrich), 10 ng/ml bFGF (Royan Biotech), 10 ng/ml GDNF (Sigma-Aldrich), and 10 ng/ml LIF (Gibco).

Histological analysis

We performed the histological analysis for morphological assessment, according to previous study (Moradian & Movahedin, 2025). Briefly, cultured testicular samples were collected and fixed in Bouin's fixative for 4 h at room temperature. The fixed tissue samples were then embedded in paraffin and serially sectioned into 5-µm-thick slices. Subsequently, hematoxylin and eosin (H&E) staining was carried out for histologic analysis under a light microscope (Olympus, Japan). Finally, we quantified the number of seminiferous tubules in the testicular tissues based on parameters such as the adhesion of cells to the basement membrane, cell cohesion, proportion of pyknotic nuclei (Moradian & Movahedin, 2025) (15 fields in 5 histological sections for each group, and results were expressed as the number of areas per mm^2.

Gene expression analysis

We performed qRT-PCR method for the anti-apoptosis gene [B-cell lymphoma 2 (*Bcl2*)], apoptosis genes [Bcl-2-associated X protein (*Bax*), tumor protein P53 (*P53*) and Fas], in testicular tissues of the all groups. Testicular tissue total RNA was extracted and purified with a total RNA purification kit (Takara). Then, 2 µg of total RNA was used for cDNA synthesis using the using the cDNA synthesis kit (Fermentas) according to the producer's protocol. QRT-PCR reactions were conducted using SYBR Green Master Mix (Jena Bioscience) and a real-time PCR system (Corbett Life Science; Rotor-Gene 6000 instrument). 2 µl of diluted cDNA template (25 ng) were added to the reaction mixture's final volume (20 µl). Each sample was run in three biological replicates to ensure technical reproducibility. The qRT-PCR thermal cycling conditions were as follows: an initial denaturation step at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing at 57°C for 30 s. A melting curve analysis was performed at 72°C for 30 s to ensure primer specificity, and a final extension step was conducted at 72°C for 5 min. The samples were obtained from three separate biological replicates. Gene expression was determined using the $2^{-\Delta\Delta CT}$ method and fold changes were normalized to the β-actin. Table 1 presents the primer sequences used for qRT-PCR analysis.

Statistical analysis

All experiments were performed in at least three independent replicates. Results are presented as the mean ± standard error of the mean (SEM). Statistical significance among groups was assessed using two-way ANOVA followed by Tukey's post-hoc test. The data were analyzed by Prism software. $p < 0.05$ was considered statistically significant.

Table 1. Primers for qRT-PCR analysis

Primer name	Accession number	Primer sequence	Length
Bax	NM-017059.2	F: 5' TTTGCTACAGGGTTTCATCCAG 3' R: 5'GTCCAGTTCATCGCCAATTC 3'	139bp
Bcl2	NM-016993.1	F: 5' GAGAGCGTCAACAGGGAGAT3' R: 5'ACAGCCAGGAGAAATCAAACA3'	169bp
P53	NM-020022.7	F:5'AGTCACAGCACATGACGGAG3' R:5'CACCCGGATAAGATGCTGGG3'	189bp
Fas	NM-002200.7	F:5'GTCCTGCCTCTGGTGCTTG3' R:5'AGCAAAATGGGCCTCCTTGA3'	220bp
β-actin	NM-017059.2	F:5'TTACTGAGCTGCGTTTTACAC3' R:5'ACAAAGCCATGCCAATGTTG3'	270bp

Results

Histological of testicular tissue

The results of H&E staining indicated that in the FT2W group, the seminiferous tubules were damaged and interstitial tissue was disrupted with of apoptotic cells (Figure 1). Moreover, the number of abnormal tubules was significantly increased in FT2W group compared to the F2W group (Figure 2) ($P < 0.001$). The *C. comosum* extract improved the damaged testicular tissue after freeze-thaw process(Figure 1) . Furthermore, this extract caused a significant decrease in the number of abnormal tubules in the testicular tissue of FT2W+ Ca group compared to the FT2W group (Figure 2) ($P < 0.001$) .

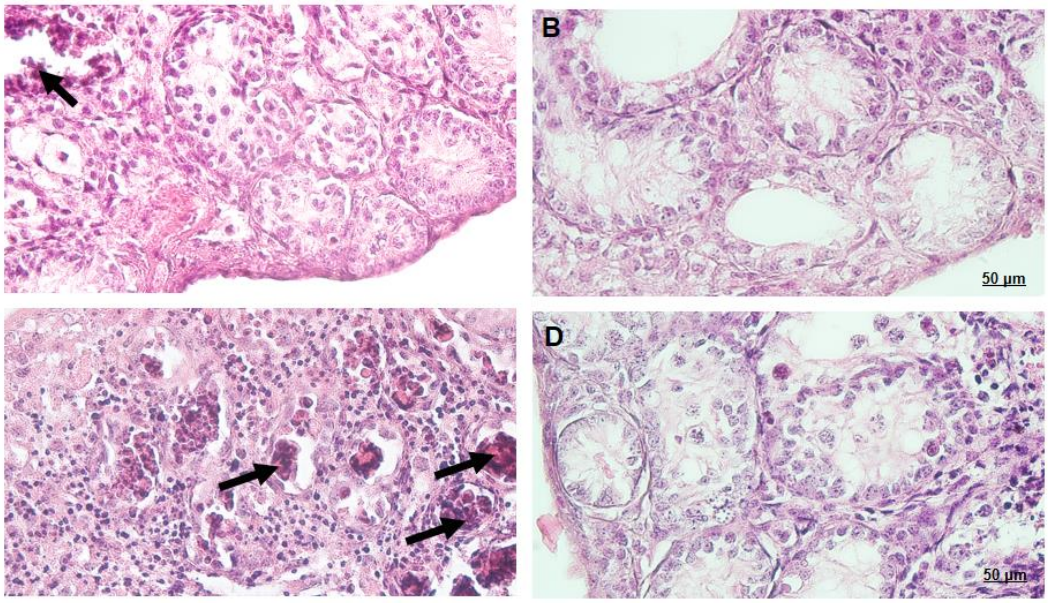


Figure 1. Histological analysis of testicular tubule: A) Freshly cultured testicular tissue B) F2W group+ 10 µg/ml *C. comosum* C) Frozen-thawed testicular tissues(FT2W) D) FT2W group+ 10 µg/ml *C. comosum*. The results of histology indicated that the the freeze-thaw process has damaged the testicular tissue, and the *C. comosum* extract reduce necrosis in testicular tissue. Arrows indicate necrotic areas.

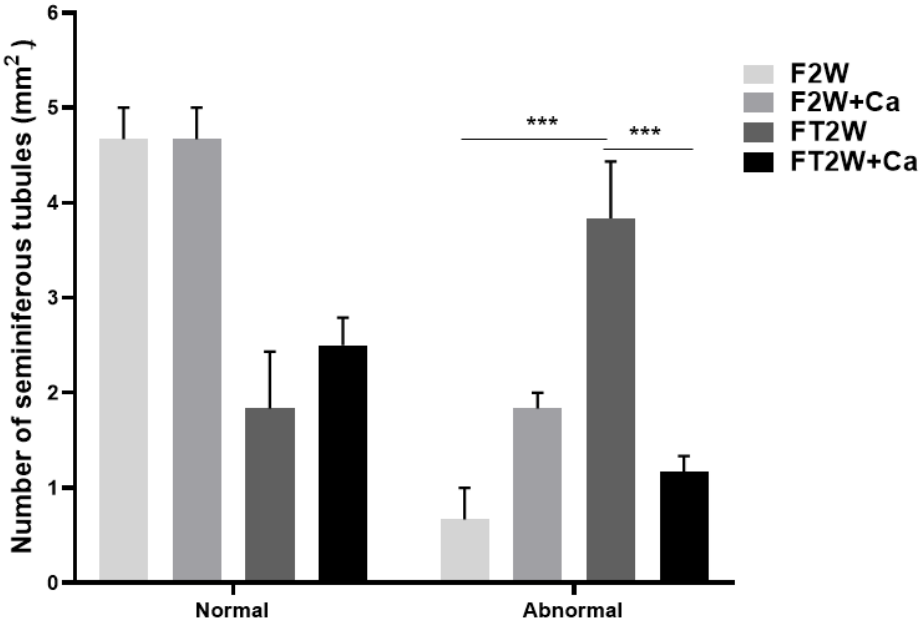


Figure 2. The number of seminiferous tubules in testicular tissue: The results demonstrated that the number of abnormal seminiferous tubules was significantly increased in FT2W group, whereas it was significantly reduced in FT2W group after treatment with the *C. comosum* extract. Results are expressed as Mean ± SE, n=5, ***$P < 0.001$

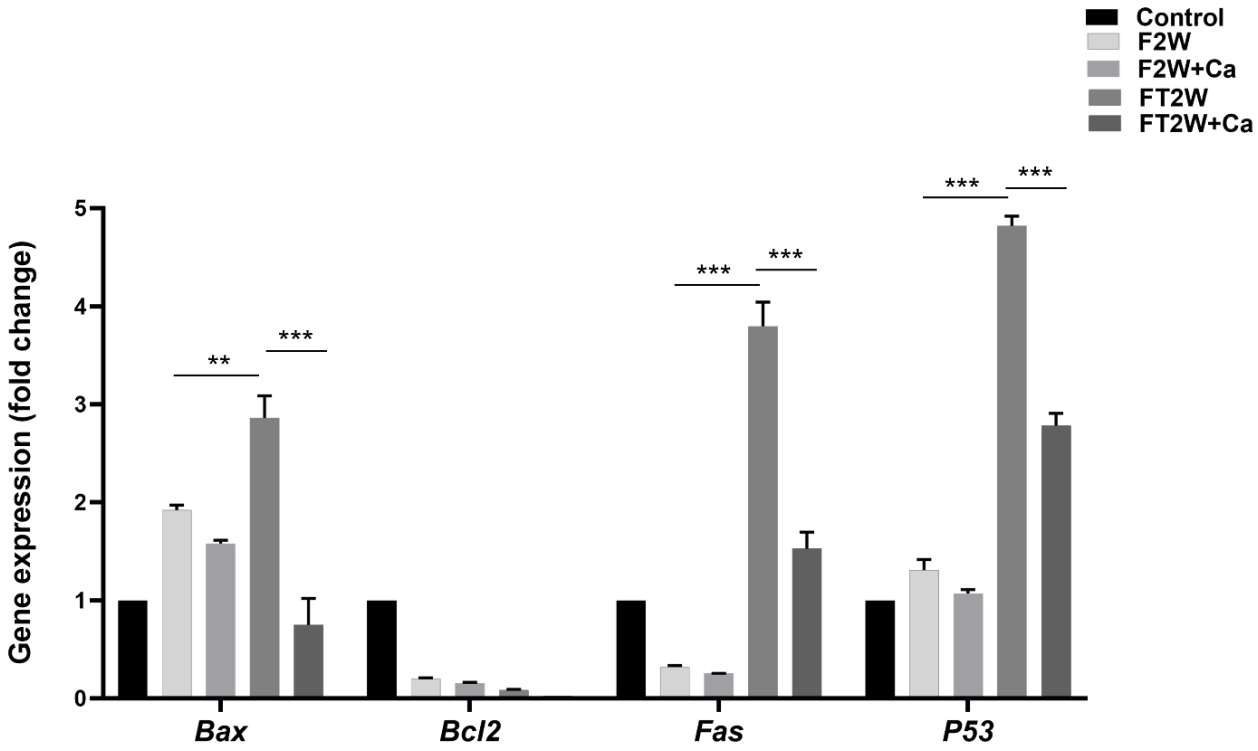


Figure 3. The expression levels of apoptosis genes: The results indicated that the expression of *Bax*, *P53* and *Fas* genes had a significant increase after the freeze-thaw process, while the *C. comosum* extract significantly decreased the expression of *P53* , *Bax*, and *Fas* genes in testicular tissue of FT2W+ Ca group. Results are expressed as Mean ± SE, n=5, ***P < 0.001, **P < 0.01.

Assessment of gene expression

The results of qRT-PCR showed that the freeze-thaw process significantly increased the expression of *Bax*, *P53* and *Fas* genes in the FT2W group compare to the F2W group (P < 0.001). Moreover, the *C. comosum* extract was significantly decreased in the expression of *P53*, *Bax*, and *Fas* genes in neonatal testicular tissue of the FT2W+ Ca group compared to the FT2W group (P < 0.001) (Figure 3). The expression of *Bcl2* showed no significant alterations among the experimental groups . Therefore, *C. comosum* extract was associated with decreased expression of *Bax*, *Fas*, and *P53*, suggesting a potential modulatory effect on apoptosis-related pathways.

Discussion

Recently, the incidence of infertility in men has enhanced due to various reasons (Nordkap et al., 2012). In cancer patients, the use of chemotherapy drugs leads to damage to testicular tissue and infertility (Bahrehbar et al., 2021; Eslami et al., 2023; Zi et al., 2022). Testicular tissue transplantation is a suitable treatment method for patients who freeze their testicular tissue before chemotherapy (Mohaqiq et al., 2019). Freezing-thawing of testicular tissue increases oxidative stress and lead to damage to spermatogenesis (Boroujeni et al., 2019; Ozkavukcu et al., 2008). Therefore, it is essential to a method to reduce oxidative stress, and medicinal plants are suitable due to antioxidant properties.

Several studies showed that antioxidants of plant extracts lead to improving in the process of spermatogenesis in different stages including increase in the number of leydig and sperm cells, sperm survival and sperm normal morphology (Kenji & Kou, 2010; Mohammadi et al., 2008; Naseri et al., 2011). *C. comosum* has many compounds that these compounds had antioxidant properties (Belaabed et al., 2017).

Considering the prevalence of infertility in men with cancer, and the antioxidant properties of *C. comosum*, we investigated the effect of the *C. comosum* on development spermatogenesis in fresh and freezing/thawing testicular tissues of six-day-old mice. Six-day-old mice were selected because their testes are still immature and germ-cell development is ongoing at this stage. This model enabled us to investigate the effects of vitrification and the potential protective effects of *C.comosum* extract on developing testicular tissue.

1. In this study, histological evaluation indicated the degeneration of seminiferous tubules in the testicular tissue of FT2W group . Furthermore, the *C. comosum* extract decreased the number of abnormal tubules in the testicular tissue of FT2W+ Ca group . In line with our results, previous a study showed the freeze-thaw process induced structural alterations in the seminiferous tubules of immature male mice. They also reported that, vitamin E protects the seminiferous tubules against oxidative stress resulting from freeze–thaw process. (Kushki et al., 2016). Gholami et al. showed that the freeze-thaw process caused damage to the seminiferous tubules in the testis of immature mice. They reported that selenium, due to its antioxidant activity, could reduce the oxidative stress effects of the freeze-thaw process and decrease seminiferous tubules damage(Kushki et al., 2015). Previous studies have showed that antioxidants can enhance the process of spermatogenesis(Arab et al., 2019; Jung et al., 2021). In line with previous stuides results, our results showed that the *C. comosum* extract improved the damaged testicular tissue after freeze-thaw process.
2. Our results of gene expression indicated that the expression of *P53*, *Bax*, and *Fas* genes had a significant increase after the freeze-thaw process, while the *C. comosum* extract decreased the expression of *Bax*, *P53* and *Fas* genes in testicular tissue of neonatal mice. Notably, although *Bcl2* expression was evaluated, no significant alterations were detected. This indicates that the observed cytoprotective effects of *C. comosum* are likely driven by the modulation of pro-apoptotic gene expression.
3. A study showed that the *Tribulus terrestris* extract led to a downregulation of *Bax* and *P53*
4. genes in the testicular tissue of cisplatin-treated mice (Zahra & Keshtmand, 2018) . Ebrahimi et al, evaluated the effects of N-acetylcysteine antioxidant on the immature mouse testicular tissue in vitro. They found that N-acetylcysteine can increase the expression of *Bax* gene vitrified immature mouse testicular tissue (Nikoosokhan et al., 2025). Khalil et al, reported that the resveratrol reduced the *Bax* and *P53* genes in cadmium chlorid-induced testicular damage (Eleawa et al., 2014).
5. Several studies have demonstated that different plant extracts, including Satureja montana (Abd El Tawab et al., 2014), Moringa olifera (Mostafa-Hedeab et al., 2023), *Ceratonia siliqua (Khani et al., 2020)* and *Eruca sativa*(Abd-Elsalam et al., 2021), reduced apoptosis in damaged testicular tissues due to their antioxidant properties. In line with previous stuides results, our results showed that the *C. comosum* extract had significantly decreased the expression of apoptosis genes in testicular tissue of FT2W +Ca group. Our findings suggest that *C. comosum* extract is associated with the downregulation of apoptosis-related genes and a reduction in histological abnormalities in neonatal testicular tissue. Therefore, we demonstrated that *C. comosum* exerts antioxidant effects and contributes to maintaining testicular tissue integrity by regulating apoptosis-related genes.

Author Contributions Statement

FN, MM, FS and **AE** designed the experiments**; FN** performed experiments and writing manuscript, **MM** Supervised**,** directed and managed the study**; FS and AE** discussed the results and final approved of the manuscript.

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This work did not receive any funding.

Data availability statement

All data supporting the results and analysis in this research will be available by Mansoureh Movahedin when it is needed.

Ethical Approval

All animal experiments were approved by the ethical committee of of Islamic azad university (Ethical code: IR.I AU.SRB.REC.1348.115).

Consent for publication

All authors have read and approved the final of the manuscript. We are interested that my manuscript publish in Pathobiology Research**.**

Conflicts of Interest

The authors declare no conflicts of interest directly relevant to the content of this article.

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Artificial intelligence (AI)-assisted technologies

The authors declare that no AI-assisted technologies were used in the preparation of this manuscript.

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