Amol University of Special Modern Technologies

Caspian Journal of Veterinary Sciences

doi: 10.22034/cjvs.2026.592993.1088

Journal homepage: https://cjvs.ausmt.ac.ir/

The protective effects of *Hypericum perforatum* extract on reproductive complications induced by experimental cholestasis in adult male rats

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Article Info

Article history:

Submit Date: 21 July 2026

Accept Date: 7 August 2026

Online Date: 11 November 2026

Keywords:

Cholestasis

Hypericum perforatum

Oxidative stress

Sperm

Testis

Abstract

Cholestasis is a complex hepatobiliary disorder characterized by impaired formation and secretion of bile, leading to the accumulation of toxic bile acids and consequent injury to hepatic and extrahepatic organs, including the reproductive system. *Hypericum perforatum* (St. John's Wort) is a medicinal plant containing hyperforin, hypericin, flavonoids, and phenolic compounds with strong antioxidant and anti-inflammatory properties that may confer protection against cholestasis-induced reproductive dysfunction. The present study evaluated the protective effects of *H. perforatum* extract on reproductive complications induced by experimental cholestasis in adult male rats. Twenty Wistar rats were divided into four groups: control, sham-operated, Bile duct ligation (BDL), and cholestasis + treatment. Experimental cholestasis was induced by bile duct ligation. Animals in the treatment group received *H. perforatum* extract orally for four weeks post-surgery. At the end of the experimental period, testicular histopathology and sperm count were assessed. BDL-induced cholestasis resulted in severe testicular degeneration accompanied by a significant reduction in sperm count. Treatment with *H. perforatum* significantly improved testicular histoarchitecture and restored sperm count compared to untreated cholestatic animals ($p < 0.05$). These findings suggest that *H. perforatum* exerts protective effects likely mediated through antioxidant and anti-inflammatory mechanisms, modulating oxidative stress, and inflammatory pathways involved in cholestatic injury.

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Introduction

Cholestasis is a significant decrease in bile flow. The liver is the primary organ affected by cholestasis (Ommati *et al.,* 2019). Cholestasis is a hepatobiliary disorder defined as the impairment of bile formation, secretion or flow. It leads to the accumulation of bile acids and bilirubin in the liver, causing hepatocellular injury and systemic complications (Hirschfield *et al.,* 2010; Ommati *et al.,* 2019). This pathogenic process disturbs enterohepatic circulation, alters canalicular transporter expression and activates detoxification and inflammatory pathways, with profound metabolic and immune effects that extend beyond the liver. The systemic milieu thus generated influences hormonal and immune homeostasis, which in turn contributes to multi-organ involvement in affected individuals (Trauner and Boyer, 2002; Saad and Mahmoud, 2014).

Cholestasis in males may affect reproductive physiology by alterations in the hypothalamic–pituitary gonadal axis and testicular function, leading to decreased testosterone production, impaired spermatogenesis, and possible hypogonadism (Foresta *et al.,* 2008). These alterations may present as decreased serum testosterone, altered semen quality, and testicular histopathological alterations such as germ cell loss and inflammatory infiltration, indicative of endocrine and metabolic disorders associated with hepatic injury. Reproductive toxicity induced by cholestasis is multifactorial and includes oxidative stress, hormonal imbalance affecting gonadal steroidogenesis, hydrophobic bile acid accumulation, and inflammatory signaling. Bile acids can damage testicular cells like Sertoli cells and Leydig cells, as well as the barrier, resulting in germ cell apoptosis and damaged sperm quality. Redox-sensitive pathways and altered nuclear receptor signaling regulate transporter expression and testicular responses, leading to a complex reproductive toxicity phenotype linking hepatic pathology to male fertility outcomes (Yoshioka *et al.,* 2004; Sedes *et al.,* 2018; Monrose *et al.,* 2021).

Hypericum perforatum (St. John's Wort) is a well-studied medicinal plant, whose biological activity is related to a multi-component phytochemical profile, where hyperforin, hypericin and various flavonoids represent the main components. These components have hepatoprotective, antioxidant, and anti-inflammatory actions that directly intersect with cholestasis pathophysiology via modulation of oxidative stress, bile acid homeostasis, and inflammatory signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) and nuclear receptor systems such as pregnane X receptor (PXR) and farnesoid X receptor (FXR) (Martinot *et al.* 2017; Nobakht *et al.,* 2022; Mohagheghzadeh *et al.,* 2023).

A greater amount of evidence suggests *H. perforatum* has an extensive spectrum of pharmacological activities. These are antidepressant, anti-inflammatory, antioxidant, antimicrobial, antiviral, analgesic, and antidiabetic activities. Moreover, the plant exhibits anti-cancer and anti-metastatic potential, as well as neuroprotective and hepatoprotective properties. Furthermore, *H. perforatum* has a choleretic effect, stimulating the production and secretion of bile, and a cholagogue effect, promoting the expulsion of bile into the intestine. The various therapeutic effects of *H. perforatum* reveal its medicinal value (Sánchez-Muniz *et al.,* 2012; Mohagheghzadeh *et al.,* 2023).

Hyperoside is one of the most prominent flavonoid components of *Hypericum* species, which has been reported to improve cholesterol homeostasis, thus contributing to the treatment of non-alcoholic fatty liver disease (NAFLD) (Duan *et al.,* 2021). The excessive production of free radicals in the hepatic tissue can damage cellular macromolecules and is considered to be an important factor in the development and progression of various liver disorders (Hai and Kawada ,2020). The hepatoprotective potential of *Hypericum* is mostly attributable to its potent antioxidant and anti-inflammatory activities.

H. perforatum was found to have protective effects on the liver by reducing the hepatic levels of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Pérez-Ramírez *et al.,* 2019; Tokgöz and Altan, 2020). These cytokines are mainly secreted by adipocytes and are important in controlling lipid accumulation and distribution and metabolic homeostasis in the body (Popko *et al.,* 2010). Thus, suppression of these inflammatory mediators could be associated with the beneficial effects of *H. perforatum* on liver function and lipid metabolism (Popko *et al.,* 2010).

A wealth of experimental and clinical evidence

exists to confirm its efficacy in ameliorating cholestasis-induced hepatic dysfunction but standardized extract formulations and rigorously controlled studies are still crucial for optimizing therapeutic consistency, dosing and safety evaluation. Experimentally, *H. perforatum* has direct regulatory effects on the biosynthesis of testosterone, spermatogenesis and quality of sperm in the male reproductive function. These effects are mediated by antioxidant and anti-inflammatory mechanisms and modulation of cellular signaling pathways in testicular tissue. Results from experimental models and clinical observations reported improvements in key reproductive parameters such as hormonal profiles, seminiferous tubule architecture (Novelli *et al.,* 2020; Ji *et al.,* 2024). These effects provide a mechanistic rationale for its protective effect in preserving male fertility in the face of hepatic injury in the context of cholestasis-associated reproductive dysfunction. According to the well documented reproductive toxicity of cholestasis and the probable therapeutic properties of *H. perforatum*, this study was designed to investigate the efficacy of St. John's Wort in alleviating reproductive complications induced by experimental cholestasis in adult male rats.

Materials and Methods

Animals and ethical consideration

Twenty adult male Wistar rats (weight: 250 g) were purchased from the animal facility of Urmia University. Animals were housed in conventional conditions (natural light/dark cycle, 25 ± 2°C and 50 ± 10% humidity) with food and water ad libitum.

Grouping and experimental design

The sample size of five animals per group was determined based on previous similar studies investigating reproductive toxicity in BDL rat models (Saad and Mahmoud, 2014; Ommati *et al.,* 2019), which reported significant histopathological differences with n = 5-6 per group. Power analysis using G Power software (version 3.1) with an α of 0.05, power of 0.80, and expected effect size (Cohen's f = 0.85) estimated a minimum of four animals per group for detecting significant differences in histopathological scores.

Following a 15-day acclimatization period, the rats were randomly divided into four groups (n =5 per group): Group 1 (Control): Healthy rats without surgical intervention or treatment. Group 2 (Sham): Rats underwent laparotomy with isolation and manipulation of the common bile duct without ligation. Group 3 (Cholestasis): Experimental cholestasis was induced by bile duct ligation (BDL) in rats. Briefly, under general anesthesia (ketamine 90 mg/kg + xylazine 5 mg/kg, intraperitoneally), a midline laparotomy incision (approximately 3 cm) was made through the skin and abdominal wall. The liver and intestines were gently retracted to expose the common bile duct, which was identified as a thin, translucent tube running from the liver hilum to the duodenum. The common bile duct was carefully isolated from surrounding connective tissue and the portal vein using blunt dissection under a surgical microscope (20 × magnification). Two ligatures (4-0 silk suture) were placed around the common bile duct, approximately 0.5 cm apart, and securely tied. The duct was then transected between the two ligatures to ensure complete obstruction. The abdominal wall was closed in two layers (muscle and skin) using 3-0 absorbable sutures. Group 4 (Cholestasis + Therapy): Rats were subjected to BDL and subsequently treated with *H. perforatum* as Kneipp® Johanniskraut Dragees H (Würzburg, Germany) at a dose of 100 mg/kg/day by oral (PO) gavage for four weeks. The entire protocol lasted for 30 days.

H. perforatum extract (Kneipp® Johanniskraut Dragees H) used in this study was a commercially available standardized preparation manufactured by Kneipp GmbH, Würzburg, Germany. According to the manufacturer's certificate of analysis (batch number: 302648, expiry date: 12/2024), each tablet contains 250 mg of dry extract derived from *H. perforatum* L. (St. John's Wort) aerial parts (extraction solvent: ethanol 60% v/v).

Collecting samples

Animals were euthanized under deep anesthesia 24 hour after the last treatment. The testes and epididymides were carefully removed and weighed. The testes were fixed in 10% neutral buffered formalin for histological processing. The caudal epididymis was transferred immediately to pre-warmed Human Tubal Fluid (HTF) medium for sperm analysis. Sperm count was assessed using a standard hemocytometer method. Briefly, the caudal epididymis was minced in 1 ml of pre-

warmed HTF medium and incubated at 37°C for 15 minutes to allow sperm dispersion. An aliquot was diluted 1:10 with HTF medium, and sperm were counted using a Neubauer hemocytometer under light microscopy (400 × magnification). Sperm motility, morphology, and viability were not assessed due to laboratory equipment limitations and resource constraints.

Histopathological assessment

The fixed testicular tissues were dehydrated, cleared, and embedded in paraffin. Sections of 5 µm thickness were stained with Hematoxylin and Eosin (H&E). Histopathological evaluation was performed by examining seminiferous tubule structure, germinal epithelium organization, and overall testicular architecture. Histopathological evaluation was performed by two independent pathologists who were blinded to the experimental groups. Each observer examined all slides independently, and the scores were averaged. The slides were coded with random numbers, and the group allocation was revealed only after all evaluations were completed. Intra-observer and inter-observer agreement was assessed; the correlation coefficient between observers was r = 0.91 for the testicular biopsy score and r = 0.89 for the Cosentino score, indicating excellent agreement. In cases of disagreement (less than 5% of observations), a consensus was reached through discussion using a multi-headed microscope.

In the Cosentino criterion, testicular damage was scored on a scale from 1 to 4 based on the integrity of the germinal epithelium, the presence of necrotic cells, and hemorrhage, where 1 indicates normal structure and 4 represents severe damage (Cosentino *et al.,* 1986).

Testicular biopsy score

Testicular histopathological changes were evaluated using a modified Johnsen's testicular biopsy score (Johnsen, 1970). In this scoring system, each seminiferous tubule was scored on a scale of 1 to 10 based on the presence and organization of germ cells:

Score 10: Complete spermatogenesis with many spermatozoa present.

Score 9: Many spermatozoa present but disorganized germinal epithelium.

Score 8: Only a few spermatozoa present.

Score 7: No spermatozoa but many spermatids present.

Score 6: No spermatozoa and only a few spermatids present.

Score 5: No spermatozoa or spermatids, but many spermatocytes present.

Score 4: Only a few spermatocytes present.

Score 3: Only spermatogonia present.

Score 2: No germ cells, only Sertoli cells present.

Score 1: Complete absence of germ cells and Sertoli cells.

A minimum of 50 seminiferous tubules were examined per testis section (from 10 randomly selected fields at 400 × magnification), and the average score was calculated for each animal.

Mature seminiferous tubules (MST) percentage

The percentage of mature seminiferous tubules was determined by examining 50 randomly selected seminiferous tubules per testis section at 400 × magnification. Tubules were classified as 'mature' if they met the following criteria: (1) intact basement membrane, (2) presence of mature spermatids or spermatozoa in the lumen, (3) complete or near-complete spermatogenesis with all germ cell layers present, and (4) organized germinal epithelium without significant vacuolization or desquamation. The MST percentage was calculated as: (Number of mature tubules / Total tubules examined) × 100.

Biochemical analysis

Blood samples were collected via cardiac puncture under deep anesthesia at the time of euthanasia. Serum was separated by centrifugation (3000 rpm, 15 minutes, 4°C) and stored at -80°C until analysis. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin were measured using commercially available colorimetric kits (Pars Azmoon, Tehran, Iran) according to the

manufacturer's protocols using an automated biochemistry analyzer (Biotecnica, BT 3000, Italy).

Statistical analysis

Data were analyzed using SPSS software (version 18.0, IBM Corp., Armonk, NY, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Levene's test. All data were found to be normally distributed ($p > 0.05$) and variances were homogeneous ($p > 0.05$). Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) as indicated. Multiple comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for pairwise comparisons. Exact *p*-values were reported where possible; for $p < 0.001$, the exact value was reported as $p < 0.001$. Effect sizes were calculated using Cohen's f statistic for ANOVA ($f = \sqrt{(\eta^2/(1-\eta^2))}$) where η^2 is the eta-squared effect size. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed by a biostatistician who was blinded to the experimental groups.

Results

Testicular histopathology

Remarkable changes were observed in the reproductive organs during gross examination at the time of sampling. The cholestatic group (Group 3) showed a marked reduction in the size of the testes and accessory sex organs (epididymis, seminal vesicles, and prostate) with clear signs of atrophy when compared to the control and sham groups. Microscopic analysis of H&E-stained testicular sections provided detailed insight into the structural damage caused by cholestasis and the subsequent protective effects of the therapeutic intervention. In Groups 1 and 2, the testicular parenchyma presented a normal and well-preserved architecture in both control and sham-operated animals (Fig. 1).

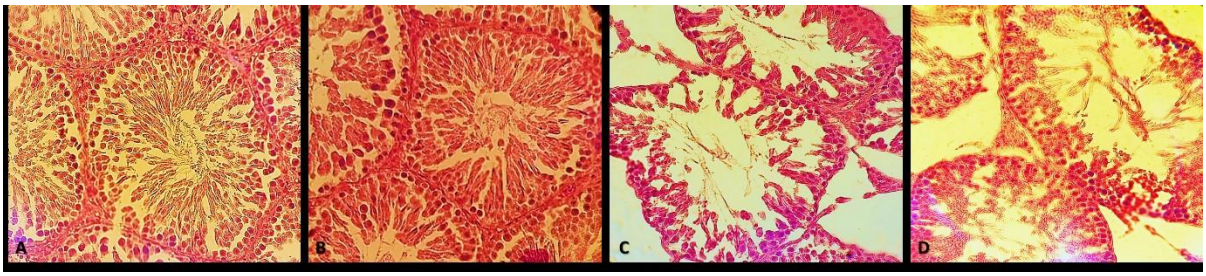


Fig. 1. Cross-section of testicular tissue: Photomicrographs of testicular sections from different experimental groups (Hematoxylin and Eosin staining, 400× magnification, scale bar = 50 µm). In control (A) and sham (B) groups, normal histoarchitecture of seminiferous tubules having active spermatogenesis was evident. Experimental cholestasis (C) resulted in marked testicular cyto-architectural disarrangements and early maturation arrest as evidenced by spermatogenic arrest at the spermatogonium/spermatocyte stage. *Hypericum perforatum* co-treatment (D) led to the pronounced cyto-histological restoration in the testicular tissue. However, hypo-spermatogenesis still could be observed in some tubules (arrows).

The seminiferous tubules were packed closely with intact basement membrane. The lineage of spermatogenesis was intact and well organized; all stages of germ cells were present, including spermatogonia, primary and secondary spermatocytes, and spermatids at different stages of differentiation. The tubular lumina were filled with many mature elongated spermatids and spermatozoa. Sertoli cells presented their normal cytoplasmic extensions and pale nuclei. The interstitial spaces looked normal with distinct clusters of Leydig cells. In Group 3 (cholestasis), induction of cholestasis resulted in severe and widespread histopathological alterations in testicular tissue (Fig. 1). The most important findings were: 1. Disorganization and depletion of germ cell: the orderly arrangement of germinal epithelium was severely disturbed with marked reduction in germ cell numbers leading to significant thinning of seminiferous epithelium. 2. Arrested spermatogenesis, a large number of seminiferous tubules showed arrested

spermatogenesis, mostly at the spermatocyte stage, with the absence of later stage spermatids and spermatozoa, indicating oligozoospermia or even azoospermia in many of the tubules. 3. Vacuolization: the cytoplasm of Sertoli cells and the intercellular spaces between germ cells contained numerous vacuoles of variable size, suggesting cellular degeneration. 4. Sloughing and necrosis: frequently observed were detached clusters of germ cells within the tubular lumen and pyknotic nuclei suggestive of apoptosis and necrosis. 5. Interstitial edema and atrophy: interstitial edema caused widening of interstitial spaces and a decrease in numbers of mature Leydig cells, indicative of interstitial atrophy. In the treatment group, co-administration of *H. perforatum* resulted in a significant improvement in cholestasis-induced histo-cytological alterations in the testicular tissue. However, mild hypo-spermatogenesis was still observed in some seminiferous tubules, indicating partial but meaningful protective effects of the intervention against cholestasis-induced testicular damage.

Spermatogenesis

The scoring systems used for testicular evaluation quantitatively supported the observed histopathological alterations. The MST was significantly reduced in the cholestatic group (14.80 ± 6.23%) compared to the control and sham groups, indicating impaired spermatogenic activity. Concomitantly, the Cosentino score was significantly increased (2.93 ± 0.05), reflecting severe testicular injury characterized by degeneration of the germinal epithelium and disruption of normal seminiferous tubule architecture (Table 1).

Cholestasis induced marked testicular damage, which was substantially ameliorated by treatment with *H. perforatum*. In the treated group, the percentage of mature seminiferous tubules was significantly increased (56.00 ± 3.60%) compared to the untreated cholestatic group ($p < 0.05$), indicating partial restoration of spermatogenic activity. Histologically, the seminiferous tubules exhibited a more organized cellular arrangement, with improved germinal epithelium integrity and reduced germ cell loss. The protective effect of *H. perforatum* was further confirmed by a significant reduction in Cosentino score (1.45 ± 0.03) compared to the cholestatic group ($p < 0.05$), indicating attenuation of tissue degeneration and preservation of testicular structure. Although not all parameters were fully restored to control levels, *H. perforatum* treatment significantly improved spermatogenic function and reduced cholestasis-induced histopathological damage.

Table 1. Histopathological evaluation of testicular tissue in control, sham, cholestatic, and cholestatic rats treated with *H. perforatum*, based on testicular biopsy score, mean seminiferous tubule (MST) percentage, and Cosentino grading system. Data were expressed as mean ± SEM. Different superscript letters indicated statistically significant differences between groups ($p < 0.05$).

Experimental groups	Testicular biopsy score	MST (%)	Cosentino score
Control	9.37 ± 0.34^{a}	90.00 ± 5.77^{a}	1.10 ± 0.05^{a}
Sham	9.10 ± 0.22^{a}	78.66 ± 11.33^{ab}	1.11 ± 0.03^{a}
Cholestasis	5.11 ± 0.17^{b}	14.80 ± 6.23^{c}	2.93 ± 0.05^{b}
Cholestasis + HP	7.33 ± 0.19^{c}	56.00 ± 3.60^{b}	1.45 ± 0.03^{c}

Biochemical confirmation of cholestasis

Serum biochemical parameters confirmed successful induction of cholestasis in BDL animals. As shown in Table 2, BDL rats exhibited significant elevations in serum ALT, AST, ALP, and total bilirubin compared to control and sham groups ($p < 0.001$). Treatment with *H. perforatum* did not significantly alter these biochemical parameters, indicating that the protective effects on testicular tissue occurred despite persistent cholestasis (Table 2).

Table 2. Serum biochemical parameters (ALT, AST, ALP, and total bilirubin) in Control, Sham, cholestasis, and cholestasis + *H. perforatum* groups. Data were expressed as mean ± SEM.

Parameter	Control	Sham	Cholestasis	Cholestasis + *H. perforatum*
ALT (U/l)	45.2 ± 3.1^{a}	48.7±4.2^{a}	385.6 ± 28.4^{b}	352.3±31.2^{b}
AST (U/l)	82.5 ± 6.4^{a}	88.3±7.1^{a}	456.2 ± 35.7^{b}	425.8±38.9^{b}
ALP (U/l)	125.3±8.9^{a}	131.7±9.5^{a}	823.4 ± 62.1^{b}	798.6±58.3^{b}
Total bilirubin (mg/dl)	0.2 ± 0.02^{a}	0.2±0.03^{a}	8.7 ± 0.9^{b}	8.2 ± 0.8^{b}

Discussion

Infertility due to cholestasis is mediated by several interconnected mechanisms that lead to testicular dysfunction. Hydrophobic bile acids accumulate locally and systemically, causing direct cytotoxicity to germ cells, stimulating mitochondrial-dependent apoptotic pathways and death receptor-mediated signaling, and degeneration of spermatogenic cells. Cholestasis elevates oxidative stress and diminishes endogenous antioxidant defenses, leading to lipid peroxidation, protein damage, and DNA fragmentation in testicular tissue, thus impairing normal spermatogenesis. In addition, the hypothalamic–pituitary–gonadal axis dysregulation and alteration of gonadotropic secretion impact the functional integrity of both Leydig and Sertoli cells, with consequent decrease of the hormonal and nutritional support necessary for germ cell development. It has also been stated that intermediate bile acid metabolites modulate nuclear receptor signaling pathways including FXRα and CAR that regulate genes essential for spermatogenesis. Consequently, a disruption of this signaling network could lead to impaired germ cell maturation and decreased sperm quality (Martinot *et al.,* 2017).

The present study indicated that experimental cholestasis induced by BDL leads to severe damage to the male reproductive system as evidenced by significant histopathological damage in the testes and significant decrease in sperm quality. Treatment with St. John's wort (*H. perforatum*) also offers a major protective effect and significantly decreases these deleterious changes. In this discussion, we seek to interpret these results in the context of multifactorial mechanisms of cholestasis-associated reproductive toxicity and potential pathways mediating the beneficial effects of St. John's wort. The reproductive dysfunction in the cholestatic group is in keeping with the well-established association of chronic liver disease with hypogonadism (Foresta *et al.,* 2008; Hirschfield *et al.,* 2010).

The fundamental disease process is complicated, with many mechanisms that interact with each other. A significant contributing factor is the systemic accumulation of hydrophobic bile acids, which are specifically cytotoxic. Experimental evidence shows that these bile acids may induce germ cell apoptosis via activating death receptor–mediated signaling and mitochondrial-dependent apoptotic pathways (Baptissart *et al.,* 2014; Saad and Mahmoud, 2014). This direct cytotoxic effect might clarify the observed degeneration of seminiferous tubules, including germ cell exfoliation, nuclear pyknosis and disruption of the normal architecture of the seminiferous epithelium. Also, cholestasis is highly associated with increased systemic oxidative stress. which plays a major role in tissue injury and reproductive dysfunction (Baptissart *et al.,* 2014). Further, an excess of reactivity oxygen species under these conditions can increase cellular damage in the testes, stimulate lipid peroxidation and impair spermatogenic processes (Yoshioka *et al.,* 2004). The findings support the concept that cholestasis mediates male reproductive toxicity through a combination of bile acid-mediated cytotoxicity and oxidative stress, whereas St. John's wort appears to improve these effects, possibly through its antioxidant and cytoprotective properties. The liver's reduced capacity to neutralize reactive oxygen species (ROS) results in their overflow into the systemic circulation, thereby establishing a pro-oxidative state that is particularly harmful to the testes. Testicular tissue, characterized by a high metabolic demand and a rich content of polyunsaturated fatty acids in sperm cell membranes, is highly susceptible to oxidative injury (Saad and Mahmoud, 2014).

Such oxidative stress promotes lipid peroxidation, induces sperm DNA damage, and disrupts the function of essential somatic cells, including Sertoli and Leydig cells (Yoshioka *et al.,* 2004). Dysfunction of Leydig cells consequently leads to reduced testosterone synthesis, which compromises the hormonal support required for normal spermatogenesis and the structural integrity of accessory reproductive organs, as demonstrated by their atrophy in the present study.

The protective effects of St. John's wort (*H. perforatum*) are largely due to its well-established pharmacological properties. The plant is rich in bioactive constituents, mainly hypericin and hyperforin, which have been reported in previous studies to exhibit strong antioxidant and cytoprotective activities (Novelli *et al.,* 2020; Nobakht *et al.,* 2022). Based on these earlier reports, it is plausible that these compounds may play a role in preventing oxidative stress-mediated testicular damage. However, this mechanism is

speculative in the context of the present study, as oxidative stress markers were not directly measured. Beyond its antioxidant effects, *H. perforatum* has also shown significant anti-inflammatory and anti-apoptotic properties that could contribute to its protective role in cholestasis-associated testicular injury. It is known that inflammatory processes contribute to cholestatic damage with systemic consequences. Modulation of inflammatory pathways is important in tissue preservation. Evidence shows that *H. perforatum* can reduce inflammatory signaling pathways such as NF-kB activation and inhibit the production of pro-inflammatory cytokines such as TNF-α and IL-6. The anti-inflammatory effect may explain the better preservation of the testicular microarchitecture in the treated group. Histopathologically, experimental cholestasis showed severe disorganization of the seminiferous tubules and early maturation arrest characterized by loss of germ cells at the spermatogonium/spermatocyte stages. On the other hand, co-treatment with *H. perforatum* resulted in remarkable restoration of testicular cytoarchitecture, with re-establishment of a more organized seminiferous epithelium and partial recovery of spermatogenic activity. Overall, these structural improvements are indicative of a significant protective effect, although some of the seminiferous tubules still presented mild hypospermatogenesis (Martinot *et al.,* 2017).

Besides that, the decreased germ cell degeneration and nuclear abnormalities histologically observed could also be the outcome of anti-apoptotic mechanisms, such as the modulation of apoptosis-regulating proteins and the inhibition of caspase pathways. Overall, these effects are in agreement with the histological recovery observed and support the role of *H. perforatum* in the attenuation of cholestasis-induced testicular damage.

The present study findings indicated that cholestasis caused by BDL has a detrimental effect on testicular histology and spermatogenic activity. The treatment with *H. perforatum* significantly ameliorated these pathologic changes and contributed to the maintenance of the integrity of the testicular tissue. The beneficial effects found in cholestatic injury might be due, at least in part, to the antioxidant and anti-inflammatory effects of *H. perforatum* by reducing cellular damage. The results emphasize the importance of *H. perforatum* as a potential adjuvant to prevent male reproductive function in cholestasis collectively. However, further studies are needed to confirm the specific molecular mechanisms of its protective effects and to evaluate its long-term potency and optimal dose in experimental models of reproductive dysfunction.

Several limitations of this study should be acknowledged. First, while the histopathological findings suggest protective effects of *H. perforatum*, we did not measure oxidative stress markers (MDA, SOD, CAT, GPx, GSH) or inflammatory cytokines (TNF-α, IL-6, IL-1β) in testicular tissue or serum. Consequently, the proposed antioxidant and anti-inflammatory mechanisms remain speculative. Future studies should include comprehensive biochemical analyses to confirm the mechanistic pathways involved. Second, the small sample size (n = 5 per group) limits the statistical power of our findings. Third, reproductive hormone analysis (testosterone, LH, FSH) was not performed, which would have provided further insight into the hormonal mechanisms underlying the observed effects.

The relatively small sample size (n = 5 per group) may limit the generalizability of our findings and reduce statistical power for detecting smaller effect sizes. Future studies with larger sample sizes are recommended to validate these findings.

Serum reproductive hormones (testosterone, LH, FSH) were not measured in this study, limiting our ability to assess the hormonal mechanisms underlying the observed testicular changes. Cholestasis is known to disrupt the hypothalamic-pituitary-gonadal axis, and measurement of these hormones would have provided valuable mechanistic insight. Future studies should include comprehensive hormonal analyses to evaluate the effects of *H. perforatum* on reproductive endocrine function.

The evaluation of reproductive function was limited to sperm count and testicular histopathology. Sperm motility, morphology, and viability-standard reproductive endpoints-were not assessed. This represents a significant limitation, as these parameters provide important information about sperm functional quality. Future studies should include comprehensive sperm analysis including motility (computer-assisted sperm analysis), morphology (eosin-

nigrosin staining), and viability (hypo-osmotic swelling test or live/dead staining).

Acknowledgments

The authors express gratitude to the staff of Central Library of Urmia University, Urmia, Iran, for facilitating access to scientific resources.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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