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Effects of the Ethanol Extracts of *Ficus deltoidea* leaves on the Reproductive Parameters in Male Mice

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Abstract

BACKGROUND: Indonesia, and in particular East Kalimantan, has a very high diversity of flora that has the potential to be used as traditional medicine. One type of flora is the leaves of *Ficus deltoidea* Jack. At present, there are no available data about the impact of *F. deltoidea* leaf ethanol extract on the male reproductive system.

AIM: The present study aims to investigate the effect of *F. deltoidea* leaf ethanol extract on several parameters of reproductive function in male mice, including changes in testicular biochemistry, reproductive hormones profile, and histopathology of the testes after subchronic exposure.

METHODS: In total, 25 male mice were divided into five groups: The control group and four treatment groups that received extract doses of 125, 250, 500, and 1000 mg/kgbw for 28 days, respectively. At the end of the treatment, surgery was performed, weight of body and reproductive organs (testis, epididymis, and seminal vesicles) were measured, and testicular biochemistry, reproductive hormone profile, antioxidant activity, and testes histopathology were analyzed.

RESULTS: In the subchronic toxicity test, there were no significant changes in body weight or in weight and relative weight of reproductive organs. Levels of testosterone, luteinizing hormone and follicle-stimulating hormone, protein, cholesterol, and activity of enzymes in the testes (alkaline phosphatase, lactate dehydrogenase, and glutamyltransferase) and activity of the enzyme superoxide dismutase increased significantly in the treated mice when compared to control mice ($p < 0.05$). Glycogen levels were not significantly different, but lipid peroxide (MDA) decreased significantly, though it did not change the histological structure of the testes.

CONCLUSION: Ethanolic extract of the leaves of *F. deltoidea* Jack does not cause toxic effects and even has a beneficial effect on the reproduction of male mice by increasing fertility, reproductive hormones, and antioxidant activity, and it does not change the histological structure of the testes.

Introduction

Plant materials have long been used for medicinal purposes, both as supplement for body maintenance and as therapy for illnesses such as diarrhea, lower respiratory tract infections, the common cold, and skin infections [1], [2]. In recent years, consumption of natural products has increased globally [3]. The World Health Organization has found that 80% of the world's population, especially in the developing countries, uses traditional medicine [4]. Thus, the safety and efficacy of medicinal plants utilized in traditional medicine should be investigated [5].

Ficus deltoidea, a member of the Moraceae family, is a perennial plant that grows in woods, either terrestrially or epiphytically. The plant is known by several names in different places, including Mas Cotek in Malaysia, Tabat Barito in Indonesia, Agoluran in the Philippines, and Kangkalibang in Africa [6]. The fruit of Tabat Barito is useful for relieving headaches and toothaches, and the powdered roots and leaves have been used to treat wounds and relieve rheumatism [7]. Conventionally, this plant is also consumed to help strengthen a woman's uterus after giving birth [6] and serves as a libido booster for both men and women [8]. Although *F. deltoidea* has displayed various pharmacological properties, safety information on this plant is still limited. Acute and subchronic toxicity studies of the ethanolic extract of *F. deltoidea* leaves showed no signs of toxicity in male mice (*Mus musculus*) after oral treatment with a single 2000 mg/kg body weight dose, while in the subchronic toxicity test, there were

no behavioral change and no significant changes in body weight or in the hematological and biochemical parameters of serum [9].

Other extracts from the genus Ficus, which has over 900 species and is known for its ability to modify the pH of the sperm microenvironment in mice growing in captivity, have been demonstrated to increase sperm production and pH in the sperm microenvironment in growing mice (*Ficus sycomorus*) [10] and, at low concentrations, to enhance spermatogenesis or to treat azoospermia (*Ficus capensis*) [11]. The sexual reproduction activity benefits of the Ficus genus are generally associated with the presence of several bioactive compounds, namely, saponins and alkaloids [12], [13]. In addition, its flavonoids and anethole are estrogenic compounds [14] that can suppress serum testosterone levels and reduce sperm production. In the phytochemical test, it was found that the ethanol extract of *Ficus deltoidea* Jack leaves contained alkaloids, phenolics, flavonoids, coumarins, and steroids [9]. According to a previous study, plant extracts and secondary metabolites exhibit a variety of biological properties, including antihypertensive, anti-inflammatory, hepatoprotective, anti-tumor, antioxidant, anti-diarrheal, antispermatogenic, and spermatogenic activities, and can increase or decrease serum testosterone levels [15].

However, there are no available data about the impact of *F. deltoidea* leaf ethanol extract on the male reproductive system. This study aimed to investigate the effect of *F. deltoidea* leaf ethanol extract on several parameters of male mice reproductive function, including biochemical parameters in the testes, reproductive hormones profile, antioxidant activity, and histopathology of the testes after subchronic exposure.

Materials and Methods

Leaf extract preparation

The leaves were washed, dried, and then blended. Extract was procured through maceration into 96% ethanol solvent (1 kg of leaves: 4 L ethanol) and stirred from time to time at room temperature for 2 × 24 h. After filtering the substance, the filtrate was accommodated, and the residual filtering was soaked in a fresh solvent. The procedure was repeated until the filtrate turned a clear color. The ethanol extract was concentrated using a rotary evaporator to create a paste of *F. deltoidea* leaf ethanol extract, which was kept at 4°C until it was utilized to treat test animals.

Animal treatment and tissue sampling

The animals used were 25 male mice weighing between 20 and 35 g and aged around 8 weeks. Animals were housed in conventional cages, kept under standard photoperiod (12 h light/dark cycle; temperature 23°C; relative humidity), with food and drink *ad libitum* for 1 week prior to beginning the trial. Animals were weighed at baseline and weekly to observe possible changes in body weight throughout the experiment. We conducted animal research in accordance with the Animal Research: Reporting of *in vivo* experiments guidelines.

The animals were placed into five groups, with each group consisting of five individuals. The extract was suspended in 0.5% CMC with an administration volume of 1%, and the suspension was kept at room temperature. The extract solution was delivered orally once a day for 28 days using a gastric probe, with extract dosages of 0, 125, 250, 500, and 1000 mg/kgbw provided every day for the duration of the study. Toward the conclusion of the therapy, the animals were required to fast overnight before being anesthetized. Blood was drawn from the orbital sinus, collected in an Eppendorf tube without EDTA, left at 37°C for 30 min, and centrifuged at 3000 rpm for 20 min at 4°C for serum separation and stored at –20°C for hormone analysis. All animals were sacrificed by cervical dislocation and dissected. The testicles, epididymis, and seminal vesicles were removed immediately, washed with ice-cold physiological saline solution (0.9%, w/v), and weighed to determine the absolute and relative weights (organs/body weight × 100). The testes were then put in Bouin's solution for histological assessment on one side of each animal. Tissues were fixed in paraffin, sliced to 5 m thickness, stained with hematoxylin-eosin, and examined under a light microscope. Testicular organs from the other side were processed and stored at –20°C until they were analyzed. Testicular tissue stored at –20°C was further crushed and homogenized in 1 ml of PBS buffer (pH 7.4). The homogenate was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected, transferred to Eppendorf tubes, and stored at −20°C until analysis for hormone and testicular biochemistry.

Reproductive hormone analysis

Serum and testicular samples were analyzed for the hormones FSH, LH, and testosterone using the ELISA method. This assay procedure was followed as specified following the instructions in the ELISA kit based on the hormone assay Bioresource, USA, Catalog number: MBS494055 protocol. The absorbance was measured with an ELISA reader (HBS-1101 Mode, ID SN11013810124E China). Serum hormone assays were obtained from plotted graphs of absorbance versus standard hormone concentrations.

Testicular biochemical analysis

The homogenized testicular samples were then analyzed for their biochemical content, measured using

a biochemistry autoanalyzer (Olympus 640 Japan): Cholesterol, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), and UV–VIS Spectrophotometer (752N China): Protein Assay Kit (Catalog Number PC0010 Solar Bio) and glycogen (Catalog Number BC0340 Solar Bio). The parameters of antioxidant enzymes and lipid peroxidase were superoxide dismutase (SOD) (Catalog Number BC0170 Solar Bio) and malondialdehyde (MDA) (Catalog Number BC0020 Solar Bio) kinetic methods, as specified in the respective commercial kits.

Statistical analysis

All experimental results are presented with mean standard errors of the mean (n = 5). The statistical significance of the differences between the control and treatment groups was determined using a one-way ANOVA followed by a *post hoc* LSD test. Statistical significance was defined as a comparison with a p = 0.05.

Results and Discussion

The results of this study generally indicated that the ethanolic extract of *F. deltoidea* leaves in male mice was not toxic in terms of body weight and reproductive organs, testicular biochemistry, reproductive hormone profile, and histological structure of the testes (Tables 1-3 and Figure 1). These results appear to be similar to the previous studies, which also found that *Ficus asperifolia* extract has androgen-like activity that does not affect changes in vital organ morphology and behavior [16].

Acute and subchronic administration of *F. deltoidea* extract to male mice did not show clinical signs of toxicity [9]. Subchronic effects of the administration of ethanolic extract of *F. deltoidea* leaves for 28 days on body weight, absolute organ weight (g), and relative organ weight (%) to body weight of male mice are presented in Table 1. In this study, all groups showed normal weight gain, but there was no significant difference in the extract treatment group compared to the control group. According to Farombi *et al*. [17] and Sakr and Nooh [18], normal weight gain is a sign of optimal and efficient health homeostasis and the possibility of no metabolic disturbances or toxicity.

Except for the weight of the seminal vesicles, there was no statistically significant change (p > 0.05) in the absolute weight of the testicles and epididymis (g) between the treatment groups and the control group. Furthermore, it was discovered that there was no statistically significant difference (p > 0.05) in organ weight relative to body weight between the treatment and control groups, with the exception of the relative weight of the seminal vesicles, which decreased slightly in the treatment group compared to the control group. However, all of these small fluctuations in relative organ weight values remained within the normal range and were found to be sporadic. Plant extracts can stimulate tissue regeneration and thus may have a positive impact on body weight and these organs. Measurement of body weight and reproductive organs is one of the tests to determine the presence of a toxic effect of each test material on the reproductive organs [19]. This parameter can also be used to determine the ability of the testes to perform their regular functions. A study by Wankeu-Nya *et al*. [20] mentioned that changes in relative organ weight can occur as a result of the inflammatory process or cell constriction. Increasing the organ-weight ratio implies either inflammation or a rise in the secretory capabilities of the organ, while decreasing these parameters indicates either constriction of the cells or cellular constriction. Meanwhile, Morakinyo *et al*. [21] stated that the weight and size of the reproductive organs usually depend on the secretory fluid from Sertoli cells and the mass of spermatogenic cells. The organ weight results showed that the extract had no toxic effect on internal organs. However, it is possible that the chemical compounds in the extract may have a toxic effect. Therefore, it is crucial to carry out a chemical profile before the toxicity evaluation to justify the relationship between the compound and bioactivity.

As indicated in Table 2, an ethanolic extract of *F. deltoidea* leaves increased testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels as compared to the control group (p 0.05). LH in males causes testicular Leydig cells to produce testosterone, which increases testicular growth and activity, in particular spermatogenic activity [22]. The present study found that increased testosterone levels were correlated with normal testicular and epididymal function [23], whereas FSH is essential for sperm production by enhancing Sertoli cell function. These results are in accordance with the previous research from Daud *et al*. [24] which revealed that the methanol extract of *F. deltoidea* at 50 mg/kg body weight showed a significant increase in sperm quality and testosterone production (p < 0.05) in male rats, and those stated by

Table 1: The effect of ethanolic extract of *Ficus deltoidea* leaves on the body, organ weights, and relative organ weights of male mice

Group	Body weight		Testes		Epididymis		Vesicula seminalis	
mg/kgbw	Initial	Final	(g)	(%)	(g)	(%)	(g)	(%)
0	16.05 ± 0.90	24.77 ± 1.65^{a}	0.11 ± 0.01^{a}	0.44 ± 0.04^{a}	0.03 ± 0.01^{a}	0.14 ± 0.03^{a}	0.04 ± 0.00^{a}	0.19 ± 0.02^{a}
125	29.24 ± 2.11	33.94 ± 1.22^{b}	0.13 ± 0.00^{a}	0.38 ± 0.01^{a}	0.04 ± 0.00^{a}	0.21 ± 0.02^{a}	0.04 ± 0.00^{ab}	0.13 ± 0.01^{ab}
250	19.17 ± 2.64	25.00 ± 2.89^{ab}	0.10 ± 0.02^{a}	0.40 ± 0.07^{a}	0.03 ± 0.00^{a}	0.14 ± 0.02^{a}	0.04 ± 0.00^{bc}	0.15 ± 0.01^{ab}
500	20.71 ± 0.57	28.34 ± 0.41^{ab}	0.22 ± 0.10^{a}	0.78 ± 0.35^{a}	0.08 ± 0.05^{a}	0.30 ± 0.16^{a}	0.03 ± 0.00^{c}	0.11 ± 0.01^{b}
1000	26.04 ± 3.23	31.83 ± 3.11^{ab}	0.12 ± 0.02^{a}	0.37 ± 0.04^{a}	0.05 ± 0.01^{a}	0.17 ± 0.03^{a}	0.03 ± 0.00 ^{c}	0.11 ± 0.02^{b}

Data was represented as Mean ± SEM. One-way ANOVA, followed by LSD test. The different letter indexes (a, b, c) in the same column indicate significantly different P < 0.05 versus control.

Zaid *et al*. [25] that *F. deltoidea* causes increased levels of gonadotropin hormone (FSH) in female rats.

Furthermore, testicular biochemical results showed that the ethanolic extract of *F. deltoidea* leaves in male mice increased the concentration of protein, cholesterol, and enzyme activity in the testes (ALP, LDH, GGT, and antioxidant enzyme activity. SOD was significant in the treated mice when compared to control mice ($p < 0.05$), but glycogen levels were not significant, while lipid peroxide (MDA) decreased significantly (Table 3).

Table 2: The effect of the ethanolic extract of *Ficus deltoidea* leaves on hormone profiles and morphometric parameter of male mice

Group mg/kgbw	Testosterone (mg/ml)	FSH (mIU/ml)	LH (mIU/ml)	Thickness of epithelium tubules seminiferous (μm)
0	16.37 ± 0.29^{a}	1.07 ± 0.18^{a}	7.37 ± 0.52^{a}	0.48 ± 0.018^{a}
125	18.05 ± 1.47^{ab}	1.39 ± 0.14^{a}	8.78 ± 1.36^{ab}	0.49 ± 0.001^{a}
250	20.56 ± 0.57^{bc}	2.23 ± 0.19^{ab}	11.44 ± 0.69^{bc}	0.57 ± 0.015^{b}
500	23.94 ± 0.46^{cd}	3.92 ± 0.69^{b}	13.02 ± 0.39^{c}	0.59 ± 0.015^{b}
1000	25.01 ± 1.05^{d}	3.75 ± 0.54^{b}	10.86 ± 0.39^{bc}	0.62 ± 0.011^{b}

Mean ± SEM. Followed by superscript letters (a, b, c, d) in the same column shows significantly different $P < 0.05$

The results in this study showed that the protein increased after administration of the extract for 28 days. Kasturi *et al*. [26] noted that testicular protein is required for spermatogenesis and sperm maturation. The extract's rise in testicular protein will provide nutrients for the development of sperm cells, increasing male fertility. Meanwhile, cholesterol is a crucial precursor for steroidogenesis, such as testosterone conversion under the direction of the LH hormone, and is also linked to proper testicular function [27], [28]. A significant increase in testicular cholesterol may imply an increase in testicular function in male mice. These results are in accordance with Nurudeen and Ajiboye [29] and Yakubu and Afolayan [30], which documented a significant increase in the testicular cholesterol levels of male Wistar rats given aqueous extract of *Bulbine natalensis* stems due to the increased anabolic and androgenic activity of the plant. The previous research performed by Ngadjui *et al*. [12] who revealed that the alkaloids and saponins contained in the extract of *F. asperifolia* have androgenic potential. Numerous studies have shown that alkaloids increase testicular cholesterol while saponins may increase LH levels or bind to enzymes involved in steroidogenesis, thereby stimulating endogenous testosterone levels [31], [32]. Furthermore, after administration of the extract testicular glycogen concentration increased, but not significantly. Testicular glycogen is widely associated with the supply of energy needed for Sertoli cells and spermatogonia in various phases of spermatogenesis, and increased levels of glycogen can also increase protein synthesis in spermatogenic cells due to their dependence on glucose for energy supply [33].

Moreover, LDH, GGT, and ALP are enzymes that play important roles in the testes; changes in their biochemical activity greatly affect the process of mammalian spermatogenesis. These enzymes are, therefore, recognized as markers of testicular function, especially in spermatogenesis, due to their role in the function and survival of spermatogonial stem cells [34], [35]. In this study, increased levels of the enzymes LDH, ALP, and GGT act as an indicator of spermatogenic potential, showing that the extract did not damage the testes, including germ cells and spermatogenic cells.

Furthermore, LDH is a germ cell-specific enzyme involved in spermatogenesis and sperm maturation. LDH also plays an important role in meiotic energy metabolism [36]. Thus, increased testicular LDH activity in treated animals provides an energy boost that promotes spermatogenesis by increasing spermatocyte maturation and transformation into spermatozoa. Similarly, increased LDH activity can cause a shift in tissue from aerobic to anaerobic respiration, which is favorable for spermatozoa metabolism [37]. In addition, carbohydrates have been recognized to be a significant source of energy for testicular animals that might related to the spermatozoa metabolism [38]. El-Kashoury *et al*. [39] also reported that reduced *in vivo* LDH activity could inhibit androgen production.

Testicular ALP, meanwhile, is an important enzyme that is directly involved in the movement and distribution of substances needed for steroidogenesis. In this study, the increased activity of testicular ALP in extract-treated animals may indicate an ability of the extract to promote steroidogenesis and spermatogenesis, as it provides a supply of essential biosynthetic materials and nutrients to Sertoli cells and germ cells [40], [41]. Furthermore, GGT plays a role in spermatogenic cells and promotes sperm maturation [3]. GGT is also an enzyme marker of Sertoli function and is involved in the secretion of fluid into the seminiferous tubules that carry spermatozoa into the rete testis [42]. The observed increase of GGT activity in the treated animals may be a sign of Sertoli cell stimulation.

Spermatogenesis and steroidogenesis are also susceptible to oxidative stress. Oxidative stress has been identified a cause of infertility that induces sperm dysfunction because it is caused by reactive oxygen species. Under normal conditions, the activity

Table 3: The effect of the ethanolic extract of *Ficus deltoidea* leaves on testicular biochemical parameters and antioxidant enzymes activities of male mice

Group mg/kgbw	Cholesterol (mg/dL)	Protein (mg/g)	Glycogen (mg/g)	LDH (U/L)	GGT (U/L)	ALP (U/L)	SOD (U/mL protein)	MDA (nmol/mg protein)
0	4.80 ± 0.37^{a}	0.53 ± 0.003^{a}	0.55 ± 0.22^{a}	446.00 ± 5.99^{a}	1.80 ± 0.37^{a}	3.80 ± 0.20^{a}	6.54 ± 1.41^{a}	31.15 ± 1.94^{a}
125	8.00 ± 0.32 ^{b}	0.55 ± 0.005^{b}	1.25 ± 0.26^{a}	476.00 ± 3.63^{a}	2.00 ± 0.45 ^{a}	4.60 ± 0.24^{a}	18.15 ± 3.84^{ab}	25.17 ± 7.27^{ab}
250	8.60 ± 0.40^{b}	0.55 ± 0.008^{b}	1.14 ± 0.33^{a}	514.40 ± 6.95^{b}	2.40 ± 0.24^{ab}	9.60 ± 0.51^{b}	29.92 ± 6.23^{bc}	13.01 ± 1.52^{b}
500	9.60 ± 0.51^{b}	0.56 ± 0.008^{b}	1.26 ± 0.55^{a}	1041.60 ± 16.40^{c}	2.60 ± 0.24^{ab}	17.20 ± 0.37^{c}	24.20 ± 5.26^{b}	21.07 ± 4.21^{ab}
1000	11.60 ± 0.51^{c}	0.58 ± 0.012^{b}	1.40 ± 0.27^{a}	1084.40 ± 5.42^{d}	3.80 ± 0.37^{b}	32.00 ± 1.67^{d}	41.87 ± 1.09^{c}	9.68 ± 1.89^{b}

Mean ± SEM. Followed by superscript letters (a, b, c, d) in the same column shows significantly different $P < 0.05$. LDH: Lactate dehydrogenase, GGT: Gamma glutamyltransferase, ALP: Alkaline phosphatase, SOD: Superoxide dismutase, MDA: Malondialdehyde

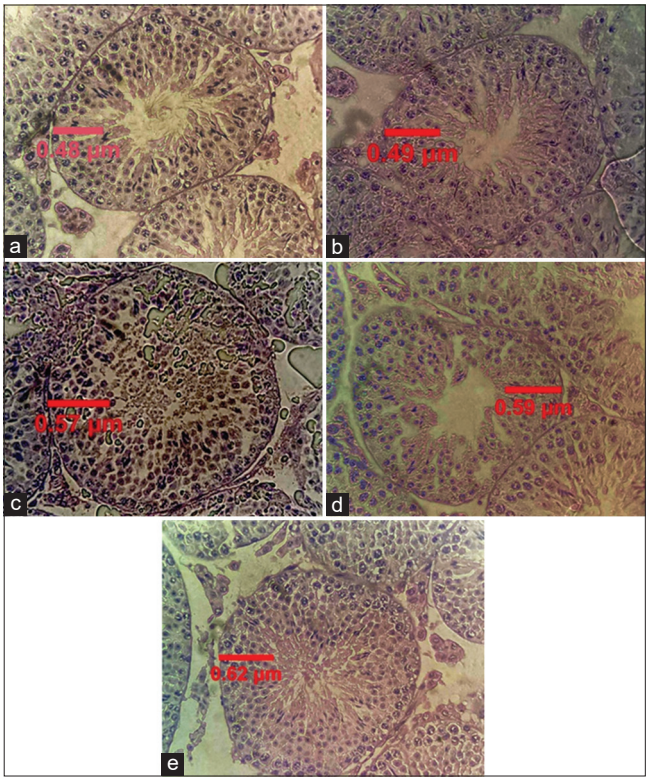

Figure 1: Testis histology structure of the control mice and mice treated with Tabat Barito. HE staining, ×100. Control group (a); treatment with extract: 125 mg/kgbw (b); 250 mg/kgbw (c); 500 mg/kgbw (d); (e) treatment with extract (1000 mg/kgbw)

of antioxidant enzyme activities, such as SOD, maintains free radical scavenging potential. SOD is an antioxidant enzyme that is considered the first line of defense against the harmful effects of free radicals in cells and plays a role in protecting spermatozoa against lipid peroxidation [43], [44]. In addition, MDA, the main end product of lipid peroxidation, has been used to assess the level of oxidative stress in tissues [43]. The decrease in MDA levels and the increase in antioxidant enzyme activity in the extract treatment group in this study indicated that the extract contained antioxidant secondary metabolites. The results of this study are in accordance with those of Haredy *et al*. [45] who documented that the activity of antioxidant enzymes in the seminiferous tubules was significantly increased by *F. carica* treatment. This is also supported by the results of Omar *et al*. [46] that found that *F. deltoidea* efficacy is attributed to its high phenolic and flavonoid content. In addition, the antioxidant research revealed that flavonoids supplied 85% of the antioxidant activities of *F. deltoidea*. This antioxidant component of *F. deltoidea* thus enhances testicular function, as seen through increased testosterone output.

Histopathological results showed that the ethanolic extract of *F. deltoidea* leaves was not toxic to the histological structures of the testes in male mice (Figure 1). Our findings are consistent with prior research indicating that *Momordica cochinchinensis* did not alter the shape and behavior of essential organs [47]. The histological observations revealed that there were no significant differences in testicular tissues for the group treated with the ethanolic extract of *F. deltoidea* leaves compared to the control group.

Histopathology is an indicator that is used to predict the dysfunction of a tissue or organ [48]. Histopathological examination, especially that of the testes, is recommended as an indicator of male reproductive toxicity [49]. Furthermore, histopathological examination of the testes in the controls showed normal tissue histological structure, a normal spermatogenesis process in the seminiferous tubules, a lumen filled with spermatozoa, and interstitial tissue that appeared dense and well organized (Figure 1a). In addition, the extract did not affect any reproductive parameters, including the histology of the testis compared to the control. Histological examination of the testes revealed that the treatment group's seminiferous tubules had normal spermatogenic and Sertoli cell organization without histopathological abnormalities (Figure 1b-e). There was a substantial difference in the thickness of the germinal epithelium of the seminiferous tubules in the treatment group compared to that of the control group (Table 2). At present, the results of this study support the safe use of doses for pharmacological ethanolic extract of *F. deltoidea* leaves. However, to determine potential chronic harmful effects of *F. deltoidea* leaves on the male reproductive system, a longer term experimental investigation of the ethanolic extract of the leaves should be conducted to detect any adverse effects after extended intake.

Conclusion

The ethanolic extract of *F. deltoidea* leaves has a beneficial effect on the reproduction of male mice by increasing fertility, reproductive hormones, and antioxidant activity and does not change the histological structure of the testes.

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