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APHRODISIAC ACTIVITY OF *TRIBULUS TERRESTRIS* EXTRACT IMPROVES MALE REPRODUCTIVE SYSTEM PARAMETERS AND FERTILITY

S. Malekzadeh[1,2*], H. K. Jashni[3] and F. Hooshmand[4]

[1]Department of Biology, Shiraz Branch, Islamic Azad University, Shiraz, Iran
[2]Department of Biology, Jahrom Branch, Islamic Azad University, Jahrom, Iran
[3]Department of Anatomical Sciences, School of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran
[4]Department of Pathology Sciences, School of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran
*Corresponding Author's Email: Samira_malekzade@yahoo.com

ABSTRACT

Since *Tribulus Terrestris* (TT) is known as an aphrodisiac and natural muscle-enhancing nutrition, this study aimed to investigate the effects of TT on the male reproductive system, including sex hormones, testicular histology, and sperm parameters. In this study, 35 adult male Wistar rats were randomly divided into five groups of seven rats including Control, Sham (normal saline, 2.5 ml), TT1, TT2 and TT3 (2.5, 5 and 10 mg/kg body weight, respectively). One day after receiving the last TT administration (by gavage once daily for 60 days), rats were sacrificed, testes were removed, blood samples were taken from the heart, and sperm analysis was performed. Results showed that administration of TT extract was significantly increased serum hormonal levels (Testosterone, FSH in 2.5 and 10 mg/kg, respectively), sperm parameters (sperm count and motility in 5 and 10 mg/kg of TT), and histology of the testis (the mean number of Sertoli (5 and 10 mg/kg of TT), Leydig in 2.5 mg/kg of dose) in rats ($p<0.05$). TT extract improves reproductive system parameters and fertility in the male rats. TT may also have beneficial effects on the male reproductive system by increasing the Testosterone hormone levels, sperm motility and testis histology.

Key words: *Tribulus Terrestris,* Fertility, Spermatogenesis, Sperm Motility, Testosterone

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INTRODUCTION

Aphrodisiacs are substances that increased sexual desire and performance which have direct effect on exciting male libido, maintaining reproductive activities, restoring healthy tissue function, and regulating the nerves of the glands and neuroendocrine system to maintain sexual power with the state of mind and body content (Dutta and Sengupta, 2018). Of course, the effectiveness of medicinal plants must be proven by clinical trials. Male Sexual Dysfunction (MSD) is the repeated inability to achieve normal sexual intercourse and interference with cycle of sexual response, leading to many problems such as physical illness, psychological disease (depression, anxiety, etc.), and feeling of inadequacy mainly in males. In men, many factors lead to MSD, including androgen deficiencies (hyperprolactinemia, testosterone deficiency, etc.), penile disease (Peyronie's, phimosis, priapism, smooth muscle dysfunction, etc.), neurological disorders (Parkinson's disease, Alzheimer's disease, spinal cord and cerebral trauma, etc.), pelvic surgery (to correct arterial or inflow disorder), systemic diseases (cardiac, hepatic, renal pulmonary, cancer, and post-organ transplant, etc.), drugs (anti-hypertensives, psychiatric medications, anti-androgens, etc.), lifestyle (cigarette smoking, chronic alcohol abuse, etc.), and aging (decrease in hormonal level with age, etc.) (Goel and Maurya, 2020).

Tribulus terrestris L. (TT), also known as Bindii, Gokshura and Puncture Vine, has long been used in traditional Chinese and Indian medicine to treat many disease such as inflammation, cough, oedema, ascites, leucorrhoea, and urinary tract infections. It is also commonly claimed that TT improves muscle strength in men, but its specific effect on fertility is unclear. So far, no long-term studies (60 consecutive days) on the three body sources, including testis tissue, sex hormone levels, and sperm parameters in rats, have been scientifically proven (60 days, once a day). Laboratory mice and rats offer the opportunity to prove this important hypothesis with at specific doses and durations in specific animals. high dose and long duration intake may also reveal liver toxicity, especially in athletes. It is worth nothing that reproductive system and sperm/oocyte health are highly sensitive to toxicity (Shojaeifard *et al.,* 2016; 2017). Studies have shown that TT contains steroids, saponins, flavonoids, protodioscins, quercetin, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, nitrate

potassium, aspartic acid, and glutamic acid (Akram *et al.,* 2011). TT extracts have many pharmacological effects, including cardioprotective, hepatoprotective, antiatherosclerotic, antitumor, antiarthritic, antioxidant, antibacterial, analgesic, and anti-inflammatory effects, and can be used to treat many diseases (Figueiredo *et al.,* 2020). There are a number of drugs used to regulate the male reproductive system and fertility. Formulated with Chinese herbal medicines has long been experienced in many cultures, but it needs to be verified by scientific experiments.

One study showed that the administration of TT (10 mg/kg) and Anacyclus pyrethrum (100 mg/kg) increased the sperm count, sperm viability and sperm motility. Also, histological examination showed a significant increase in spermatogonial leydig cells, and spermatid (Haghmorad *et al.,* 2019). Clinical studies have shown that administration of TT extract (7.5 mg/kg) significantly improves sexual performance in women (Akhtari *et al.,* 2014). Laboratory evidence also shows that plant extracts including Vitex agnus-castus, Glycyrrhiza spp., Cimicifuga racemosa, Paeonia lactiflora, Cinnamomum cassia, and TT reduce the LH, prolactin, fasting insulin, and testosterone. It may be recommended for women with oligo/amnorrhea, hyperandrogenism, and polycystic ovary syndrome (Arentz *et al.,* 2014). This study also showed that TT extract had a positive effect on spermatogenesis in sheep during the breeding season. Also, TT extract also increased spermatozoid count, sperm viability and motility within 40 days (Kistanova *et al.,* 2005). An alcoholic extract of Tribulus alatus (50 mg/kg) significantly increased serum testosterone levels, thereby increasing androgens (El-Tantawy *et al.,* 2007).

The aim of this study was to investigate the protective effects of TT extract on fertility and reproductive system parameters in male rats. In addition, these factors were analyzed by measuring sperm parameters (sperm count and motility), histopathology of testis (sertoli, leydig and spermatid cells count) and serum hormone levels (Testosterone, FSH, and LH) after administration of TT extract to male rats within 60 days.

MATERIALS AND METHODS

Ethics: All animal experimental protocols were performed under the supervision of the Local Ethics Committee of Medical Science University. Every efforts is made to minimize animal suffering and reduce the number of animals used.

Experimental design: Eight-week- old male Wistar rats weighing 180±20 g were randomly housed in individual cages with controlled humidity (55-60%) and temperature (22±2 ºC) under 12:12 light/dark cycle of room. Rats were randomly divided into five groups including, control (without any herbal treatment), sham (normal saline, 2.5 ml), and groups III, IV and V (2.5, 5 and 10 mg/kg body weight of Tribulus terrestris extract, respectively). Force feeding was performed once a day for 60 consecutive days. All animals were evaluated for performance according to the protocol (National Institutes of Health, 1985). Also, food and water were available ad libitum.

Tribulus terrestris extract: Tribulus terrestris plants were purchased and was powdered at the Botanical Laboratory of the Shiraz School of Medicine. The powder was stored in 80 ml of 70% hydroalcohol (per 100 grams of powder) in the percolation system for 72 hours. A crystallized dry extract was obtained under vacuum for 24 hours. The crystallized dry extract was then dissolved in a certain amount of saline. Rats were fed (by gavage) at 2.5, 5 and 10 mg/kg body weight dose for 60 consecutive days.

Determination of Serum Testosterone, FSH and LH: At the end of the study, blood samples were taken from the heart and plasma was separated by centrifugation at 3000 rpm for 10 minutes. Serum testosterone, FSH and LH levels were measured by Radio Immuno Assay (RIA) according to the instructions provided in the kit.

Sperm parameters: For sperm analysis, sperm are removed from the cauda epididymis, after which the sperm suspension is diluted in formaldehyde fixative (10% formalin in PBS). Dilutions (approximately 10 µl) were transferred to a haemocytometer and sedimented spermatozoa were evaluated under a microscope (Seed *et al.,* 1996).

Histology of Testis: Testes were removed by incision in the peritoneal region under ether anesthesia and getting out of the life cycle. Testes were placed in 10% formalin and were transferred to the histological laboratory for tissue section preparation and examination. Histological slides were prepared to a thickness of 5 microns, thickness, stained with hematoxylin and eosin (H&E), and examined by light microscope to identify the cross-sectional seminiferous tubules (Sertoli cells, leydig cells and spermatids) of all groups. Ten sections of each group were examined.

Statistical analysis: Data were analyzed by one-way ANOVA followed by Tukey analysis using the Statistical Package for the Social Sciences (SPSS) software (IBM SPSS Statistics V23 Core System; Armonk, N.Y., USA). Further, data were expressed as Mean±Standard deviation (SD) and were considered with $P<0.05$.

RESULTS

Determination of Serum Testosterone, FSH and LH level: The results of this study showed that treatment of the rats with TT extract had a significant effect on the levels of serum FSH and Testosterone but no significant

effect (p<0.05) on the levels of serum LH levels in male rats (Table 1). Furthermore, serum Testosterone levels in the TT1 group (2.5 mg/kg) were significantly increased compared to the control and sham groups. Furthermore, serum FSH levels in the TT3 group (10 mg/kg) were significantly increased compared to the control and sham groups.

Sperm parameters: The results of analysis of sperm parameters showed that subsequent administration of *Tribulus Terrestris* extract significantly improved sperm quantity and quality in the TT groups compared to the control group (Table 2; p<0.05). Mean sperm counts in TT2 and TT3 groups were significantly increased after administration of *Tribulus Terrestris* extract compared to control and sham groups (p<0.05). Also, the the average percentage of sperm motility was significantly increased in TT2 and TT3 groups compared to the control group (p<0.05). All summary data are shown in Table 2.

Histology of Testis: The result of evaluating the number of seminiferous tubule cells, including spermatid, sertoli and leydig cells, was evaluated, followed by administration of *Tribulus Terrestris* (Table 3; P<0.05). The mean number of sertoli cells in TT2 and TT3 groups was significantly increased compared to control group (P<0.05). Also, the mean number of leydig cells in the TT1 group was significantly increased compared to the control group (P< 0.05) (Figure 1).

Table1- Comparison of the serum sexual hormone levels of male rats treated with *Tribulus Terrestris* extract between different groups (p < 0.05).

	N	Testosterone (ng/dl)	FSH (mlμ/ml)	LH (mlμ/ml)
Control	7	0.40±0.10	17.01±2.84	0.60±0.12
Sham	7	0.55±0.06	17.69±3.90	0.62±0.10
TT1 (2.5 mg/kg)	7	1.70±0.67*#	21.10±3.34	0.92±0.10
TT2 (5 mg/kg)	7	0.62±0.05	22.90±0.72	0.71±0.10
TT3 (10 mg/kg)	7	0.91±0.30	28.02±2.65*#	0.89±0.11

Note: All the values are expressed as Mean ± SD. *P<0.05, control vs. Exp; # P<0.05, Sham vs. Exp

Table 2- Comparison of the sperm parameters (sperm count and motility) follow by administration of *Tribulus Terrestris* extract between different groups (p<0.05).

	Sperm count×10^6	**Motile sperms (%)**	**Semi motility sperms (%)**	**Immotile sperms (%)**
Control	25.14±1.48	51±0.01	30±0.01	20±0.01
Sham	25.16 ±1.07	51±0.01	30±0.03	18±0.02
TT1 (2.5 mg/kg)	61.57 ±3.53	67±0.08	12±0.01*	10±0.01*#
TT2 (5 mg/kg)	82.5 ±4.84*#	73±0.02*#	15±0.01	11±0.00
TT3 (10 mg/kg)	77.85 ±7.05*#	74±0.04*	13±0.02	12±0.02

Note: All the values are expressed as Mean ± SD. *P<0.05, Control vs. Exp; # P<0.05, Sham vs. Exp

Table 3- Comparison of the numbers of seminiferous tubule cells (Sertoli, Leydig and Spermatid cells) treated with *Tribulus Terrestris* extract between different groups (p<0.05).

	N	**Sertoli cells**	**Leydig cells**	**Spermatid**
Control	7	21.85±0.47	14.52±0.77	33.48±1.63
Sham	7	22.25±0.58	15.63±0.68	33.37±0.75
TT1 (2.5 mg/kg)	7	22.28±0.50	18.33±1.89*	31.77±1.07
TT2 (5 mg/kg)	7	23.48±0.52*	14.64±1.12	31.81±1.26
TT3 (10 mg/kg)	7	23.71±0.43*	14.25±0.76	35.45±1.56

Note: All the values are expressed as Mean ± SD.*P<0.05, control vs. Exp; # P<0.05, Sham vs. Exp

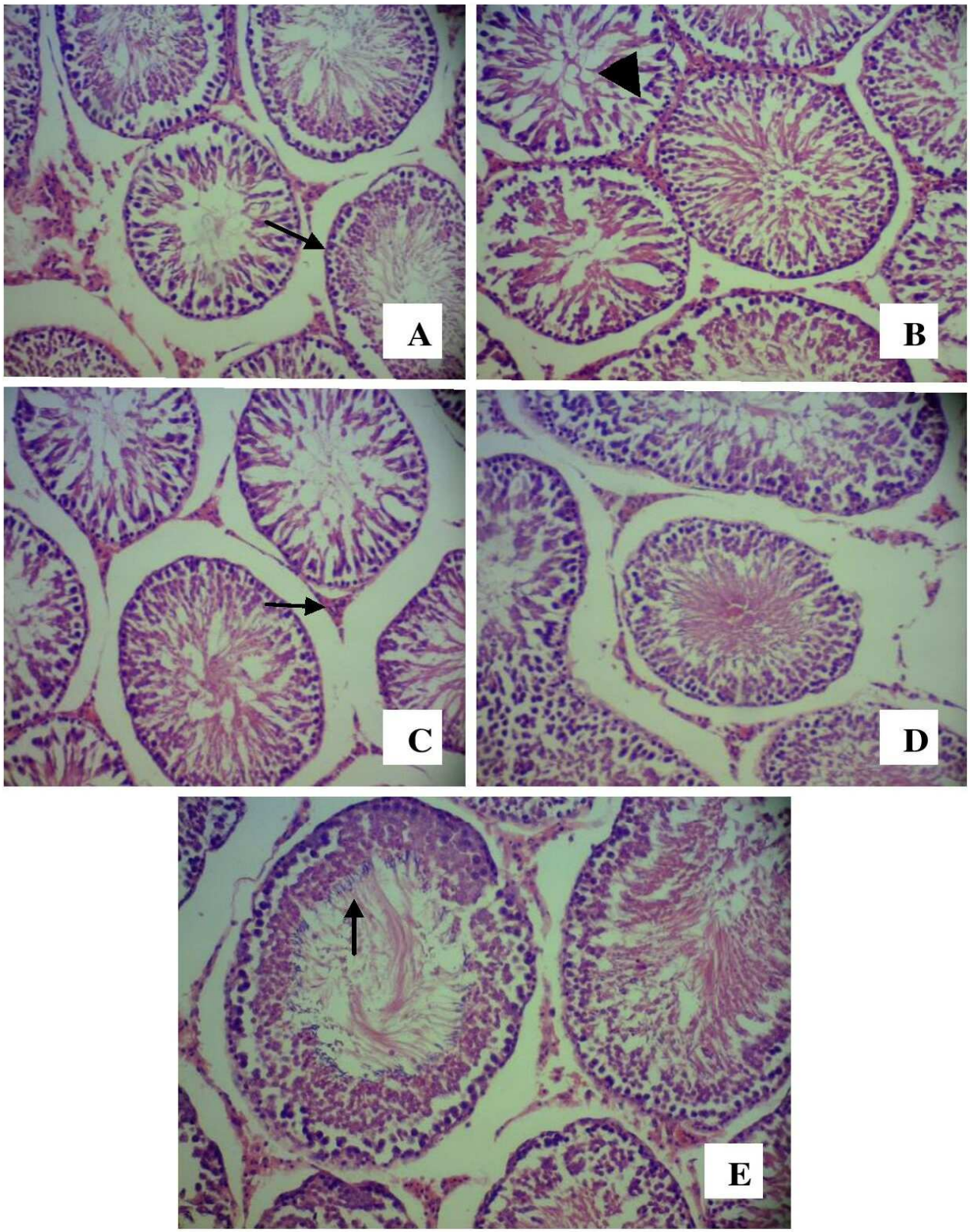

Figure 1- Photomicrograph of the seminiferous tubule cells in the transverse section in different groups (H&E; ×400). A: Control; B: Sham; C: TT1 (2.5 mg/kg TT extract), D: TT2 (5 mg/kg TT extract) and E: TT3 (10 mg/kg TT extract). Look at the arrows to indicate the primary spermatocyte (in A), sertoli (in B), leydig (in C), and full of spermatid (in E) cells in seminiferous tubules.

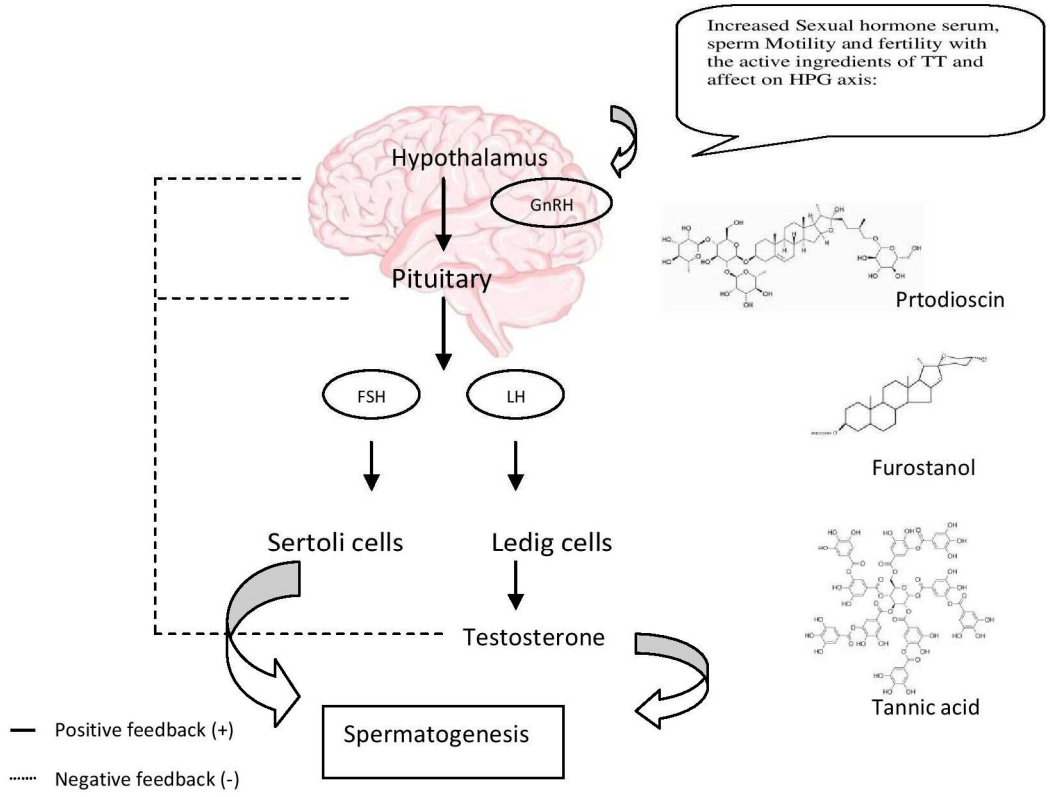

Figure 2- The hypothalamic-pituitary-gonadal (HPG) axis regulated male reproductive function and spermatogenesis. Also, TT which having combinations like Protodioscin, Frustanol, Tannic acid, etc. increased spermatogenesis and male fertility.

DISCUSSION

The results of this study showed that TT extract significantly improved fertility and the ability of the reproductive system performance in rats ($P<0.05$). Our results of this study also showed that administration of TT extract significantly increased sperm parameters (sperm count and motility at 5 and 10 mg/kg TT), histopathology (sertoli count at 5 and 10 mg/kg of TT and mean number of leydig cells at dose 2.5 mg/kg, and serum hormone levels such as Testosterone only at 2.5 mg/kg and FSH only at 10 mg/kg). LH levels were not statistically significant. It is clear from these results that mild administration of TT at low doses (2.5 mg/kg) can affect Testosterone. Higher dose may be effective in strengthening muscles, but lower doses in this increased Testosterone levels in rats within 60 days (once daily). A previous publication showed that administration of TT significantly increased primary spermatocytes within 60 days at a dose of 10 mg/kg (Karimi Jashni *et al.,* 2012).

The hypothalamic-pituitary-gonad (HPG) axis controls male reproductive function and spermatogenesis. Moreover, disturbance of the HPG axis affects the quality and quantity of semen parameters (Figure 2). In addition, some active TT gradients such as protodioscin, frustanol and tannic acid affect the gonadal system. One study examined the effect of TT extract on the sex hormones testosterone (T), dihydrotestosterone (DHT) and dehydroepiandrosterone sulphate (DHEAS) in various animal models (primates, rabbits and rats). They also demonstrated the role of TT extract in treating male erectile dysfunction (Gauthaman and Ganesan, 2008). Administration of Tribestan containing 250mg TT to humans and animals improved testosterone levels and libido and stimulated spermatogenesis within 60–90 days (Koumanov *et al.,* 1982; Tomova *et al.,* 1981). After administration of PTN to a hypogonadal patient, testosterone and lutinizing hormone levels increased within 30–90 days (Koumanov *et al.,* 1982). Consistent with these studies, our results showed that administration of TT at dose of 2.5 and 10 mg/kg, respectively, significantly increased testosterone and FSH.

Moreover, sperm count and motility improved within 3 months after TT treatment in patients with oligospermia (Balanathan *et al.,* 2001). Moreover, the 8 weeks of treatment with TT extract increased sexual behavior parameters in castrated rats (Gauthaman *et al.,* 2002). This study showed that incubation sperm from 40 healthy male volunteers with 20, 40, and 50 mg/mL of TT extract significantly increased total sperm motility, progressively motile sperm count, and curvilinear velocity after a retention time of 60-120 minutes. Furthermore, TT extracts (40 and 50 mg/mL) do not affect sperm DNA fragmentation after the 120-minute retention time, in vitro (Khaleghi *et al.,* 2017). One study reported that administration of TT extract increased sperm concentration and motility and decreased abnormal morphology in mice (Adaay *et al.,* 2012). Consistent with these studies, our results showed that TT extract significantly improved sperm motility and number in all experimental groups (2.5,5 and10 mg/kg).

One study showed that administration of TT after cadmium poisoning in rats had a protective effect against testicular injury. This is due to the antioxidant effects of TT, which stimulates the testosterone production from leydig cells (Rajendar *et al.,* 2011). Also, one study showed that testosterone levels in castrated rats were significantly increased after TT administration (Gauthaman *et al.,* 2003). They suggested that TT could be administered to humans during andropause, when sex hormone levels decline. The 3 weeks Treatment with 750 mg/day TT improved male sexual function in diabetic and non-diabetic men (Adimoelja and Adaikan, 1997). TT protodioscin also has the ability to stimulate and increase testosterone production. In this study, saponin-enriched TT extracts were shown to have protective effects (anti-glycation, antioxidant and anti-proliferative effects) on human tumor cell lines (Figueiredo *et al.,* 2020).

Ingestion of natural antioxidants significantly increases sperm count and sperm motility in infertile men while reducing the oxidative stress (Walczak-Jedrzejowska *et al.,* 2013; Singh *et al.,* 2016). Protodioscin from TT has a positive effect on spermatozoa maturation in patients (Adimoelja and Adaikan, 1997). A major cause of oxidative stress is ROS, which is one of the major causes of idiopathic male infertility and sexual dysfunction (Cocuzza *et al.,* 2007). Daily administration of 70 mg/kg TT daily for 20 days significantly increased the mean body weight, the weight of the paired testes and the seminiferous tubules weights in two-week-old rats (Bashir *et al.,* 2009). Tannins are one of the phytochemical constituents of TT. The study showed that the intake of tannins as herbal medicine has health-enhancing effects on serum biochemical indicators and female reproductive hormones. Also, hydrolysable tannin intake is sufficient to treat obesity and polycystic ovary syndrome (Manzoor *et al.,* 2020). Quercetin is another TT phytochemical compound with antioxidant activity. Furthermore, this study showed protective effects of 5-50 µm quercetin on boar semen and spermatozoa and motility after 24, 48 and 72 hours of semen storage (Tvrdá *et al.,* 2020). Furthermore, one study showed that TT extract (11 mg/kg) was protective against testicular injury in a cyclophosphamide mouse model within 14 days (Pavin *et al.,* 2018). Incubation of sperm suspension with Tannin-rich plant extract improved fertilization of boar *in vitro* for 1 hour. (Galeati *et al.,* 2020). One study showed that administration of ethanolic TT extract (100, 300, or 500 mg/kg) improved sperm quality parameters such as percentage of sperm motility and percentage of normal cells (Keshtmand *et al.,* 2014). There is evidence that TT improves male sexual behavior and male sperm count and quality (Arsyad, 1996). Administration of TT enhanced the fertilization effect and improved testicular morphology and histology in humans (Seth, 1994), immature sheep (Georgiev *et al.,* 1988), fish (Cek *et al.,* 2007), and rats (Dimitrov *et al.,* 1987). TT administration significantly increased the number of spermatids, soermatogonia, and spermatocytes in the seminiferous tubules, which was associated with the effects of protodioscin (Viktorov *et al.,* 1994). In this study, TT extract was showed to improve semen quality, sperm production, erectile function, libido and the testosterone levels in a ram, which may be related to saponins causing increased LH hormone from the pituitary (Hussain *et al.,* 2009).

One study (Martino-Andrade *et al.,* 2010) demonstrated that TT has no intrinsic hormonal activity, as it was unable to stimulate endocrine-sensitive organs in either male or female rats. However, the study was conducted over 28 days and serum testosterone levels did not change. This study contradicts our results that showed that TT extract significantly increased testosterone levels after administering 2.5 mg/kg of TT extract within 60 days. However, our results showed no effect of TT extract on LH hormone levels. This study showed that TT methanol extract increased serum-free testosterone levels in male mice (Hussain *et al.,* 2009). In addition, aqueous TT extracts have aphrodisiac properties and show protective effects on erectile function. They also reported that these properties may be due to increased androgen production and nitric oxide release from the nerve endings of the corpus cavernosum (Gauthaman *et al.,* 2003). One study reported that the weight of the rat increased after administering 6mg/kg TT fruit extract for 30 consecutive days (Singh *et al.,* 2009). The study investigated that effect of TT alcohol extract and aerobic exercise to improve the oxidative stress index after H2O2 intoxication in male rats (Rasolifoshazeh *et al.,* 2020). The feed of the sheep with TT showed positive nitrogen balance. This is because increasing the testosterone level helps to increase muscle strength, power and gains of muscle size (Sebata *et al.,* 2005). One study reported that

even if took TT supplements for five weeks, it had no effect on strength, body mass, or fat free mass in elite rugby league players (Rogerson *et al.,* 2007).

In general, we have shown that Chinese herbal medicine could affect fertility and reproductive system. In addition, the intake of TT extracts has significantly increased testosterone levels (in TT1), seminiferous tubule cells like leydig (in TT1) and sertoli (in TT2 and TT3), and sperm parameters (sperm count and motility in both TT2 and TT3). In addition, TT administration adjusted the HPG axis, improving the male fertility and sexual state of men, as well.

Conclusion: As a whole, this study has shown that the administration of the TT extracts improves male reproductive ability. The administration of the TT extract increased the serum testosterone of the male rat and FSH level, improved sperm count and motility, and improved the histology of testis. We also recommend taking it for MSD conditions. However, taking high dose of TT should be considered and potential adverse effects on other tissues should be investigated.

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