Potential Mitigation of Spirotetramat-Induced Reproductive Toxicity by *Tribulus terrestris* in Domestic Pigeons (*Columbia livia domestica*)

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**ABSTRACT**

*Tribulus terrestris* is traditionally used to treat various diseases. The current research aimed to study the protective effects of methanolic extract of *Tribulus terrestris* (TT) against Spirotetramat (SPT) induced reproductive toxicity in adult male domestic pigeons (*Columbia livia domestica*). For ten consecutive weeks and under an artificial photoperiod (19L: 5D), thirty male pigeons weighing approximately 309.20 ± 14.41g were divided equally into six groups as follows: CT served as the control, SPT group orally given with 15 mg/kg BW/day, TT100 and TT50 groups orally administrated with 100 and 50 mg/kg BW/day of TT respectively, and SPT+ TT100 and SPT + TT50 groups. Testicular volume and body weight were measured each two weeks. Whereas histopathological profile and luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T), total cholesterol (TC), HDL-C, LDL-C, and triglyceride (TG) were evaluated at week 10. The obtained data reveal that under a long photoperiod of 19L:5D, sexual activity lasted only 06 weeks in the control and TT groups, with a significant increase in testicular volume followed by spontaneous gonadal regression up to week 10. But testicular weights were superior in TT pigeons compared to the control during all experiments. However, the administration of SPT has suppressed gonadal growth and delayed photo-refractoriness. Sex hormones levels revealed a significant increase in LH and FSH levels in all groups compared to controls. However, a significant depletion in testosterone levels. Nevertheless, there was a substantial increase in TC, HDL-C, and LDL-C levels. Furthermore, co-administration of TT with SPT restored the lowered testicular volume, relative testicular weight, and T level but decreased the TC, HDL-C, and LDL-C levels. Finally, the histopathological investigation revealed degenerative changes in testes and gonad damage in the SPT group. However, TT reduced the damage induced by SPT. In conclusion, TT could be beneficial in preventing SPT reproductive toxicity and improving sex hormone synthesis.

**INTRODUCTION**

Exposure to environmental or xenobiotic substances may induce reproductive toxicity in living species (Oladele et al., 2016). Pesticides may also affect reproductive functions, including congenital abnormalities, reduced fecundity, infertility, and altered development (Collotta et al., 2013). SPT is a new tetramic acid-based insecticide that belongs to the keto-enol pesticide family. It has a unique mode of action that interacts with lipid synthesis (Gong et al., 2016b). Most infertile males have low sperm quality, as shown by reduced sperm counts, aberrant sperm geometrometry, and lower sperm motility (Haghmorad et al., 2019). Male infertility is caused by sperm abnormalities in the pre-testicular, testicular, and post-testicular phases (Dimitriadis et al., 2017). Many researchers have assessed SPT’s environmental and non-target species impacts. According to the results of their study, Huiming et al. (2012) concluded that SPT is absorbed and metabolised differently in different organs and tissues. In addition, Gutbrod et al. (2020) found that acetyl-CoA carboxylase activity was reduced by SPT exposure. Furthermore, Liu et al. (2011) report that rats were given 100 mg/kg/d SPT lost weight and had liver and genital damage after seven days. According to other research, SPT treatment can induce toxicity in zebrafish and oxidative stress in zebrafish.
Ovaries. SPT exposure can affect FSH-r and LH-r gene expression and oocyte size and maturation, among other things (Liu et al., 2011). Apoptosis of cells and gonad histological inflammation showed that exposure to SPT caused gonad damage in zebrafish and that SPT altered the endocrine (Zhang et al., 2020b).

Medicinal plants have been traditionally employed for their health-improving characteristics for many years. The belief that natural medicines might improve general health and help people overcome chronic diseases has fueled a global interest in plant-based supplements (Neychev and Mitev, 2016). There is a strong interest in plants that enhance male fertility due to the widespread male fertility deficiency caused by multiple factors, including environmental pollutants. Therefore, herbal remedies are preferred for treating male infertility, because natural antioxidants found in plants to treat many diseases were shown to cure male infertility without adverse effects (Safarnevadeh and Rastegarpanah, 2011). Studies have shown that phytotherapy, also known as plant medicine can increase testosterone levels and improve male fertility (Bahmanpour et al., 2012; Zang et al., 2015; Fedail et al., 2016; Zhu et al., 2017). TT of the Zygophyllaceae family is a widely spread plant across the globe. It has antibacterial, anti-inflammatory, aphrodisiac, antioxidative, hepatoprotective, cardiotoxic, anthelmintic, hypolipidemic, and diuretic properties (Chhatre et al., 2014; Singh et al., 2012; Hamidi et al., 2019; Tian et al., 2019). In addition, TT is commonly used in traditional Chinese medicine to treat various diseases, including coronary artery disease, post-stroke syndrome, cancer, hypertension, atherosclerosis, and myocardial infarction (Shahid et al., 2016). There are diverse active compounds in its various parts, including alkaloids, steroidal saponins, glycosides, flavonols, and flavonoids (Chhatre et al., 2014). TT influences spermatogenesis, as shown by changes in the testicular tubule, such as increased tubular volume, total tube length, and seminiferous epithelium height (Zhu et al., 2017). The TT extract increased the cytoplasmic, nuclear, and individual volume of Leydig cells in male Wistar rats (Neylanne et al., 2015). Several clinical examinations have shown that TT increases reproductive activity, including increased hormone levels such as estradiol, with testosterone being only slightly influenced (Gauthaman et al., 2002). In the testis, seminiferous tubules spermatogenesis is a multistep process highly regulated by hormones (Meri et al., 2013; Walker and Cheng, 2005). Furthermore, TT is a testosterone booster; TT saponins seem to bind with hypothalamus receptors that detect sex hormones, in part inhibiting the receptors directing to the hypothalamus, distorting the body’s sex hormone concentrations as lower than they certainly are (Sun et al., 2003). The hypothalamus signs of starting the synthesis of LH; When LH levels are augmented, the average production of testosterone also surges. The participation of TT in the enhancement of male sexual dysfunctions is well-recognized. TT has a protecting effect against cypermethrin (Poonam et al., 2013) and cadmium (Rajendar et al., 2011) induced testicular injury in the rat. According to (Haghmorad et al., 2019), treatment with TT and Anacyclus pyrethrum improved sex hormone levels and sexual indices. In addition, when Anacyclus pyrethrum and TT are used together, sexual parameters in Wistar rats, such as sex hormone concentrations, sperm quality, and male Wistar rat histarchitecture, improve. Thus, the primary objective of this study is to evaluate the protective effect of TT against reproductive toxicity induced by SPT by assessing body weight, testicular volume, relative testicular weight, sex hormones (FSH, LH, and Testosterone) levels, and lipid parameters (total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride) levels in the serum of pigeons.

MATERIALS AND METHODS

Chemicals

The Spirotetramat formulation (Movento®, 150 OD. CAS No. 203313-25-1, purity 98.5%) was purchased from Bayer Crop Science (Germany) (Augsburg, Germany). A dose of 15 mg/kg/day was applied in this study, according to LD50 of Spirotetramat to birds is above 2000 mg/Kg (Maus, 2008).

Plant material and preparation of the extract

Aerial parts of Tribulus terrestris (1.5kg) were purchased from a herbal market (Setif-Algeria, January 2021). The plant sample was identified by a botanist (Dr Nora Sakhraoui, University of Skikda); 1 kg of the Tribulus powder was macerated in a hydro-methanolic solution (80%) for 24 h. Eventually, the extract was filtered using a paper filter. Finally, methanol was evaporated below the vacuum evaporator (Heating bath, cat N=18100984, RE-100 Pro) at 45°C to obtain a solid residue. Two doses of 50 and 100 mg/kg of the plant were applied to the animals at the treatment time.

Animals

Thirty adult males of domestic pigeons (Columba livia domestica) weighing 309.20±14.41g were obtained from Sikka (North-East of Algeria) at the end of January. At the arrived time, animals were housed in polypropylene cages measuring 90x90x90 cm (Animal House, Department of Biology, University of Skikda). For two weeks, they
were acclimated in standard conditions 23±2°C, adequate aeration, and humidity of 50±10%). The pigeons were fed a standard chow diet (20% protein, 10% fat, and 70% carbohydrates) and given water ad libitum.

**Experimental design**

Thirty male pigeons were divided into six groups, each of five which they were orally treated for ten consecutive weeks as follows: (1) Group (CT): was used as a control and treated with distilled water. (2) Group (SPT) treated with Spirotetramat (15 mg/kg/day). (3) Group (TT100) treated with TT (100 mg/kg/day). (4) Group (TT50) treated with TT (50 mg/kg/day). (5) Group (SPT+TT100) treated with Spirotetramat (15 mg/kg/day) combined with TT (100 mg/kg/day). (6) Group (SPT+TT50) treated with Spirotetramat (15 mg/kg/day) combined with TT (50 mg/kg/day).

All animals were held below long artificial photoperiod (19L:5D) using an electrical clock.

**Body weight and testicular volume**

Gonadal status and body weight of animals were estimated each two weeks of the treatment. The testes were observed from a small incision between the last two ribs after a local anaesthetizing with viscous lidocain. The sizes of the left testis were assessed to the nearest 0.5 mm.

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\text{Testicular volume (V)} = \frac{4}{3} \pi a^2 b
\]

a is ½ the width, and b is ½ the length (long axis).

**Samples collection**

After ten weeks of treatment under a long photoperiod (19L:5D) pigeons were sacrificed. Blood samples were obtained and centrifuged at 4000 x g for 10 min. Testes were immediately collected, washed in distilled water, weighed, and fixed in 10% formol solution.

**Analytical procedures**

FSH, LH and testosterone (T) were assessed by Enzyme-Linked Fluorescent Assay (ELFA) using ELFA (VIDAS-BIOMERIEUX) automate.

Using a Beckman-Coulter Synchron LX20 PRO (Beckman-Coulter Inc, Fullerton, CA) and Syncron system reagents, total serum cholesterol (TC), high-density lipid-cholesterol (HDL-C), low-density lipid-cholesterol (LDL-C), and triglyceride (TG) levels in serum were determined in all groups.

**Histopathology examination**

Fixed tests were successively treated in ethanol, xylene, and paraffin designed for histological investigation.

Testes tissues entrenched in paraffin wax and attached on glass slides were segmented into 4 μm thick sections, discoloured with hematoxylin and eosin, air-dried, and observed under a light microscope (Zeiss, 400X).

**Statistical analysis**

We used Graph pad prism version 9.2.0 (GraphPad Software, LLC, CA, USA) for statistical tests analysis of results. All values were expressed as mean±standard deviation (SD) of five animals. Data from different groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for inter-group comparisons. All results were considered statistically significant when P<0.05.

**RESULTS**

**Body weight variations**

The evolution of the body weight during the study under a long photoperiod (19L:5D) is shown in Figure 1A. In the beginning, the experimental pigeons had an average body weight of all pigeons at the commencement of the

**Fig. 1. The effect of spirotetramat, extract of Tribulus terrestris (TT100, TT50) on body weight (g) and testicular volume (mm³) of male pigeons (Columba livia domestica) (n=5) during 10 weeks of treatment under a long photoperiod (19L:5D). SPT: Spirotetramat (15mg/kg.BW/day), TT100: Tribulus terrestris (100mg/kg.BW/day), TT50: Tribulus terrestris (50mg/kg.BW/day). Values= Mean ± SD. Methanolic extract of TT caused a significant increase in the body weight of pigeons. SPT induced a decrease in testicular volume. However, methanolic extract of TT could improve the testicular volume significantly.**
treatment was 309.20±14.41g. Data obtained revealed a significant increase in body weight in the TT50 and TT100 groups compared to the control (P<0.001). However, starting the fourth week, the SPT group’s mean body weight was lower than the control group.

**Testicular volume variation during the experiment**

The variations in testis size evaluated during the current study are presented in Figure 1B. In the beginning, animals had a mean testicular volume of 648.5±85.12 mm³. Control pigeons kept under long artificial photoperiod (19L:5D) had completed their reproductive cycle. The gonadal development lasts six weeks with a higher volume of 1011.76 mm³. TT 100 mg and TT50 mg groups showed a higher significance (P < 0.01 and P < 0.001, respectively) in the testicular volume than the control. However, during a long photoperiod of (19L:5D), pigeons exposed to SPT revealed a lower testes volume. But, administration of combined TT+ SPT significantly increased the testicular volume (P < 0.001).

**Sex hormones levels**

FSH, LH, and testosterone levels in the serum of pigeons treated under a long photoperiod (19L: 5D) for ten (10) weeks consecutive are mentioned in Figure 3. This figure revealed that the FSH and LH concentrations were significantly (p < 0.001) increased in TT100, TT50 groups, and SPT groups (p < 0.05 and p < 0.001, respectively) compared to the control. While, serum testosterone levels were significantly (p < 0.001) decreased in the SPT exposed pigeons. However, administration of TT100 and TT50 mg/kg combined with SPT 15 mg/kg restored the lowered serum testosterone level provoked by SPT. The TT100 and TT50 mg/kg treated pigeons showed significant (p < 0.001) elevated serum testosterone levels.

**Biochemical parameters levels**

TC, HDL-C, LDL-C, and TG levels in the serum of pigeons treated for ten weeks under a long photoperiod (19L:5D) are shown in Figure 4. Results showed a significant increase (p < 0.001) in TG, HDL-C, and (P < 0.05) in HDL-C serum levels in SPT exposed pigeons. All animals treated with TT combined with SPT significantly reduced the serum’s higher TC, HDL-C, LDL-C, and TG levels induced by SPT exposition.

**Histopathology examination**

Histological photomicrograph in the control group revealed a regular testis architecture (Fig. 5A1, A2). This group had generally formed normal seminiferous tubules with a consecutive stage of spermatogenesis. Normal epithelial tissue (thin, abundant spermatogenic cells and spermatozoa) was visible. Testes of the SPT group showed
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**DISCUSSION**

The current study investigated the protective effects of TT methanolic extracts against SPT-induced reproductive toxicity in male pigeons. For this, two doses (50 and 100 mg/kg/day) of TT and 15 mg/kg/day of SPT were applied for ten weeks to domestic male pigeons (*Columba livia domestica*) under a long photoperiod (19L:5D). Bodyweight, testicular volume, testes mass, sex steroid hormones (FSH, LH, and testosterone), cholesterol, HDL-C, LDL-C, TG levels in the serum, and testicular histopathology were evaluated to detect the testicular lesions.

Bodyweight is controlled by balancing food intake and energy expenditure (*Simpson et al.*, 2008). In fatty acid biosynthesis, acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA, the first step in fatty acid biosynthesis. Inhibition of this enzyme reduces fatty acid synthesis while...
Fig. 6. Histological photomicrograph structure of testis of TT50, SPT+TT100 and SPT+TT50 treated pigeons under a long photoperiod (19L: 5D) showing:

TT50 group (A1 and A2) Normal testicular tissue, normal seminiferous tubules, and a higher number of Spermatozoa and epithelial germ cells are observed. Our examination revealed that SPT+TT100 (B1 and B2) and SPT+TT50 (C1 and C2) groups showed a testis tissue that seemed close to the control. The extract has achieved in partially decreasing the damage induced by SPT. Magnification:

A1, B1, C1, C2 = X10. A2, B2 = X40. Stain H and E.

increasing fatty acid oxidation (Harwood, 2005). Our results suggest that the decrease in body weight following SPT administration in Columba livia pigeons may be due to the inhibition of acetyl-CoA carboxylase. Gong et al. (2016) revealed that SPT has substantially affected fertility in adults by inhibiting the acetyl-CoA carboxylase. In addition, a previous study demonstrated that acetyl-CoA carboxylase inhibitor therapy reduced adipose tissue and caused weight loss (Harwood, 2005). However, our findings showed a significant increase in body weight following TT administration. These results are accordant with Gautaman et al. (2003), Bashir et al. (2009), and Abadjieva et al. (2019), who found a significant rise in body weight in rats fed with different doses of TT extract. The obtained results suggest that the androgenic effect of TT causes an increase in appetite (Abadjieva et al., 2019).

Additionally, the present study revealed that under a long photoperiod (19L: 5D), domestic pigeons showed a fully reproductive cycle characterized by whole mature testes at the 6th week and followed by spontaneous gonadal regression. These results are accordant with Slimani et al. (2018). Moreover, Wingfield and Farner, (1993) found that for birds that increased photoperiod, an increase in gonadotrophin-releasing hormone (GnRH) secretion, which increases gonadotropin secretion and thus gonadal maturation hormones such as LH and FSH, which in turn induces gonad growth and steroid hormone production. However, the study showed that SPT treatment reduced testes size and disrupted the reproductive cycle. It is possible that SPT interfered with testicular function indirectly or directly by disrupting hypothalamic or pituitary gland activities (Recio et al., 2005; Mitra and Maitra, 2018). Furthermore, Zhang et al. (2020) have reported that SPT impaired gonad development and caused gonad injury. Damsgaard et al. (2016) indicate that Sertoli cell degeneration lowers the testicular volume. In this study, oral TT and SPT+TT co-treatment enhanced testicular volume and relative testicular weights. Other studies by Neylanne et al. (2015) and Bashir et al. (2009) found an increase in testes weight, total tube length, tubular volume, and seminiferous epithelium height in rats treated with TT extracts.

It is known that pesticides disrupt gonadal, adrenal, and thyroid function (Diamanti-Kandarakis et al., 2009; Slimani et al., 2011, 2014; Pandey et al., 2017). The present research showed a significant increase in FSH and LH levels. However, a decrease in testosterone levels following SPT exposure. Dandona and Rosenberg (2010) and Zhang et al. (2020) have reported a deficiency in testicular development, low serum testosterone and high LH and FSH concentrations. Moreover, Zhang et al. (2020) demonstrated that SPT exposure could reduce plasma estradiol-2, testosterone, 11-ketotestosterone, and numerous other genes, including hsd involved in testosterone production, which became inactive after SPT exposure. Testosterone biosynthesis is dependent on cholesterol availability; the rise in testosterone levels found in the present investigation following the SPT administration could be due to SPT’s indirect effect on testosterone biosynthesis. TT extract administration at two doses revealed a significant increase in testosterone, FSH, and LH levels compared to the control group. Also, combining TT with SPT significantly restored the high
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decrease in serum testosterone induced by SPT. These results are similar to Shalaby and Hammouda (2014), Pavin et al. (2018), Sanagoo et al. (2019), and Kamenov et al. (2017) findings. Our results suggest that TT extract contains antioxidant effects (Amin et al., 2006), and it also has an aphrodisiac effect that can increase testosterone production (Gauthaman et al., 2003). The capacity of TT to inhibit the generation of oxygen free radicals prevents testicular tissue peroxidation. We further proposed that TT may protect Sertoli cells by increasing testosterone synthesis in the testicles (Gauthaman and Ganesan, 2008). Furthermore, TT improves reproductive function in male rats by naturally boosting LH secretion, leading the body to produce excess testosterone (Adimoelja, 2000; Shalaby and Hammouda, 2014). Sharma et al. (2020) found that TT Methanolic extracts increased LH, enhancing Leydig cell number and function. Therefore, the saponin in TT leaves stimulates the pituitary gland to produce more LH. Because luteinizing hormone makes testosterone, it could help with sperm production, erectile dysfunction, and sexual satisfaction (YJ et al., 2001).

Several studies have shown that HDL-C cholesterol is the primary substrate for testosterone synthesis in rats (Charreau et al., 1981; Chu et al., 2003). Cholesterol is a precursor for steroid synthesis and is essential for male reproduction (Yokoyama, 2000; Parton and Hancock, 2004). Wise et al. (1993) found a positive correlation between testosterone and cholesterol in boar serum. But a recent study found that low testosterone causes severe hypercholesterolemia (Cai et al., 2015). The obtained results showed a significant decrease in serum T levels following SPT exposure. This suggests that SPT indirectly inhibits testosterone synthesis by inhibiting the enzyme acetyl-CoA carboxylase, which is involved in fatty acid synthesis and cholesterol accumulation. In our study, Only TT treatment did not affect TC, LDL-C, HDL-C, or TG levels. But when TT was combined with SPT, TG levels decreased significantly. Similar findings were reported by Chu et al. (2003), Altug et al. (2009), and Hussain et al. (2009), who found that TT could decrease serum TC and TG levels. In addition, previous studies showed that people with dyslipidaemia had reduced LDL-C and TC levels after using tribestan (Doncheva et al., 2006). Moreover, TT saponins have modulated lipid metabolism (Yang et al., 1999) and hyperglycemia (Li et al., 2002). Furthermore, Lirette et al. (1993) indicate that the saponins and flavonoids in TT inhibit enzyme processes that generate cholesterol. As a result, when SPT lowers TC, LDL-C, and HDL-C levels, TT becomes a very effective hypolipidemic compound.

Finally, the histological analysis revealed that SPT exposure caused irregularities and atrophy of seminiferous tubules, with aberrant spermatozoa in the tubular lumen. According to Zhang et al. (2020a), interstitial connective tissue hyperplasia and widening and the absence of seminiferous tubule walls were observed after SPT exposure, indicating that SPT exposure could cause lobule histology changes, which in turn could disrupt average sperm production. Additionally, our results showed a diminution in Sertoli cell numbers with vascular congestion and fibrous thickening. Many studies have reported that the seminiferous tubule and interstitial cells were injured, and the seminiferous epithelium was destroyed after SPT exposure Zhang et al. (2020a). However, co-treatment of TT with SPT restored regular testicular morphology and reduced SPT-induced damage. Our findings corroborate Bashir et al. (2009) findings of increased spermatogenetic cysts and late stages of spermatogenesis after TT treatment. Also, a study performed by Kamboj et al. (2011) and Kumar and Singh (2016) found that TT’s antioxidant properties can help decrease free radical damage.

CONCLUSION

Spirotetramat at 15 mg/kg/day for 75 days may be caused gonadal damage, endocrine system alteration and inhibition, and lipid metabolism disruption. On the other hand, treatment with *Tribulus terrestris* reduced testicular damage, improved sexual hormone (T, FSH, and LH) production, and regulated lipid metabolism. Therefore, *Tribulus terrestris* has higher antioxidant activity and could be considered a first-line treatment for male reproductive toxicity and other endocrine disorders.

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IRB approval

The study was approved by the ethics committee of University of 20 August 1955 - Skikda, Algeria.

Ethical statement

All procedures applied in this study, including animal housing and experimentation, were in accord with the conformist guidelines of the Animal Ethics Committee of Skikda University.
Statement of conflict of interest

The authors have declared no conflict of interest.

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