Acta Nat. Sci. (2023) 4(1): 68-76
DOI: 10.29329/actanatsci.2023.353.07
e-ISSN: 2718-0638

Original Research Paper
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ACTA NATURA ET SCIENTIA PRENSİP

The Effects of Diets Including Different Amount of *Tribulus terrestris* Supplementation on Spermatological Parameters and Fertilization Ability of Male Rainbow Trout (*Oncorhynchus mykiss*)

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Please cite this paper as follows:
Kılınç, D., Yorulmaz, B., & Öğretmen, F. (2023). The Effects of Diets Including Different Amount of *Tribulus terrestris* Supplementation on Spermatological Parameters and Fertilization Ability of Male Rainbow Trout (*Oncorhynchus mykiss*). *Acta Natura et Scientia*, *4*(1), 68-76. https://doi.org/10.29329/actanatsci.2023.353.07

ARTICLE INFO

Article History
Received: 02.05.2023
Revised: 30.05.2023
Accepted: 06.06.2023
Available online: 20.06.2023

Keywords:
Tribulus terrestris
Rainbow trout (*Oncorhynchus mykiss*)
Seminal plasma
Sperm quality
Sperm motility

ABSTRACT

The aim of this study was to examine how varying doses of *Tribulus terrestris* supplementation affected the fertilization ability and spermatological characteristics of male rainbow trout (*Oncorhynchus mykiss*). Male fish (mean weight of 497±5.29 g and a mean length of 33.5±3.4 cm) were fed with four experimental diets containing different amounts of *T. terrestris* (0, 100, 250, 500 mg/kg feed) for 90 days in duplicates. The motility time analysis of all groups indicates that the motility times of male rainbow trout fed with *T. terrestris* increased. Besides, the fertilization rate of the control group is lower than the groups that fed with *T. terrestris* supplementation. In comparison with the control group without any additives, especially the fertilization rate of the trout fed with 250 mg/kg was 95% ($p<0.05$). Furthermore, this study also examined how *T. terrestris* supplementation affects the blood plasma of male rainbow trout and it was found that the male rainbow trout fed with *T. terrestris* supplementation had the highest LH hormone value at 250 mg/kg.

INTRODUCTION

So far, plant supplementation has been tested for diverse uses, including the drugs, cosmetics and food supplement industries (Bhuvaneswari & Balasundaram, 2006). It has been reported that medicinal plants are powerful agents in comparison to chemical drugs and the compounds of medicinal plants enhance the therapeutic effects while reducing the toxicity and side effects of drugs. Besides, they help to accelerate gastrointestinal absorption process (Platel et al., 2002).

In recent years, there have been varieties of research conducted in the field of aquaculture which aim to decrease the use of chemicals and, as alternatives to those chemicals and antibiotics, many

different additives have been started to use as phytobiotics (Yeganeh et al., 2017; Elsabagh et al., 2018). The phytobiotics' analyses indicated that because they are harmless, they are extensively used as aqua-feed additives to support gaining weight of fish, improve feed conversion ratio in the fish farms and enhance growth performance (Yeganeh et al., 2017).

Plants are safe and cheap with their numerous benefits in animal nutrition such as supporting antibacterial, antiviral and antioxidant actions, activating appetite and feed intake, improving the secretion of digestive enzyme activation and stimulating immune responses (Citarasu, 2010).

Tribulus terrestris is an herbal supplement that grows in various regions of the word such as Europe, Australia, India and especially South Africa. It is an annual, flowering plant which belongs to a widespread family known as *Zygophyllaceae* family, consisting of about 250 species and 25 genera. *T. terrestris* is a crawling herbal plant that can grow as tall as one meter and usually grows in sandy soils and arid climates. The term Tribulus has its roots in the Greek word "tribolos", referring to a fruit with spiky projections. To treat various health conditions, the fruits of this plant are used in Bulgaria, in Ayurvedic medicine in India, and traditional Chinese medicine (TCM) (Pokrywka et al., 2014).

T. terrestris supplementation contains various compounds that possess both chemical structures and biological properties. These include alkaloids, terpenoids steroidal saponins, polyphenol carboxylic acids, tannins, and flavonoids. Flavonoids are commonly found natural compound providing hepatoprotective and antioxidant benefits (Miller, 1996). Besides, flavonoids are also known for their broad range of biological and chemical functions which include the ability of radical scavenge radicals (Kavitha et al., 2011). Furthermore, people utilize *T. terrestris* to enhance their muscle-building capacities. Also, the dominant saponin in *T. terrestris* is protodioscin which plays an important role in supporting the production of testosterone (Ganzera et al., 2001).

Tribulus terrestris has been found to exhibit antioxidant and hepatoprotective properties in different animals such as *O. mossambicus* (Kavitha et al., 2011). Moreover, as reported that to increase the testosterone hormone level in animals, these natural aromatic plants are used (Cek et al., 2007). In addition to all these, *T. terrestris* also improves sperm motility, stimulates sexual desire and enhances secretion of LH and testosterone (Bucci, 2000; Kavitha et al., 2011).

There are various bioactive components like steroids, alkaloids, flavonoids and phenolic aid in enhancing the antimicrobial activity, appetite, the growth, and the immunity of the cultured fish (Chakraborty et al., 2015). In their research, Cek et al. (2007) claimed that *T. terrestris* positively impacts growth performance, sex reversal and survival rate of various fish species like *Poecilia reticulate*, *Cichlasoma nigrofasciatum* and *Clarias gariepinus*. Besides, the results of the study found out that treated progenies exhibited better growth rates, more successful sex reversal and spermatogenesis compared to untreated ones.

Yet, *T. terrestris* as a natural supplement has been used for different purposes such as improving sexual activities, general health condition, feed utilization and treating male infertility. However, there is no study has been carried out on the impacts of *T. terrestris* on male rainbow trout. Hence, the major goal of this present study is to find out the influences of *T. terrestris* in different amounts on fertilization ability and spermatological parameters of male rainbow trout (*Oncorhynchus mykiss*).

MATERIAL AND METHODS

Study Area and Fish

The experiments were conducted on a commercial trout farm, located on the southwestern part of the Turkey. A total of 300 fish and including 60 replacement fish were selected among brood stock candidates (with a mean weight of 497±5.29 g and a mean length of 33.5±3.4 cm) of the farm in September.

Preparation of Herbal Extracts and Experimental Diets

T. terrestris fruit was obtained from a local market in Turkey. To prepare the extract, 100 g of the fruits were finally ground before mixing 1 liter of 90% ethanol. After heating the mixture at 80°C for 2 hours, through Whatman No. 2 filter paper, they filtered. Under reduced pressure, using a rotary evaporator, the filtrates were evaporated. The fruit extract yield was 20% (wt/wt). To yield 50% (wt/wt) of the crude extract, the aqueous extracts were filtered and then lyophilized. *T. terrestris* fruit extracts are protected and preserved in a refrigerator to use for this study.

The desired (100, 250, 500 mg) concentration of lyophilized dry 10g extract of *T. terrestris* was dissolved in 1 L 90% ethanol and sprayed on a commercial trout pellet (Abalıoğlu fish feed) while mixing the diet continuously for a homogenous distribution of *T. terrestris*. Then, the feeds were dried under vacuum to evaporate alcohol. The feeds were freshly prepared every week and were kept at +4 °C until feeding fish. For the experiment, 30 males were randomly selected for one of the 8 net pens ($2\times2\times2m=8m^3$) and placed into 2 concrete ponds. Each cage was installed one month before the experimental feeding and adaptation to the cages was achieved with standard care.

Experimental feeds were given for 90 days (from October to December). The fish were evaluated in bi-weekly. Before weighing, all fish were anesthetized with 500 ppm phenoxy ethanol. The live weights of the fish were measured in grams (g) with Cas brand precision scales and their lengths were measured in centimeters (cm) with a 1cm precision using a ruler.

Fish were fasted for two days before the feeding to ensure fecal contamination during milking for gamete uptake. Gametes were taken from normal male and female brood stocks by abdominal massage method into glass tubes and plastic containers. After semen was stripped from each cage, sperm samples were kept in plastic containers containing ice at 2-4°C for analysis and transported to the laboratory under controlled conditions. In the laboratory, spermatological characteristics such as sperm quantity, density, total spermatozoa, and percentage of motility, motility duration, osmolality and pH were determined.

Experimental Analysis

A micro pH meter probe (WTW 3110 GmbH, Germany) was used to measure the pH of semen, while within the help of a Gonotec Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany), osmolality measurements were conducted.

Hormonal Analysis

The hormonal levels found in blood serum are measured through various indices such as LH (ng/mL), testosterone (ng/mL), FSH (ng/mL) and were analyzed by centrifugation at 8000 rpm for 5 minutes and stored in liquid nitrogen.

Semen Analysis

Semen analysis was applied according to the method described by Tekin (1994). The following parameters were estimated: motility divided into motility percentage, motility duration and vitality. Sperm motility was determined and recorded in triplicates using a video camera (AxioCam ICc 5, Germany) attached to a phase-contrast microscope (Zeiss Axio Scope A1, Carl Zeiss Microscopy, Germany) at 400× (Rurangwa et al., 2004). The progressive motility (%) and the durations of progressive motility (s) were analyzed. Determination of sperm motility percentages were conducted by measuring the time until forward movement stopped and circular movement began. An arbitrary scale with 10% interval increments was used to assess sperm motility percentages. Non-motility was recorded as 0% (modified from Borges et al., 2005).

Seminal Plasma Composition Analysis

The samples were preserved at a temperature of −20°C to conduct biochemical and ionic analyses. an AbbottAeroset autoanalyzer (Chicago, IL, USA), along with its original kits, was used to measure the parameters of cholesterol, glucose, K^+, Cl^-, Na^+, $Ca2^+$ and $Mg2^+$. Furthermore, the seminal plasma pH level was measured with the standard probe of the WTW pH meter 3110 set2 device. Osmolality values were

determined by Gonotech Osmomat 030 crycoscopic osmometer.

Fertilization Process

To prevent differences that may occur during the fertilization stage and also to prevent contamination of the eggs, it was given importance not to mix urine, blood and feces. After milking process, the ovarian fluid was filtered. Fertilization of eggs was put in 12 cm aluminum containers. Freshly milked eggs were mixed with sperm for one minute using bird feathers. After that 100 ml of activation solution (60 mM $NaHCO_3$; 50 mM Tris; pH=9.5). After that 100ml of activation solution (60 mM $NaHCO_3$; 50 mM Tris; pH=9.5) and waiting for 3 minutes, the eggs were washed with 13°C hatchery water and filled with fresh water again. The eggs were left to swell for one hour then plate in incubation pens with a continuous flow system. The fertilization was performed 3 times for each sperm groups.

Statistical Analysis

Nonparametric Mann–Whitney U tests were used due to the unequal variance and sample size, followed by Kruskal–Wallis tests to show the differences of the variables in terms of the periods of spawning seasons (Mann & Whitney, 1947). One-way analysis of variance (ANOVA) was used to analyze the effect of treatment on different parameters and Tukey's multiple comparison test was used (Kirk, 1968). The effects of treatment on different parameters. Results are shown as mean±SD and statistical significance level was set as $p<0.05$ and all statistical analyses was conducted using SPSS version 11.5.

RESULTS

Hormonal Analysis

The effects of dietary supplementation of *T. terrestris* extract on male rainbow trout's LH, testosterone, FSH are given in Table 1. There were no significant differences ($p<0.05$) among experimental groups for testosterone, FSH and LH. The highest level of testosterone was recorded in TT100 followed by TT250 and TT500, while the lowest value was recorded in CG ($p<0.05$). The highest level of FSH was recorded in TT500 followed by TT250, CG, while the lowest value was recorded in TT100 ($p<0.05$). Finally, the highest level of LH was recorded TT250, followed by TT500 and TT100, while the lowest value was recorded in CG ($p<0.05$).

Semen Analysis

Results of the effect of dietary supplementation of *T. terrestris* extracts on semen parameters of the male rainbow trout are presented in Table 2. It showed significant differences ($p<0.05$) among all experimental groups. The greatest vitality was achieved in TT500 followed by TT250 and TT100, while the lowest vitality was recorded in CG. When the percentage and average duration of motility data were analyzed, it showed that the greatest level of average motility duration was recorded in TT250 followed by in TT500 and TT100, while the lowest value was recorded in CG. Considering all sampling periods, it showed significant differences ($p<0.05$) among all experimental groups. It was observed that all groups and linear motility times were below the literature average, whereas linear motility gained in samples with *T. terrestris* added was better. As a result of the examination of each group's semen parameters, as it is given in Table 2, the duration of the period of highest vitality mean (49.6 s) was recorded in 500 mg/kg, TT-reinforced group, while the lowest one (35.2 s) was saved in CG.

Seminal Plasma Composition Analysis

Results of seminal plasma composition parameters are presented in Table 3. Table 3 shows the presence of no significant differences ($p>0.05$) among experimental groups for osmolality values. There was a clear relationship between seminal plasma osmolality and sperm motility. The osmolality of the seminal plasma and the motility of spermatozoa are mainly affected by the ionic content, pH and osmolality values surrounding the cells.

The suitability of fish sperm to gain motility in the testicles depends on the ion composition, osmolality and pH values of the seminal plasma in the reproductive canals.

Table 1. Effect of oral administration of *T. terrestris* extract on testosterone, FSH and LH of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Testosterone(ng/ml)	3.19[a]	3.59[a]	3.47[a]	3.44[a]	0.05
FSH (ng/ml)	5.17[a]	5.12[a]	5.37[a]	5.57[a]	0.05
LH (ng/ml)	3.59[a]	3.80[a]	3.95[a]	3.84[a]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table 2. Effect of oral administration of *T. terrestris* extract on semen parameters of male rainbow trout

	CG	TT100	TT250	TT500	p-value
Mean Motility (%)	85[a]	87[a]	92[ab]	93[ab]	0.05
Mean Motility duration (s)	21.75[a]	24.41[a]	27.16[b]	25.25[ab]	0.05
Mean vitality (s)	35.2 [a]	39.8 [ab]	40.4 [ab]	49.6 [b]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table3. Effect of oral administration of *T. terrestris* extract on seminal plasma composition of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Osmolality mOsm/kg	0.269[a]	0.284[a]	0.286[a]	0.288[a]	0.05
pH	7.39[a]	7.47[a]	7.55[a]	7.65[ab]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<0.05).

Table 4. Effect of oral administration of *T. terrestris* extract on reproductive performance of the male rainbow trout

	CG	TT100	TT250	TT500	p-value
Fertilization	92.33[a]	96.11[b]	95.22[b]	94.33[a]	0.05
Incubation	80.55[a]	84.77[ab]	87.11[b]	84.11[ab]	0.05
Hatching	70.37[a]	75.25[ab]	80.25[b]	77.12[ab]	0.05

Note: *Significant differences between groups (one-way ANOVA, p<.0.05).

The pH values among all experimental groups showed significant differences (p<0.05). The highest pH value was recorded in TT500, while the lowest one was observed in CG.

Reproductive Performance

Results of the effect of dietary supplementation of *T. terrestris* extract on reproductive performance parameters (Table 4) showed the presence of significant differences (p<0.05) among experimental groups for fertilization, incubation and hatching. The greatest effect of *T. terrestris* on fertilization values was recorded in TT100 followed by TT250 and TT500, while the lowest value was observed in CG. In addition, while the highest value of incubation in TT250 was recorded, TT100, TT500 and CG followed it respectively. When the hatching values of male rainbow trout were observed, the highest value was recorded in TT250 followed by TT500, TT100 and CG.

DISCUSSION

This study aimed to investigate how different amounts of *T. terrestris* affect male rainbow trout's reproductive efficiency. To achieve this goal, the effects of *T. terrestris* were examined in the diet of *O. mykiss* males. This study revealed that *T. terrestris* extract significantly increased sperm quality, fertilization, incubation and hatching rates of trout.

T. terrestris is rich in bioactive compounds such as protodioscin and steroidal saponins and it was proposed that steroidal saponinsis the primary active constituent stimulating the production of hormone (Kavitha et al., 2011). According to the consequences of this present study, *T. terrestris* extract affects testosterone production and also has positive effect on LH values compared to control group. A variety of studies have been carried out to examine the

influences of *T. terrestris* supplementation causing changes in the hormonal values of living beings.

Moghaddam (2013) studied the effect of *T. terrestris*, a traditional Unani drug used to increase sexual activity, on the gonadotropin levels and sex hormone of dependent male rats fed a diet with the addition of *T. terrestris*. A significant increase in hormones ($p<0.05$) in the treated dependent group was reported and oral consumption of *T. terrestris* also caused to antagonize a significant decrease in sex hormones and gonadotropins. Besides, there are varieties of clinical research supported the findings of this study, has demonstrated that *T. terrestris* extract positively impact the concentration levels of hormones such as estradiol, improves reproductive function, libido and ovulation by minimally affecting testosterone (Dimitrova et al., 2012).

Cek et al. (2007) found a significant correlation between sperm reproduction and motility. It has been stated that increased sperm motility improves the reproductive performance of men; therefore, motility plays a crucial role in assessing the reproductive capacity. In this study, comparative results of spermatological features of motility were examined and accordingly, it was determined that *T. terrestris* supplemented diet with a dosage of 250 mg was the most effective amount for improving semen motility times in rainbow trout. Besides, according to the quantity given in the duration of the period of highest vitality 49.6 s (500 mg/kg, TT-reinforced group) and the lowest 35.2 s (control group) was recorded.

Similarly, Hassona et al. (2020) conducted a study to examine the influences of *T. terrestris* nutritional supplement on reproductive potential and growth performance of male Nile tilapia. In their study, sperm analysis, sperm concentration, viability and motility were investigated. The study revealed that *T. terrestris* had the most significant effect on semen parameters, respectively TT750, TT500 and TT250 as compared to the control group ($p<0.05$). Haghmorad et al. (2019) analyzed the viability and motility of semen in their study on improving fertility parameters by adding *T. terrestris* to the diets of male rats. The viability and motility of the semen was evaluated by the Eosin-Nigrosin method under the light microscope. Accordingly, it was observed that the viability and motility rate of the semen of rats fed with *T. terrestris* extract increased.

Studies have been conducted on the fact that the viability periods of spermatozoa change in various activation solutions (Jayaprakas & Bimal Lal, 1996). Khaleghi et al. (2017) found in laboratory studies on the improvement of sperm parameters in humans by *T. terrestris* extract. 40 volunteer healthy men were divided into 4 groups and incubated at 20, 40, 50 mg/ml *T. terrestris* respectively in three groups other than the control group. *T. terrestris* extract was found to be effective in inhibiting motility of sperm in comparison to control group. Additionally, the extract increased the number of progressive motile sperm in particular and improved the viability of spermatozoa. Grigova et al. (2008) conducted a study to examine the effects *T. terrestris* extract adding in the water of the breed on the quality of sperm of the white Plymouth rock. The results showed that the concentration of spermatozoid, sperm motility and the ejaculate volume of the chickens in the treatment applied by adding *T. terrestris* extract increased.

In this study, besides spermatological parameters, the results of *T. terrestris* extract according to fertilization rates were analyzed. As mentioned above, it was reported that fertilization rates were higher in all male rainbow trout fed with *T. terrestris* extract, but especially in those with 100 mg and 250 mg extract added. It is seen that the fertilization rates of the animals fed with *T. terrestris* extract are higher when compared to the control groups. Haghmorad et al. (2019) studied how *T. terrestris* extract influences the fertility abilities of male rats. It was observed that the hormones affecting fertilization increased significantly in rats fed with TT extract compared to the control group.

Another study conducted to investigate the effects of orally administrating an alcoholic extract of *T. terrestris* on fertility of male rabbits. The study comprised fifteen male rabbits of mixed breed, aged between 4-5 months and weighing 1.5–1.7 kg. It was reported that male rabbits' fertilization parameters were enhanced by the use of alcoholic extract at doses of 75 mg/kg/day, 150 mg/kg/day. The 150 mg/kg/day

dose was observed to be the most effective in improving male rabbits' fertilization level (Meeni, 2016).

The literature review confirmed that many plants are used as fertility regulators around the world (Bhatia et al., 2010). There are several medicinal plants that are commonly utilized as agents to improve fertility (Sumalatha et al., 2010). *T. terrestris* is one of these non-hormonal plant species with tremendous medicinal properties and has been shown to support fertilization by increasing sexual behavior (Gauthaman, 2002).

CONCLUSION

The present results demonstrated that *T. terrestris* supplementation of diet improves spermatological parameters of male rainbow trout. It was revealed that *T. terrestris* extract causes significant increase in the fertilization, sperm quality, and hatching rates of male rainbow trout. Considering the previous experience, it has been observed that it can be effective not only in the breeding season but also in the off-season production.

The results of this study have drawn attention to the importance of *T. terrestris* plant extract, used as an alternative to chemical, leads to increase the reproductive and fertilization performance of cultured fish of rainbow trout. The use of synthetic hormones for the stimulation of the reproduction of farm animals has numerous consequences. The use of hormones is sometimes ineffective and often causes permanent hypo function of the hypothalamic-pituitary-gonadal axis. Apart from this, synthetic hormones and their derivatives accumulate in animal products (milk, meat, etc.) and become dangerous for human health. Therefore, it is necessary to prefer ecologically clean reproductive stimulants in order to obtain high quality and reliable products for human health.

As mentioned, supplementation of phototherapeutic plants such as *T. terrestris* to the diets of cultured fish, which is also the subject of this study, improves gamete quality, supports the increase of fertilization affecting positively production. It was concluded that incorporating medicinal plants into fish can effectively contribute to sustainable, cost-effective and secure fish farming practices.

ACKNOWLEDGEMENTS

The study presented here is a component of the Ph.D. thesis of the first author conducted under the guidance of the first and second authors.

Compliance with Ethical Standards

Authors' Contributions

DK: Collected and transported samples to the laboratory, researched and reviewed literature, designed and wrote manuscript, determined and organized sampling field, prepared and designed laboratory experiments, performed analyzed of data, drafted and also edited of manuscript.

BY: Supported literature review and performed drafting.

FÖ: Chose and determined of this study area, managed field sampling, controlled and checked laboratory experiments, performed and managed data analysis.

All authors read, checked and approved the last version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The research has received approval from the relevant institutional and/or national ethics committee and it has been carried out in compliance with the ethical principles outlined in the 1964 Declaration of Helsinki, as well as any subsequent revision or equivalent ethical standards. The authors affirmed that they adhered to all pertinent international, national, and/or institutional protocols concerning the treatment and utilization of animals. It was declared that this study complies with research and publication ethics.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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