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Early Gonadal Differentiation, Sex Ratio, and Growth Performance of Nile Tilapia (*Oreochromis niloticus*) with *Tribulus terrestris* Extract Supplementation

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Abstract

One way to increase the production of Nile tilapia (*Oreochromis niloticus*) is to breed dominant male monosex tilapia. Natural steroid hormones derived from Tribulus (*Tribulus terrestris*) are safe and environmentally friendly. This plant includes flavonoid compounds, steroid saponins, and aphrodisiacs. This study aims to evaluate the effect of Tribulus seed extract on early gonadal differentiation, sex ratio, and growth performance of tilapia juveniles. The study was conducted for 60 days at the Technical Implementation Unit of Freshwater Aquaculture Fisheries Service (UPTD PBAT) of Kota Agung, Tanggamus. The study used a Completely Randomized Design (CRD) with five treatments: 17α-methyltestosterone 0.0005 g/L (P1), and different doses of Tribulus extract 0 g/L (P2); 0.005 g/L (P3), 0.01 g/L (P4), 0.02 g/L (P5) with three replications. Ten-day-old larvae were immersed in the treatment solutions for 12 hours weekly, repeated three times, and water quality was monitored during the first month to ensure optimal conditions. The larvae were initially fed *Artemia* four times per day for 20 days and switched to a commercial diet until the end of the rearing period. Histological analysis of gonad sex differentiation was performed at different stages, and the sample was taken 10-60 days after hatching to determine the sex ratio of each treatment group. The treatment significantly affected the early gonad development and sex ratio ($P<0.05$), while it did not significantly affect the growth performance and survival rate ($P>0.05$). The best dose to increase the percentage of males in Nile tilapia was the 0.01 g/L Tribulus seed extract.

INTRODUCTION

Tilapia (*Oreochromis niloticus*) is one of the most widely studied sexual species in the context of sex control, and one of its forms is sex reversal (Baroiller and D'Cotta, 2018; Silva *et al*., 2023). In tilapia farming, sex reversal targets monosex males. The way

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to produce male monosex tilapia is by sex reversal (masculinization) technique, in which the technology directs sex differentiation to males. This is achieved when the gonads of the fish are not yet differentiated (Bardhan *et al*., 2021). According to Nivelle *et al*. (2019), sexual differentiation of tilapia occurs between 10 and 30 days after fertilization. Commonly used methods are immersion, injection, and oral. The immersion method is considered more effective during the larval and juvenile stages because it can reduce stress levels in fish (Heltonika *et al*., 2023).

The monosex technique is done by administering synthetic or natural hormones. Natural steroid hormones derived from plants are safer and more environmentally friendly. Some natural ingredients commonly used in fish include honey in guppies (*Poecilia reticulata*) and siamese fighting fish (*Betta* sp.) (Sarida *et al*., 2010; Lubis and Fitriani, 2017), coconut water in siamese fighting fish (Dwinanti *et al*., 2018), purwoceng extract in guppy (Matondang *et al*., 2018), pine pollen flour in tilapia (Abaho *et al*., 2022) and Tribulus extract in tilapia (Ghosal and Chakraborty, 2020; Matter *et al*., 2024).

Tribulus (*Tribulus terrestris*) is an herbaceous plant belonging to the group of flavonoids, which includes steroid saponins and contains aphrodisiac compounds that are considered the most effective, good, and safe in increasing testosterone hormone production (Chhatre *et al*., 2014; Al-Garadi *et al*., 2022). Previous studies reported the application of this extract on cichlid fish (*Cichlasoma nigrofasciatum*) through the seed immersion method showed that the highest percentage at a dose of 0.3 g/L which is 87.23% (Cek *et al*., 2007a). Also, a similar study was conducted on tilapia using the feeding method, which showed the highest results at a dose of 2 g/kg of feed of 90% males (Ghosal *et al*., 2015) and was tested again in 2017 and obtained 91.53% (Ghosal and Chakraborty, 2020).

So far, the use of Tribulus extract through immersion in tilapia seeds has never been done, so it is necessary to study the stages of differentiation and gonadal development of tilapia seeds through the administration of Tribulus extract with the immersion method that can provide real results in obtaining the highest percentage of males in tilapia seeds. This study is expected to provide an alternative method to create a monosex population of male tilapia which has been done using synthetic hormones that are expensive and difficult to degrade by the environment.

METHODOLOGY

Ethical Approval

The test subjects utilized were 0-day-old tilapia larvae resulting from the breeding of sultana strain tilapia parents obtained from the Technical Implementation Unit of Freshwater Aquaculture Fisheries (UPTD PBAT) in the Western Region of Tanggamus Regency, with a total of 70 individuals for each replication. These larvae were reared for 10 days, followed by a weekly treatment regime for 30 days. Subsequently, after the immersion treatment, the tilapia larvae were maintained without administering hormones for 60 days. The experimental animals were handled following appropriate care standards, ensuring an optimal environment free from harmful bacteria or toxic substances.

Place and Time

This research was conducted from April to June 2020 at the Technical Implementation Unit of Freshwater Aquaculture Fisheries Service (UPTD PBAT) in the Western Region of Tanggamus District, Lampung, and the Fisheries Cultivation Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Lampung.

Research Materials

The materials and equipment used in this research were a container (two types: 45 L and 12 L), a digital scale (Superior mini digital platform scale I-2000, China), a binocular microscope (Leica DM 500, Singapore), cover glass, object glass, 1.5 ml

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Eppendorf tube, 1 L beaker glass, thermometer, pool, aerator. The larvae were obtained from UPTD PBAT Western Region, Tanggamus, NaCl 100%, tissue, water, ethanol absolute 96% (Merck 1.00983.2511, Germany), *T. terrestris* powder (Flozindo), alcohol 70%, commercial feed pf 500, 17α-Methyltestosterone (Argent Labatories Inc., Philipines).

Research Design

The research design utilized was the Complete Randomized Design (CRD) method, incorporating five treatments with three replicates each. The treatments were as follows: (P1) 500 µg/L concentration of 17α-Methyltestosterone (positive control), (P2) 0 g/L concentration of Tribulus extract (negative control), (P3) 0.005 g/L concentration of Tribulus extract, (P4) 0.01 g/L concentration of Tribulus extract, and (P5) 0.02 g/L concentration of Tribulus extract.

Work Procedure

Preparation of *T. terrestris* Extract

Tribulus powder (100 g) was prepared, placed in a glass jar, and mixed with 1 liter of 90% ethanol solvent. The mixture was stirred in a water bath (80°C) for 2 hours, then cooled and filtered using filter paper to obtain the filtrate. The filtrate was concentrated using a rotary vacuum evaporator at 85 rpm with a temperature of 45°C (Do *et al*., 2013; Sasikumar *et al*., 2014). The resulting thick extract was stored in a dark bottle at -20 °C until use.

Immersion Treatment

At the specified treatment dose, 70 larvae tilapia eleven days old were placed in immersion containers containing a solution. The larvae were immersed for 12 hours and subsequently transferred to maintenance containers. This immersion process was repeated weekly until the larvae reached one month of age. Weekly siphoning was conducted, and water quality was assessed during each sampling to ensure optimal conditions were maintained.

The Maintenance of Fish

Feeding was done when tilapia larvae were 5 or 6 days old after release from the yolk. The larvae were then fed with *Artemia* for up to 20 days and the larvae began to be fed with commercial powder until the end of rearing using the *ad satiation* method. The frequency of feeding was done 4 times a day when the larvae were fed with *Artemia* at 08:00 am, 1:00 pm, 5:00 pm, and 9:00 pm and 3 times a day when the larvae were fed with commercial feed.

Histology Gonadal Sex Differentiation and Sex Ratio

Histological analysis of gonadal sex differentiation was performed by sampling 15 fish at 10 days post-hatching (dph), followed by 8 fish at 20, 30, and 40 dph from each treatment. Subsequently, a sex ratio analysis was conducted by sampling 20 fish at 60 dph from each treatment. The fish were anesthetized with clove oil at a concentration of 300 ppm (Fernández-Lázaro *et al.*, 2022), after which their length and weight were measured. The trunk was then dissected and placed in a 10% neutral buffered formalin (NBF) solution for 24 hours, rinsed with 70% ethanol, and stored at room temperature until further processing. Histological preparation was conducted at the Lampung Veterinary Center, involving standard procedures where samples were dehydrated in an ascending alcohol series, embedded in paraplast, and cross-sectioned at a thickness of 5 μm. The histological sections covered the entire length of the abdominal cavity and were stained with hematoxylin-eosin.

Data Collection

The gonadal sex differentiation, sex ratio, specific growth rate, and survival rate of tilapia fry were observed in this study. The gonadal sex differentiation of tilapia juvenile was observed every ten days from 10 to 40 dph, meanwhile, the other variables were calculated according to the following equations:

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$$SGR = \frac{\ln Wt - \ln W0}{t} x100$$

Description:
SR = specific growth rate (% day^{-1})
Wt = final weight
W0 = initial weight
t = number of rearing days

$$\text{Sex ratio}(\%) = \frac{\text{number of male fish}}{\text{numbers of final fish}}$$

$$SR(\%) = \frac{Nt}{N0} x100\%$$

Description:
SR = survival rate (%)
Nt = Number of fish survived at the end of experiment
N0 = Initial number of fish stocked

Data Analysis

Data were analyzed quantitatively with Microsoft Excel 2016 numerical processing software application and SAS 9.4 statistical data processor at 95% confidence level with analysis of variance. Further tests for significant differences were carried out with the Duncan test. Data analysis of early gonad development and differentiation of tilapia seeds were analyzed qualitatively by observing gonad morphology based on the criteria found and analyzed descriptively.

RESULTS AND DISCUSSIONS

Gonadal Sex Differentiation of Nile Tilapia (*Oreochromis niloticus*)

The study investigated the effect of different doses of *T. terrestris* (TE) extract on the gonadal sex differentiation of Nile tilapia juveniles. Table 1 illustrates the gonadal differentiation stages at various days post-hatching (dph) across five treatments (P1-P5). At 10 dph, all gonads were undifferentiated, while differentiation into presumptive male and female gonads began at 20 dph. By 60 dph, a clear distinction between male and female gonads was evident, with a higher number of male gonads observed in all treatment groups, particularly in P1, which used a synthetic hormone (17α-methyltestosterone) (19 males) compared to P2- P5, which used various concentrations of TE.

Figure 1 illustrates the progression of gonadal differentiation in Nile tilapia from 10 to 60 dph under different concentrations of TE extract. At 10 dph, the tilapia gonads were undifferentiated, showing a clearer arrangement of a pair of gonads, germ cells, and somatic cells, including steroid-producing cells (SPC) (Figure 1a). As previously mentioned by Zhao *et al*. (2022), in newly hatched larvae, primordial germ cells are found along the dorso-median region, specifically from the peritoneal wall at the dorsal root along with the undeveloped mesentery. By 20 dph, presumptive female gonads began differentiating, characterized by the appearance of germ cells and steroid-producing cells (SPC) near blood vessels (Figure 1b). At 30 dph, female gonads showed more advanced differentiation, including unique somatic cell assemblages initiating ovarian cavity (OC) development, clusters of somatic cells, and germ.

Table 1. Gonadal Sex Differentiation of Nile Tilapia (*Oreochromis niloticus*) under Different Doses of *Tribulus terrestris* Extract.

Stage of Gonadal Sex Differentiation	Treatments (Dosages of *Tribulus terrestris,* mg/L)																				
	P1-P5	P1				P2				P3				P4				P5			
	Days Post Hatching																				
	10	20	30	40	60	20	30	40	60	20	30	40	60	20	30	40	60	20	30	40	60
Undifferentiated	10	3	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	1	-	-	-
Presumptive Female		0	1	-	-	2	3	-	-	2	2	-	-	1	1	-	-	2	1	-	-
Female		-	0	-	-	-	-	2	4	-	-	2	3	-	-	1	2	-	-	1	2
Presumptive Male		1	4	1	-	0	1	3	-	0	1	3	-	1	1	4	-	1	2	4	-
Male		-	-	3	19	-	-	-	15	-	-	-	14	-	-	-	18	-	-	-	18
Intersex					1				1				3				0				0
Not Available*	5	3	2	3	0	5	3	2	0	4	4	2	0	4	5	2	0	3	4	2	0

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*Gonad is not available in the preparate.

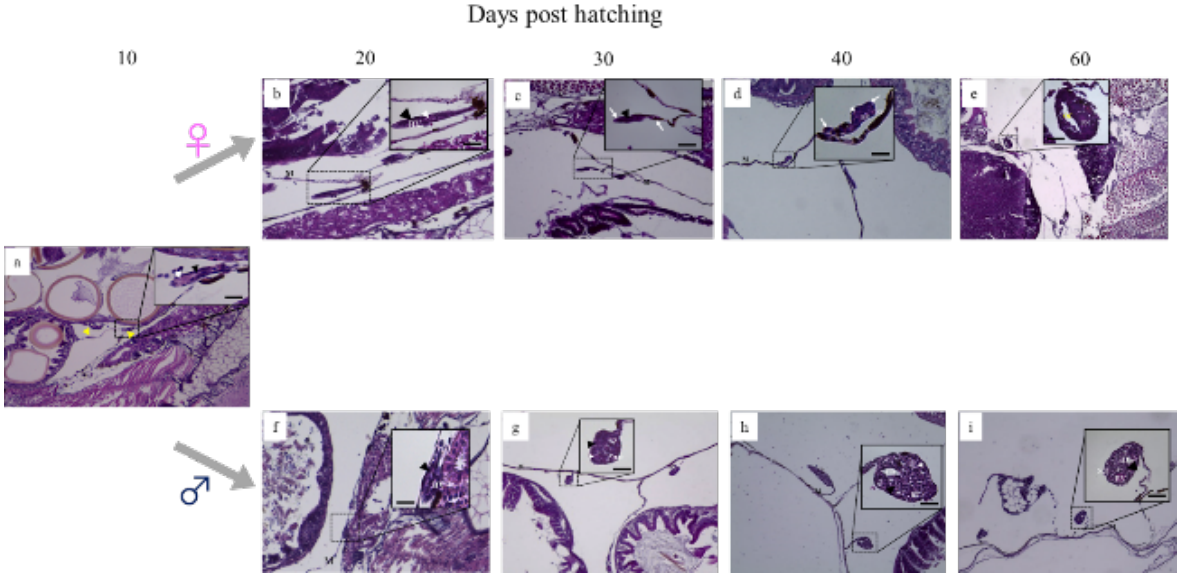

Figure 1. Schematic illustration of the gonadal sex differentiation process in female and male Nile tilapia (Oreochromis niloticus) at various dosages of *Tribulus terrestris*.

Description: At 10 days post-hatch (dph), the gonads of tilapia are undifferentiated, with a clearer arrangement of a pair of gonads observed, along with germ cells and somatic cells, including steroid-producing cells (SPC) (a). By 20 dph, presumptive female gonads begin differentiating, characterized by germ cells and SPC near blood vessels (b). At 30 dph, the gonads show more advanced differentiation, with unique somatic cell assemblages initiating ovarian cavity development, clusters of SPC, and germ cells along the gonad`s periphery (c). By 40 dph, female gonads continue developing, featuring primary oocytes, the ovarian cavity, and perinuclear oocytes by 60 dph (d, e). In the male tilapia, gonadal differentiation follows a distinct pathway. At 20 dph, undifferentiated gonads show the presence of somatic cells and blood vessels (f). By 30 dph, presumptive male gonads begin differentiating, with the formation of a putative efferent duct (ED) in the stromal tissue, along with visible germ cells and SPC (g). At 40 dph, male gonads are further differentiated, with the ED, germ cells, SPC, and blood vessels becoming more defined (h). By 60 dph, male gonads show a fully developed ED, spermatogonia, and continued presence of SPC and blood vessels, indicating the progression towards functional male reproductive structures (i).

Cells along the gonad's periphery (Figure 1c). These observations align with findings by Kobayashi *et al*. (2012) and Zhao *et al*. (2022), who noted that early gonadal differentiation in fish can be detected by the presence of specific cell types and their organization. By 40 dph, female gonads continued developing with primary oocytes (PO) and the formation of the ovarian cavity (Figure 1d). At 60 dph, the development reached a stage featuring perinuclear oocytes, indicating mature female gonadal structures (Figure 1e).

For male tilapia, gonadal differentiation followed a distinct pathway. At 20 dph, the gonads remained undifferentiated but showed the presence of somatic cells and blood vessels (Figure 1f). By 30 dph, the differentiation of male gonads began with the formation of a putative efferent duct (ED) in the stromal tissue, accompanied by visible germ cells and SPC (Figure 1g). At 40 dph, male gonads showed further differentiation with a more defined ED, germ cells, SPC, and blood vessels (Figure 1h). At 60 dph, male gonads exhibited a fully developed ED, spermatogonia, and a continued presence of SPC and blood vessels, indicating the

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progression toward functional male reproductive structures (Figure 1i).

The patterns observed align with previous studies on sex differentiation in tilapia, where primordial germ cells (PGCs) in female gonads (XX) proliferate after 9 dph, while the number of germ cells in male gonads (XY) remains unchanged from 9-14 dph (Nakamura *et al.*, 1998; Kobayashi *et al*., 2002, 2012). The initial signs of sex differentiation between 20–26 dph, characterized by ovarian cavity formation in genetically XX gonads and efferent duct development in genetically XY gonads, support findings by Kobayashi *et al*. (2012). The study further corroborates that meiosis in the ovaries begins between 25-30 dph, while spermatogenesis starts between 50-70 dph, consistent with earlier research (Nakamura *et al*., 1998; Kobayashi *et al*., 2002; D'cotta *et al*., 2001; Kobayashi *et al*., 2012). Further, this case is similar result with Hidayat *et al*. (2024) study using climbing perch (*Anabas testudineus*), the study only shows differentiated gonads in females starting at 18-21 dph with sign of genital ridges and the formation of the ovarian cavity, meanwhile male climbing perch may not have differentiated gonads until age 26 dph, leading to speculation that sexual differentiation develops faster in female fish.

The results demonstrate that the use of *T. terrestris* extract, particularly at higher doses (P4 and P5), significantly promotes the development of male gonads in Nile tilapia, suggesting a potential androgenic effect like synthetic hormones like 17α-methyltestosterone (P1). These findings are consistent with previous studies, such as Abaho *et al*. (2022), who reported increased male ratios in fish treated with plant-based phytoestrogens. Moreover, the decrease in the number of intersex individuals at higher TE concentrations supports the hypothesis that TE enhances gonadal differentiation toward a male phenotype.

Additionally, the study suggests that *T. terrestris* extract may have a masculinizing effect on tilapia gonadal differentiation, as higher dosages tend to produce more male-dominated sex ratios. This finding underscores the potential influence of external factors, including natural extracts, on fish sex differentiation. The ability to manipulate sex ratios in aquaculture using herbal supplements like *T. terrestris* could have significant practical implications, enhancing production efficiency and meeting specific market demands for tilapia aquaculture.

Sex Ratio and Intersex

By 60 dph, the differentiation was complete, with the treated groups showing varied sex ratios (Table 2). The treatment with 0.01 g/L of *T. terrestris* extract resulted in the highest percentage of male tilapia, confirming the extract's efficacy in promoting male differentiation. This outcome supports the study by Aldaddou *et al*. (2022) and El-Kady *et al*. (2022), which highlighted the effectiveness of plant-based extracts in achieving desired sex ratios in aquaculture, providing a sustainable alternative to synthetic hormones. Also, based on the results of this study, the use of tribulus extract on the tilapia sex ratio (Table 2) was able to increase the sex ratio of tilapia compared to the control treatment (P2) ($P < 0.05$). This study used a lower dose compared to previous studies. Due to the extraction process of Tribulus using 90% ethanol, the concentration obtained is higher than the extraction using 70% ethanol, as done by Ghosal *et al*. (2020) and Çek *et al*. (2007b). Interestingly, the presence of intersex individuals in some groups indicates that further optimization of dose and treatment duration may be necessary to achieve more consistent results.

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Table 2. Sex ratio of male and intersex tilapia fry (*O. niloticus*) for 60 days. Different superscripts in the same column indicate significant differences at a 95% confidence level (P<0.05).

Treatments	Sex ratio	
	Male	Intersex
P1 (17α-MT 500 µg/L)	97.10±5.02[a]	2.90±5.02[a]
P2 (TE 0 g/L)	72.63±11.52[b]	2.67±4.62[a]
P3 (TE 0.005 g/L)	66.31±14.23[b]	4.76±8.25[a]
P4 (TE 0.01 g/L)	92.75±6.64[a]	0
P5 TE (0.02 g/L)	92.93±6.41[a]	0

The success of masculinization is thought to be due to the content of protodioscin and protogracillin in Tribulus extracts (aphrodisiacs) that can work in increasing testosterone and can produce red blood cells and oxygen transport that can lead to specific immune system health (Fernández-Lázaro *et al.*, 2022). *T. terrestris* is a medicinal plant that is reported to increase testosterone levels and precursor testosterone levels by affecting androgen metabolism (Aldaddou *et al.*, 2022). This plant is also reported to be highly effective in producing monosex populations of *O. niloticus* (El-Kady *et al.*, 2022). This plant extract was found to exhibit dose-independent devariability in many functions and benefits (Adewale *et al.*, 2014; Tian *et al.*, 2021).

The male tilapia sex ratio was not 100% successful in both MT and Tribulus extract treatments due to labile factors, the dose given, and immersion time. In this study, intersex tilapia was found, namely the presence of male and female gonadal cells in one individual. According to Susanto *et al.* (2021), the administration of steroid hormones at very low doses will not be able to form a maximum male population and will cause the formation of intersex individuals. The occurrence of intersex fish is generally due to the administration of sub-optimum low doses of steroid hormones (Bhattacharya and Munshi, 2021).

The Specific Growth Rates (SGR) and Survival Rates (SR)

The specific growth rates (SGR) and survival rates (SR) of tilapia fry across the different treatments are summarized in Table 3. The SGR of male tilapia juveniles had no difference with the treatments where the SGR ranged from 5.91% to 6.16%, while the SGR for female tilapia juveniles varied widely, with values ranging from 1.91% to 5.95%. Then, there were no statistically significant differences in SR across all treatments. These results indicate that there is insufficient evidence to confirm a significant influence of Tribulus extract application on the SGR of both males and females. This could be due to steroidal saponin in the Tribulus, which has negative effects on growth rate inhibition to the presence of steroid saponin in Tribulus, which can inhibit growth by disrupting respiration when absorbed in the gills, leading to decreased appetite (Ezraneti and Fajri, 2016).

Additionally, antinutritional factors in tribulus, such as hydrocyanic acid, phytate, nitrate, and oxalate, may reduce the bioavailability of key nutrients (Ștefănescu *et al.*, 2020). Interestingly, the SGR did not differ significantly across treatments, indicating that TE does not negatively impact growth performance compared to the control and synthetic hormone treatments. However, a slight reduction in survival rates was noted with increasing TE concentrations, which could be attributed to potential toxic effects at higher doses, as observed in similar studies by Janalizadeh *et al.* (2015) using *B. splendens* and Hajibeglou

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and Machanlou (2024) using *Oncorhynchus mykiss*.

Table 3. The specific growth rate and survival rate of tilapia fry for 60 days. Different superscripts in the same column indicate significant differences at a 95% confidence level (P<0.05).

Treatments	Specific Growth Rate (%)		Survival Rate (%)
	Male	Female	
P1 (17α-MT 500 µg/L)	6.04±0.82[a]	1.91±3.31[a]	70.43±20.40[a]
P2 (TE 0 g/L)	5.93 ±0.54[a]	5.83±0.79[a]	71.67±23.11[a]
P3 (TE 0.005 g/L)	6.12±0.88[a]	5.95±1.36[a]	67.62±23.96[a]
P4 (TE 0.01 g/L)	5.91±0.09[a]	2.90±2.51[a]	64.62±20.57[a]
P5 TE (0.02 g/L)	6.16±0.58[a]	3.49±3.02[a]	59.19±18.19[a]

CONCLUSION

The findings of this study indicate that the treatment significantly affected the early gonad development and sex ratio (P<0.05), while it had no significant effect on the growth performance and survival rate (P>0.05) of Nile tilapia fry. The best dose to increase the percentage of males in juvenile Nile tilapia was the treatment with Tribulus seed extract at 0.01 g/L.

CONFLICT OF INTEREST

The conflict of interest contains a declaration that there is no conflict of interest among all authors upon writing and publishing the manuscript.

AUTHOR CONTRIBUTION

The study was conceptualized by MS. The methodology and experimental portion were carried out by LL and AYP. Data curation and analysis were conducted by MS, LS, and AYP. The manuscript was drafted by MS and reviewed and revised for publication by YE and YTA.

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