Comparative assessment of natural and synthetic reproductive inhibitors in *Oreochromis niloticus*

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Abstract: Nowadays, natural sources of sex reversal agents are preferred over synthetic ones in fish farming due to their reliability and economic value. This study compared the effectiveness of 17*α*-methyltestosterone (MT), *Carica papaya* seed meal (PSM), and common carp testes (CCT) with tilapia (*Oreochromis niloticus*) in terms of sex reversal, hematological parameters, gonadal histology, enzymatic activity, and overall growth. A 90-day trial was conducted with 560 tilapia fry (2-3 days old) distributed into one control and six treatment aquaria, each with two replicates. Fries were fed with a control diet (T0) or one of six experimental diets (T1-T6) containing different MT, PSM, or CCT levels for 30 days, followed by the control diet for 60 days. MT induced the highest male proportion (85% and 75% in T2 and T1, respectively), while PSM and CCT enhanced tilapia's growth and carcass composition. Histological analysis revealed gonadal deformities in MT and PSM treatments, which might lead to sterility. The gonadosomatic index (GSI) was reduced in natural treatments as compared with synthetic ones. Hematological parameters did not show any adverse effects of PSM and CCT. Protease and amylase activities were higher in PSM and CCT than in MT, indicating better digestion and feed absorption. PSM and CCT are biodegradable, locally available, and eco-friendly alternatives to synthetic hormones to change sex in tilapia. Natural sources (plant and animal by-products) are preferable to synthetic sources as they are less expensive and control prolific breeding in tilapia.

Keywords: sex reversal, natural reproductive inhibitors, synthetic reproductive inhibitors, *Oreochromis niloticus*, *Carica papaya*
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1 Introduction

Aquaculture is one of the most dynamic sectors in global food production and is crucial for global food needs as it provides affordable and high-quality food for billions of people across the world[1]. Following the global consumption trend, there has been a rise in the production of fisheries and aquaculture (which in 2020 reached 214 million t). By 2030, aquaculture is expected to account for more than 50% of global fish consumption[2]. At least 845 million people worldwide depend on fish for their nutritional needs. Fish are a valuable source of nutrition for humans due to their rich nutritional profile, which includes essential macronutrients such as proteins, lipids, and ash, as well as a wide range of micronutrients, including vitamins and minerals[3].

The contribution of tilapia production and farming in global aquaculture has increased significantly in the last three decades in terms of quality and quantity. Tilapia's share of the world's aquaculture production rose from 1.9% in 1987 to 5.3% in 2017 by weight and from 1.5% to 4.4% by value over the same period, highlighting its role in ensuring global food security[4]. The Nile tilapia has several advantages, including quick breeding cycles, suitable spawning, swift development, efficient feed conversion, remarkable adaptability to its surroundings, and feeding of a variety of organic food sources or low-cost synthetic feeds, as well as subtle taste and strong appeal in the market[5]. Despite the aforementioned qualities, tilapia mature and breed quickly, and these traits can cause problems by overcrowding ponds and limiting growth. As a result, small fish are unsuitable for the market and consumers. Mono-sex stocks in aquaculture have several advantages, including controlling unwanted reproduction, minimizing conflicts, enhancing survival rates during harvesting, and potential growth benefits[6]. By manipulating the sex of tilapia, aquaculture can reap boundless rewards. To control unwanted spawning, fish are subjected to various methods and techniques that hinder their sexual development[7]. Sex reversal in the case of Nile tilapia is changing the sex from female to male, which has advantages such as faster growth, larger size, and reduced reproduction. But it only works within a critical time of sex differentiation (development of the gonads)[8].

Different methods such as hand-sorting, androgen-induced hormone change, hybridization, environmental alteration (like temperature change), and gene or chromosome modification are being used to get all male tilapia[9]. Male tilapia grow faster as compared to females. In aquaculture, using androgenic hormones to achieve all-male stock is common. In hormonal sex inversion, different synthetic androgens (19-methyltestosterone, fluoxymesterone, methyltestosterone, 17-methyltestosterone, androstenedione, and dihydrotestosterone) are being used[10]. Among synthetic steroids, 17*α*-MT is commonly used for sex reversal, and its results are good for producing all-male tilapia, but this hormone has side effects on the environment and non-target species. Also, the cost of this hormone is an obstacle to producing a mono-sex population[11]. The debate over this issue has prompted the

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exploration of new alternatives for sex reversal, such as phytochemicals and other natural sources that are biodegradable, come at low cost, and can interfere with the endocrine system.

Carica papaya is an excellent example of a reproductive suppressant, decreasing male and female sex hormones and causing delaying maturation in tilapia[12]. Papaya has phytochemicals and is appreciated for its antioxidant, digestive, and nutraceutical properties due to its bioactive components[13]. *Caricain & carpasemine* enzyme, saponin, and oleanolic acid 3-glycosides are active chemicals in papaya seed meal (PSM), which have been shown to cause sterility in male rats and have been utilized to suppress prolific reproduction of *O. niloticus* by reversing the sex of fish in favor of males[14]. Glucosides in PSM can also destroy gonadal cells of tilapia[15]. The predominant biologically active constituent found in *C. papaya* seeds, known as Benzyl isothiocyanate (BITC), has demonstrated its role in inducing the contraceptive impact. Studies have revealed that papaya seed powders can stimulate growth and induce sex reversal in various freshwater fish species[16]. Another alternative to produce male tilapia is natural androgen that exhibits endocrine disruptive activity originating from fish testes. Fish testes (natural sources of testosterone) are also important in sex reversal[17-19]. Fish testes are cost-effective and locally available sources to incorporate in feed. Natural sources are potential substitutes for synthetic hormones in aquaculture because they bring valuable pharmacologically active metabolites with a variety of benefits such as immunity boost, growth promotion, antioxidant enhancement, antidepressant effects, survival rate, digestive enhancements, appetite stimulation, and improved food conversion ratio[20]. This is a new trend in aquaculture to use animal by-products (fish testes). Only a few studies have evaluated the use of fish testes powder as a natural source for masculinization. This research aimed to precisely investigate how various levels of papaya seed and fish testes powder, administered at specific time intervals, influence factors such as growth performance, proximate body composition, sex ratio, hematology, enzymatic activity, and gonadal history.

2 Materials and methods

2.1 Experiment design and condition

Tilapia fry at 2-3 days old (n=560) were procured from a local fish hatchery. Fries were randomly selected and apportioned into one control and six treatment groups, each with two replicates. This experiment was completed in two phases. In phase 1 (treatment phase), treatment diets were fed to tilapia twice a day for one month, while in phase 2 (rearing phase), fish were reared under a control diet (32% CP) for two months, and growth parameters were calculated fortnightly throughout the study of three months. Finally, the body composition and reproductive parameters of tilapia reared under treatment and control diets were examined. Major physicochemical factors were monitored daily, and water temperature was maintained between 20°C-24°C[21].

2.2 Feed preparation and protocol

Fresh papaya seed (ripe fruit) and fish testes (mature stage) were collected from the local market and sun-dried for 3-4 days till they were completely dried, ground separately into fine powder form, and added in basic feed ingredients. For the formulation of the diet, the basic ingredients listed in Table 1were weighed, ground, measured based on percentage composition, and thoroughly mixed; after adding water, the dough was prepared, and 2 mm pellets were formed. The pelleted diets were dried, packed in an airtight bag, labeled, and stored until use[22]. The experimental setup followed a "Complete Randomized Design" (CRD) approach, consisting of one control group (devoid of hormones and containing 32% CP) and six different experimental diets. The experimental diets included the incorporation of the 17α-methyltestosterone hormone at concentrations of 60 and 70 mg/kg, along with Papaya seed meal at 6 and 7 g/kg of feed and testes powder at 70% and 80%. All feed mixtures were preserved until they were utilized.

Table 1 The composition of ingredients in basal and experimental diets

Feed Ingredients/g	Control (without hormone)	PSM1	PSM2	MT1	MT2	CCT1	CCT2
Fish Meal	28	28	28	28	28	28	28
Rice Polish	16	16	16	16	16	16	16
Canola Meal	19	19	19	19	19	19	19
Wheat Bran	18	18	18	18	18	18	18
Rice Broken	12	12	12	12	12	12	12
Fish Oil	5	5	5	5	5	5	5
Vitamin Premix	1	1	1	1	1	1	1
Papaya seeds powder/g·kg^{-1}	--	6	7	--	--	--	--
17α- Methyl Testosterone/ mg·kg^{-1}	--	--	--	60	70	--	--
Common carp Testes/%	--	--	--	--	--	70	80

2.3 Growth indices

Growth related parameters were measured by the following formulas[23,24]:

Specific growth rate,

$$\mathrm{SGR} = \left(\frac{\ln W_t - \ln W_0}{t}\right) \times 100\% \tag{1}$$

where, W_t is the final weight, g; W_0 is the initial weight, g; t is the time period of experimental trial.

$$\text{Condition factor (K)} = \frac{\mathrm{W} \times 100}{\mathrm{L}^3} \tag{2}$$

where, W is the body final weight, g; L is the final total length, cm.

$$\mathrm{PER} = \frac{\text{Final body weight} - \text{Intial body weight}}{\text{Amount of protein intake}} \tag{3}$$

2.4 Chemical body composition

Carcass composition (ash, moisture, protein, and fat) was also evaluated at the end of the feeding trial. According to AOAC[25], protein was measured by Kjeldal, and Soxhlet apparatus was used to determine fat[26].

2.5 Identification of sexes

To determine the sex reversal ratio, ten individuals were randomly selected at maturity and dissected to examine the gonads, as shown in Figure 1.

The gonado-somatic index was determined by the following method[27]:

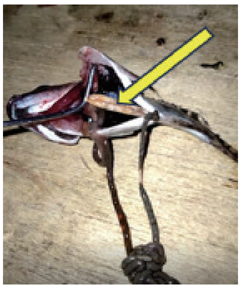
$$\mathrm{GSI} = \frac{\text{Gonadal weight}}{\text{Body weight} - \text{Gonadal weight}} \times 100\% \tag{4}$$

2.6 Hematological and digestive indices

At the end of trial, blood sample was collected (3 fish per aquarium) from the caudal receptacles after starved for 24 h[28]. A plastic syringe was used to draw blood; first aliquot was mixed with EDTA. An automated cell counter was used to analyze the hematology indices red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), and hematocrit (Hct) of all samples (Sino thinker. sk9000, US).

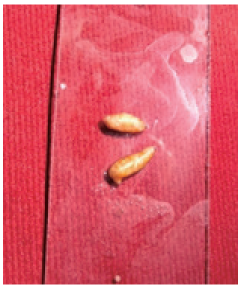
To determine the digestive indices samples of liver and gut, samples (3 fish per aquarium) were homogenized and centrifuged at

8000 r/min for 5 min after being washed with saline solution (0.90%; pH 7.5). The supernatant was stored at 80°C for future use. Protease activity was calculated after evaluating the non-specific protease activity of sigma using casein. At A540 and A714 nm, amylase activity was measured by spectrophotometer according to the method of Wang et al.[29]Proteolytic activity was measured using the casein hydrolysis method of Kunitz, as modified by Walter[30,31].

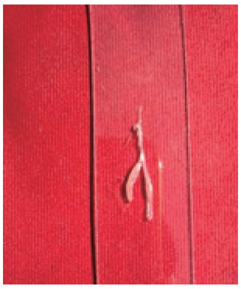


a. Observation of gonads

b. Observation of gonads



c. Overies of fish



d. Testes of fish

Figure 1 Dissection of fish to observe the gonads after 90-day trial

2.7 Histological analysis

The histological study was carried out by the method of Gewaily and Abumandour[32]. In this study, 0.25 mL/L of clove oil was used to anesthetize the fish, and gonads of the fish were removed for histological examination. Small pieces (5 mm) of ovarian and testicular tissues were fixed in 10% neutral buffer formalin for 24 h. The samples underwent dehydration at 4°C by submerging them in increasing concentrations of ethanol, followed by methyl benzoate for clearing and then paraffin for embedding. Testicular and ovarian sections were stained by hematoxylin and eosin and mounted on slides for microscope examination (Nikon Phase Contrast Dry, 0.90, Japan).

2.8 Statistical analysis

After finding the conceivable results, information on growth, body composition, gonad histology, hematology, and enzymatic activity were subjected to statistical analysis ANOVA, ±SE values were computed by utilizing SPSS[33], and the difference between means (p<0.05) was calculated by Tukeys's HSD test. The sex ratio was determined through the Chi-square test.

3 Results

3.1 Growth parameters

Growth performance, protein efficiency ratio, specific growth rate, and condition factor of tilapia fed with the control and test diets are listed inTable 2. Fish fed with PSM and CCT showed a significant increase in final body weight (p<0.05). Highest body weight ($5.29±0.04^{abc}$) was observed in fish fed PSM2, with no significant difference in fish fed with CCT2 (p>0.05). It is noted that no differences were found in protein efficiency ratio (PER), specific growth rate (SGR), and condition factor (CF) between control and other treatment groups. Overall, PSM-treated groups (T3 and T4) showed the best results, followed by groups treated with testes powder. Both of these (PSM, CCT) are natural and preferable over synthetic sources. All other zootechnical performances (PER, SGR, CF) were not significantly different among all the treatments.

Table 2 Effect of experimental diets on growth parameters of *O. niloticus*

Growth index	T0	T1	T2	T3	T4	T5	T6
	Control	MT1/60 mg·kg⁻¹	MT2/70 mg·kg⁻¹	PSM1/6 g·kg⁻¹	PSM2/7 g·kg⁻¹	CCT1/70%	CCT2/80%
IW/g	$1.800±0.000^{q}$	$1.810±0.020^{q}$	$1.790±0.010^{q}$	$1.800±0.010^{q}$	$1.820±0.010^{q}$	$1.840±0.020^{pq}$	$1.850±0.020^{pq}$
FW/g	$5.210±0.010^{abc}$	$5.110±0.030^{bc}$	$5.070±0.070^{c}$	$5.230±0.040^{ab}$	$5.290±0.040^{abc}$	$5.190±0.060^{ab}$	$5.220±0.010^{abc}$
CF	$1.490±0.100^{a}$	$1.530±0.090^{a}$	$1.450±0.070^{a}$	$1.520±0.090^{a}$	$1.500±0.070^{a}$	$1.480±0.110^{a}$	$1.490±0.120^{a}$
PER	$0.095±0.005^{a}$	$0.105±0.005^{a}$	$0.100±0.010^{a}$	$0.095±0.005^{a}$	$0.105±0.005^{a}$	$0.100±0.000^{a}$	$0.105±0.005^{a}$
SGR/%·d⁻¹	$1.170±0.020^{a}$	$1.140±0.040^{a}$	$1.150±0.020^{a}$	$1.200±0.010^{a}$	$1.160±0.040^{a}$	$1.145±0.015^{a}$	$1.170±0.030^{a}$

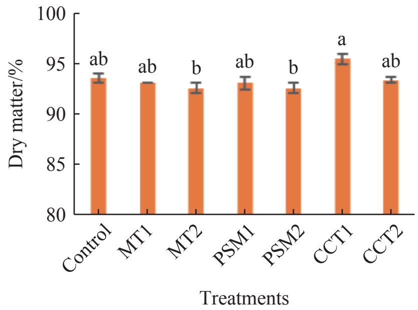
Note: IW: Initial Weight; FW: Final Weight; IBW: Increase in Body Weight; CF: Condition Factor; PER: Protein Efficiency Ratio; SGR: Specific Growth Rate; Values (Mean±SEM). Rows with different letters represent statistically significant differences (p<0.05), same as below.

3.2 Proximate body composition

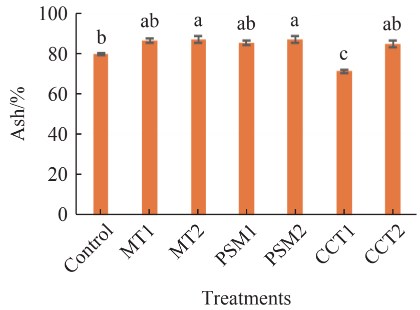
Changes in dry matter, total ash, crude protein, total crude lipid, and nitrogen-free extract in *O. niloticus* under control and treatments were recorded. The significant reduction (p<0.05) in ash level was observed in CCT1. Dietary CCT2 inclusion significantly affected (p<0.05) crude protein ($41.32±0.320^{a}$) and crude fat contents ($26.44±0.24^{a}$). However, dry matter had no difference among all treatments (p>0.05). Assessment of nitrogen-free extract revealed no significant trend across all treatments except CCT2 (Figure 2). Values are displayed as (Mean±SEM). Statistically significant differences are shown by letters (p<0.05). The common carp testes (CCT) treatment has remarkable results as compared to other treatments, followed by PSM-treated groups.

3.3 Masculinization

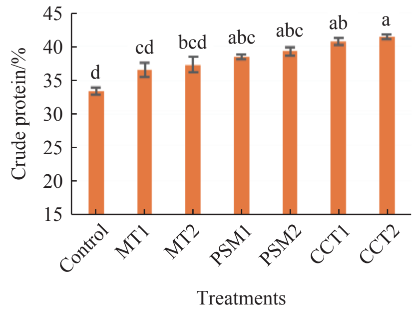
Reproductive analysis of *O. niloticus* revealed significant differences (p<0.05) between the control and other treated groups. The highest percentage of females ($55.00±1.43^{a}$) was observed in the control group, whereas MT2 and MT1 treatments had a significantly higher male percentage, i.e, ($85.00±4.72^{a}$) and ($75.00±1.70^{ab}$), respectively, followed by CCT2 treatment ($70.00±4.98^{ab}$). However, there was no significant difference among these three groups (p> 0.05). Sterility (55%) was observed in PSM-treated groups (Table 3). It is noted that PSM1 and PSM2 had the highest values of sterility; however, no significant difference was observed among these treatments. Results of the GSI of the experimental fish from the different treatment groups showed the maximum value ($2.13±0.01^{a}$) in MT2 group, while the PSM groups had the lowest. The male-to-female (M:F) ratio in the control group that only fed on the control diet was expected to be 1.0:1.0 (M:F), and the actual ratio of 1.0:1.2 was not significantly different from expected. In all the treatments, the highest sex ratio of male-to-female (5.6:1.0) was obtained in the methyltestosterone-treated group (MT2). However, when the fish were fed with PSM, they became more sterile. The male: female: sterile ratio was 1.25:1.00:2.75 (M:F:S) in PSM1. However, the male-to-female ratio was 2.3:1.0 (M:F) in *O. niloticus* fed with CCT2. PSM was able to skew the sex ratio in favor of sterile (55%). In all the treatments, the highest sex ratio of male-to-female (5.6:1.0) was obtained in the methyltestosterone-treated group (MT2).



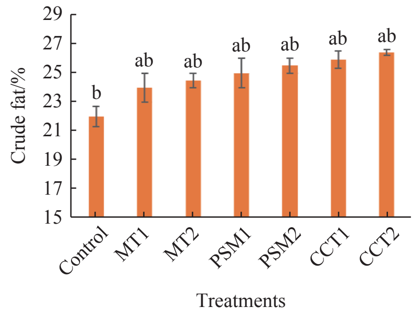
a. Dry matter



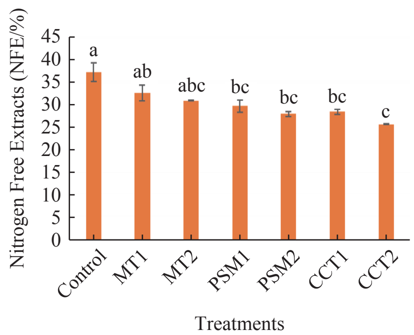
b. Ash



c. Crude protein



d. Crude fat



e. Nitrogen free extract

Figure 2 Proximate body composition (%) of nutrients in *O. niloticus* fed on control and treatment diets

Table 3 Sex percentage, sex ratio, and gonadal index of males, females, and sterile *O. niloticus* fed different diets (Mean±SEM)

Variables	T0		T1		T2		T3			T4			T5		T6	
	Control		MT1 (60/mg·kg⁻¹)		MT2 (70/mg·kg⁻¹)		PSM1(6/g·kg⁻¹)			PSM2 (7/g·kg⁻¹)			CCT1 (70%)		CCT2 (80%)	
	M	F	M	F	M	F	M	F	S	M	F	S	M	F	M	F
Sex/%	45.00±2.52^{cd}	55.00±1.43^{a}	75.00±1.70^{ab}	25.01±1.39^{cd}	85.00±4.72^{a}	15.00±0.79^{e}	25.00±1.01^{e}	20.0±0.33d^{e}	55.00±1.15^{a}	30.00±1.44^{de}	15.00±0.46^{e}	55.0±1.79^{a}	60.00±2.72^{bc}	40.00±2.54^{b}	70.00±4.98^{ab}	30.00±1.21^{c}
Sex ratio	1.0:1.2		3.0:1.0		5.6:1.0		1.25:1.00:2.75			2.0:1.0:3.6			1.5:1.0		2.3:1.0	
GSI	1.530±0.020^{b}	1.930±0.020^{ab}	1.830±0.000^{3a}	2.010±0.010^{ab}	1.790±0.010^{a}	2.130±0.010^{a}	0.710±0.010^{c}	0.930±0.020^{d}	0.500±0.020^{a}	0.680±0.020^{c}	0.890 ±0.020^{d}	0.490±0.010^{a}	1.390±0.040^{b}	1.545±0.065^{c}	1.485±0.040^{b}	1.815±0.080^{b}

Note: GSI-gonadosomatic index; F-female; M-male; S-sterile.

3.4 Hematological studies

The hematological parameters of *O. niloticus* exposed to different treatments are summarized inTable 4. The results showed a significant increase in RBCs (p<0.05) in CCT-fed groups. In contrast, white blood cells (WBCs) were reported as maximum in PSM1 (3.15±0.05^{a}) and minimum on MT2 (1.55±0.07^{c}) (p<0.05). Hemoglobin in PSM1 was found to be significantly higher (p>0.05), while in control and MT-treated groups no significant difference was observed. The concentration of hematocrit also remained statistically highest for PSM2 compared to other treatment groups. It is noted that mean corpuscular hemoglobin (MCH) was higher in the PSM1-treated group, while mean corpuscular volume (MCV) showed the best results in MT1. However, MCHC values were not significantly (p>0.05) different among all groups.

Table 4 Hematological parameters of *Oreochromis niloticus* fed with 17*α*-methyltestosterone, papaya seed meal, and common carp testes

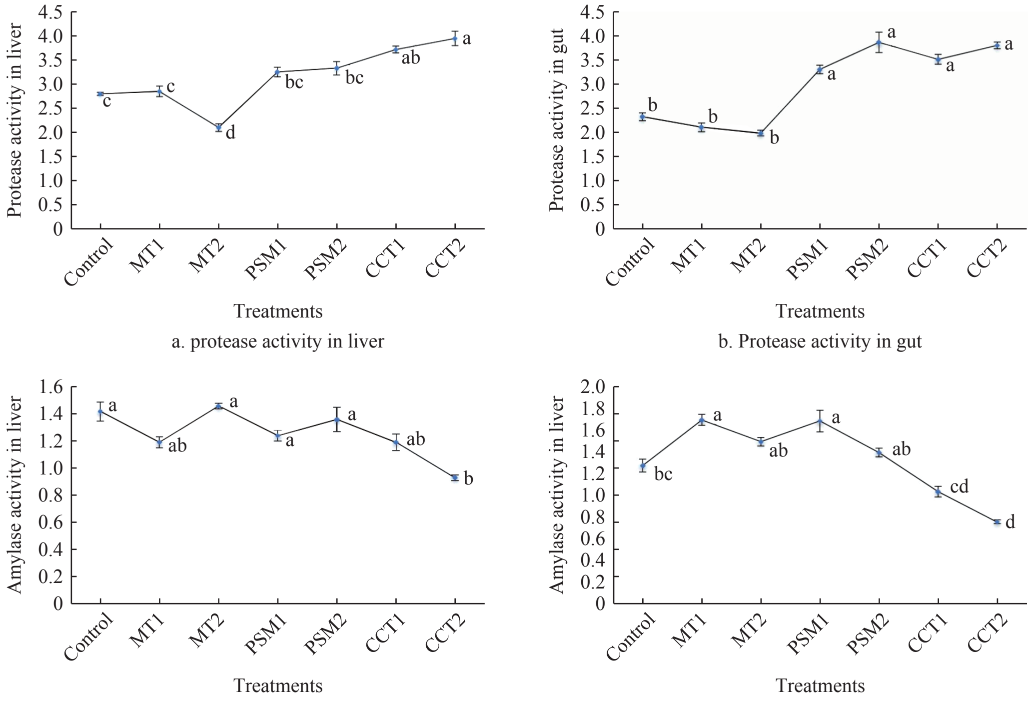
Parameters	T0	T1	T2	T3	T4	T5	T6
	Control	MT1 (60/mg·kg⁻¹)	MT2 (70/mg·kg⁻¹)	PSM1 (6/g·kg⁻¹)	PSM2 (7/g·kg⁻¹)	CCT1 (70%)	CCT2 (80%)
RBCs (×10^{6}/cell·mm^{-3})	2.43±0.14^{ab}	1.65±0.06^{c}	1.45±0.05^{c}	2.25±0.05^{b}	2.45±0.05^{ab}	2.76±0.11^{a}	2.86±0.12^{a}
WBCs (×10^{3}/cell·mm^{-3})	2.23±0.06^{b}	1.75±0.05^{bc}	1.55±0.07^{c}	3.15±0.05^{a}	2.05±0.12^{bc}	1.98±0.15^{bc}	1.76±0.11^{bc}
Hb (g·dl^{-1})	7.67±0.37^{c}	8.20±0.22^{c}	7.90±0.28^{c}	12.50±0.41^{a}	11.09±0.61^{ab}	10.23±0.13^{b}	12.10±0.20^{ab}
Hematocrit	19.43±0.96^{c}	24.60±0.52^{bc}	23.30±0.68^{c}	32.45±2.31^{ab}	34.30±1.78^{a}	32.50±1.19^{a}	31.60±1.51^{ab}
MCV	79.95±3.64^{d}	149.09±5.83^{a}	160.68±5.34^{a}	144.22±5.80^{ab}	140.00±6.76^{abc}	117.75±5.46^{bc}	110.49±4.05^{c}
MCH	31.56±1.27^{d}	49.69±2.39^{ab}	54.48±1.83^{a}	55.50±1.49^{a}	40.18±0.87^{c}	37.06±0.96^{cd}	42.30±0.53^{bc}
MCHC	39.47±1.14^{a}	33.33±1.71^{a}	33.90±1.81^{a}	38.52±1.45^{a}	32.30±1.71^{a}	31.47±1.69^{a}	38.29±1.27^{a}

Note: Red Blood Cells-RBCs; White Blood Cells-WBCs; Hematocrit-Ht; Hemoglobin-Hb; Mean-corpuscular-volume-MCV; Mean-corpuscular-hemoglobin-MCH; Mean corpuscular hemoglobin count-MCHC.

PSM-treated groups showed the most significant results as compared to other treatments, followed by the testes powder groups.

3.5 Enzymatic activity

The results of digestive enzyme activity tests are shown in Figure 3. Tested diet CCT2 significantly (p<0.05) improved liver protease activity (3.97±0.15[a]), while PSM2 had a higher value of gut protease activity (3.88±0.21[a]) as compared to control. However, no significant difference was observed for gut protease activity in PSM- and CCT-treated groups. No significant differences were observed between fish fed MT2, PSM1, PSM2, and control groups in terms of the amylase activity in liver. However, MT1 and PSM1 retained the highest amylase activity in gut, and the lowest value was observed in CCT2.

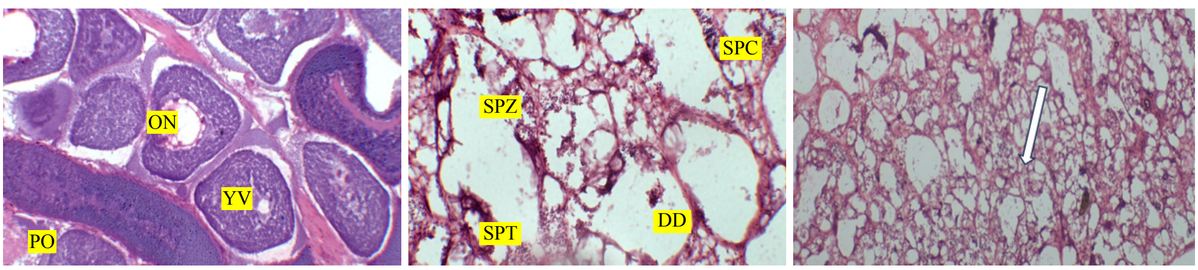


Note: Values are displayed as (Mean±SEM). Significant difference between dietary conditions is shown by letters (p<0.05). PSM- and CCT-treated groups showed significant results for protease activity in gut. Similarly, highest gut amylase activity was observed in MT1- and PSM1-treated groups.

Figure 3 Protease and amylase activity in *O. niloticus* liver and gut after 90-day trial fed with control and experimental diets

3.6 Histological examination

Ovary and testes revealed varying levels of evolution based on histological morphology, as shown inFigures 4a-4c. The histological study of the ovary in the CCT1 group showed primary oocyte with visible nuclear yolk and nucleus. CCT showed no negative impact on the fish gonads' histology. However, it was observed that fish treated with PSM2 testes had degenerative gonads, including deformed primary spermatozoa and ductus deference. In all spermatogenic stages, PSM showed unadorned reduction. MT2 showed almost diffused structure of gonads.



Note: CCT-treated groups are preferred due to their availability, while PSM is preferable in context of sterile fish. ON=ovarian nucleus; YV=yolk vesicles; PO=primary oocyte; ST=spermatid; SPZ=spermatozoa; SPC=spermatocytes; DD=ductus deference.

Figure 4 Histological appearance of fish after 90-day experimental trial fed with control and treatment diets

4 Discussion

Oreochromis niloticus is one of the most widely cultured fish species in the world due to its fast growth, high adaptability, and high market demand. However, the high spawning rate of *Oreochromis niloticus* is a challenge for aquaculture and fish management. This species has a high fecundity and can reproduce throughout the year, leading to overpopulation, competition for food, and environmental degradation. Therefore, there is a need to regulate the reproduction of *O. niloticus*. Synthetic androgens, mainly MT, have been applied as sex reversal agents to produce all-male fish populations for years. However, despite their high effectiveness, their safety could be improved and subject to regulations. The main objective of this study was to find natural alternatives to synthetic sources and check their effect on the reproductive performance and growth of Nile tilapia (*Oreochromis* *niloticus*), a commercially important fish species. Various androgens have been utilized to produce a mono-sex population. Nowadays, 17*α*-MT is being widely used to induce sex reversal in tilapia, but due to high prices and other constraints, other natural sources that are biodegradable and have inhibitory effects are also being considered. These include many plants such as *Carica papaya* (PSM), a tropical fruit that has been reported to have anti-fertility properties in mammals; animal sources such as testes of boar, bull, and ram; and fish testes such as Cyprinus carpio testes (CCT), a natural source of gonadotropin-releasing hormone (GnRH) that can inhibit gonadal development and maturation in fish. It was expected that PSM and CCT would inhibit the uncontrolled reproduction in tilapia, with a positive effect on the growth and immunity of the fish compared to MT. İnanan et al.[34]described how sex reversal (SR) alters the oxidative status of rainbow trout semen. Specifically, SR female trout exhibited better semen quality in terms of higher TAC, protein concentration, and catalase activity compared to normal female trout. İnanan and Acar[35] evaluated mature SR female rainbow trout obtained through three different steroid hormones [ethisterone (ET), 11β-hydroxyandrostenedione (HD), and 17*α*-methyltestosterone] in terms of spermatological parameters and health status. Spermatozoa concentration and motility characteristics were evaluated in the SR females. Seminal plasma parameters, including pH, testosterone, and gonadotropins, were measured. Additionally, the blood parameters of SR females were compared with those of normal males (NM). The GSI of SR females by HD was noted to be greater, indicating a higher proportion of body weight devoted to the gonads, a potential indicator of reproductive readiness. The health status was assessed through various blood parameters. The hormone levels measured in both seminal plasma and serum were significantly the lowest in the ET group. This indicates distinct hormonal profiles associated with different sex reversal methods. The findings suggest that using HD for obtaining SR females should be preferred due to better quality spermatozoa and health status. İnanan and Yılmaz[36]also explained that preference for all-male or all-female progeny can vary among different fish species based on their biology, reproductive characteristics, and growth patterns. Controlling the reproductive capabilities of fish populations is crucial in preventing unwanted breeding in aquaculture settings. Producing a uniform sex in a population can enhance efficiency in terms of growth rates, feed conversion, and overall management. The practice of using SR females and incorporating androgen hormones into the diet at the larval stage is a common and effective approach in rainbow trout aquaculture to achieve the desirable outcome of producing all-female progeny. This is advantageous in terms of uniform growth and avoiding the energy expenditure associated with sexual maturation in males. In SR females, the concentration of spermatozoa was reported to be 5-6 times greater than that of normal males. This suggests that SR females, which were likely females who underwent sex reversal to function as males, exhibited significantly higher sperm production than biologically male individuals.

4.1 Growth performance

As shown by this study, the growth performance of *O. niloticus* was significantly improved by PSM treatment, followed by CCT treatment. This study revealed that *Oreochromis niloticus* had remarkably better growth and weight gain when they were fed papaya seeds at 6 g/kg and common carp testes at 80%, as papaya seeds contain a high percentage of protein (28.3%) and fiber (22.6%). They also have various phytochemicals, such as saponins (1.2%), flavonoids (0.4%), benzyl isothiocyanate (0.07%), benzyl glucosinolate (0.03%), and carpaine, which are all essential growth promoters and bactericides[37,38]. Among other natural sources, carp testes also improve the growth performance of tilapia by increasing the protein and lipid contents, as they contain 12.9% protein and 1.8% lipid, as described by Ding et al.[39]Furthermore, the 17*α*-MT at 70 mg/kg dose level had notably improved growth performance compared to the control group due to its stimulation of the production of growth hormone and insulin-like growth factor. This is in agreement with the research conducted by Mehrim et al.[40] and Zaki et al.[41]

4.2 Proximate analysis of body meat

The findings of the recent study indicate that when fish were fed with PSM and CCT in their diet, their body composition was outstandingly increased. The body meat of fish treated with 7 g/kg of PSM exhibited the most remarkable levels of both crude protein and crude lipid, closely trailed by those treated with 80% of CCT. This is attributed to the influence of crude protein and fat on fish metabolism and hormonal balance, ultimately enhancing their growth, respectively, as noted by George et al.[42]and Radwan et al.[12] Moreover, carp testes serve as a valuable reservoir of testosterone, a steroid hormone known for its role in regulating protein synthesis and lipid metabolism. As a result, it plays a significant role in increasing fish body mass (Khanal et al.)[17] 17*α*-MT also has a pronounced effect on crude protein and lipid retention in fish bodies. Alam et al.[43] and Asad et al.[44] reported a similar increasing trend which was noted in both lipid and protein contents due to the incorporation of 17*α*-MT. This effect can be attributed to the anabolic reactions triggered by 17*α*-MT, which influence the expression of genes related to muscle development and lipid metabolism[45]. In our current study, the fish treated with 7 g/kg of PSM exhibited the highest levels of gross energy. A similar pattern was also observed in tilapia by Ugonna et al.[20]and Omeje et al.[38], where feeding the fish with PS led to an increase in GE.

4.3 Fish sex distribution

Among the synthetic and natural sources in fish feed, *C. papaya* seed diet was the most effective in causing 55% sterility of *Oreochromis niloticus*, followed by 30% masculinity, as papaya seeds harbor caricacin, carpasemine, and oleanolic glycoside. These bioactive components function as non-steroidal aromatase inhibitors, potentially inducing masculinization. The findings of our study are consistent with previous research by Yadav et al.[46] and Iipinge et al.[16] that has demonstrated the sterility and masculinizing effects of papaya seed, respectively, due to the presence of active alkaloids. Meanwhile, the 17*α*-MT (70 mg) and CCT (80%) showed the highest percentage of males at 85% and 70%, respectively, followed by papaya seed (PSM), as both MT and CCT are male androgens that stimulate male characteristics as well as significantly promote the growth of tilapia due to the anabolic effect of the androgen. These results agreed with Zaki et al.[41] and Apenuvor et al.[45], who claimed that 90% males were obtained at the high dose of 17*α*-MT. The maximum male population in tilapia was observed by Khanal et al.[17]and Meyer et al.[47]when they were fed carp testes, bull testes, and hog testes, respectively.

4.4 Gonadal somatic index

GSI in fish is a metric that represents the relative weight of the gonad to the total fish weight. As the study went on, the fish that nourished on PSM, CCT, and 17*α*-MT diets had a remarkable drop of GSI in their reproductive organs, for both males and females, as they consumed more PSM and CCT. This shows that PSM and CCT have active compounds that have pronounced hyperplasia and degenerative effects on germ cells compared to MT. A similar decreasing trend in GSI was observed in PSM-, CCT-, and 17α-MT-treated fish[12,42,46].

4.5 Hematology

Rahman et al.[48] stated that one way to evaluate the health of fish is to measure the hematological profile that reflects their physiological state. In the current study, the RBC, Hb, and PCV values were slightly different among PSM and CCT groups, signifying that natural sources such as plants did not elicit any negative effect on the fish's physiological status, as described by Anjusha et al.[49] Hemoglobin is a protein that transports oxygen and carbon dioxide in RBCs, and abnormal changes in its number may indicate anemia or stress. In a recent study, fish exposed to 17α-MT showed a substantial decrease in their hematological markers (p>0.05). The decline in RBC values has been noted in fish when exposed to exogenous hormone (MT). The reason may be that there is enough oxygen in the water, but fish will not be able to take in much of it because of their low erythrocyte counts. This study is in line with Witeska et al.[50], who state that fish with anemia may have lower values for all red blood cell markers. An increase in some hematological parameters may be due to erythropoietin activity. Testosterone stimulates its activity, so in CCT groups, the presence of testosterone reflects a significantly higher value of RBCs compared to other treatments. These findings are similar to those described by Ben et al.[51] All treatment groups had higher values of MCV, MCH, and MCHC than the control, but there were no significant differences (p>0.05) among the treatments. The high number of WBCs in the papaya seed (PS) group may show adequate immune responses; this finding is in line with Irshad et al.[52]

4.6 Digestive enzyme activity

Enzymes play a crucial role in digestion, demonstrating a potential impact on feed utilization and growth performance. Tian et al.[53]described how the study of digestive enzymes is a reliable tool to comprehend the digestive processes and nutritional status of fish. The highest protease enzyme activity in the gut was observed in PSM2, while in the liver, CCT2 achieved the highest protease activity. However, MT2 retained the highest amylase activity in the gut, while MT1 retained the highest amylase activity in the liver. Magouz et al.[54]stated that the enhancement in growth performance could be associated with the increased digestibility of nutrients and efficient feed utilization. The activities of digestive enzymes (amylase and protease) were the main factors that helped fish digest plant-based feed efficiently without dropping the feed intake. Results revealed that as protease activity increases, amylase activity decreases. Similar results were observed in genetically improved farmed tilapia (GIFT) juvenile and in Pangasiodon pangasius; they both showed that amylase activity decreased linearly and exponentially with increasing dietary crude protein content, which may be explained by lower carbohydrate (starch) availability in the digestive tract of higher dietary CP-fed animals. Similarly, lowered amylase activity in higher protein-fed groups was reported by Singha et al.[55]when fed to *Oreochromis niloticus* with varying dietary crude protein. Mozanzadeh et al.[56]demonstrated that there was a positive correlation between digestive enzyme activities and the growth of fish.

Ogunji et al.[57]reported that protease activity increased significantly in fish when fed a blood meal-based diet, owing to the fact that blood meal contains high protein levels. There is evidence that types of protein sources ingested may affect digestive enzyme secretion. Iqbal et al.[58]reported a significant increase in protease activity in *Labeo rohita* fed a plant-based protein diet. This study is in line with our findings that natural sources increase the enzymatic activity of the fish by accelerating its metabolism.

4.7 Histological examination

To protect the health of humans and the environment from the negative effects of aquaculture, fish farmers are using more natural sources, i.e., plant extracts and animal testes instead of synthetic hormones. These natural sources are organic, safe, cheap, and easy to make, and they can help produce fish in a sustainable way[59,60]. The growth and development of reproductive organs can be affected by them. Histological examination of fish fed a papaya seed diet showed lesions in seminiferous tubules and ultimately degenerative testes. The findings reveal that PS disruptive effects can appear in direct or indirect ways; papaya seeds have a compound called oleanolic acid 3-glucoside, which is a type of saponin. This compound can reduce or prevent the fertility of animals or humans[18]. This can also change the amount of sex hormones that are available in the body by increasing the production of a protein that binds to them. This evidence is in agreement with the observations of Farrag et al.[61]and Omeje et al.[38]Carp testes are a source of natural androgen, which is a male sex hormone. Carp testes developed the genetic females into phenotypic males with functional testes. Herein, carp testes change the fry sex in trends of males (70%) at high dose levels without any changes in testes (Perera et al.)[62] Recent research found the detrimental effect of MT on the testes followed by interlobular septa in ductus deference. These findings coincided with Apenuvor et al.[45]and Zaki et al.[41], who reported the deterioration in testes and dispersion of sperm.

5 Conclusions

Regarding environment biosafety and human health, this study discovers that *Carica papaya* seed (PSM) and Cyprinus carpio testes (CCT) are more effective and natural alternatives to synthetic hormones such as 17α-methyltestosterone. *Carica papaya* caused sterility in *Oreochromis niloticus* and significantly increased growth rate and protein retention in fish compared to 17α-methyltestosterone. Biological resources are preferable to artificial means of controlling the proliferation of *Oreochromis niloticus*, as they are an affordable, biodegradable, and accessible option for fish farmers and researchers alike. It is also essential to consider the environmental impact of different methods while exploring natural alternatives to synthetic hormones. Further studies on fish hematology and enzymatic activity are required to fully understand the effect of fish testes on fish immunity and blood parameters.

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