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The Effect of Dietary Supplementation of *Ulva reticulata* Extract on Reproduction of the Male Red Tilapia (*Oreochromis* sp.)

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ABSTRACT

Plant extract is widely used in aquaculture due to the presence of several secondary metabolites that act as precursors for steroid hormone synthesis in animal reproduction. In this context, *Ulva reticulata* Forsskal is a marine plant with potential physiological effects on fish reproduction. Therefore, this study aimed to assess the effect of different *U. reticulata* extract doses on the reproductive capabilities of the male red nile tilapia (*Oreochromis* sp.). A completely randomized design was used with different doses of plant extract. These treatments included JK without *U. reticulata* extract (control), as well as JA, JB, JC, and JD with extract doses of 0.75, 1.5, 2.25, and 3%, respectively. The parameters evaluated included testis weight, gonad maturation percentage, gonadosomatic index (GSI), hepatosomatic index (HSI), testosterone concentration, sperm motility, and sperm viability. The results showed that the administration of *U. reticulata* extract at 2.25 to 2.45% proved to be the most effective treatment. This dose caused a threefold increase in gonad maturation, with a weight of 1.38g, a GSI of 2.14%, a 100% GML IV percentage, sperm motility of 82.50%, and sperm viability of 88.97% at weeks 6 and 7 of optimum observation. Furthermore, it reduced IHS value by 0.76% and increased testosterone concentration to 33.23ng/ mL at week 8. Based on the results, feeding *U. reticulata* extract could improve the reproductive performance of the male red tilapia.

INTRODUCTION

Aquaculture is expected to increase by 62% between 2010 and 2030 to meet the rising demands of animal protein consumption **(FAO, 2018)**. This underscores the significant role of aquaculture in providing animal protein sources **(Stankus, 2021)**. Therefore, productivity in aquaculture must increase every year through innovation in reproductive performance to ensure the sustainability of the stock supply based on the number of new individuals and efficient maintenance time **(Jatiswara *et al.*, 2020)**. Fish

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reproductive performance can be improved through various nutritional, environmental, and hormonal methods **(Estrada-Godinez *et al*., 2022)**.

Steroid hormones greatly influence the reproductive performance of fish. During reproduction, *gonadotropin-releasing hormone* (GnRH) regulates gonadotropin secretion from the pituitary gland. Gonadotropins are then released into the bloodstream and act on the testes to produce *follicle-stimulating hormone* (FSH) and *luteinizing hormone* (LH) in the target organs. Sertoli cells secrete FSH, which stimulates testosterone to regulate the growth and development of spermatogenesis during reproduction **(Yaron & Levavi-Sivan, 2011)**. Meanwhile, theca cells secrete LH, which regulates sperm maturation **(Bhat *et al*., 2021)**. Gonadotropins (GtHs), including FSH and LH, play a significant role in spermatogenesis **(Li *et al*., 2020)**. Hormones can accelerate the development and maturation of fish sperms **(Caldas *et al*., 2021)**.

Synthetic hormonal manipulation is widely practiced in several fish species, enhancing male reproduction **(Budi *et al*., 2023; Closs *et al*., 2023)**. However, the use of synthetic hormones has harmful effects, including potential risks to consumer health and negative effects on the environment **(Hoga *et al*., 2018; Jenila *et al*., 2023; Khatun *et al*., 2024)**. Alternative efforts with the same objectives are needed to enhance male fish reproductive performance using natural steroids derived from plant extract **(Jatiswara *et al*., 2020)**. Steroid hormones derived from plants have the same physiological effects as those found in animals **(Abaho *et al*., 2022)**.

Plant extract contains several bioactive compounds including steroids/terpenoids, which serve as precursors in the synthesis of steroid hormones in animals **(Tarkowská, 2019)**. Alkaloid compounds in plant extract have androgenic effects that can stimulate testosterone hormone production to meet gonadal availability **(Dada & Ogunduyile, 2011; Schiano *et al*., 2022)**. Therefore, plant extract has potential applications in fish reproduction with several advantages including safety, availability, cost-effectiveness, and environmental friendliness **(Ghosal *et al*., 2015)**. In this context, *Ulva reticulata* is suspected to have physiological effects on fish reproduction **(Arini, 2021)**. It is a green seaweed commonly found in several Indonesian waters **(Tega *et al*., 2020)**. Qualitatively, the bioactive compounds in the extract consist of flavonoids, saponins, tannins, and phenols with weak activity, while alkaloids and steroids/terpenoids are considered to have a moderate activity. Moreover, *U. reticulata* extract contains sitosterol (19.01mg g^{-1}) and stigmasterol (35.00mg g^{-1}) **(Tarigan *et al*., 2023)**.

Sitosterol and stigmasterol are characteristic plant sterols that share a structure similar to animal cholesterol. Both compounds are converted to cholesterol, which is the main component in the biosynthesis of reproductive hormones **(Tarkowska, 2019)**. These compounds act as sexual tonics in the hormonal system, activating the hypothalamic-pituitary-testis axis through positive feedback mechanisms **(Yurnadi & Suryandari, 2006; Teixeira *et al*., 2023; Chavda *et al*., 2024)**. Thus, *U. reticulata* extract has the potential to be used for reproduction. The bioactive compounds work

synergistically to affect male reproduction. This is supported by the results of **Okab *et al*. (2013)**, stating that supplementation with 2% *U. lactuca* increased testosterone concentration and sperm motility in male rabbits. The use of 10% *U. reticulata* flour also increased gonad weight of pigs **(Purbiantoro *et al*., 2014)**.

The use of terrestrial plant extract in fish reproduction has been widely reported. Administration of *Java chili* extract at 187.5mg/ kg enhanced reproductive performance and sperm quality in male catfish **(Elisdiana *et al*., 2016)**. Furthermore, administration of *Purwoceng* extract (*Pimpinella alpine*) at 5g/ kg in feed improved the reproduction of catfish (*Clarias* sp.) **(Bertha *et al*., 2016)**. Dietary supplementation of *Tribulus terrestris* extract at 500- 750mg/ kg diet also improved the growth and reproductive performances of the male Nile tilapia **(Hassona *et al*., 2020)**. The use of marine plant extract, including *U. reticulata*, as feed for fish reproduction has not yet been addressed. The male red tilapia (*Oreochromis* sp.) was used as the test animals due to several advantages, including easy maintenance and a short reproductive cycle **(Abaho *et al*., 2022)**. Consequently, this study aimed to assess the effect of administering different doses of *U. reticulata* extract on the reproductive capabilities of the male red tilapia.

MATERIALS AND METHODS

1. Preparation of *U. reticulata* extract

U. reticulata Forsskål, 1775 from Moudolung waters, East Sumba, NTT, Indonesia was identified morphologically at the Integrated Laboratory of Oceanographic Research of National Research and Innovation Agency (BRIN), Ancol-Jakarta. Extraction was carried out using the maceration method with 96% ethanol solvent at a ratio of 1:15 **(Zaghbib *et al*., 2022)**. Subsequently, simplisia up to 1000g was dried, mashed, and placed into a dark glass jar container soaked in 15 liters of 96% ethanol. The maceration process was carried out for 5 days (5 × 24h) using ethanol at room temperature under dark conditions. The product obtained was filtered using Whatman 0.2 paper, and *U. reticulata* extract was concentrated using a rotary evaporator at 40^0C.

2. Feed formulation

A commercial feed with a protein content of 33% supplemented with *U. reticulata* extract at a predetermined dose was used. The extract was mixed using the coating method with carboxymethyl cellulose (CMC) as an adhesive. A 1kg feed was produced by adding 1% CMC as an adhesive and stirring until homogenous. Subsequently, 100mL of water was added to the feed and stirred until it was evenly coated. The mixed feed was then air-dried for 1h and stored for further use.

3. Fish and experimental design

Maintenance was carried out using an outdoor container of seven units, each of which was blocked using three sections (hapa) with a mesh size of 4 × 4mm and a size of 100 × 70 × 100cm. This study was conducted using the male red tilapia weighing 20- 30g

sourced from Babakan Dramaga Pond, Bogor. Each bulkhead was filled with 15 samples, and the duration of maintenance was 56 days. The doses of *U. reticulata* extract used followed the method of **Okab *et al*. (2013)**, with slight modifications. The treatments included JK without extract *U. reticulata* 0 (control), as well as JA, JB, JC, and JD with extract doses of 0.75, 1.5, 2.25, and 3%, respectively. Feeding was performed until satiation with the frequency of twice a day in the morning and evening. During fish rearing every two weeks, observations of the measured parameters were made, and water changes of 50% were performed. All maintenance and experimental procedures were approved by IPB University Animal Care and Use Committee (No. 251-2022 IPB).

4. The experimental measurements

4.1 Testicular weight

Testicular weight was measured every 2 weeks during the rearing period. The body weight of two fish was measured, followed by an anesthesia procedure using a solution of 2-phenoxy-ethanol up to 0.4mL L^{-1}. Subsequently, surgery was performed to remove the testicular organs. The testes were then cleaned and weighed using a digital scale.

4.2 The percentage of gonad maturity level (GML)

The percentage of GML was assessed every 2 weeks during the maintenance period. This calculation provided insight into the proportion of fish with mature gonads during the study. The percentage of individuals with mature gonads was calculated using the formula:

$$\text{Percentage of individuals with mature gonads (\%)} = \frac{\text{Number of individuals with mature gonads} \times 100}{\text{Total number of individuals}}$$

4.3 Gonad somatic index (GSI) and hepato somatic index (HSI)

Observations of GSI and HSI were conducted every 2 weeks during maintenance. These observations were performed by weighing the testes and liver organs using a digital scale. GSI was calculated using the formula:

$$\text{GSI (\%)} = (\text{Gonad weight} / \text{Body weight}) \times 100$$

HSI value was calculated using the formula:

$$\text{HSI (\%)} = (\text{Liver weight} / \text{Body weight}) \times 100$$

4.4 Concentration of testosteron levels

Concentration of testosterone levels in the fish blood was measured at weeks 0, 4, and 8. Blood was collected using a syringe that had been rinsed with an anticoagulant solution to prevent clotting. About 4mL of blood was taken at the base of the tail fin, and then collected in a microtube. Subsequently, the collected blood was centrifuged at 5000rpm for 10min to separate the cells from the plasma. The blood plasma (supernatant) obtained was collected in a microtube and stored at -20^0C. Measurement of testosterone concentration was performed through Enzyme-linked Immunosorbent Assay (ELISA) using the Fish Testosterone Kit BT Lab E0001Fi.

4.5 Sperm motility

Sperm motility assessments were conducted every 2 weeks during the maintenance period. Observations were carried out under a microscope at 400 x magnification. Sperms were categorized based on active movement, and the total count was calculated using the formula:

$$\text{Sperm motility (\%)} = \frac{\text{(Sperm motility – sperm inmotility)}}{\text{Total sperm}} \times 100$$

4.6 Sperm viability

Sperm viability was measured every 2 weeks during the maintenance period. Observations were carried out under a microscope at 400 x magnification, while classification was based on the percentage of live sperm in the total count. Sperm viability was calculated using the formula:

$$\text{Sperm viability (\%)} = (\text{Live sperm / total sperm}) \times 100$$

4.7 Histological analysis of testes

Histological observations of fish testes were conducted at the end of the maintenance period. One fish specimen was collected from each treatment group for histological analysis, which provided detailed information on the cellular composition and structure of the testes, allowing for the evaluation of reproductive development. Histological analysis of the testes was performed by the conventional paraffin section method. The collected sperms were placed in a covered vial, and an appropriate amount of Bouin's fixating solution was added. The air in the bottle was drawn with a 5mL syringe to fully fix the tissue cells for 24h. The fixed tissues were rinsed with distilled water several times until the yellow color disappeared. Dehydration was carried out with graded ethanol of 30, 50, 75, 85, and 95% with anhydrous ethanol for 3h each, and transparent with xylene (xylene I: 2h, xylene II: 1h). The tissues were dipped and embedded in paraffin, made into sections with a thickness of 5μm, stained using hematoxylin and eosin, then observed and photographed with a Leica optical microscope.

5. Statistical analysis

Statistical analysis of the experimental results such as testicular weight, GSI, HSI, concentration of testosterone levels, sperm motility, and sperm viability was conducted using one-way ANOVA to determine significant differences among all treatments with a confidence interval of 95%. Treatments showing significant differences ($P< 0.05$) were further analyzed using Duncan's multiple range test. The percentage of GML and histological gonad images were analyzed descriptively in the form of images.

RESULTS

1. Testicular weight

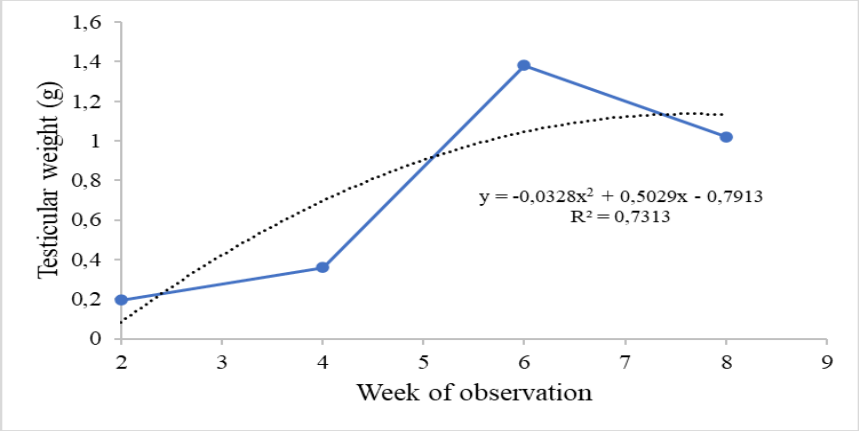
The male red tilapia fed a dose of *U. reticulata* extract showed an increase in testicular weight occurring in each week of observation. Different doses of the extract had a significant effect on testicular weight at week 6 ($P< 0.05$). The testicular weight of

the treatment groups was higher than that of JK control from weeks 2 to 8. The highest value of 1.35g was observed in JC treatment at week 6 (Table 1).

Table 1. Testicular weight of the male red tilapia with different doses of *U. reticulata* extract during rearing.

Week of observation	Doses of *U. reticulata* extract				
	JK (0 %)	JA (0.75%)	JB (1.5%)	JC (2.25%)	JD (3%)
2	0.13±0.01[a]	0.18±0.01[b]	0.17± 0.03[ab]	0.20± 0.02[b]	0.24± 0.04[c]
4	0.19±0.06[a]	0.26±0.04[ab]	0.30±0.12[ab]	0.36±0.14[b]	0.30±0.04[ab]
6	0.21±0.11[a]	0.57±0.18[b]	0.59±0.12[b]	1.38±0.43[c]	0.60±0.05[b]
8	0.45±0.08[a]	0.65±0.10[b]	0.93±0.12[c]	1.02±0.22[c]	0.95±0.22[c]

Description: Data are shown as mean ± standard deviation. Numbers followed by different letters in the same row indicate significant differences ($P< 0.05$).



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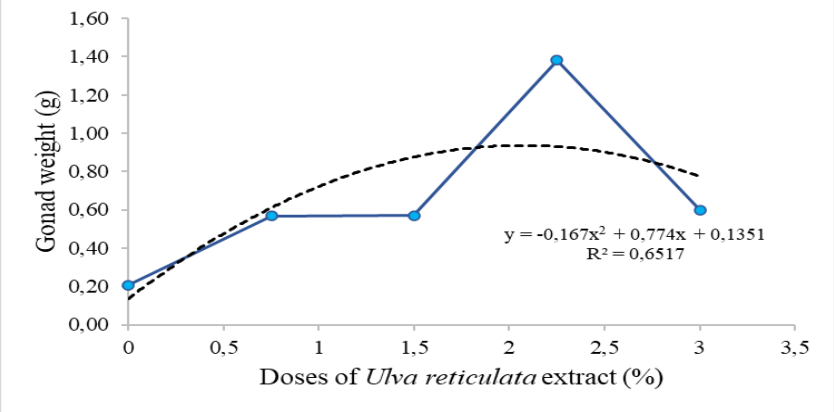

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Fig. 1. Relationship between (a) the week of observation and (b) dose of *U. reticulata* extract on testicular weight of the male red tilapia

Fig. (1) shows the relationship between the week of observation and the addition of *U. reticulata* extract on the testicular weight of the male red tilapia fish at week 6. The optimal relationship between the week of observation and the dosing of *U. reticulata* extract for increasing testicular weight was observed at week 7.6, as described by the equation $y = -0.0328x^2 + 0.5029x + 0.7913$ (Fig. 1a). Meanwhile, optimal dosing of *U. reticulata* extract, yielding a maximum increase in testicular weight was found between 2.25- 2.31%, as described by the equation $y = -0.167x^2 + 0.774x + 0.1351$ (Fig. 1b). This implied that the optimal dose range in the 7.6-week observation period was between 2.25 and 2.31%, effectively increasing the testicular weight of the male red tilapia.

2. The percentage of gonad maturity level (GML)

The percentage of GML in fish refers to a certain stage before and after spawning. Data from GML composition of the male red tilapia during the 8-week rearing are presented in Fig. (2).

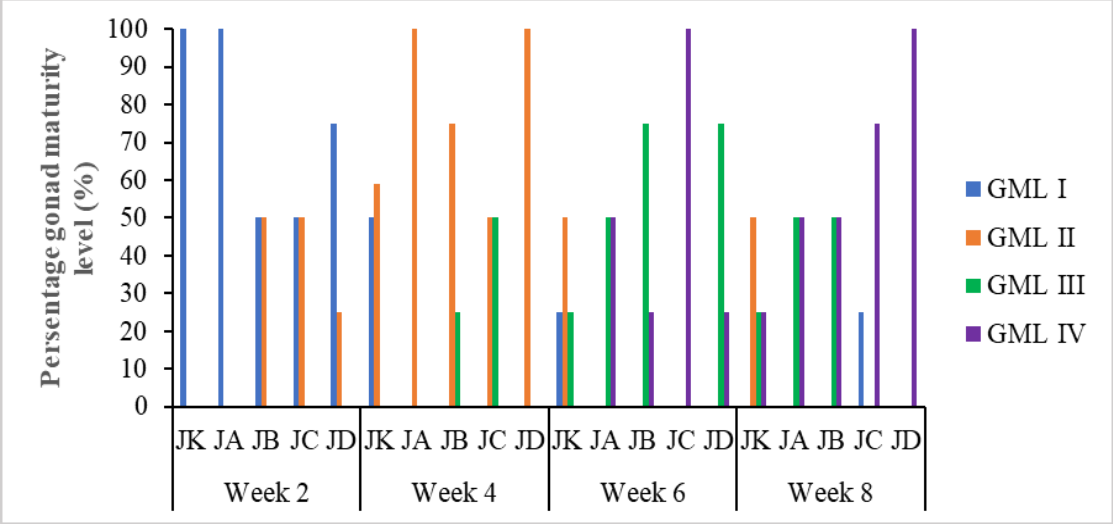


Fig. 2. GML composition of the male red tilapia fish with different doses of *U. reticulata* ethanol extract during maintenance. note: JK: without *U. reticulata* ethanol extract (control); JA: *U. reticulata* ethanol extract dose 0.75%; JB: *U. reticulata* ethanol extract dose 1.75%; JC: *U. reticulata* ethanol extract dose 2.25%; JD: *U. reticulata* ethanol extract dose 3%

Fig. (2) shows that the administration of different doses of *U. reticulata* extract to the male red tilapia led to various growth rates and gonad development to maturity during the 8-week observation period. In JK treatment, all fish gonads were still in stage I (GML I) during week 2 of observation with no significant development. At week 4, the gonads started to develop, with a higher percentage reaching stage II (GML II) although some remained in GML1. By week 6, a few gonads were already in stage III (GML III), but some were still in GML II and I. At week 8, some gonads were already at stage IV (GML

IV) while some were in GML II and III. The percentage of GML II was higher than III and IV.

In JA treatment, week 2 observations showed that all fish gonads were still at GML I, with no progression to II. At week 4, all gonads had developed into GML II stage, and at week 6, gonads were found to be at GML III and IV, with no remaining at II. By week 8, the gonads were still at GML III and IV maintaining the same percentage.

JB treatment at week 2 showed no GML I gonads but rather II and III with the same percentage. At week 4, gonads were found in GML II and III, where the percentage of II gonads was higher than in III. Observation at week 6 showed gonads in GML III with a small percentage progressing to IV. The percentage of GML III gonads was found to be higher than IV. Furthermore, at week 8, gonads were still found in GML III and IV, maintaining the same percentage.

In JC treatment, at week 2 of observation, gonads experienced development with equal percentages of GML II and I. At week 4, further development was observed with an equal percentage of GML III and II. Furthermore, at week 6, gonads grew faster with 100% reaching GML IV, and none in III. By week 8, GML IV gonads were prevalent with a smaller percentage of I.

In JD treatment by week 2 of observation, gonads had developed to include both GML II and I, with a higher percentage of I. At week 4, gonads had progressed into GML III, but some remained in II with the same percentage as III. By week 6, gonads were found in GML IV and III, with a higher percentage of III. Furthermore, at week 8, 100% of gonads were in GML IV with none at III. These results show that administering a dose of *U. reticulata* extract to the male red tilapia can accelerate gonad development.

3. Gonad somatic index (GSI) and Hepato somatic index (HSI)

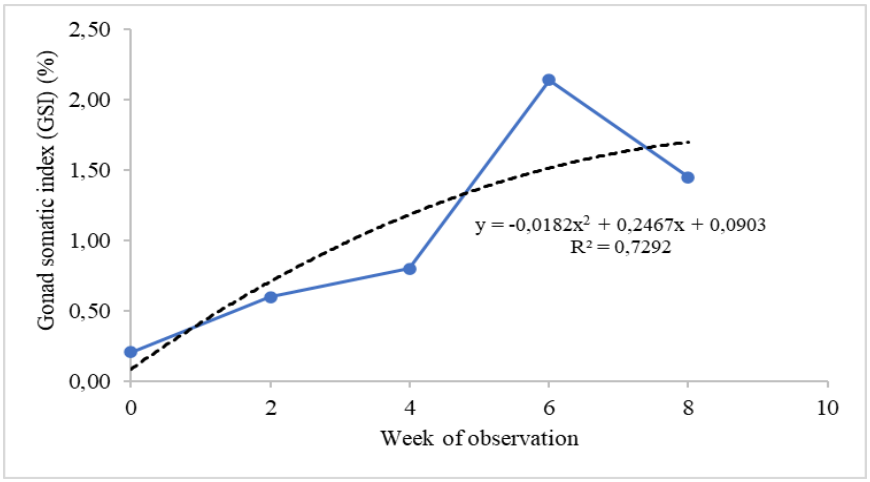
GSI and HSI values of the male red tilapia treated with *U. reticulata* extract for 8 weeks of rearing are shown in Table (2). The pattern of the relationship between the week of observation and the addition of *U. reticulata* extract to the testicular weight at week 6 is presented in Fig. (3).

Table (2) shows that GSI value increased from week 2 to 8. The dose treatment yielded a significant effect on GSI value at observation weeks 6 to 8 ($P< 0.05$). The highest GSI value was observed in JC treatment at 2.14% during week 6, while the lowest was found in JK treatment at 0.54%. However, HSI values in the male red tilapia fluctuated during each week of observation. The dose of *U. reticulata* extract had a significant effect on HIS value from weeks 6 to 8 ($P< 0.05$). The highest HSI value was observed in JK treatment (1.10 %), while the lowest was found in JC treatment (0.76 %) at week 8.

Table 2. GSI and HSI values in the male red tilapia with different doses of *U. reticulata* extract during rearing

Week of observation	Doses of *U. reticulata* extract				
	JK (0 %)	JA (0.75%)	JB (1.5%)	JC (2.25%)	JD (3%)
			GSI (%)		
2	0.47± 0.02^{a}	0.05 ± 0.02^{a}	0.61± 0.09^{ab}	0.59± 0.03^{ab}	0.73± 0.08^{b}
4	0.49± 0.06^{a}	0.53± 0.07^{a}	0.66± 0.09^{a}	0.80± 0.17^{a}	0.66± 0.05^{a}
6	0.54± 0.16^{a}	1.04± 0.23^{a}	0.99± 0.12^{a}	2.14± 0.36^{b}	0.92± 0.07^{a}
8	0.67± 0.09^{a}	1.05± 0.11^{b}	1.30± 0.05^{ab}	1.45± 0.12^{c}	1.38± 0.17^{bc}
			HSI (%)		
2	1.02± 0.14^{a}	1.18± 0.06^{a}	1.11± 0.06^{a}	1.05± 0.06^{a}	1.00± 0.10^{a}
4	1.04± 0.04^{ab}	1.43± 0.26^{b}	1.22± 0.15^{ab}	1.00± 0.03^{ab}	0.93± 0.04^{a}
6	1.40± 0.10^{ab}	1.26± 0.11^{ab}	1.71± 0.27^{b}	0.89± 0.23^{a}	1.34± 0.07^{ab}
8	1.10± 0.15^{b}	0.87± 0.07^{ab}	0.99± 0.55^{ab}	0.76± 0.55^{a}	0.98± 0.03^{ab}

Description: Data are shown as mean ± standard deviation. Numbers followed by different letters in the same row indicate significant differences (P< 0.05).



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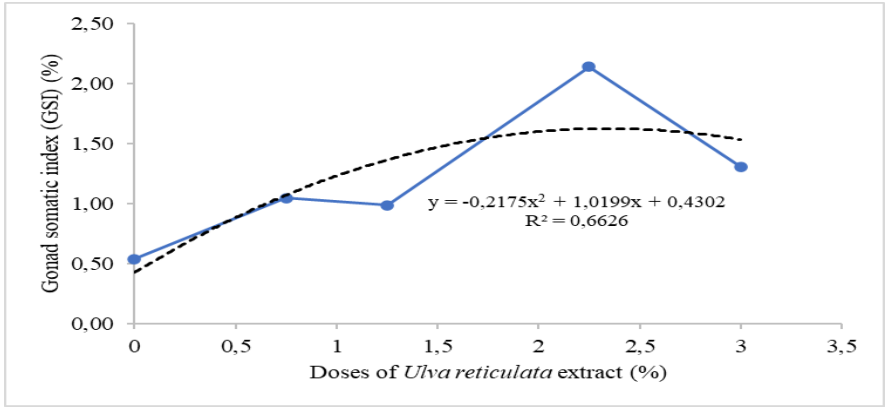

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Fig. 3. Relationship between (a) the week of observation and (b) dose of *U. reticulata* extract on GSI value of male red tilapia.

Fig. (3a) shows the relationship pattern between the week of observation and the dosing of *U. reticulata* extract, leading to the optimum GSI value at week 7, as described by the equation $y = -0.0182x^2 + 0.2467x + 0.0903$. Fig. (2b) shows the relationship pattern between *U. reticulata* extract dosing and the increase in testicular weight. The optimal dose range for the increase was 2.25- 2.34%, as described by the equation $y = 0.2175x^2 + 1.0199x + 0.4302$ (Fig. 3b). This shows that the optimum dose limit for achieving maximum GSI value in the male red tilapia is between 2.25- 2.34% at week 7 of observation.

4. Concentration of testosterone levels

Fig. (4) shows the concentration of testosterone levels in the male red tilapia treated with different doses of *U. reticulata* extract during rearing. The dose treatments had a significant effect ($P< 0.05$) on the concentration of testosterone from weeks 4 to 8 compared to JK. The highest concentration was found in JC at 33.23ng/ mL compared to the other treatments at week 8.

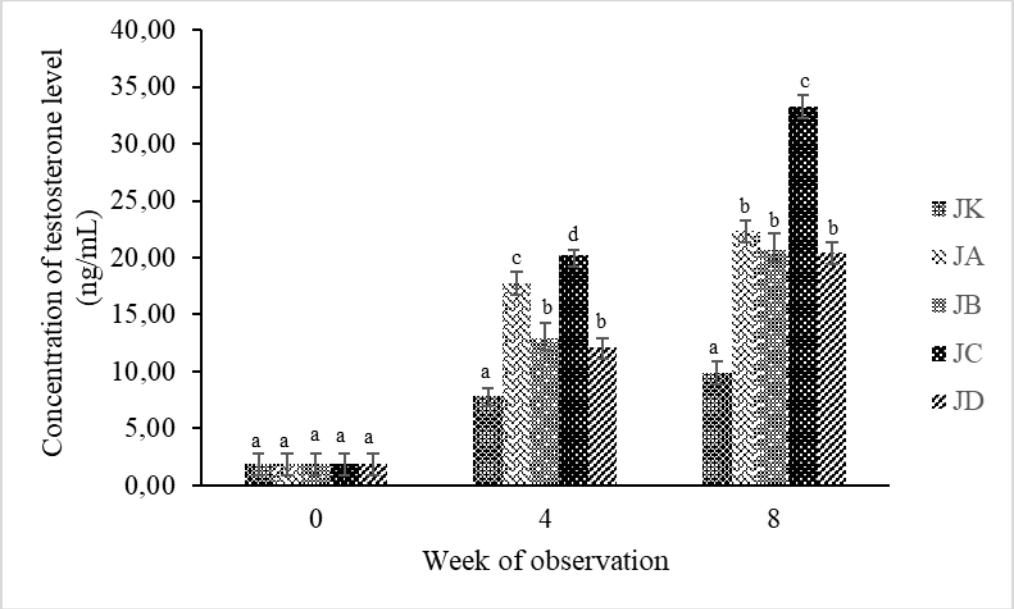


Fig. 4. Concentration of testosterone level in the male red tilapia fish with different doses of *U. reticulata* extract during rearing.

Description: Data are shown as mean ± standard deviation. Graphs followed by different letters in the same column indicate significant differences ($P< 0.05$).

5. Sperm motility

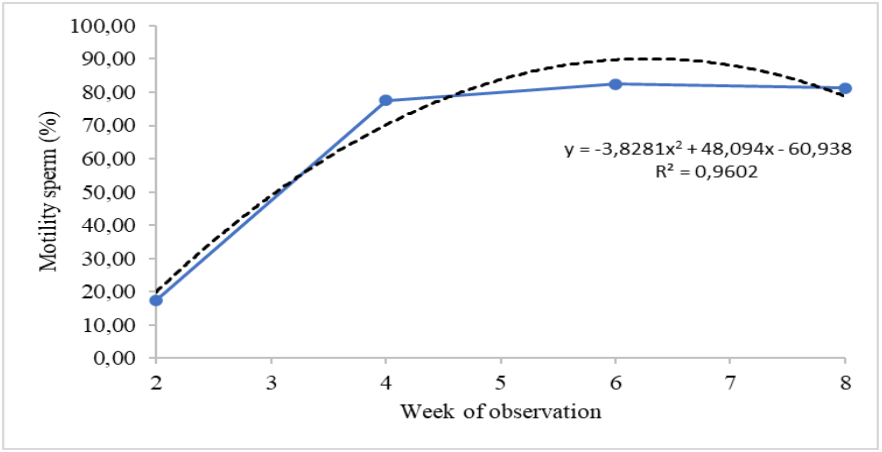
Table (3) shows the percentage of sperm motility for the male red tilapia after administering doses of *U. reticulata* extract during rearing. The relationship between the week of observation and the addition of *U. reticulata* extract dose to the sperm motility of the male red tilapia at week 6 is presented in Fig. (5).

Table 3. Sperm motility in male red tilapia with different doses of *U. reticulata* extract during rearing

Week of observation	Doses of *U. reticulata* extract				
	JK (0 %)	JA (0.75%)	JB (1.5%)	JC (2.25%)	JD (3%)
2	15.00± 2.88^{a}	17.50± 2.55^{a}	20.00± 2.00^{a}	17.50± 2.20^{a}	16.25± 2,20^{a}
4	42.50± 5.50^{a}	73.75± 2.50^{b}	70.00± 3.53^{b}	77.50± 2.50^{b}	77.50± 2,50^{b}
6	47.50± 2.50^{a}	77.50± 2.05^{b}	80.00± 2.04^{b}	82.89± 1.44^{b}	77.50± 2,15^{b}
8	68.75± 1.25^{a}	78.75± 2.39^{b}	77.50± 1.44^{b}	81.25± 2.39^{c}	76.75± 1,21^{b}

Description: Data are shown as mean ± standard deviation. Numbers followed by different letters in the same row indicate significant differences (P< 0.05).

Table (3) shows that the dose treatment of *U. reticulata* extract had a significant effect (P< 0.05) compared to JK for each week of observation. However, the extract dose had a significantly different effect at week 8. The highest sperm motility of 82.89% was observed in JC treatment at week 6. Sperm motility in JC treatment group increased by 18.1% compared JK.



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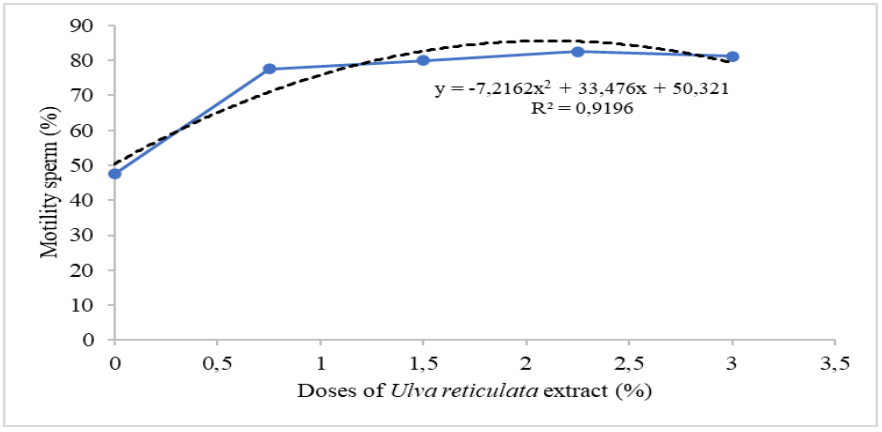


b

Fig. 5. Relationship between (a) the week of observation and (b) dose of *U. reticulata* extract on sperm motility of the male red tilapia

Fig. (5a) denotes the relationship pattern between the week of observation and *U. reticulata* extract dosing on sperm motility, with the optimal point occurring at week 6.2, as described by the equation y = -3.8281x2 + 48.094x + 60.938. Fig. (5b) shows that the optimal dose for increased sperm motility was 2.25- 2.31%, as described by the equation y = -7.2162x2 + 33.476x + 50.321. These results indicate that the optimal dose of *U. reticulata* extract to achieve maximum sperm motility in the male red tilapia is between 2.25 and 2.31% at week 6.2 of observation.

6. Sperm viability

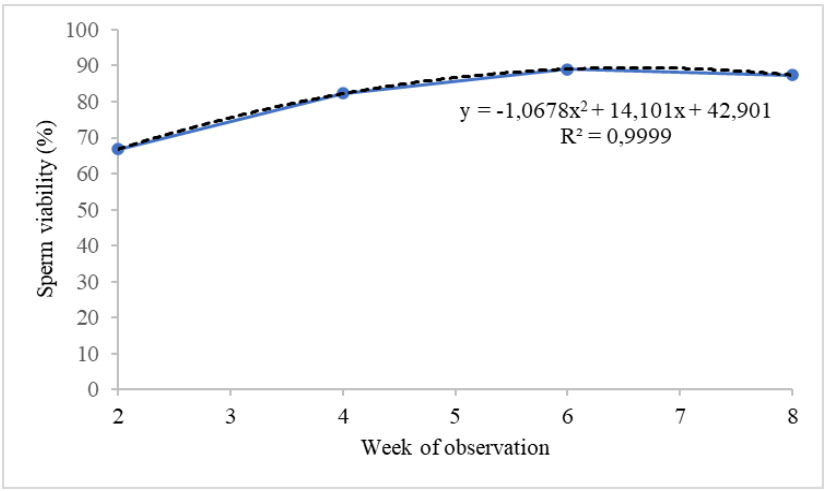
Table (4) exhibits sperm viability results in the male red tilapia administered doses of *U. reticulata* ethanol extract during rearing. The relationship pattern between the week of observation and the addition of *U. reticulata* extract on the sperm viability at week 6 is presented in Fig. (6).

Table 4. Sperm viability in male red tilapia with different doses of *U. reticulata* extract during rearing

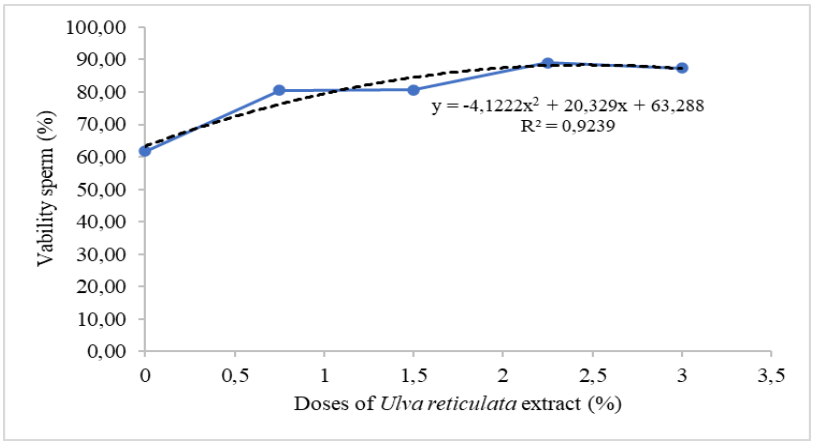
Week of observation	Doses of *U. reticulata* extract				
	JK (0 %)	JA (0.75%)	JB (1.5%)	JC (2.25%)	JD (3%)
2	21.00± 1.33^{a}	29.40± 1.44^{b}	30.32± 0.67^{b}	31.11± 0.86^{b}	29.78± 0.74^{b}
4	48.75± 1.25^{a}	72.70± 1.60^{b}	79.32± 1.39^{c}	82.32± 1.08^{c}	88.26± 1.73^{d}
6	61.79± 1.06^{a}	80.47± 0.40^{b}	80.70± 0.59^{b}	88.97± 1.15^{d}	84.69± 2.06^{c}
8	77.24± 2.08^{a}	86.15± 0.82^{b}	85.90± 2.12^{b}	87.40± 2.02^{b}	88.61± 2.31^{b}

Description: Data are shown as mean ± standard deviation. Numbers followed by different letters in the same row indicate significant differences ($P< 0.05$).

Table (4) displays that sperm viability increased with higher doses of *U. reticulata* ethanol extract. The dose treatment had a significant effect ($P< 0.05$) on sperm viability from weeks 4 to 6 compared to JK. The highest fish sperm viability was observed in JC treatment (99.97 %) at week 6. The increase in JC treatment was 13.1% compared to JK. The relationship pattern between the week of observation and *U. reticulata* extract dosing on sperm viability reached the optimum point at week 6.5 of observation, as described by the equation y = -1.0678x2 + 14.101x + 42.901 (Fig. 6a). Meanwhile, the relationship pattern between *U. reticulata* extract dosing and sperm motility reached an optimum value with an extract dose range of 2.25 to 2.45%. The relationship was described by the equation y = -4.1222x2 + 20.329x + 63.288 (Fig. 6b). These results imply that the optimal dose to achieve maximum sperm motility in the male red tilapia is between 2.25-2.45% at week 6.2 of observation.



a



b

Fig. 6. Relationship between (a) the week of observation and (b) dose of *U. reticulata* extract on sperm viability of the male red tilapia

Histological analysis of testes

Histological testicular development was assessed at the end of the study period. The testicular features of the male red tilapia treated with *U. reticulata* extract progressed toward maturity. Testicular development was assessed from the distribution of spermatozoa stages presented in Fig. (7).

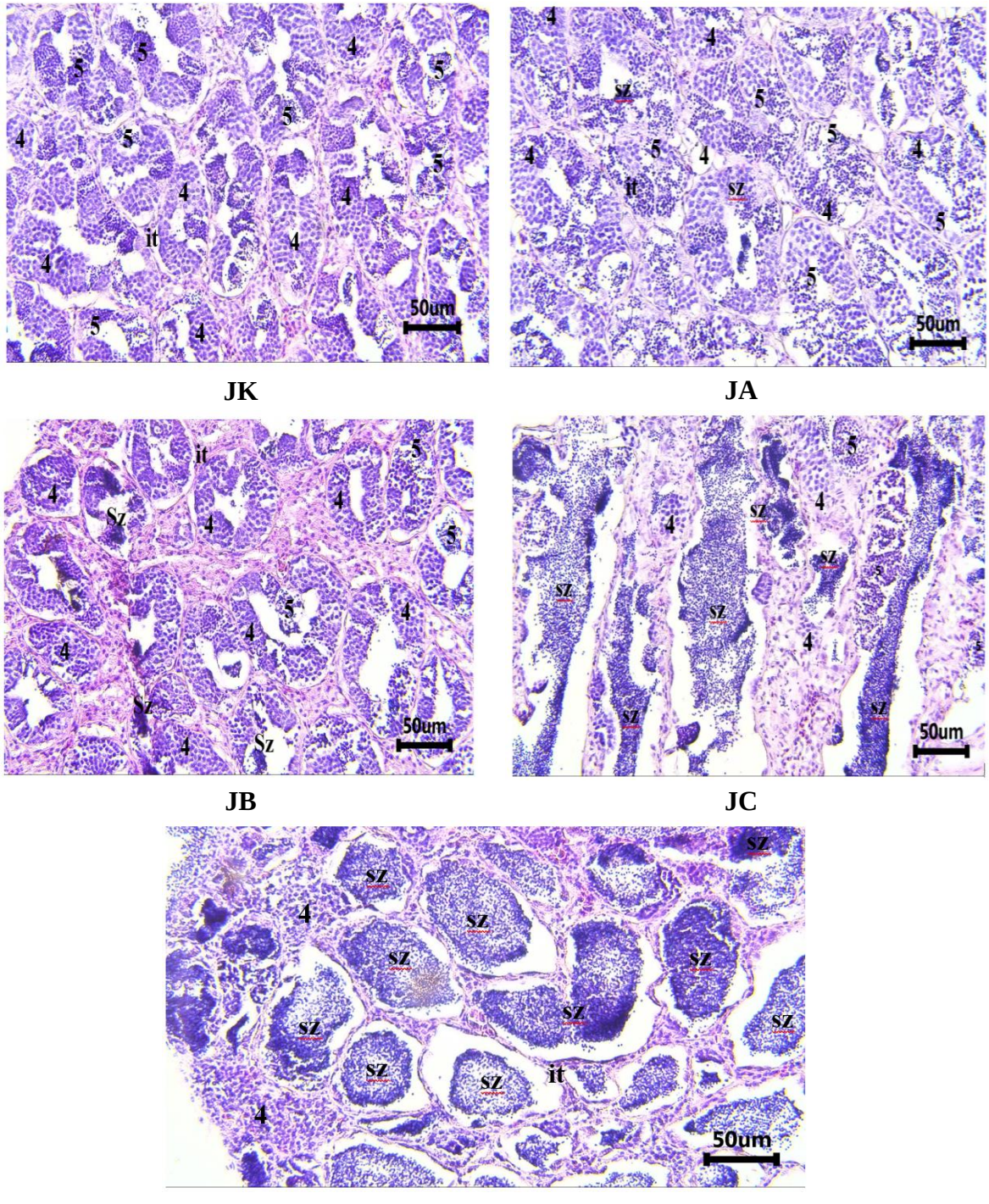


Fig. 7. Histology of the testes at the end of the study with different doses of *U. reticulata* extract in diet during maintenance. JK: without *U. reticulata* extract (control); JA: 0.75% *U. reticulata* extract dose; JB: 1.75% *U. reticulata* extract dose; JC: 2.25% *U. reticulata* ethanol extract dose; JD: 3% *U. reticulata* ethanol extract dose with 10X magnification. Description: spermatozoa (Sz), interstitial tissue (it), secondary spermatocytes (4), and spermatids (5)

Fig. (7) shows that treatment with *U. reticulata* extract induced rapid development toward maturity, evidenced by the presence of spermatozoa. Meanwhile, in JK treatment, testicular development occurred very slowly and the testes remained at the secondary spermatogonia stage. The formation of spermatids also occurred along with limited distribution of spermatozoa. In JA treatment, testicular development was in the stage of secondary spermatogonia and spermatids, with limited distribution of spermatozoa. In JB treatment, the distribution of spermatozoa was found, also with the development of secondary spermatogenia and spermatids. For JC treatment, the distribution of spermatozoa ready for spawning and spermatids was greater than that of the secondary spermatogonia stages. Meanwhile, in JD treatment, a large distribution of spermatozoa was observed with few secondary spermatogonia. As shown in Fig. (8), the best testicular development was found in JC treatment, characterized by a greater distribution of spermatozoa ready for spawning compared to other treatments.

DISCUSSION

The use of plant extract in aquaculture has witnessed a consistent increase due to the ability to reduce the application of chemicals and synthetic drugs **(Abaho *et al*., 2022)**. This function is attributed to the presence of primary and secondary metabolites that work synergistically on fish physiology **(Beltrán & Esteban, 2022)**. Other predominant compounds are steroids, including sitosterol and stigmasterol, which have the same physiological action as steroid hormones in fish **(Tarkowská, 2019)**. Consequently, plant extract finds applications in various aspects of animal physiology, such as the growth, health, and reproduction of fish **(Elisdiana *et al*., 2016; Madibana *et al*., 2017; Abo-Raya *et al*., 2021)**. **Elisdiana *et al*.** (**2016**) showed that the use of 187.5mg/ kg of *Javanese chili* extract enhanced the physiological parameters of the male Siamese catfish. Furthermore, the provision of Purwoceng extract at 5g/ kg increased GSI and spermatocrit values in the male catfish **(Bertha *et al*., 2016)**.

The extract from *U. reticulata*, a type of green seaweed, contains secondary metabolites, such as flavonoids, saponins, and phenols, which have weak activity, while alkaloids and steroids have a moderate activity. The extract also contains sitosterol (19.01mg g^{-1}) and stigmasterol (35.00mg g^{-1}). Furthermore, the brine shrimp lethality test (BSLT) showed no toxicity, indicating safety for use in aquatic animals **(Tarigan *et al*., 2023)**. Sitosterol and stigmasterol are converted to cholesterol, which is a primary component in the biosynthesis of reproductive hormones **(Tarkowska, 2019)**. Cholesterol is then transformed into pregnenolone by cytochrome P450 enzymes during the biosynthesis of steroid hormones **(Tredau, 2022)**.

The results showed that the use of *U. reticulata* extract in feed significantly influenced parameters such as testis weight, GML, GSI, sperm motility, and sperm viability in the male red tilapia ($P< 0.05$). Testis weight increased significantly with different doses of extract in feed during the rearing period compared to JK group. The highest testis weight of 1.20g was observed in JC treatment at week 6. Similarly, **Purbiantoro *et al*. (2014)** reported that the administration of *U. reticulata* flour at 10%

increased the gonad weight of sea cucumbers by 5.26%. An increase in testicular weight indicates testicular development, particularly spermatogenesis **(Assem *et al.*, 2016)**. This is consistent with the observed percentage of gonad maturation in the male red tilapia during the rearing period. Administration of *U. reticulata* extract doses induced faster gonad maturation compared to JK group at weeks 6 and 8. The highest gonad maturation percentage was observed in JC treatment, reaching 100% stage IV gonad maturation (GML IV) in week 6. However, at week 8 of observation, there was a decrease in the percentage of GML IV by 75% and GML I by 25% (Fig. 3). These results indicate that the administration of *U. reticulata* extract can accelerate gonad maturation, resulting in a higher percentage of GML IV compared to JK group at week 6 of rearing.

Based on the results, the administration of *U. reticulata* extract increased GSI in each treatment of the male red tilapia during the rearing period. The highest GSI value was observed with JC treatment, reaching 2.14% at week 6 of observation (Fig. 5). The increase in GSI values and gonad maturation percentage can be attributed to the presence of secondary metabolites in *U. reticulata* extract, such as alkaloids and steroids (sitosterol and stigmasterol), which influence the reproductive performance. This is supported by **Schiano *et al*. (2022)**, stating that secondary metabolites found in seaweed work synergistically to enhance reproductive performance by affecting the actions of the hypothalamus-pituitary gland and gonads in fish. **Arini (2021)** also reported that seaweed extracts had physiological effects on reproduction. The secondary metabolites present in *U. reticulata* extract synergistically work in reproductive hormone biosynthesis, thereby influencing rapid gonad formation and growth. Furthermore, rapid gonad growth leads to an increase in gonad weight, affecting both GSI values and maturation percentage. This finding is in line with the result of **Ciji *et al*. (2021)**, stating that during gonad development, changes occur in gonad weight, causing an increase in GSI value. **Pham and Le (2020)** also found that GSI values increased with gonad maturation in fish. However, HSI values showed a different trend with an increase occurring from week 2 to 4, followed by a decrease from week 6 to 8 after the administration of *U. reticulata* extract in the feed. The highest value was observed in JK treatment (control) at 1.10%, while the lowest was found in JC treatment at 0.76%. The fluctuation observed is closely related to energy reserves and allocation during gonadal growth and development, thereby affecting liver weight and HSI values. Energy reserves stored in the liver influence the HSI values during the reproductive phase of fish **(Sharma & Ram, 2020)**.

The blood testosterone concentration in the male red tilapia was studied, and the treatment dose of *U. reticulata* extract produced significantly higher values compared to JK ($P< 0.05$). As shown in Fig. (5), the blood testosterone concentration in JC treatment group was higher than JK. The increase in testosterone concentration was directly proportional to sperm quality in the male red tilapia. Furthermore, treatment with *U. reticulata* extract had a significant effect ($P< 0.05$) on sperm motility and viability compared to JK. For the treatment group, the motility and viability of fish sperm

increased from week 2 to 8 during the rearing period (Figs. 10, 13). This indicates that the administration of *U. reticulata* plant extract can increase the testosterone concentration and sperm quality in fish. Similarly, **Okab *et al.* (2013)** reported that supplementation with *U. lactuca* at 2% increased the testosterone concentration and sperm quality in male rabbits. In addition, the supplementation with *Caulerpa lentillifera* extract increased sperm motility in rats **(Khairuddin *et al.*, 2020)**. The increase in blood testosterone hormone levels in fish administered *U. reticulata* extract can be attributed to to the presence of secondary metabolites such as sitosterol and stigmasterol, which are converted into cholesterol, the main precursor in the biosynthesis of steroid hormones including testosterone during steroidogenesis. The compounds sitosterol and stigmasterol activate signals in the hypothalamus-pituitary-gonad axis through a positive feedback mechanism during the reproductive phase. This is supported by **Yurnani and Suryandari (2006)**, stating that sitosterol and stigmasterol compounds from plants could act as precursors in steroid hormone biosynthesis, providing signals to the hypothalamus-pituitary-gonad axis during the reproductive process. Additionally, alkaloids present in *U. reticulata* extract presumably stimulate testosterone secretion during the fish reproductive phase. These compounds have androgenic effects that can stimulate testosterone production to meet the demands of gonads **(Dada & Ogunduyile, 2011; Schiano *et al.*, 2022)**.

Aside from sterols and alkaloids, the antioxidant content of *U. reticulata* extract presumably plays a role in improving sperm quality. **Tarigan *et al*. (2023)** reported that the extract had antioxidant activity classified in the strong category. The antioxidant contents, derived from secondary metabolites, namely flavonoids, vitamins, and minerals **(Siddik *et al.*, 2023)** prevent the oxidation process in sperm cells and stimulate testosterone during spermatogenesis **(Yungsang & Wanxi, 2011; Dewantari, 2013; Félix *et al.*, 2020)**. **Maulida *et al.* (2024)** reported that antioxidant concentration increased sperm motility and viability in *Anabas testudineus*. Furthermore, **Kalikiy *et al.* (2019)** stated that zinc (Zn) mineral supplementation improved the sperm quality and quantity of *Pangasianodon hypophthalmus* catfish.

Gonad histology is a reproductive parameter that provides a direct picture of growth and development **(Mansour *et al.*, 2022)**. Fig. (8) shows that testicular development in the group treated with *U. reticulata* extract at a dose of 2.25% (JC) was faster toward maturity compared to other treatments. Generally, the development of fish testes is related to testosterone concentration. Testosterone is a steroid hormone that regulates the formation and growth of secondary spermatocytes and the differentiation of spermatids into spermatozoa. A previous study also found that high blood testosterone concentrations support sperm formation and maturation. In addition, alkaloid compounds contained in *U. reticulata* extract can increase testosterone levels, supporting the formation and development of the male red tilapia sperm. **Elisdiana *et al*. (2016)** stated that the Javanese chili extract containing alkaloids increased the testosterone

concentration, thereby affecting the process of sperm development and maturity in the testes of the catfish.

CONCLUSION

In conclusion, administering *U. reticulata* extract in fish feed enhanced reproduction by increasing testosterone concentration. The optimum dose ranging from 2.25 to 2.45% increased the testicular weight, GSI, GML IV percentage, as well as sperm motility and viability at weeks 6 to 7 of observation. Furthermore, this dose reduced IHS value and improved testosterone concentration by 33.23ng/ mL at week 8.

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