



Bitter Melon (*Momordica charantia* L) Fruit Extract Affects The Gonad Development and Ovulation of Female Tilapia (*Oreochromis niloticus*); Study of Gonadal Morphology and Histopathology

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Abstract

One of the problems in the cultivation of tilapia (*Oreochromis niloticus*) is the ability to reproduce early and quickly mature gonads so that the fish kept are difficult to reach the size of market demand. This study aims to determine the effect of various doses of bitter melon fruit extract on the development of female gonads and to study the histopathological changes in the structure of the gonad. This research was carried out in July-September 2021. There were 600 juveniles of tilapia used in this study. Tilapia rearing was carried out in 12 containers (each 200 L volume of water) for 60 days. The experimental design was carried out with 4 treatments of bitter melon extract (BME) consisting of 0 g, 0.1 g, 0.2 g, and 0.3 g for 1 kg of feed and 3 replicates. Parameters observed were gonad morphology, gonad somatic index, and histopathology of the gonad. The gonadal maturity index for all treatment has not significant ($P > 0.05$) but the morphology of the gonad showed differences in size and color. Based on histopathology analysis shows structural changes in the form of oocyte cells undergoing necrosis and increased oocyte atresia in BME treatment.

Keywords: Antifertility; Bitter Melon, Tilapia, Gonad, Morphology, Histopathology.

1. Introduction

Tilapia (*Oreochromis niloticus*) is one type of freshwater cultured fish that has important economic value, is much in demand by the community, is easy maintenance, has relatively fast growth, and is tolerant of unfavorable aquatic environmental conditions so that it has been widely cultivated throughout Indonesia. Tilapia production is also growing rapidly, which is indicated by data from the Ministry of Maritime Affairs and Fisheries regarding tilapia aquaculture production in 2015-2019 with an average of 1 million tons per year Pusat Data Statistik dan Informasi Kementerian Kelautan dan Perikanan (KKP) RI, 2020).

Although tilapia is an easy fish to care for, there is a very common problem faced by aquaculture farmers, namely the ability of tilapia to reproduce at a young age due to the rapid maturation of the gonads making it difficult to reach large sizes. Although monosex technology has begun to be developed, monosex seeds are still difficult to obtain by cultivating farmers in the regions. The use of sex reversal technology in fish can be carried out using synthetic steroid hormones that are often used, namely 17 α -methyltestosterone (17 α -MT) for masculinization because the success of using

synthetic hormones is very high (can reach 97-100%) when compared to physical treatment. However, the use of synthetic steroid hormones is still being debated, and the pros and cons related to food safety, in this case, the residue that is feared is still stored in the fish's body (Suseno *et al.*, 2020). The community also complains about the use of drugs that are not specifically used in cultivation, one of which is using birth control pills that are commonly used by humans as a contraceptive. Therefore, cultivators think of using these drugs to inhibit reproduction and increase growth. It is feared that people who consume fish fed with a mixture of birth control pills will have a negative effect and cause unplanned reproductive inhibition for both men and women (Manshuri, 2013).

Efforts besides the use of synthetic hormones are the use of probiotics. Phytobiotics are plants that contain chemical compounds that are beneficial to living things (Fujaya, 2021). One of the methods used is the administration of herbal extracts which aim as anti-fertility agents to prevent early reproduction. According to Mulyani *et al.* (2016), herbal is a plant term that has medicinal properties. Indonesian people have long known and used medicinal plants as an effort to overcome health problems. Knowledge of

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medicinal plants is based on experience and skills passed down from generation to generation.

Bitter Melon fruit can be considered an herbal plant that has the potential to be an anti-fertility agent. The advantages of using these plants include low toxicity, ease to obtain, cheapness, and few side effects. As a plant of the Cucurbitaceae family, bitter melon fruit also contains ingredients belonging to triterpene glycosides or cucurbitacin (Cholifah *et al.*, 2014). The active compound contained in bitter melon, namely cucurbitacin which belongs to the terpenoid glycoside group, is thought to work to inhibit the development of spermatogenic cells through cytotoxic effects and through hormonal effects (Cholifah *et al.*, 2014). Bitter gourd also contains active flavonoids which can act as anti-fertility for both male and female animals (Ifeanyi *et al.*, 2011).

2. Material and Methods

The test animal used was juvenile tilapia measuring ± 8 cm. Juvenile tilapia was obtained from hatcheries at the Takalar Brackish Water Cultivation Center. There were 600 test animals studied, with a density of 50 juvenile tilapia per tank. As well as prepared 400 tails in stock tanks for each treatment. Before stocking, the test fish were disinfected using vitomolt plus at a dose of 10 ppm for 30 minutes.

The test fish were sorted to homogenize the size. Fish were weighed and measured in length as initial data. Next, the fish are stocked into a conical tub that has previously been filled with water and the water quality is measured. The weighing was done using an electric scale with an accuracy of 0.01 gram and the initial length of the fish was measured using a ruler with an accuracy of 0.1 cm as initial data.

The container used in this study was a conical tub with a volume of 250 L, consisting of 12 pieces filled with 200 liters of water. The water used is fresh water obtained from drilled wells from the Hatchery Laboratory, Faculty of Marine Sciences and Fisheries, Hasanuddin University. Before use, all containers are cleaned using chlorine. The water to be used is also disinfected with chlorine after being filtered using a 10 μ m filter bag, the filtered water is disinfected using 100 ppm chlorine, and left for 24 hours, after that the chlorine is neutralized using thiosulfate, then fully aerated for 24 hours, the treated water closed until used before stocking the test fish, media water was given vitomolt plus at a dose of 2 ppm.

The feed used in this study was commercially produced feed with nutritional content including, 14-15% protein, 5% minimum fat, 5% max fiber, and 12% max water content which is commonly used by aquaculture farmers for tilapia. Extraction of Bitter melon made by extraction standard with ethanol as a solvent. The test feed was prepared by: bitter melon

extract dissolved in 100 ml of water, for 1 kg of feed each. The amount of bitter melon extract (BME) was adjusted to the concentration of the treatment. Furthermore, the solution is sprayed on artificial feed evenly. Dry and store in a closed container until use. Before feeding, the feed is moistened with enough water to swell slightly.

During the rearing of the test fish, they were given artificial feed containing vitomolt as much as 5% of the weight of fish biomass per day with the frequency of feeding 2 times a day (07.00-08.00 am and 05.00-06.00 pm). Feeding was done manually or stocked directly into each experimental unit. Visual observations were made every day to control fish development. The rest of the feed and feces siphon every day before the next day's feeding. Changes in water as much as 50-70% are done every week.

This study consisted of 4 treatments, and each treatment consisted of 3 replications. Thus, this study consisted of 12 experimental units. The treatment that was tried was the difference in the dose of BME namely: 0, 0.1, 0.2, and 0.3 g for each kg of feed.

The data displayed includes data on the gonad maturity index and gonad histopathology of female tilapia after administration of bitter melon fruit extract.

2.1. Gonad Maturity Index

According to Rahayu (2017), Gonad Maturity Index can be calculated using the following formula:

$$IKG = Wg/Wt\%$$

Where:

IKG = Gonad Maturity Index (%)

Wg = Gonad Weight (g)

Wt = Fish Weight (g)

2.2. Gonad Histopathology

The parameters observed in this study were to identify tissue damage to female gonad cells treated with bitter melon extract at different doses after 60 days of maintenance. Knowing the type of egg and sperm cells observed based on development refers to El-Sakhawy *et al.* (2011) and El-Saba *et al.* (2013).

The preparation of histology preparations was carried out starting with sampling the gonads of fish on days 0, 30, and 60. The gonads were taken 3 females per treatment. Gonad retrieval is done by dissecting the fish in the abdomen vertically starting from the anus leading to the vertebrae, then horizontally leading to the ventral fin. After the belly of the fish is exposed, the gonads can be observed and removed by slowly separating them from the digestive tract to avoid damage to the gonads. Then the gonads were weighed with an electric scale with an accuracy of 0.0001 g on a previously weighed paper. Then sample preparations for observation of gonadal histology for the entire sample collection and then 3 slides of each sample were made.

Stage 1 Gonad fixation was carried out by immersing the target organs in 4% paraformaldehyde solution for 2 days (2x24 hours), then the tissues were transferred to 70% alcohol until further processing.

Stage 2 Trimming (selection of tissue) ie the fixed tissue is cut using a sharp and sterile scalpel so that the tissue is not damaged in the process. After the trimming process is carried out, the cut tissue is inserted into the cassette. The cassette containing tissue is then immersed in distilled water for one minute in order to avoid shrinkage of the tissue due to prolonged exposure to air, inserting the selected tissue which has been fixed in the tissue into the tissue cassette.

Stage 3 Processing and embedding is done by inserting the tissue cassette into the tissue processor. The tissue in the tissue cassette is then dehydrated by placing the tissue in a graded alcohol solution, namely 70%, 80%, 90%, 95%, and 100% alcohol. Alcohol 70% to 80% each for 1 day. Then 90% and 95% 12 hours respectively. Then 100% (1) and 100% (2) each 1 hour. Then clear into xylol I and xylol II, each for 15 minutes. Then the infiltration stage, namely the tissue cassette is put into liquid paraffin I and II at a temperature of 56°C for 1 hour each. The next stage is embedding, namely printing the tissue in liquid paraffin by placing the specimen on top of the mold and then filling it with paraffin. The position of the specimen to be cut must be facing down against the mold. Then put the pink cassette on top of the mold and add paraffin.

Stage 4 Cutting After the preparation can be cut, it is cut with a microtome with a thickness of 5 μ m. The tissue pieces are then placed on a slide that has been given distilled water and numbered. Then the slides were placed in an incubator at 40° C for 1 day before staining.

Stage 5 Tissue staining is immersed in xylol I solution for 30 minutes then immersed in xylol II solution for 30 minutes, then put into 100% alcohol I, 100% II, 95%, 80%, and 70% respectively. for 1 minute. Then soaked in distilled water for 15 minutes so that the hematoxylin staining can stick well. Then proceed with inserting the preparation into the eosin dye solution for 10 minutes. The next step is to put the preparation into an alcohol solution of 70%, 80%, 90%, 95%, 100%, 100%, xylol I, and xylol II respectively for 1 minute except for xylol I and II for 30 each. minute. After that, the preparation was dried and given 1-2 drops of Entelan adhesive, then carefully covered with a cover slip until no air bubbles were formed, then stored for several minutes until the adhesive dries and is ready to be observed under a microscope.

After the results of the histological preparations were completed, observations were made under a microscope, using a subjective lens magnification of 10x and objective lenses of 4x, 10x, and 40x. Observations and images were taken using an Olympus CX43 microscope with the help of a Dino-lite microscope camera and Dino-Capture 2.0 software. From the results of the histology images will be observed and identified the type of gonadal cells and the damage that occurs histologically which is the result of this study.

2.3. Water Quality

Supporting parameters are measuring water quality, temperature using a thermometer, pH using a pH meter, and DO meter. This water quality measurement can indirectly affect important fish activities.

2.4. Statistical Analysis

Data on the gonad maturity index of tilapia were analyzed by Analysis of Variance (ANOVA) while data on the level of female gonad maturity, gonadal maturity index, and gonadal histopathology were analyzed descriptively in the form of tables and figures. Data analysis using computer software package SPSS version 26.0 program.

3. Results and Discussion

3.1. Results

The average gonadal maturity index in tilapia during 60 days of rearing in each treatment can be seen in the following table 1.

Table 1. The average gonad maturity index of female tilapia reared for 60 days was treated with a dose of bitter melon extract

Treatment (g BME/kg of feed)	Gonad Somatic Index (%)		
	Day_0	Day_30	Day_60
(A) 0	0,28±0,08	2,47±1,68	2,7±1,69
(B) 0,1	0,28±0,08	1,62±0,96	2,39±2,06
(C) 0,2	0,28±0,08	1,15±0,68	3,19±2,03
(D) 0,3	0,28±0,08	1,69±0,59	1,47±0,68

The average gonadal maturity index obtained during the study was between 0.28% - 3.19%. The results of analysis of variance (ANOVA) showed that the effect of various doses of bitter melon extract from 0 g to 0.3 g in diet had no significant effect ($p>0.05$) on the average gonadal maturity index of tilapia.

3.2. Gonad Histopathology

Histopathology of female tilapia gonads after 60 days of rearing for each treatment can be seen in Figure 1.

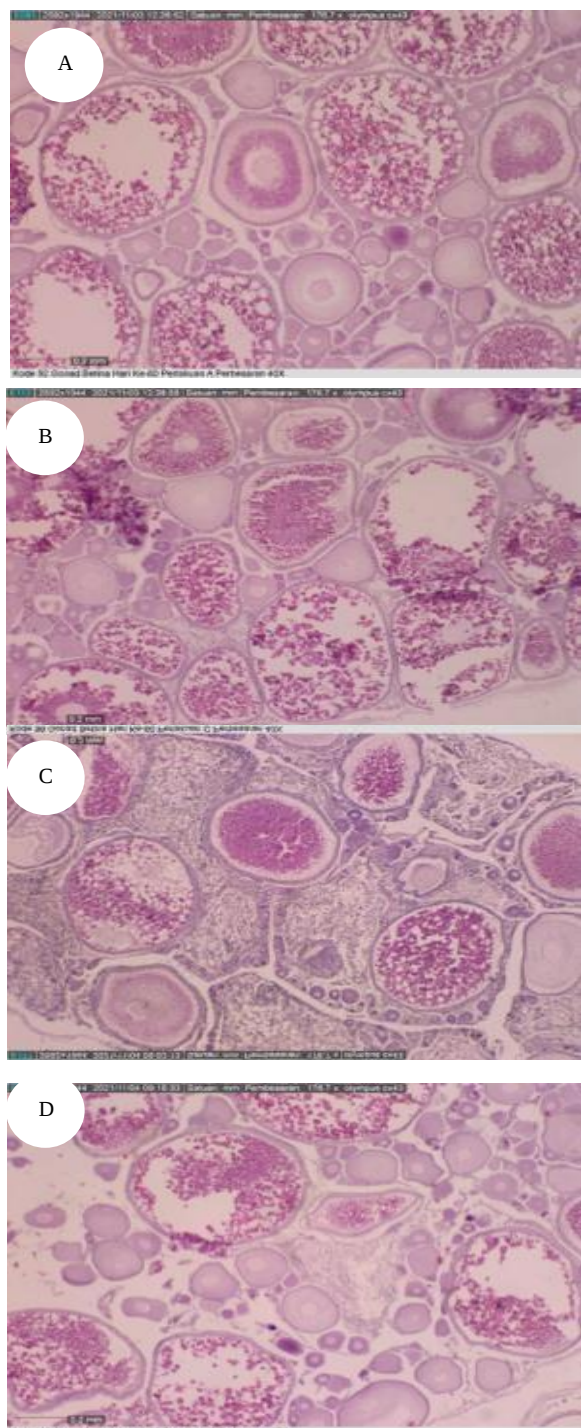


Figure 1. Histopathology of female gonad after 60 days of farming

Figure 1 showed, In treatment A, various oocyte stages were seen starting from previtellogenic, vitellogenic, and mature oocytes. The number of oocytes increased compared to the previous day. The yolk granules are seen filling the cavities of the mature follicles, and the oocyte stages are evenly distributed at each stage. In treatment B, there was also an increase in the number of oocytes at various stages of oocyte development. Oocytes in this

treatment showed that there were oocytes lacking egg yolk granules. In treatment, C showed various stages of oocyte development. It can be seen that there are oocytes that have experienced quite a severe atresia and have turned into connective tissue or stromal tissue so that they show differences from previous treatments. And In treatment D, it was seen that the presence of necrotizing oocytes was dominant and atretic oocytes were seen in the treatment and connective tissue or stroma was also seen. However, it was not as severe as in treatment C. This treatment also consisted of various kinds of oocyte development.

3.3. Water Quality

Water quality parameters measured during the study included temperature, pH, dissolved oxygen, and ammonia content in the water, which were in the appropriate range for tilapia (Table 2).

Table 2. Water quality of tilapia rearing media for 60 days of rearing.

Water Quality Parameters	Days to-			Optimal	Reference
	0	30	60		
pH	7.9	7.3-7.73	7-7.64	6.5-8.5	SNI No.7550 (2009)
Suhu (°C)	26-28	26-28	26-28	25 - 30°C	
DO (ppm)	4.80	2.88 - 6.40	2.24 - 6.72	≥3	
Ammonia (ppm)	0,0002	0,002-0,006	0,005-0,017	< 0,02	

3.4. Discussion

The size of the IKG value is related to gonadal growth (Fadli *et al.*, 2016). This study showed that the highest average gonadal maturity index was in treatment C (0.2-gram bitter melon extract) and the lowest average gonadal maturity index was in treatment D (0.3-gram bitter melon extract). The slow growth of the gonads is thought to be due to the cytotoxic effect of bitter melon extract which affects the hormones estrogen and progesterone in inhibiting the ovulation process (Cahyadi, 2009). This low average gonadal maturity index is also thought to be influenced by the saponin content contained in the bitter melon which is used as a contraceptive estrogen, namely to prevent pregnancy by inhibiting the ovaries from releasing eggs. According to Kellis *et al.* (1984) in Agustina *et al.* (2013), flavonoid substances synthesized by bitter melon fruit can inhibit the aromatase enzyme which the aromatase enzyme functions to catalyze the conversion of androgens to estrogens. With the inhibition of these enzymes, the amount of androgens will increase. High concentrations of androgens will cause negative feedback to the anterior pituitary by not releasing FSH and/or LH. The non-release of FSH and LH results in disturbances in the process of follicular maturation,

ovulation, and the formation of the corpus luteum, so flavonoids can be referred to as antigonadotropins which can cause antifertility. Giving the right dose is thought to stimulate the inhibition of the ovulation process effectively. However, high doses are suspected to have a negative effect on both the gonads and other organs (Adimoelja (1987) in Agustina *et al.* (2013).

Based on the histological observations of the female tilapia gonads, showed that there was damage to the oocyte cells that developed in different treatments. This is presumably due to the effect of the addition of vitomolt extract of bitter melon with different doses, it is known that the content of bitter melon is one of them is cucurbitacin which is a type of Momordica that has a cytotoxic effect and functions as an inhibition of the formation of oocyte cells. These microscopic results also showed the structure of the oocyte cells that underwent changes at different treatment doses (Table 4) after the 60th day of maintenance. In line with that. According to Laurence (1993) in Jerald *et al.* (2012), any compound that has estrogen activity can show anti-fertility activity by suppressing gonadotropin secretion with consequent inhibition of ovulation.

Estrogen and progesterone are the hormones responsible for the histological and functional modification of the genital tract in females. The cause of this change in cell structure is thought to be the cytotoxic effect of this bitter melon extract. These results have a positive impact on inhibiting gonadal development and reducing the mating process and fertilization of tilapia eggs in the cultivation process. The C and D treatments were seen to experience more severe damage than the other treatments. Giving the right dose will stimulate the inhibition of tilapia oocyte cells effectively. However, giving a low dose does not have a real effect on oocyte cell inhibition, while giving a dose that is too high has a negative effect on other organs. Previous studies also reported that administration of high doses of bitter melon extract showed changes in the histological condition of the gonads of *Tilapia zillii* females who experienced oocyte damage and were abnormal so it was believed that bitter melon herbal ingredients could be anti-fertility agents (Akin-obasola and Jegede, 2014).

In histology observations, identification of the developmental stage of oocyte cells is known as the process of oogenesis and abnormalities in oocytes. Oogenesis is the term used to describe the processes by which mature oocytes are formed. Ovaries have various kinds of oocyte development ranging from previtellogenic oocytes, and vitellogenic oocytes to developing mature oocytes. Microscopically, the oocyte is spherical in shape. Small oocytes will become large and develop into mature oocytes. The Tilapia ovary tissue consists of various stages of oocyte development including small size, chromatin

nucleolar oocytes and perinucleolar oocytes having a medium size, cortical alveolar oocytes, and vitellogenic oocytes with the incorporation of yolk granules having a large size, mature oocytes showing immediate maturation and spawning. Oocytes have various stages of oocyte development (Jirarach *et al.* 2001). Tilapia spawning cycles tend to be highly asynchronous which results in variations in gonadal development, even within the same size (Bromage and Coward (1999) in Neves *et al.* 2009).

Oocyte damage can be seen microscopically in Figures 9 and 10. This damage includes abnormal Oocyte Necrosis and Atresia. On the 30th day of observation, treatments A and B did not show any damage to the oocytes. However, in treatments C and D, there were cells that were damaged in several oocytes. Furthermore, on the 60th day in treatment A, the oocytes were perfectly mature without any serious abnormalities. In contrast to the treatment given bitter melon extract, it was seen that there were oocytes that had suffered damage or abnormalities such as oocyte necrosis and quite a lot of atresia. Oocytes undergoing necrosis are characterized by the decrease and disintegration of the yolk granules in the oocyte. In addition, there are oocytes that experience abnormal atresia. Atresia is a degenerative process, most often from vitellogenic eggs. Atresia is characterized by the disintegration of the nucleus, breakdown of the vitelline sheath, and an increase in the number and size of follicular cells (granulosa), melting of the yolk granules with follicular cells. The degeneration of follicular cells after absorption of the yolk is complete and finally, the cells form connective tissue (Blazer, 2002). There are two known atretic oocytes, namely hypertrophic and cystic atretic oocytes. Hypertrophic atretic oocytes are oocytes characterized by increased cells both in size and development. However, oocyte follicles collapse or rupture and spread to degenerate into stromal tissue or connective tissue, while cystic atresia oocytes are oocytes that experience a thickened epithelial lining and follicles and shrinkage or decrease in oocyte mass (El-Saba *et al.* 2013). In this study, oocyte damage was thought to be due to the treatment given to tilapia, namely the addition of vitomolt extract of bitter melon given orally. Severe damage occurred as the dose of bitter melon extract increased. Oocytes that experienced this stage of atresia occurred more when the dose of bitter melon extract was higher than feeding without a dose of bitter melon extract. Bitter melon extract itself is known to have active substances that provide a cytotoxic effect. Thiagarajan *et al.* (2019) stated that the active substances in *M. charantia* extract such as cucurbitacin which is a type of protein that causes toxic effects and can interfere with the biological pathways of zebrafish embryos that stop their development. The study of Sharanabasappa *et al.* (2002) explained that histological observations of the effect of the bitter melon extract on mice on day 30

showed a decrease in the number of developing follicles, Graafian follicles, and corpora lutea, and an increase in the number of atretic follicles in the histological section. Quoted by McNatty et al. (1976) in Sharanabasappa *et al.* (2002) The effect of the content of bitter melon gives the effect of decreasing ovarian glycogen which was observed in his research. This is due to the reduced availability of ovarian estrogen. A reduction in the number of developing follicles and Graafian follicles is also associated with decreased ovarian estrogen availability because these follicles are the main source of estrogen in the ovaries. Furthermore, although follicular atresia is common in rat ovaries, an increase in the number of atrophic follicles in the ovaries of experimental animals indicates the unavailability of the required amount of gonadotropins. Also related to the gonads of the tilapia studied, the atresia oocyte that occurs is natural. However, the condition of increasing atresia that increases indicates the effect of the treatment given.

4. Conclusion

From the results of this study, it was concluded that the administration of vitomolt and bitter melon fruit extract in tilapia feed has the potential to change and damage the structure of gonad tissue. The effect of bitter melon extract at a dose of 0.3 grams showed good results on the gonadal maturity index and also gave changes to the histopathology results. Histopathological results revealed structural changes in the form of oocyte cell experiencing necrosis and increased oocyte atresia. These results also provide opportunities for bitter melon extract as a natural anti-fertility agent.

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