PROPOLIS, THE ALTERNATIVE NATURAL MATERIAL FOR SEX REVERSAL IN TILAPIA

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ABSTRACT

Growth of tilapia male is better than female. Practically, cultured of all male population are more efficient than that of mixed sex population. Sex reversal with 17α-methyltestosterone hormone is the most popular technique to make all or nearly all male tilapia. But, alternative substitution materials for sex reversal must be found due to limited application of this synthetic hormone that caused of its carcinogenic potential on human body. Besides that, 17α-methyltestosterones are also suspected as the unfriendly material for environment. Several early studies were conducted to find the alternative materials for sex reversal in tilapia. One of the highly potential material found is aromatase inhibitors, the materials which are function to inhibit the secretion of aromatase, the catalyst enzyme in the bio-synthetic of estrogen from androgen. Due to the chemical characteristic materials and relatively high price of aromatase inhibitor, we tried to find the natural material which has similar composition or function to that compound. One of the natural suspected materials which has similar function with aromatase inhibitors is propolis. Chrysin as apart of flavonoid compound found in the propolis shows the most potent inhibitors of aromatase. Early study showed that application of propolis up to 3 mL.kg^{-1} of feed resulted the highest percentage of male in tilapia. Based on that study, the optimum dosage of propolis for sex reverse of the tilapia has not been known. This study was conducted to know the optimum dosage of a commercial propolis for sex reversal in tilapia, especially in NIFI red tilapia. The dosages proposed in this study were 3, 4, 5 and 6 mL propolis.kg^{-1} of feed. Sex reversal was carried out in aquarium for 35 days. Rearing of fingerling fish for 2 months was conducted in hapas suspended in earthen pond. The result showed that application of 5 mL propolis.kg^{-1} of feed resulted the highest proportion of male, which up to 76.67%. Application of all propolis dosages in this study did not affect to the survival rate and the abnormality of fish.

KEYWORDS: red tilapia, sex reversal, propolis

INTRODUCTION

All tilapia male culture are more effective and efficient than that of mixed sex population because of growth of tilapia male is better than female. In the growing pond, tilapia male use all or nearly all energy for growth while the female share their energy for gonad developments. Besides that, at the spawning period, the female keep the recruits in the mouth so that there are no input energy for them (Hepher & Pruginin, 1981).

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To produce all or nearly all male population of tilapia, several techniques were developed, such as manual sexing, interspecific hybridization, sex reversal, and YY supermale. From those, one of the most popular applied technique is sex reversal with androgenic hormone, such as the synthetic hormone 17α-methyltestosterone (mt). This technique produced all or nearly all male tilapia (Bowker *et al*., 2007). Nevertheless, the use of the synthetic hormone has not been approved becaused of the food safety reason. This opinion assumed that the synthetic hormone 17α-mt was carcinogenic material for human. Besides that, this material was suspected not eco friendly (Contreras-Sancez *et al*., 2001).

One of several substitution materials for sex reversal is aromatase inhibitor (AI) compounds. The function of AI is to inhibit the secretion of aromatase, the catalize enzyme in estrogen synthesis. Early studies in sex reversal with aromatase inhibitor were conducted by several authors. Kwon *et al*. (2000) reported that application of aromatase inhibitor (fadrozole) in feed with dosage of 500 mg.kg^{-1} of feed in 5 days after hatching of larvae produced more than 80% male of tilapia. Other studies which conducted by Nurlaela (2002), Astutik (2004), and Barmudi (2005), each reported that dipping of red tilapia larvae at 5, 9, and 13 days after hatching in 20, 30, and 1,500 mg imidazole, another kind of synthetic aromatase inhibitor, per liter for 10 hours produced 82.22%, 57.97%, and 73.09% male, respectively. The recent study which conducted by Ariyanto *et al*. (2010) showed that application of imidazole in oral sex reversal with 25 mg/kg of feed for 28 days, successfully improved the percentage of male tilapia, up to 87%. Nevertheless, due to the synthetic material which may have a negative side effect and relatively high price of those materials, we tried to find the natural material which is relatively saver and cheaper one.

One of the natural suspected material which has same the function with aromatase inhibitor is propolis. Propolis contains the active ingredient which has indirect effect to the differentiation of sex. The compounds which can be found in propolis are flavonol, flavonoid, phenol, and other aromatic compounds (Greenaway *et al*., 1990). Chrysin as apart of flavonoid compound shown to be the most potent inhibitors of aromatase (Ta & Walle, 2007).

Early studies in sex reversal with propolis was conducted by Ukhroy (2008) and Anwar (2009). Ukhroy (2008) reported that oral application with dosage 60 µL of propolis.kg^{-1} of feed in guppy, *Paecilia reticulate* resulted in percentage of male population about 55.17%. Anwar (2009) reported that oral application of the same material with dosage ranged 0, 0.2, 1.2, 1.8, 2.4, and 3 mL.kg^{-1} of feed in red tilapia resulted in percentage of male population about 60.62%, 64.89%, 62.92%, 65.75%, 65.85%, and 69.71%, respectively. This results showed the dosage application of propolis in tilapia related to the proportion of male in population but it did not show the optimum level of the dosage. This study was conducted to know the optimum dosage of propolis trough orally application for sex reversal in tilapia, especially in NIFI red tilapia.

MATERIAL AND METHOD

The fish used in this experiment were NIFI red tilapia larvae which were 7 days age after hatching. This larvae were the progeny of 5th generation of NIFI red tilapia collection of Research Institute for Fish Breeding in Sukamandi, West Java. To optimize the condition of the brood-fish, all male dan female were reared separately in different ponds.

The propolis used in this experiment was the commercial propolis Diamond, which consists of 20% extract propolis and it contains more than 23.000 mg/L bio-flavonoid.mL^{-1}.

Experimental Design

This study was carried out with complete randomized design (CRD). The testing factor was propolis dosage for sex reversal with orally application at level is 3, 4, 5, and 6 mL.kg^{-1} of feed. Fish given the same feed without adding propolis were used as the control population. All treatments were conducted in four replicates.

Spawning Brood-Fish

The number of spawned brood-fish was 10 of males and 20 of females which each was selected from separates brood-stock ponds. The brood-fish were then pooled in a concrete pond with sizing 25 m^2 and were fed twice daily with commercial pelleted feed containing 40% of crude protein. The feeding rate was 2%-3% from total biomass of fish. Eggs were collected from the spawned female brood-fish

after 2 weeks. The eggs were then incubated in the artificial hatching trays for 3-5 days. Methylen blue was added in this media to anticipate the fungus spread along the incubation periods.

Rearing and Stocking Larvae

In the incubation trays, the eggs fish hatched in 3-5 days. The larvae obtained from the trays were quickly collected and pooled in an aquaria which was installed with an artificial aeration. The density of larvae in this aquaria was 50 larvae.L^{-1} of water. For indogenous feeding period, the larvae were not be given a feed. Life foods, such as *Moina* and *Daphnia* were fed to this larvae since 3rd to 6th day after hatching. In the 6th day after hatching, 100 larvae were stocked in each 15 units of aquarium sizing 60 cm x 30 cm x 35 cm for sex reversal treatment. All aquarium were installed with an artificial aeration from electric hi-blower.

Enrichments of Feed with Propolis

The feed used in this experiment was a commercial powder feed containing 40% of crude protein. The propolis was diluted in 250 mL of 70% ethanol before applying in the feed. The apropriate diluted propolis with each treatment was sprayed to the feed and then it was dried in the room temperature. All feeds which were enriched with propolis were packed in plastic pocket and stored.

Sex Reversal Treatments

Seven days after hatching larvae of NIFI red tilapia were fed with commercial powder feed treated with propolis ad satiantion. The feed was given three times each day for 35 days. Along this treatment period, all aquarium were siphoned and the water was exchanged about 20%-30% every day. In the 36th day, all fish in each aquaria were transferred to 15 units of happa sized 2 m x 1 m x 1 m which were installed in an earthen pond. The water depth in the happa was about 80 cm. For 2 months fingerling rearing period, all fish were fed twice each day with a dietary 28%-32% of crude protein from commercial pelleted feed, at 5% from total biomass of fish.

Sex Identification

Sex identification of fish was conducted at the end of fingerling rearing period. Histological analysis was used to identify the proportion of male and female of the fish. Thirty samples were collected from each replicate in each treatment. Fresh gonadal tissue was dissected out from the live fish, fixed in Bouin's fixative and embedded in paraffin wax. Tissue cross-sections were prepared as thin as 4-6 µm with a microtome. The sections were stained with haematoxylin and eosin and mounted on glass microslides. The slides were examined and photographed using a stereomicroscope (Nikon Optiphot, Nikon Corp., Tokyo, Japan).

Paramaters and Data Analysis

The percentage of male and female proportion was used as the main parameter. The other parameters such as the survival rate and the morphologically abnormality of NIFI red tilapia population were observed. As the supporting data, water quality in the aquarium and happa, i.e. temperature, pH, dissolved oxygen, and ammonia were measured every two weeks.

Analysis of variance (Anova) was used to explore the effect of the treatment to the proportion of male and female of fish. Duncan Multiple Ranged Test (DMRT) was used to identify the differences among the treatments. The data of water quality in the aquarium and in the hapa were analyzed descriptively.

RESULT AND DISCUSSION

Proportion of Male and Female of Fish

Histological section of gonadal tissue of NIFI red tilapia orally treated with propolis was presented in Figure 1. Percentage of male and female of NIFI red tilapia in each treatment based on histological analysis was described in Figure 2.

All populations of tilapia which were fed with treated feed have high percentage of male compare with the control population (Figure 2). Further more, all population of tilapia fed with treated feed have percentage of male which were significantly different from the female. Analysis of variance showed that oral application of propolis during the sex differentiation in fish affected the proportion of male and female of NIFI red tilapia. Duncan multiple ranged test showed that the propolis dosage of 5 mL.kg^{-1} of feed was significantly different from 3 and 4 mL.kg^{-1} of feed ($P<0.05$), but it was

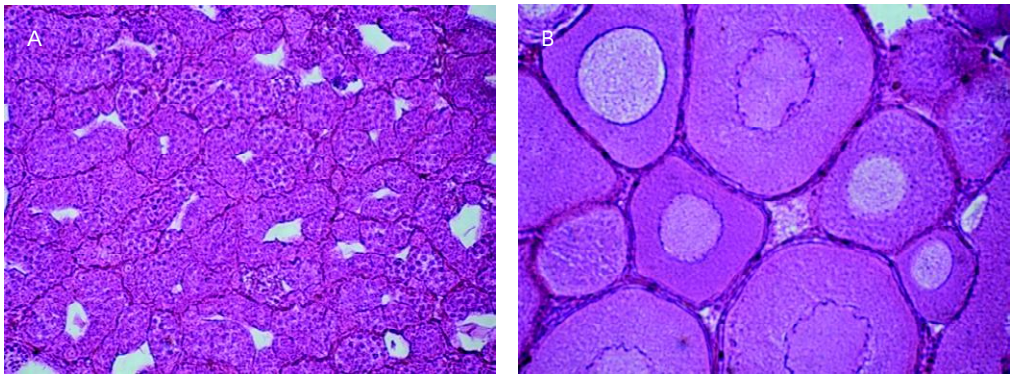

Figure 1. Histological section for male and female proportion analysis. A: testes and B: ovary

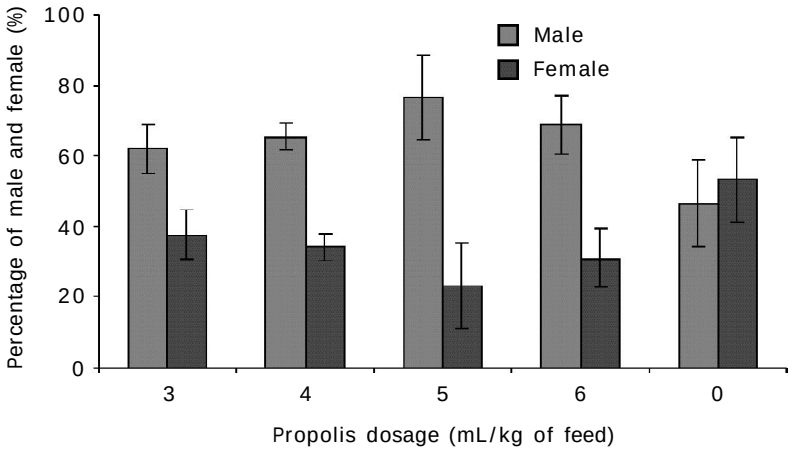

Figure 2. Percentage of male and female of NIFI red tilapia treated with orally different dosage of propolis

not significantly different from 6 mL.kg^{-1} of feed ($P>0.05$).

The success of one material for sex reversal in organism depends on several factors i.e. age of the organism, time and duration of treatment, and several environment factors (Nagy *et al*., 1981). Hunter & Donaldson (1983) also explained that the success of sex reversal depends on gonad development interval time, especially when gonad was being in labile period, so it's easy to be influenced by that material. According to Yamazaki (1983), sex reversal which was conducted in different species must be done in specific time and duration. This pattern was related to specific sex differentiation period in each species. Brodie (1991) explained that in tilapia, aromatase, the enzyme which catalized in the estrogen synthesis, was detected in 7 days after hatching larvae. Furthermore, Kwon *et al*. (2000) also explained that differentiation of sex in tilapia will be occurred until 37 days after hatching. Based on this hypothetic, orally application of propolis for sex reversal in NIFI red tilapia in this experiment may be conducted at the optimum period to get the maximal results.

Aromatase is the catalyst enzyme in estrogen synthesis from the androgen. Application of propolis in feed as the aromatase inhibitor affected the sex differentiation of fish because the material inhibits the brain aromatase biosynthetic of estrogen from androgen. The action mechanism of this material is to inhibit the transcription of mRNA aromatase. No transcripted mRNA aromatase affected to decreased of the translation mechanism for

aromatase secretion. This condition will decrease the rate of androgen conversion to estrogen. Furthermore, the low estrogen ratio from androgen will be directed to un-activating of the aratase gene transcription as the feed-back (Sever *et al*., 1999). Ankley *et al*. (2002) reported that administered of aromatase inhibitor significantly decreased the activity of the brain aromatase in fathead minnow (*Pimephales promelas*). This report agrees with the study conducted by Lee *et al*. (2002) in protandi black porgy (*Acanthopagrus schlegeli*). This study reported that aromatase inhibitor significantly decreased the brain aromatase activity and in the same time increased both of the 11-ketotestosterone and LH, which knowing as the specific hormone product in male.

The same suspected phenomenon accurred in this study, in which decreasing activity of the brain aromatase in NIFI red tilapia affected to the ratio of estrogen and androgen. Orally application of propolis in this experiment successed to increase the percentage of male tilapia. Chyrsin, the material contained in propolis was suspected as the aromatase inhibitor. Chrysin as apart of flavonoid material will inhibit the activity of the brain aromatase in fish (Ta & Walle, 2007). The optimum concentration of propolis for sex reversal in NIFI red tilapia indicated with the highest percentage of male was 5 mL.kg^{-1}. The concentration of propolis dosage below 5 mL.kg^{-1} was not effective and neither was the highest concentration (Yamazaki, 1983), stated that, high concentration of additional material in fish food have a side effect on both of its fish and human as the fish consums.

Relatively high price of propolis is one of the main constrain in the development of it to be a commercial material for sex reversal in fish. For this reason, there is necessary to explore the cheaper natural material which has the same or maybe higher ability to direct the differentiation of sex in fish, especially in red tilapia.

Survival Rate

Survival rate is one of indicator parameters for the tolerance ability of fish to the environment condition. Survival rate of NIFI red tilapia in this experiment was shown in Figure 3.

Analysis of variance showed that survival rate of NIFI red tilapia treated with different dosage of propolis was not significantly different from the control population ($P>0.05$). This result indicated that mortality of NIFI red tilapia in this experiment was not be affected by the treatment. Anwar (2009) also reported that orally application of propolis for sex reversal in red tilapia did not affect to its survival rate.

Several earlier studies, application of aromatase inhibitor did not effect to the mortality rate of the fish nor the prawn. Kwon *et al*. (2000) reported that there is no statistical relationship between aromatase inhibitor treatments with mortality rate of fish. This result was supported by several studies in several

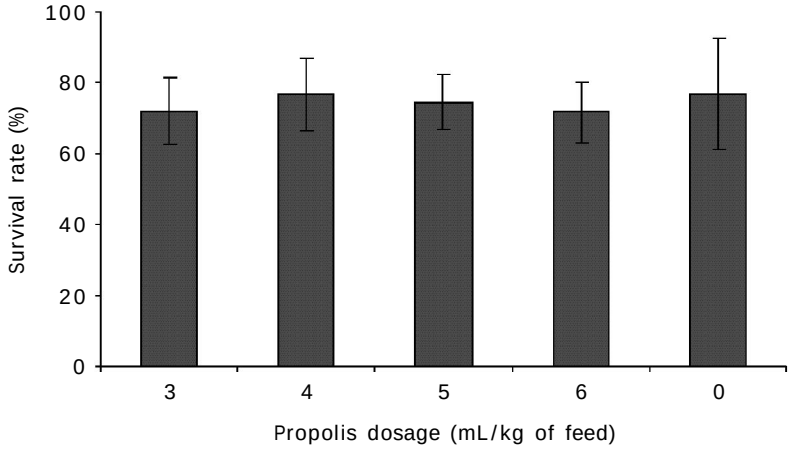

Figure 3. The survival rate of NIFI red tilapia treated with orally different dosage of propolis

species of fish and prawn such as by Piferrer *et al*. (1994) in salmon, Sarida (2005) in giant prawn, Ukhroy (2008) in guppy, and Anwar (2009) in tilapia.

The relative high mortality of fish in this experiment was suspected due to genetically or environmentally effects, excluded the treatments. Genetically, all fish used in this experiment was obtained from natural mass spawning of one population of brood-stock. This spawning model potentially occured the unwanted inbreeding of its offspring.

In the other hands, intensive feeding rate and feeding frequency during the sex reversal treatment period affected to the water turbidity in the aquarium. It was caused by the high aeration which formed an up-welling wave. This condition affected the mal function of the syphoned technique for exchanged water to maintain the water quality.

The low of dissolved oxygen in happa for several time may effect the high mortality of fish (Table 1). Boyd (1990) explained that for normal development, aquatic organisms need the optimal condition, especially the dissolved oxygen. The poor water quality affects to the life of aquatic organism. Stress, anorexia, and susceptible to diseases and parasitism are the example of effect of poor water environment. In the extrim case, poor water will be affected to the accute and mass mortality of the organisms (Schwaiger, 2001 *in* Almeida *et al*., 2008).

Morphologically Abnormality

The percentage of morphologically abnormal fish in each treatment was relatively low, ranged from 0.73% - 1.95% (Figure 4). Among the treatments, application of different dosage of propolis did not significantly different affect to the percentage of morphologically abnormality of fish.

Abnormality showed the performance of fish which has morphologically abnormal. The abnormal performances of fish such as the spinal deformities and hypertrophy of mouth maybe caused of several factors, such as the genetic, the treatments, and the environments factors excluded the treatments (Dongen, 2006). As It has been explained previously, all fish which were used in this experiment were obtained from natural mass spawning of one population of brood-stock. Several study reported that natural mass spawning in the domesticated population potentially increased the inbreding rate of the population. Tave (1999) explained that the effect of inbreeding depression in domesticated fish is decreased production phenotype, such as growth rate and survival rate, while increasing the number of deformed offspring.

Statistic analysis showed that there is no significant differences of the abnormality percentage between the treated populations and its control ($P>0.05$). Based on this result, abnormal fish in this experiment was not caused by the treatments. Abnormality in both of the treated and the control populations maybe caused by genetic factor, especially the inbreeding depression effect or environment factors exclude the treatments, such as the low water quality in aquarium, poor water environment in pond and maybe the ethanol used in the enrichment of feed with propolis. Almeida *et al*. (2008) explained that environmental stress can rise or decrease the developmental stability of individuals, which may result in reducing performance of fitness componens.

Water Quality

Results of water quality analysis during the treatment and fingerling fish rearing periods were presented in Table 1. The values are presented the minimal and maximal measured val-

Table 1. Water quality of media in aquarium during the treatment period and of media in happa during the fingerling fish rearing period

Periods	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Ammonia (mg/L)
Aquarium	27.6-29.3	7.4-8.5	4.46-6.29	0.013-0.030
Happa	26.3-29.8	7.5-8.0	1.3-5.7	0.043-0.053
Literature*)	25.0-30.0	7.0-8.0	> 3.0	< 1.0

*) Boyd (1990)

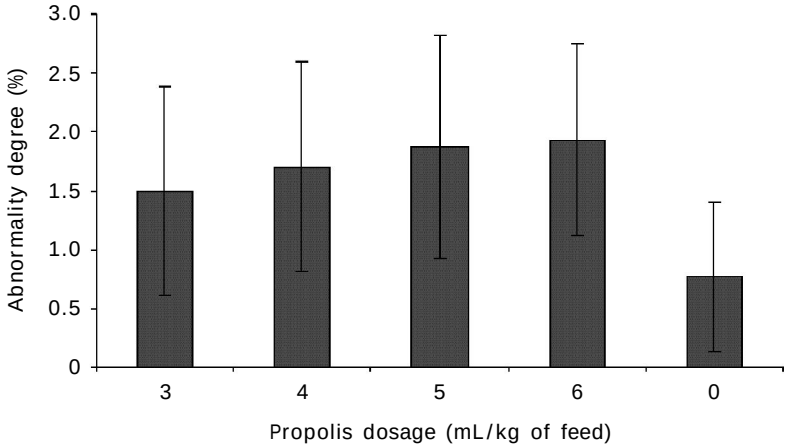

Figure 4. The abnormality degree of NIFI red tilapia treated with orally different dosage of propolis

ues obtained from all analysis during those periods.

Generally, water quality of all media during this study was suitable for normal life of tilapia. The minimal value of dissolved oxygen in happa occured in early morning. The low of dissolved oxygen which reached under the minimal oxygen requirement for tilapia may cause relatively high mortality of fish in this experiment.

CONCLUSION

1. Orally application of 5 mL propolis in 1 kg of feed resulted the highest percentage of male of NIFI red tilapia.
2. Orally application of propolis for sex reversal in NIFI red tilapia did not affect to both of survival and abnormality rate of the population.

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