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Masculinization of tilapia *Oreochromis niloticus* using extract of pasak bumi plant *Eurycoma longifolia* through larval immersion

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Abstract
The present research was aimed to investigate the effect of *E. longifolia* extract to change the sex direction to male fish through larvae immersion. The completely randomized design consisted of different doses of *E. longifolia* extract (20, 40, 60 mg L^{-1}) compared to the control with 0.5 mg L^{-1} 17α-methyltestoterone (MT) and no immersion, each with three replications. A fifty larvae of ten days old after hatching were immersed during eight hour and reared for 60 days after treatment. The result showed that larvae immersion using *E. longifolia* extract 60 mg.L^{-1} produced 67.44 % of male significantly different compared to the control without immersion 52.71 % ($p<0.05$), while control with MT reached 72.87% of male.

Keywords: 17α- methiltestoteron, *Eurycoma longifolia*, masculinization, tilapia

1. Introduction
Culture of male mono-sex tilapia has been reported to have some advantages such as faster growth, size similarity, wild spawning in pond [1]. Masculinization could be performed by addition of androgen hormone [2-3], where 17α-methyl testosterone (MT) was commonly used. However, the use of MT has been restricted since its residue may produce detrimental effects on fish and environment [4].
Differentiation of female sex occurred when enzyme (P450 aromatase) was produced. This enzyme catalyzed the formation of estrogen from androgen, thus P450 aromatase was responsible for balance of androgen and estrogen [5]. In addition, pandian [5] stated that inhibition of P450 aromatase activity in the receptor during differentiation caused changes from female phenotype to male phenotype. Activity of cyp19 could be retarded by administration of steroid hormone or by manipulating the environmental temperature [6]. Administration of natural hormone (11β-hydroxy androstenedione) at dose of 50 mg kg^{-1} in feed for 21-35 days induced masculinization of male tilapia up to 98% [7]. Masculinization could also be performed through addition of aphrodisiac-containing plants. Previous study revealed that incorporation of purwoceng at dose of 20 mg L^{-1} could improve male population up to 73.3% through larval immersion (7-days larvae after hatching) for 8 h [8].
Pasak bumi or tongkat Ali *(Eurycoma longifolia)* has been recognized as herbal plant in Southeast Asia countries such as Indonesia, Malaysia, Thailand, Laos, Cambodia and Vietnam [9]. Administration of pasak bumi at dose of 800 mg kg^{-1} in feed (twice a day for 10 days) enhanced sexual activity of male rats [10]. Pasak bumi contained flavonoid 6.1% [11], mineral (Fe, Co, Mg, Zn), saponin, sterol, and isoprenoid that were required to synthesize steroid hormone such as testosterone [12]. This study aimed to understand effectivity of pasak bumi extract on sex direction to male fish through larval immersion.

2. Materials and Methods
2.1 Study Area
The present research was conducted from December 2015 to June 2016 in Laboratory of Fish Reproduction and Genetic, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University.

2.2 Experimental Design
Completely randomized design consisting of 5 treatments and 3 replications was used. The treatments were various concentrations of pasak bumi extracts including 20 mg L^{-1} (PB 20), 40 mg L^{-1} (PB 40) and 60 mg L^{-1} (PB 60), positive control (addition of MT at dose of 0.5 mg L^{-1} without immersion), and addition of 17α-MT as control negative (K).

2.3 Chemical Analysis of Pasak Bumi
Chemical composition of pasak bumi (powder) was determined using GC-MS (GC-MS QP 2010). The temperature was set at 400 °C.

2.4 Preparation of Pasak Bumi Extract
Pasak bumi (PB) plant (root) was obtained from Kalimantan. The root was dried and powdered. The powder was then macerated using ethanol 96% at ratio of 9:1 (ethanol 900 mL: PB powder 100 g) using *thermoshake* at 160 rpm and 37 °C for 24 h. The macerated powder was the filtered and evaporated using *rotary evaporator* at 40 °C to obtain extract. The extract (paste form) was weight as required dose.

2.5 Masculinization and Fish Rearing
Larvae were immersed for 8 h in pasak bumi extract at density of 50 larvae in each experimental unit. The larvae were then transferred into an aquarium (50×30×50 cm) and fed with shrimp pellet containing protein 45% for 3-4 times a day. The diet was 5% of larvae biomass, and given for 60 days of rearing period. The water substitution (90% of initial volume) was performed twice a day. The medium temperature was maintained at 27.6–28.7 ° C, pH 6.5-7.5, Dissolved oxygen was 2.5-6 mg/L. Sampling for growth and survival rate was carried out at immersion, after immersion. In the end of experiment, presence of fish abnormality after masculinization was observed.

2.6 Sex Identification
Sex identification was carried out in the end of rearing period (60 days) using acetocarmine staining [13].

2.7 Statistical Analysis
Parameters observed in this experiment included sex ratio, growth, and survival rate. Data were evaluated using analysis of variance (ANOVA) using SPSS 22 software at significance level of 95%. The variance of significance was verifid using Duncan test. Abnormality was observed using descriptive analysis.

3.1 Results
3.1.1 Chemical compounds of root pasak bumi
Experiment using GC-MS demonstrated that pasak bumi contained some stigmasterol compounds 19.54% of total compounds in pasak bumi root. These stigmasterol compounds were detected in some peaks at different time. They were 2-Cyclopenten-1-one-2-hydroxy-3-methyl- (CAS) Corylon, 3-Ethyl-2-hydroxy-2-cyclopenten-1-one, Cyclohexanone, 2-(hydroxymethyl)-(cas) 2(hydroxymethyl)cyclohe, 2-Furancarboxaldehyde, 5-(hydroxymethyl)- (CAS) HMF, 2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)- (CAS) 1-(4-hydroxy-3-methoxy, Benzaldehyde, 4-hydroxy-3,5-dimethoxy- (CAS) Syringaldehyde, Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy- (CAS) Coniferyl alcohol, Phenol, 2,6-dimethoxy-4-(2-propenyl)- (CAS) 4-Allyl-2,6-dimethoxyphenol, 3-(3',5'-dimethoxy-4'-hydroxyphenyl)-E-2-propenal, 9H-Xanthen-9-one, 1,3-dihydroxy-6-methoxy-8-methyl- (CAS) 6-o-methyl.

3.1.2 Percentage of sex ratio
Masculinization of male tilapia through larval immersion in PB extract at concentration of 60 mg L^{-1} resulted in 67.44±2.32% of male tilapia. This value was significantly higher ($P<0.05$) than control group (52.71±5.37%), but insignificantly different ($P>0.05$) from MT group (72.87±1.32%) as presented in Fig 1.

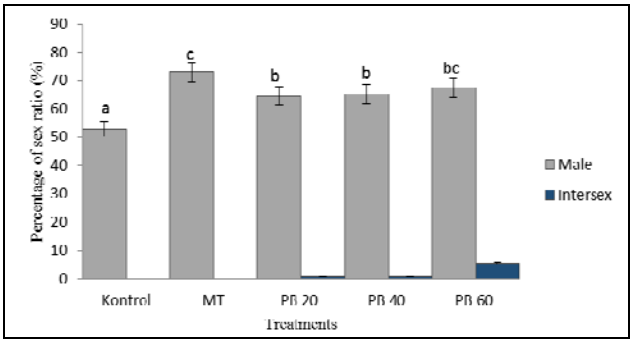


Fig 1: Percentage of tilapia sex ratio resulted from 60-days masculinization through larval immersion using pasak bumi extract.

Masculinization of tilapia with pasak bumi and / or MT could not produce 100% male, but intersex fish having prospective sperm cells and egg cells in a gonad were observed (Figure 2). Intersex fish with treatment of PB 60 was 5.83±1.44%, while lower percentage of intersex fish (0.83±1.44%) was also observed in lower dose (Fig 1). No intersex fish was observed in control and MT treatments.

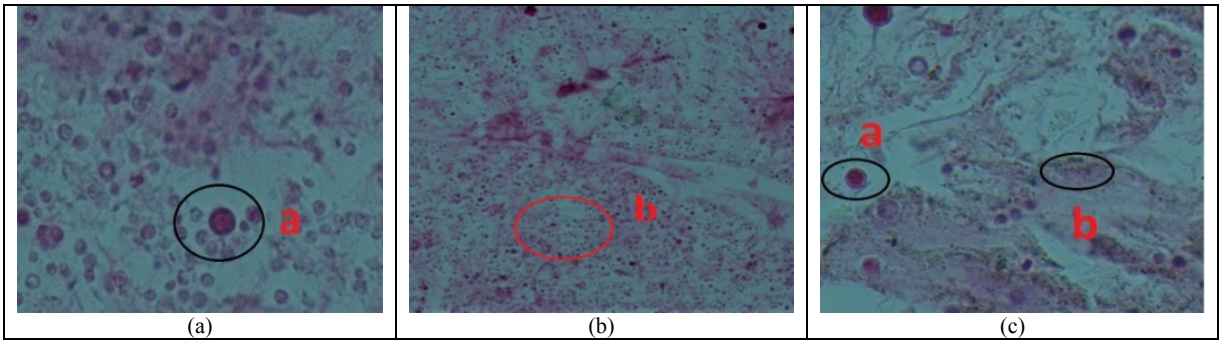


Fig 2: Gonadal tissue of tilapia with acetocarmine staining: female gonad with egg cells (a), male gonad with sperm cells (b), intersex gonad (having both sperm and egg cells).

3.1.3 Survival Rate of Tilapia

The present study found that all treatments (with immersion, MT and control) resulted in 100% of survival rate. Our data revealed that survival rate of tilapia with immersion and MT treatments was significantly different (P<0.05) from control. The highest survival rate (90%) was attributed to PB 20, while control showed the lowest survival rate (86%) as exhibited in Table 1.

Table 1: Survival rate (%) of tilapia at immersion stage and 60-days of masculinization

Treatments	Measured at	
	immersion stage	60-days of masculinization
K (-)	100	86.00 ± 2.00[a]
K (+)	100	87.33 ± 1.15[ab]
PB 20	100	90.00 ± 2.00[b]
PB 40	100	89.33 ± 2.31[ab]
PB 60	100	87.33 ± 2.31[ab]

Different superscripts following the numbers mean significant difference using Duncan test (p<0.05).

3.1.4 Tilapia Growth

Fish growth is an important parameter related to physiological condition affected by masculinization through immersion using pasak bumi extract. Absolute growth of tilapia at PB 40 was higher (P<0.05) than that of PB 20, PB 60, control and MT (Fig 3).

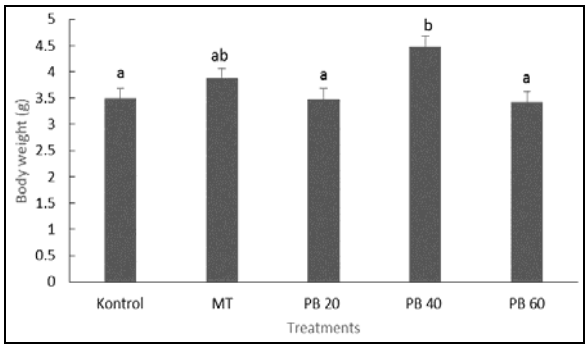


Fig 3: Absolute growth of tilapia after 60-days of masculinization through larval immersion using pasak bumi extract.

The similar result was observed in daily growth rate of fish (Fig 4). Fish growth of PB 40 was higher (P<0.05) than other treatments

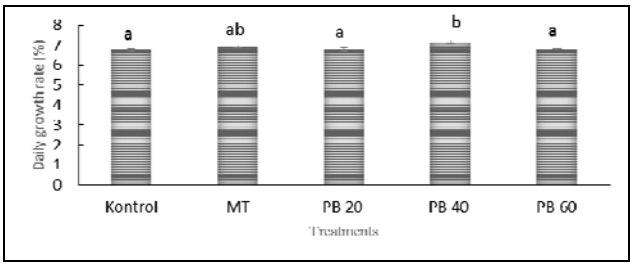


Fig 4: Daily growth rate of tilapia after 60-days of masculinization through larval immersion using pasak bumi extract

3.1.5 Tilapia Abnormality

Current experiment found morphological abnormalities including lordosis, kyphosis (Figure 5). Fish abnormalities of PB 20 and PB 40 were 0.27% of total population, while MT resulted in lower abnormalities (0.13%) as depicted in Figure 6.

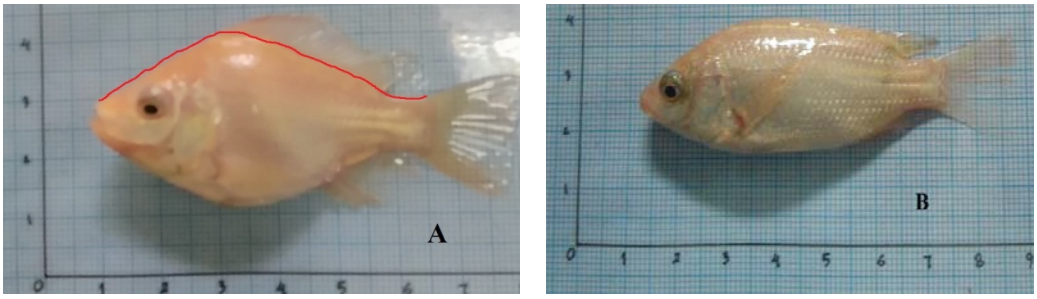


Fig 5: Morphological abnormalities of fish after 60-days of masculinization through larval immersion using pasak bumi extract; abnormal fish (a) and normal fish (b).

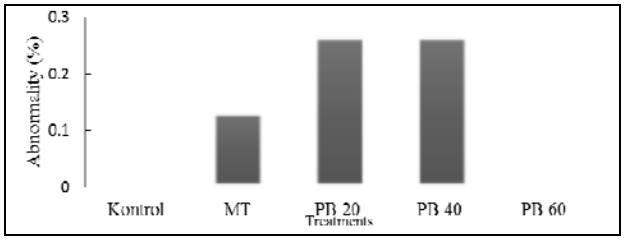
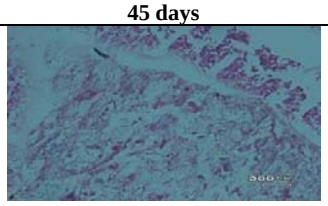
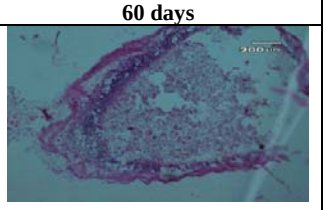
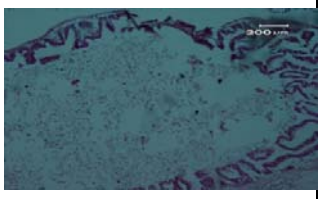
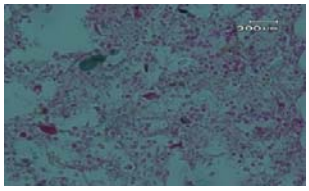
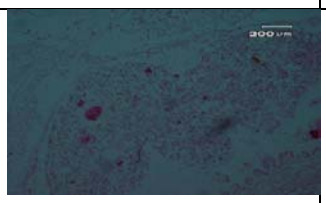
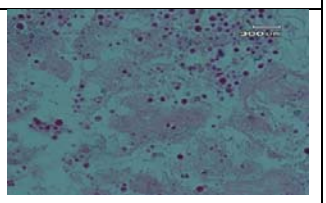
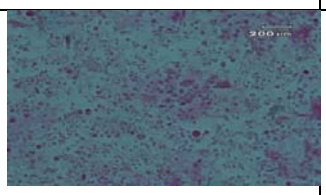
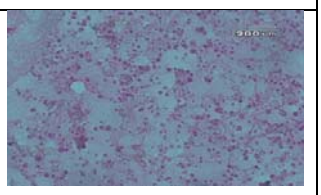
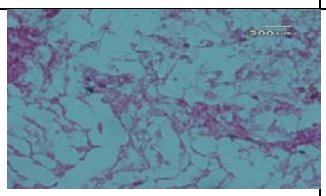
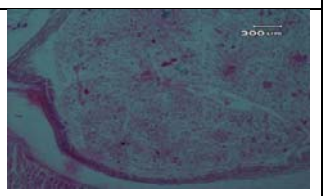


Fig 6: Fish abnormality after 60-days of masculinization through larval immersion using pasak bumi extract

3.1.6 Histology of Tilapia Gonad

Histological conditions of tilapia gonad with treatment of immersion of pasak bumi extracts showed gonad development at 45-days larvae stage. In PB 20 and PB 40, gonad development started at 45-days after hatching, while PB 60 showed no prospective gonad cells in experimental fish. In positive and negative control, the gonad cells were not developed. After 60-days hatching, the gonad of PB 20 and PB 40 treatments was developed, meanwhile PB 60, positive and negative control treatments showed initial developmental stage of gonad. (Table 2).

Table 2: Histology of tilapia gonad after immersion of pasak bumi extract

Treatments	Age of fish	
	45 days	60 days
Control (-)		
Control (+)		
PB 20		
PB 40		
PB 60		

3.2 Discussion

Experimental data from GC-MS demonstrated that pasak bumi rrot contained stigmasterol compounds. These compounds showed important role in regulating sex ratio of experimental fish. Our data revealed that immersion treatments at various concentrations (20, 40 and 60 mg L^{-1}) yielded higher male ratio than control. Increased male ratio was allegedly as a result of effects of stigmasterol compounds on affinity of androgen receptor, thus serving as androgen. Trembley & Van Der Kraak [14] found that affinity of androgen receptor could be affected by stigmasterol compounds.

This current work also found presence of intersex fish, that was associated with low concentration of stigmasterol in pasak bumi used in immersion. This was augmented by previous report of Nakamura *et al.* [15] that found addition of steroid hormone at lower dose was unable to perfectly form male sex, leading to formation of intersex. In contrast, higher concentration led to sterile gonad. Presence of intersex was also found in previous studies involving natural compounds. Putra [8] found that immersion of 8-days tilapia (at dose of 20 mg L^{-1} for 8 h) using purwoceng extract resulted in intersex of 13.3%. Ghosal & Chakraborty (16) found that intersex fish was 7.2% as consequence of *Tribulus terrestris* immersion at dose of 0.05 g L^{-1} in 3-days fish.

Our data revealed that immersion using pasak bumi extract and MT treatment showed no lethal effects on tilapia, represented by survival rate of 100% and 86-90% at masculinization and post-masculinization, respectively. Ghosal & Chakraborty [16] found the same result, that masculinization using *Tribulus terrestris* extract resulted in survival rate of 80.3–87.5%. Desprez [7] also reported that masculinization using 11β-hydroxyandrostenedione (11 βOHA4) yielded survival rate of 81-82.3% in the end of rearing period.

PB 40 showed higher growth in comparison with other treatments. However, we found that administration of pasak bumi extract showed no deleterious effects on experimental fish. Phelps & Popma [17] reported that androgen hormone demonstrated 2 physiological pathways: androgenic that induces male characteristics and anabolic that stimulates protein biosynthesis in fish.

Our current work also found fish with morphological abnormalities (Fig 6). Johnson & Katavic [18] stated that, in wild habitat, the abnormal fish was susceptible to mortality due to natural selection. However, in controllable culture and predator-free condition, larvae with morphological abnormalities may be survived. Bone abnormality was

reported in some species of cultured fish, where the abnormality of these fish was associated with both biotic and abiotic factors [19]. We hypothesized that pasak bumi contained toxic compounds to fish in excessive concentration, leading to disturbance of tilapia morphological development. This was augmented by Farrell [20], that farming area polluted with toxic compounds was responsible for fish morphological disorders.

In treatment of pasak bumi, gonad development occurred at 45-days, while gonad of control fish was underdeveloped. Presence of chemical compounds in pasak bumi induced faster development of gonad compared with control. Stigmasterol that is similar to androgen hormone could affect fish gonad differentiation. Yamazaki [21] augmented that androgen hormone would trigger formation of fish gonad.

4. Conclusion

Administration of pasak bumi in tilapia larvae immersion improved sex male ratio. In the future, we need to observe the effects of immersion with higher frequency and longer period on masculinization of tilapia.

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