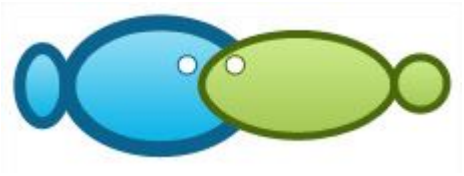

Feed enriched with methanol extract of tongkat ali *Eurycoma longifolia* Jack root for masculinization of Nile tilapia *Oreochromis niloticus*

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Abstract. Danger of residue in fish body and environment causes the use of synthetic steroid 17α-Methyltestosterone (MT) to yield monosex population of male Nile tilapia *Oreochromis niloticus* be limited. An alternative substitute of the synthetic steroid is the use of androgenic natural material, *Eurycoma longifolia* Jack plant (commonly called tongkat ali or pasak bumi or malaysian ginseng), a flowering plant in the family Simaroubaceae. This study was aimed at examining the use of feed enriched with methanol extract of *E. longifolia* root for masculinization of Nile tilapia. The study employed Complete Randomized Design with 3 treatments of *E. longifolia* root extract doses, 30, 60, and 90 mg extract kg^{-1} feed, respectively, 0 mg extract kg^{-1} feed as negative control, and 50 mg of MT kg^{-1} feed as positive control, each of which had 3 replications. *E. longifolia* root methanol extract-containing feed was given up to the next 30 days, and then from 31th day to 60th day, the test fish were fed without addition of methanol extract of *E. longifolia* root. Results showed that male sex ratio ranged from 80.36 to 82.10%, higher than that in negative control, 49.19% and lower than that in positive control of MT, 88.53%. The survival rate ranged from 86.00 to 88.00%, not significantly different from that of negative control, 84.00%, but significantly different from that in positive control of MT, 81.33%. Weight increment was not significantly different between treatments, 7-9.95 g, at the end of rearing experiment. Thus, methanol extract of *E. longifolia* root could be used for Nile tilapia masculinization.
Key Words: *Eurycoma longifolia* Jack, masculinization, male, sex ratio, survival rate.

Introduction. Synthetic steroid hormone used in, so far, Nile tilapia *Oreochromis niloticus* culture to make male population is androgen group, 17α-methyltestoterone (MT), that, in fact, has side effect due to releasing hazardous residue in fish and environment (Pandian & Kirankumar 2003). Awareness of utilizing natural products has triggered and encouraged to find alternative substitutes of the synthetic chemicals from plant extract for production development (Gabriel 2019). Plant extract is believed to be easier to access, easy to apply, and safe for human and environment due to be biodegradable (Reverter et al 2014).

The masculinization effect on *O. niloticus* using feed added with plant materials has been reported, such as the use of *Aloe vera* (Gabriel et al 2017), *Mucuna pruriens* (Mukherjee et al 2015), and *Butea superba* (Kiriyakit 2014). Other plant material that possesses androgenic effect is the root of *Eurycoma longifolia* Jack or commercially known as Tongkat Ali in Malaysia, Pasak bumi in Indonesia, Piak and Tung saw in Thailand, and Cay ba binh in Vietnam (Effendy et al 2012).

E. longifolia Jack is one of the forest-originated medicinal plants that have not been developed yet, but it has a lot of benefits. Empirical experiences show that *E. longifolia* is more commonly known as aphrodisiac (de Padua et al 1999). Several studies also found that the root of *E. longifolia* can increase sexual quality and reduce the doubtness of middle-aged male rat to do sexual activity (Ang et al 2003), and raise the

libido of male rat (Ang & Lee 2002). According to Nainggolan & Simanjuntak (2005), *E. longifolia* extract can also enlarge testosterone level in DDY-strained male rat's blood and add the number of spermatogenic cells, Sertoli cells, and Leydig cells of sexually mature rats (Rosida 2003).

Masculinization is conducted through addition of androgen hormone gonad differentiation phase of the fish. The differentiation in female fish occurs when P450 aromatase enzyme is produced. This enzyme works to catalyze the change in androgen hormone to estrogen, so that it is believed that P450 aromatase enzyme determines the equilibrium between androgen and estrogen hormones (Pandian 2011), besides the inhibition of P450 aromatase activity at the receptor level during the sex differention period makes causes changes in female phenotype to male phenotype. This study was aimed to know the effect of *E. longifolia* root methanol extract feeding at different concentrations on the sex ratio of Nile tilapia.

Material and Method

Time and place. This study was carried out for 4 months (January to April 2017) in the Laboratory of Fish Domestication and Breeding, CV. Griya Aquatica - Fish Farming, Jalan Krakatau, Palangka Raya, Central Kalimantan.

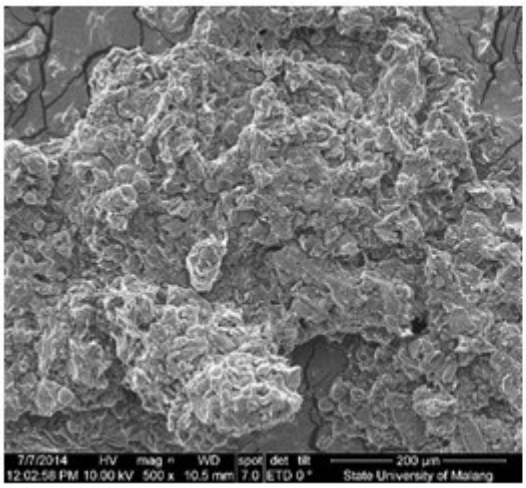
Experimental design. This study employed Complete Randomized Design with 3 treatments and 2 control treatments, each of which had 3 replications. The treatments were different doses of *E. longifolia* root methanol extract in artificial feed administered to the larvae of *O. niloticus*, 0 mg extract kg^{-1} feed as negative control TH (K-), 30 mg extract kg^{-1} feed (EPB1), 60 mg extract kg^{-1} feed (EPB2), 90 mg extract kg^{-1} feed (EPB3), and 50 mg of 17α-Methyl testosterone per 1 kg feed as positive control MT (K+).

Preparation and analysis of tongkat ali E. longifolia root. Samples of *E. longifolia* root were collected from Rakumpit district, Palangka Raya, Central Kalimantan. They were dried and ground to flour, weighed, wrapped in filter paper, and put in the Soxhlet containing methanol. Heating equipment of the soxhlet was set at 70^{o}C for 4 hours so that the solvent evaporates through condenser and go into the sample column. If the solvent accumulation in the sample column is full, the solvent will go down to the heated solvent column. The solvent cycle refluxes during extraction for about 4 hours. The extract was then evaporated using rotary evaporator at 40^{o}C to obtain viscous *E. longifolia* extract. The pasta-shaped extract was then weighed as dose needed.

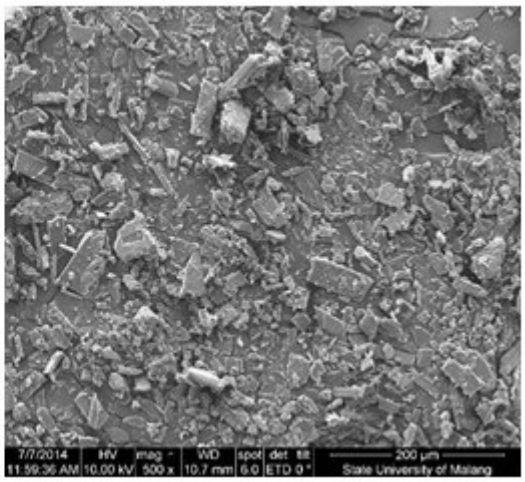
From 1,095 g of *E. longifolia* root flour, as much as 54.98 g of methanol extract was obtained with mean extract mass of 4.93%. The examination applied GC-MS as presented in Table 1.

A total of 65 g of methanol extract of *E. longifolia* root was used to determine the composition of amino acids contained in the extract (Table 2). Analysis was performed using HPLC with Triple Quadrupole Tandem Mass Spectrometry detector (LC-MS/MS). Qualitative analyses were conducted by monitoring Ion Ratio of 2 MRM pairs for each compound. Quantitative determinations were calculated by single point matrix-based calibration at the Reporting Limit.

SEM analysis was done to evaluate the surface morphology of the active compounds used. It was set at 500 magnifications that the surface morphology could be seen clearly. The sample micrograph is presented in Figure 1. The methanol extract particles of tongkat ali root form clusters in polymorphic structure (not homogenous) and have not given information on the particle shape and size yet (a). The particles 17α-methyltestesterone look evenly spread with the particle shape and size in the micron range, less homogeneous, some have elongated shapes and some form a cube structure (b).



Methanol extract of *E. longifolia* (a)



17α-methyltestesterone Aldricch Product 69240-5G (b)

Figure 1. Characterization and analysis of nanostructure using scanning electron microscope (SEM) at 500 magnifications of the solution used for *O. niloticus* masculinization.

Table 1

GC-MS outcome of *E. longifolia* root methanol extract

No.	*Name*	*Suitability factor (%)*	*Total (%)*
1.	3-(2-pentenyl)-1,2,4-Cyclopentanetrione	53	5.80
2.	Palmitic acid methyl ester	98	1.63
3.	Palmitic acid	99	10.39
4.	4-ethoxy-2,5-dimethoxybenzaldehyde	60	2.23
5.	Isofraxidin/6,8-dimethoxy-7-hidroxycoumarin	96	1.55
6.	Oleic acid methyl ester	99	1.44
7.	Linoleic acid	96	1.33
8.	Oleic acid	99	4.78
9.	Octadecanoic acid	97	1.16
10.	Benzonitrile, 4-(5-propyl-2-pyridinyl)-	58	7.95
11.	Canthine-6-one(3,4-diazafluoranthen-2(3H)-one	94	4.21
12.	m-Cresol / m-Toluol / 3-methyl pPhenol	64	8.80
13.	Isooctyl phthalate	91	1.91
14.	4-phenil-8-oxo-4,5,6,7-tetrahydrocyclopenta (b)-1,2,3-triazolo (4,5-e) pyridine	90	6.98
15.	2-methyl-4,4-diphenyl-2-imidazolin-5-one	91	3.95
16.	Methyl ester 1',2-dimethyl-ferrocenecarboxylic acid	49	4.73
17.	4,5-dihydroxy-4-(3-methyl-2-butenyl) benzoic acid methyl ester	46	2.48
18.	Unknown	25	0.93
19.	Estra-1,3,5,(10)-trienn-17-ol, 3-[(trimethylsilyl)oxy]-,acetate, (17.beta.)-	87	1.12
20.	5-heptadecatri-8(Z),11 (Z), 14 (Z)-enylresorcinol	80	1.12
21.	Piperine	99	1.12
22.	Salicylaldehyde/beta resorcylaldehyde	49	1.21
23.	Methylenetanshinquinone	41	1.23
24.	Not known	10	1.10
25.	Stigmasta-5,23-dien-3.beta.-ol	64	5.97
26.	Unknown	15	2.85
27.	Unknown	18	1.53
28.	Unknown	15	1.17
29.	Spinasterone	53	4.62
30.	Sitostenon / delta.4-sitosterol-3-on	92	3.22
31.	Aurantiamide	46	1.52

Note: Stigmasterol is 5.97% of total component.

Table 2

LC-MS/MS outcome of free amino acid of *E. longifolia* root methanol extract

No.	Measurement	Ppm (mg kg^{-1})	
		RL	Result
1.	Alanine	20.0	173
2.	Argine	20.0	2,360
3.	Asparagine	20.0	67.4
4.	Aspartic acid	20.0	633
5.	Cysteine	20.0	ND
6.	Glutamic acid	20.0	1.230
7.	Glutamine	20.0	17.5
8.	Glycine	20.0	24.4
9.	Histidine	20.0	47.2
10.	Isoleucine	20.0	48.7
11.	Leucine	20.0	25.7
12.	Lysine	20.0	32.8
13.	Methionine	20.0	ND
14.	Phenylalanine	20.0	56.7
15.	Proline	20.0	802
16.	Seleno-L-cysteine	20.0	ND
17.	Serine	20.0	69.3
18.	Threonine	20.0	42.9
19.	Tran-4-hidroxy-L-proline	20.0	10.1
20.	Tryptophan	20.0	10.4
21.	Tyrosine	20.0	183
22.	Valine	20.0	56.1

Note: ND - not detected; LoQ - limit of quantitation; RL - reporting limit = practical LoQ (RL is measured every analysis batch).

Treatment feed preparation. Feed used was flour form of PS-P-typed Hi-Pro-Vite for freshwater fish seeds produced by PT. Centra Proteina, Tbk. with nutrient composition of minimum protein 40%, minimum fat of 10%, maximum coarse fiber of 8%, and water content of 12%.

The treatment feed was made by mixing *E. longifolia* root extract at 30, 60, 90 mg kg^{-1} and 50 mg MT kg^{-1} feed into 300 mL of 70% alcohol. A magnetic stirrer was used to dissolve the extract in the alcohol for several minutes up to both materials be really homogenous. After the extract had been dissolved in the alcohol, it was put into a sprayer and sprayed on the experimental feed, stirred up to be evenly mixed and wind-dried.

Masculinization and larval rearing

Tank preparation. The culture media were 15 units of 60 x 40 x 30 cm aquaria. The aquaria were filled with drinking water from the regional company and left for one week before use. Water circulation was conducted through recirculation system. The water tank was facilitated with heater in order to stabilize the temperature between 28-30^{o}C.

Larvae stocking. Black Nile tilapia *O. niloticus* were brought from Public Hatchery Unit around Bincau village, Banjar regency, South Kalimantan. The larvae were taken from one-day old individuals (still holding yolk), reared for 5 days in the aquarium up to the yolk finished (7 days old), with individual body weight of 0.01-0.02 g. Larval density per aquarium was 50 individuals.

Feeding. Feed was added with *E. longifolia* root extract. The feed was weighed before feeding using a digital balance. Percent daily feeding was 10-30% day^{-1} administered 3-4 times a day. Feeding *E. longifolia* root extract-enriched feed was done every day following the desired treatment doses for 30 or 37 days until the yolk was finished. For rearing period from 31st day to 60th day (the end of experiment), feed without addition of *E. longifolia* extract was given with size and type suitable for larval development phase.

Water quality measurements. Water quality parameters measured were temperature, dissolved oxygen, pH, and NH_3 using thermometer, DO-meter, pH meter and spectrophotometer, respectively. Water temperature, dissolved oxygen, and pH were measured everyday, while NH_3 measurement was done once a week. Water temperature during the study ranged from 28 to 30^oC, dissolved oxygen from 5.4 to 6.8 mg L^{-1}, pH from 6.2 to 7.6, NH_3 from 0.01 to 0.032 mg L^{-1}. Growth and survival rate were recorded every 15 days up to 60 days.

Sex identification. Gonad was checked after the fish had been 60 days old by selecting 30% of the test fish samples. They were dissected and gonads were carefully removed using tweezers. To ease gonad sampling, the intestine and other organs in the body cavity were also removed. A part of the gonad was put on the object glass and chopped with scapel knife to fine particles. The gonad particles were put on the object glass and given 2 drops of acetocarmine (Guerrero & Shelton 1974). The object glass was covered with cover glass, and the gonad was ready to be observed under a binocular microscope at 40 x magnification. Some gonad sample was made histological preparate with hematoxylin-eosin (HE) staining, and then the structure was examined using explorative histological method (Gunarso 1989).

Testosterone hormone profile in fish. Measurements of testosterone level of the test fish were conducted at the beginning of the experiment (0 day), 15th day, 30th day, 40th day, and at the end of the experiment, 60th day. Testosterone level was measured using enzyme linked immunosorbent assay (ELISA) method, an immunoassay employing enzyme as label (Simanjuntak 2013).

Statistical analysis. Parameters statistically tested were sex ratio, growth, and survival rate. Data were tabulated using MS Office Excel 2010 program and ANOVA utilized STATISTICA 8 program at 95% significance level. The significantly different effects were then tested using Duncan test.

Results and Discussion

Percent of sex ratio. Percent mean male *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding is shown in Figure 2. ANOVA indicated that the application of methanol extract of *E. longifolia* root in feeding gave very significant effect on the percent of males ($p < 0.01$). Duncan test revealed that treatments EPB1, EPB2, and EPB3 did not give significantly different effect, but all treatments yielded significantly different effect from those of positive control MT (K+) and negative control TH (K-) treatment.

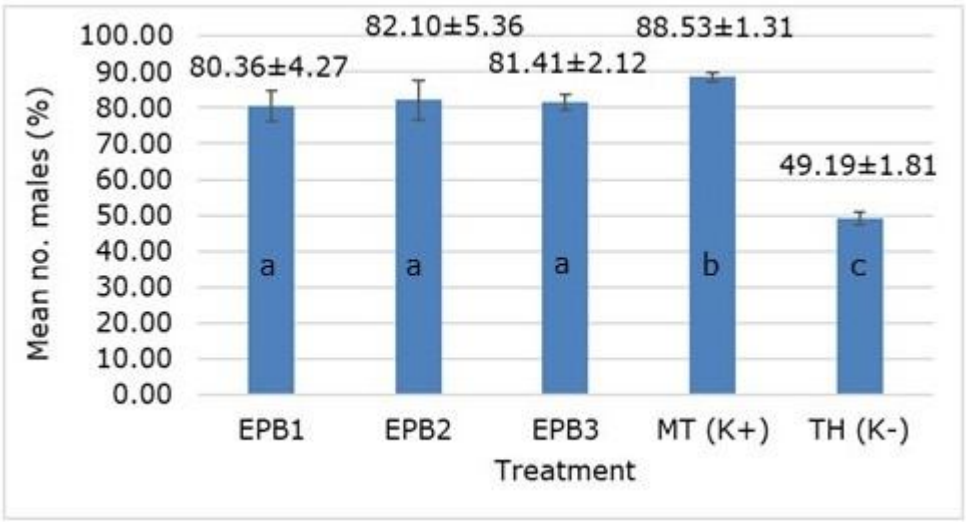


Figure 2. Percent mean male *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding.

O. niloticus intersex can be seen in Figure 3. ANOVA showed that application of *E. longifolia* root extract to feeding gave significant effect on the percent intersex of *O. niloticus* ($p < 0.01$). Duncan test did not show difference among EPB1, EPB2, EPB3, and MT (K+), but these 4 treatments gave highly significant different effect from negative control TH (K-).

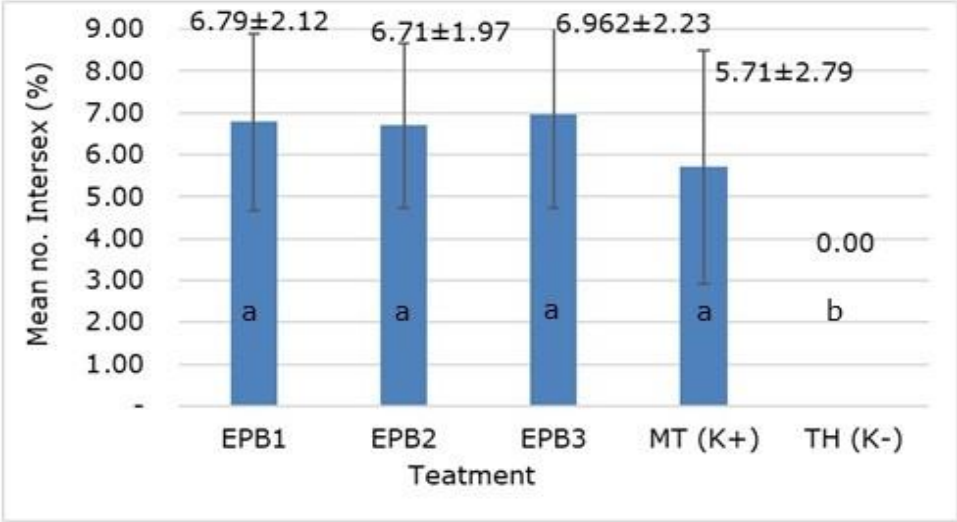
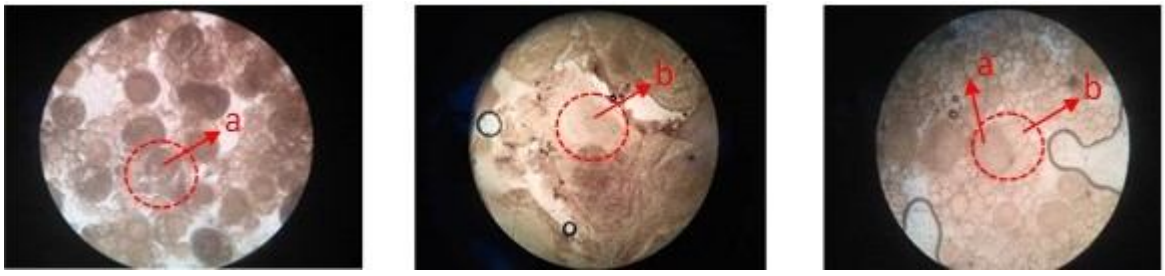


Figure 3. Percent of mean intersex of *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding.

O. niloticus has a pair of gonad, located in the body cavity, under vertebrae, restricted by thin layer. These gonads are long-shaped and flow to the sperm or ovum-releasing opening. Gonads in young fish have very small size like a thin thread, while fish with further gonad development have large-sized gonads and fat-like white color.

Observation on gonad tissue using acetocarmine staining technique as presented in Figure 4 demonstrates normal female gonad, normal male gonad, and intersex gonad. Under the microscope at 40 magnification, difference in prospective eggs, testis tissue, and prospective sperms can be seen. In male gonad, the prospective sperms appear very tiny as red dot or homogenously distributed small dots (Figure 4b). In female gonad tissue, ova look round and has uniform size, much bigger than the prospective sperms, with nucleus in the center (Figure 4a). In intersex gonad, ova and sperms can be seen in separate groups (Figure 4c).



(a) (b) (c)

Figure 4. Gonad tissue of 60 day old *O. niloticus* under acetocarmine staining; a: female gonad with ova, b: male gonad with sperms, and c: intersex gonad (having ova and sperms).

Survival rate. The survival rate of *O. niloticus* at the end of the experiment is shown in Figure 5. ANOVA revealed that application of *E. longifolia* extract under different doses significantly affect the survival rate of *O. niloticus* ($p < 0.05$). Duncan test did not show different effect among EPB1, EPB2, EPB3, and negative control TH (K-), but all these gave different effect on the survival rate from that of positive control MT (K+).

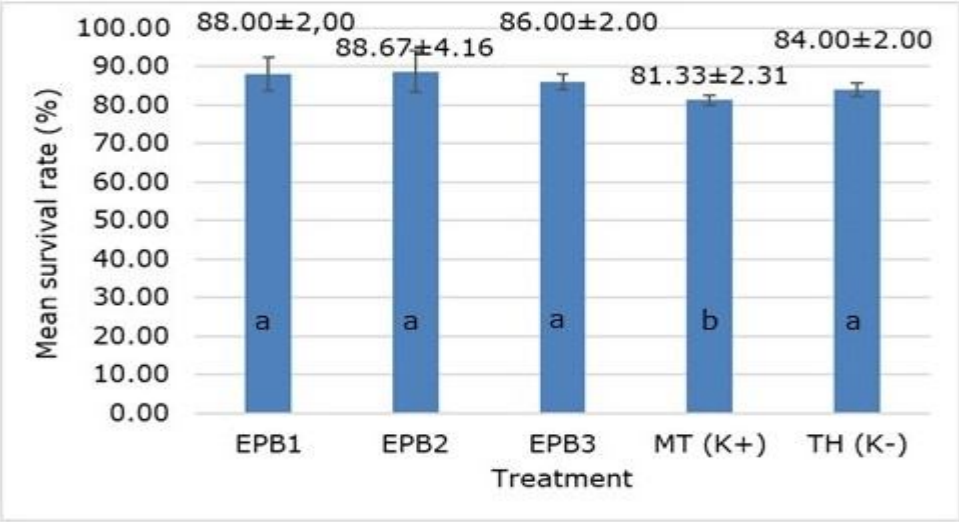


Figure 5. Mean percent of survival rate of *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding.

Growth. Daily specific growth is shown in Figure 6 and absolute growth in Figure 7. ANOVA showed that application of *E. longifolia* root extract in feeding gave very significant effect on both daily specific growth and absolute growth of *O. niloticus* ($p < 0.01$). Duncan test indicated the effect of MT (K+), EPB1, EPB2, and EPB3 applications was not significantly different, but these 4 treatments yielded very significantly different effect from that of negative control TH (K-).

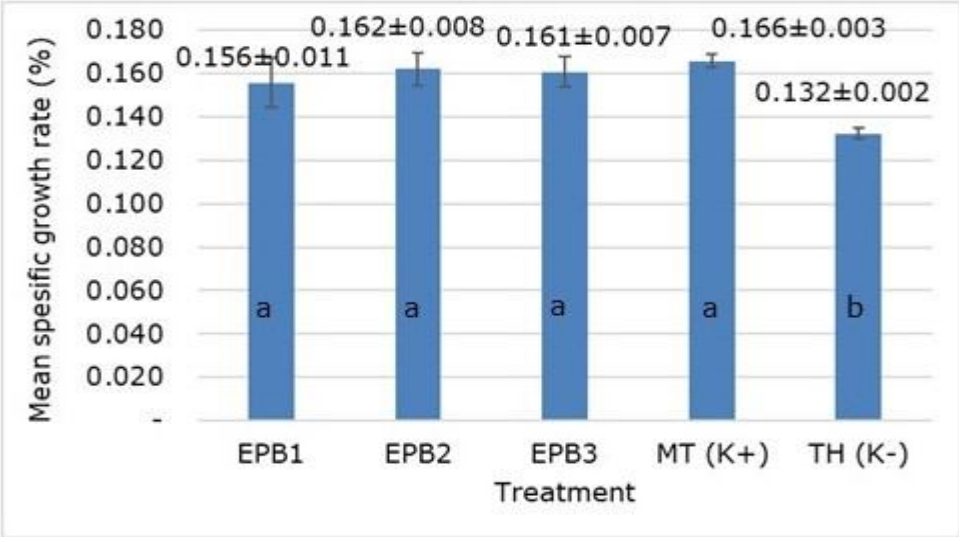


Figure 6. Daily mean growth rate (%) of *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding.

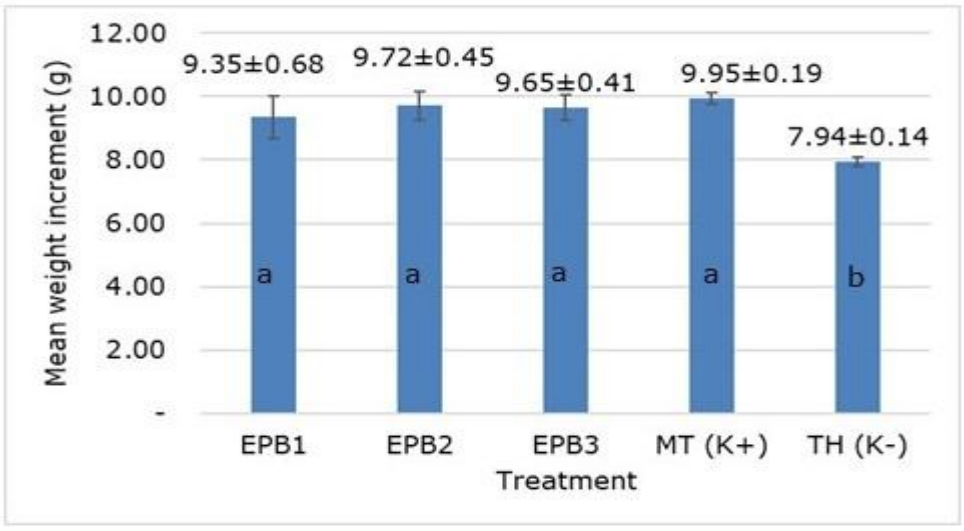


Figure 7. Mean weight increment (g) or absolute growth of *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding.

Gonad histology. Gonads of *O. niloticus* treated with methanol extract of *E. longifolia* root through feeding of 30 mg extract kg^{-1} feed, 60 mg extract kg^{-1} feed, and 90 mg extract kg^{-1} feed, negative control TH (K-) of 0 mg extract kg^{-1} feed, and positive control of 50 mg of MT kg^{-1} feed have apparently developed after 60 days of rearing experiment. The gonad exhibits *tubulus seminiferus* development (Table 3).

Hormone profile. Testosterone level in the body of fish larvae, based on ELIZA method, ranged from 0.29 to 0.48 pg mL^{-1} indicating that *O. niloticus* larvae have natural testosterone. After treated with methanol extract of *E. longifolia* root through feeding, the testosterone level of *O. longifolia* larvae treated with EPB1, EPB2, EPB3, and positive control MT drastically rose on the 15th day with the range of 0.44-0.58 pg mL^{-1}, while that of negative control slightly rose, 0.44 to 0.45 pg mL^{-1}. Testosterone levels in the fish body for treatments EPB1, EPB3, positive control MT, and negative control from day 15 to day 60 declined, while testosterone level in treatment EPB2 increased in the 15th day, then declined in day 30 to day 60. Relatively low decline pattern appears in negative control treatment, while that of positive control MT steeply occurs from day-45 to day-60. Treatments EPB1 and EPB3 possess similar decline pattern that tends to be steep in the interval of day-15 to day-30, while in treatment EPB2, the drastic decline occurs in day-30 to day-45 (Figure 8).

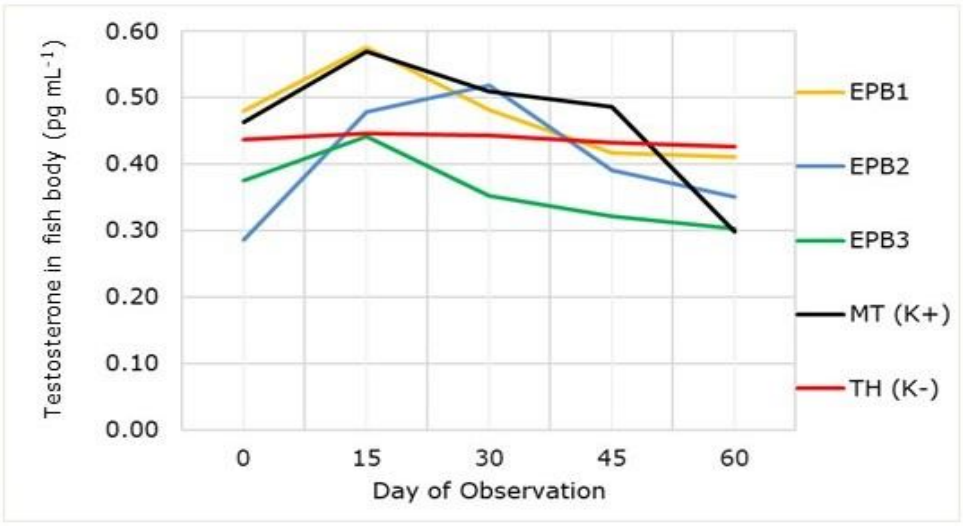
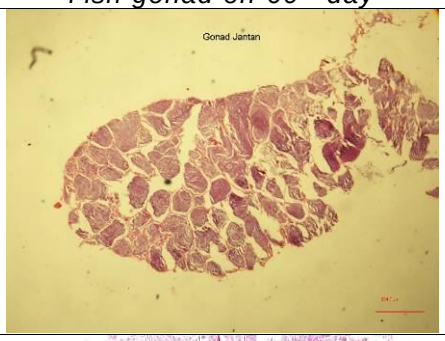
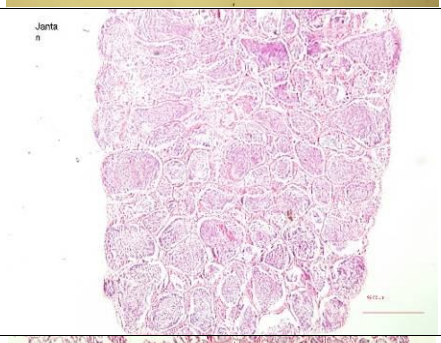
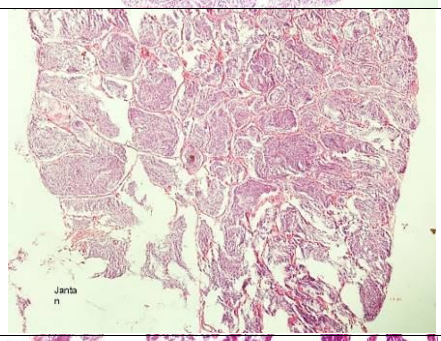
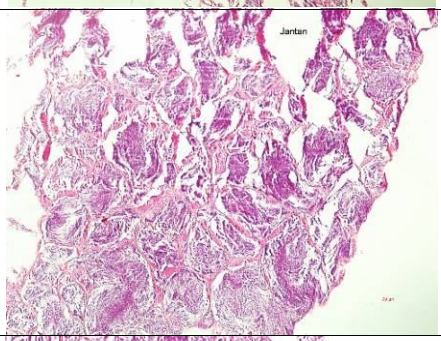
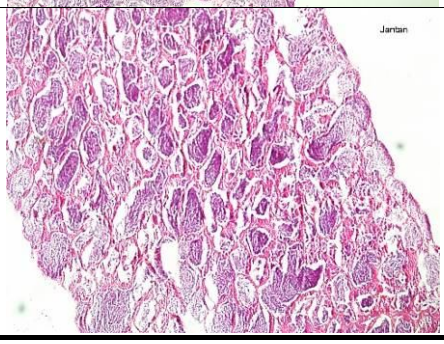


Figure 8. Testosterone in fish body (pg mL^{-1}) of *O. niloticus* treated with *E. longifolia* root methanol extact through feeding.

Table 3

Gonad histology of *O. niloticus* in 60 days after treated with methanol extract of *E. longifolia* root through feeding

Treatment	*Fish gonad on 60th day*
Negative control TH (K-)	
Positive control MT (K+)	
30 mg extract kg^{-1} feed (EPB1)	
60 mg extract kg^{-1} feed (EPB2)	
90 mg extract kg^{-1} feed (EPB3)	

Discussion. The sex ratio of male *O. niloticus* treated with 30 mg extract kg^{-1} feed, 60 mg extract kg^{-1} feed, and 90 mg extract kg^{-1} feed of *E. longifolia* root extract ranged from 80.36 to 82.10%, higher than that of negative control, 49.19%, but lower than that of positive control MT, 88.53%. Increased percentage of males in the 3 treatments could result from active compound in *E. longifolia* root extract. It could result from the presence of phytoandrogen in the form of stigmasterol in *E. longifolia* plant.

GC-MS measurements of *E. longifolia* root extract found 31 chemical compounds, 5.97% stigmasterol. This compound works to stimulate androgen hormone development of the body. Phytochemical examination showed that *E. longifolia* root extract contained saponin, phytosterol, alkaloid, and the highest intersex fish occurred in treatment of 90 mg extract kg^{-1} feed, 6.96 %, followed by 30 mg extract kg^{-1} feed, 6.79%, 60 mg extract kg^{-1} feed, 6.71%, and positive control 50 mg of MT kg^{-1} feed, 5.71%, respectively, while no intersex fish was found in negative control treatment. Figure 4c shows that sperms and ova occur in separate groups.

Very low dose application causes sex reversal to occur improperly, but the use of high concentration results in intersex (Yamazaki 1983). Nasrun (1994) found that the administration of high testosterone concentration could reduce the percent of male fish and result in intersex fish, 2.3%. This deviation could result from conversion of testosterone to estrogen as a consequence of high dose of androgen received.

The survival rate of *O. niloticus* larvae after application of *E. longifolia* root extract ranged from 88.00 to 88.67%, not significantly different from that of negative control, 84.00%, but significantly different from that of positive control MT, 81.33%. This high survival rate could result from the presence of other compounds in the methanol extract of *E. longfolia* root. It is in line with LC-MS/MS measurements (Table 2) that various amino acids occur in the extract. Amino acids arginine, histidine, leucine, glutamate, glycine, proline, serine, and tyrosine are very beneficial for androgen hormone formation, in which testosterone could play good role in libido development or spermatozoa formation.

According to Hafez &Hafez (2000), amino acids are one of the supplements that stimulate the formation of steroid hormones, such as testosterone, and spermatogenesis. Besides having androgenic feature, testosterone, in fact, has anabolic feature (Fullerton 1980), in which it can accelerate muscle growth. Application of 60 mg extract kg^{-1} feed and 90 mg extract kg^{-1} feed of *E. longifolia* root gave better growth performance than other treatments. It is in agreement with Phelps & Popma (2000) that androgen hormone has two physiological roles, androgenic one promoting male character and anabolic one stimulating protein biosynthesis in the fish body.

Chemical compounds in *E. longifolia* root extract could give stimulus on gonad formation. It is shown in the histology of tubulus seminiferus development at treatment of 30 mg extract kg^{-1} feed, 60 mg extract kg^{-1} feed, 90 mg extract kg^{-1} feed, and the positive control 50 mg of MT kg^{-1} feed. Mean tubulus seminiferus size was larger than that in negative control. According to Yamazaki (1983), androgen hormone stimulates gonad formation, and thus, it is believed that stigmasterol compound, like androgen hormone affects the gonadal development of *O. niloticus* gonad.

According to Sukmaningsih et al (2011), time needed to finish each developmental stage of spermatozoids is different, and therefore, there will be various cell combination forms of different germinal cell developments in tubulus seminiferus. Besides adaptation to spermatogenesis cycle duration, the difference could be caused by other factors, such as environment or hormones.

ELISA method was applied in this study to measure the testosterone concentration in the fish body (Memmat et al 2015). As shown in Figure 8, testosterone level drastically rose on the 15^{th} day, then quickly fell up to day-30, and stably declined up to day-60. The fish testosterone level of the negative control treatment (0 mg extract kg^{-1} feed is relatively more stable than other treatments. Fast decline of steroid level was also reported by Pandian & Kirankumar (2003) that decline of steroid level occurs very fast at the beginning and gradually gets more stable. Hormone decline level is dependent upon species, steroid purity used, detected organ, and treatment protocols. In the present study, testosterone was detected using the entire body of the fish.

Several other researchers reported that MT hormone is quickly metabolized and excreted. MT concentration in the plasm of *O. niloticus* declines at the 22th hour after feeding has been terminated (Rinchard et al 1999). After 24 hours, MT concentration in fish body decreases to 2.5-3.0% (Curtis et al 1991), and after 100 hours it becomes 1% (Johnstone et al 1983).

Conclusions. Masculinization of Nile tilapia *O. niloticus* employed methanol extract of tongkat ali *E. longifolia* Jack root through feeding yielded male sex ratio between 80.36-82.10% and survival rate of 86.00-88.00%. Weight increment ranged from 7 to 9.95 g. Hence, methanol extract of *E. longifolia* root could be utilized as an alternative substitute material of synthetic hormone 17α-Methytestosteron (MT) for *O. niloticus* masculinization.

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