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THE EFFECT OF PHYTOECDYSTEROID OF *Cycas revolua*, *Portulaca oleracea*, AND *Morus* sp. ON MOLTING PERIOD, GROWTH AND SURVIVAL RATE OF TIGER SHRIMP, *Penaeus monodon*

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ABSTRACT

The problem which has still been faced in the Artificial insemination (AI) is the slow of shrimp to molt. Ecdysteroid hormone has been reported to stimulate molting of tiger shrimp. This study aims to isolate ecdysteroid hormone from *Cycas revoluta*, *Portulaca oleracea* and *Morus* sp. and evaluate its effect on molting period, growth and survival rate of tiger shrimp. Isolation of ecdysteroid from the leaves of three species was carried out by maceration and solvent partition method. Purification of ecdysteroid used repeated column chromatography and preparative thin layer chromatography (TLC). Evaluation of the isolated phytoecdysteroid hormone effect on molting period, growth and survival rate of shrimp was done by injecting of 100 μL phytoecdyasterod (27.5 μg/shrimp) at the first somite of ventral abdomen. As the comparison, the commercial ecdysteroid (positive control) and sterile saline solution (negative control) were also injected at the concentration of 8.6 μg/shrimp and 0 μg/shrimp, respectively. Finding showed that the highest percentage of phytoecdysteroid was obtained in *Portulaca oleracea*, followed by *Morus* sp. and *Cycas revoluta* with the ecdysteroid content of 0.43%, 0.22%, and 0.09%, respectively. Pytoecdysteroid isolated from the three plants was able to shorten molting period of shrimp into 4, 4, 2, and 5 days earlier for *Portulaca oleracea*, *Morus* sp., *Cycas revoluta*, and positive control, respectively, compared to the negative control. The highest survival rate and growth were obtained at the treatment of *Portulaca oleracea*, followed by *Morus* sp. and *Cycas revoluta* with the survival rate, length and weight increase of 86%, 75%, and 25%, 4.42%, 2.26% and 2.16%, and 15.90%, 10.55%, and 8.73%, respectively.

KEYWORDS: ***Portulaca oleracea*; *Morus* sp.; *Cycas revoluta*; phytoecdysteroid; molting**

INTRODUCTION

Artificial insemination (AI) is a technique to produce families relied on the removal of both spermatophores from a single male and the application of the spermatophores over the thelycum of a ripe female. AI has been applied for the successful selective breeding in tiger shrimp by increasing the number of fertile eggs on tiger shrimp (Arnold *et al.*, 2012; Laining *et al.*, 2014). Furthermore, female tiger shrimp inseminated with spermatophore of male tiger shrimp collected from nature at the different location source produced the fertile eggs ranged from 61%-65% (Lante & Laining, 2016). Through AI, the eggs fertility can be improved. Nevertheless, it is well known that the problem faced in the AI is the low percentage of shrimp to molt. Therefore, it needs to utilize hormone in accelerating molting of tiger shrimp. Ecdysteroids, a group of polyhydroxylatedketo-steroids synthesized by the Y-organ are primarily involved in regulating the molting process (ecdysis) of crustaceans (Bart *et al*., 2006; Ghanawi & Saoud, 2012, Sorach *et al*., 2013). One of ecdysteroid derivative, 20-hydroxyecdysone (20E) has been identified as the main molting hormone in crustacean (Martin-Creuzburg *et al*., 2007). The 20E can not be only used to accelerate molt, but it can also be used to synchronized mating activity of silkworm bombyx (Rufaie *et al*., 2011).

Molting hormones have been found in both plants and animals in which the plant origin is called phytoecdysone whereas the animal origin is called zoecdysone. Several species of plants obtained to have phytoecdysone such as *Gomphrena celosioides*, *Cyathula prostrata*, *Achyranthus aspera*, *Vitex pinnata*, *V. canescent*, *Sesuvium portulacastrum,* and *Morus alba* (Putchakarn, 1992). In the present study three plants, *Cycas revoluta* (Suryati & Tenriulo, 2013) *Pertulaca oleracea* (Suryati *et al.*, 2013) and *Morus* sp., were fur-

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ther isolated their ecdysteroid and investigated their ability in stimulating of the tiger shrimp molt since their leaves extracts have been reported to contain ecdysteroid hormone which can shorten the molt cycle of tiger shrimp at the concentration of 25 mg/L and proven to accelerate molt on crab at the concentration of 100 mg/L (Chou & Lu, 1980; Herlinah *et al*., 2013). Total haemocyte count (THC) was also studied as it is known that THC is one of parameters which can be used as an indicator of stress on crustacean (Arifin *et al*., 2014). The stress is able to influence the molt activity of shrimp giving an effect on its growth process (Pratiwi *et al*., 2016). The aims of this study were to isolate phytoecdysteroid hormone from these three plants and evaluate their effects on molting period and survival rate of tiger shrimp, *Penaeus monodon*.

MATERIALS AND METHODS

Extraction and Isolation of Phytoecdysteroid

Leaves of *Cycas revoluta* and *Portulaca oleracea* were collected from the field of Research Institute for Coastal Aquaculture (RICA), Maros-South Sulawesi, Indonesia around pond in Maranak and *Morus* sp. leaves was obtained from the Garden of *Morus* sp. in Bili-Bili, South Sulawesi. The universal leaves of these three species selected were brought to Biotechnology Laboratory of RICA and separately dried using an oven at the temperature of < 40°C. Dried leaves were grounded to powder form, then suspended in 96% ethanol for 1 to 3 days. After filtration and evaporation of ethanol, the extract were partitioned with n-hexane and 75% ethanol to get n-hexane and ethanol extract. Ethanol extract obtained was evaporated and partitioned with chloroform:ethanol:water (1:1:1) to generate chloroform and water phase. After evaporation, dried chlorofom extract was suspended with a mixture of ethyl acetate and ethanol (2:1) and continued the filtration through neutral alumina. Further elution on alumina column was done by using a mixture of ethyl acetate and ethanol (2:1) to get crude ecdysteroid. Purification of the ecdysteroid was carried out by repeated column chromatography and preparative thin layer chromatography. Identification of phytoecdysteroid was done by comparison to the TLC profiling of 20-hydro xyecdysteron (standard) under UV_{254nm}. Percentage of phytoecdysteroid isolated was counted by the equation followed:

$$E\,(\%) = \frac{Wa}{Wf} \times 100\%$$

where: E = Phytoecdysteroid
Wa = The weight of fraction containing phytoecdysteroid isolated
Wf = The weight of dried powder leaves

Application of Phytoecdysteroid on Tiger Shrimp

Female tiger shrimp broodstock with an average body weight of $\geq$ 80 g were moved from Station of experimental pond of Research Institute for Coastal Aquaculture (RICA) located in Takalar to Station of shrimp breeding of RICA in Barru, South Sulawesi. Prior to experiment, shrimps were acclimatized for two weeks in 3 m^3 concrete tanks and fed with a combination of fresh feed (squid, worms and mussels) and moist pellet commercial 15% and 5% of the total body weight. For the application of phytoecdysteroid on tiger shrimp, the shrimps were randomized sampling from acclimated tanks and measured for length and weight for recording. The shrimps with the average length of 18.3-21.8 cm and body weight of 88-91 g were individually eyestalk-tagged and reared in concrete tanks of 3 m^3 with density of 10 shrimp/experiment. Postmolt shrimps, two days after moulting were injected with ecdysteroid hormone namely; treatment (A) injection of 275 μg/mL (27.5 μg/shrimp) *Pertulacaoleracea*, treatment (B) injection of 275 μg/mL (27.5 μg/shrimp) *Morus* sp., treatment (C) injection of 275 μg/mL (27.5 μg/shrimp) *Cycasrevolute*, treatment (D) injection of 86 μg/mL (8.6 μg/shrimp) commercial ecdysteroid (positive control), and treatment (E) was served as control receiving injection of 100 μL of sterile saline solution. The study was undertaken for 30 days after injection. During the experiment, the shrimps were fed with a combination of freshfeed (squid, worms and mussels) and moist pellet commercial. Feeding rate of freshfeed and pellet was15% and 5% of the total body weight, respectively. Broodstocks were fed with freshfeed three times/day in the morning at 07.00 h, in the afternoon at 17.00 h and in the evening at 22.00 h and commercial feed once/day in the morning at 10.00 h completed with adequate aeration and rearing water was changed daily at rate of 25% volume.

Data Collection

Observations done were molting, molt duration, the measurement of length and weight, survival rate, and total haemocyte count at the initial and the end of the experiment.

Molt Duration

The observation of time for molting was checked at 7.00 am., 11.00 am, and 6.00 pm. each day during 30 days of rearing and the molt duration was counted as followed:

$$M = Ta - Tf$$

where: M = The molt duration
Ta = Time at prawn molt
Tf = Time at start as day

Size Increase

After the shrimps molted in two days, they were measured length and weight for comparison with control. Size increase was counted as followed:

$$L = LA - LB$$

where: L = The increasing length or weight
L A = The length or weight after molting
LB = The length or weight at start

Survival Rate

Survival rate was determined by counting the shrimps died in the case. The percentage of survival was determined as followed:

$$S\% = \frac{NA}{NT} \times 100\%$$

where: S = Survival
NA = Number of prawns at end
NT = Number of prawns at start

Total Haemocyte Count (THC)

Total haemocyte count (THC) was measured according to modified method of Maftuch *et al*. (2013). One hundred μL of shrimp haemolymph was transferred into a tube containing 300 μL of anti-coagulan (0.01 M tris HCl, 0.25 M sucrose, 0.1 M sodium citrate, at pH 7.6). Ten μL of haemolymph from each treatment was transferred to hemocytometer and the number of haemocytes was determined microscopically. THC was calculated using the following formulation (Wootton *et al*., 2003):

$$THC\,(cell/mL) = \frac{THC \times P \times 2 \times 10^4}{N}$$

Where: THC = Total hemolymph counted
P = Dilution factor
N = Number of square counted

RESULTS AND DISCUSSIONS

Isolation of Phytoecdysteroid

Isolation of ecdysteroid from the leaves of *Morus* sp., *Pertulaca oleracea* L and *Cycas revolute* Thumb were done and showed profiling of thin layer chromatography as exhibited in Figure 1. TLC analysis of ecdysteroid from all samples showed one major band with the dark colour under UV_{254nm} light. The rate of flow (R_f) value obtained were 0.59 for both *Morus* sp. and *Cycas revoluta* and 0.58 for *Pertulaca oleracea* and standard with the solvent system of dichloromethane (DCM) and methanol (MeOH) at the ratio of 8 : 2. This is indicated that all phytoecdysteroid isolated were similar to the commercial standard (20-hydroxyecdysone, 20E). Previously, the R_f value of 20E obtained was lower (0.23, chloroform/ethanol 12:1) (Liktor-Busa, 2008) probably due to the different solvent used in the two studies.

The highest phytoecdysteroid percentage (0.43%) was obtained from *Pertulaca oleracea* L., followed by *Morus* sp. and *Cycas revolutae Thumb* with the phytoecdysteroid percentage of 0.22 and 0.09%, respectively (Table 1). Percentage of phytoecdysteroid could be qualitatively predicted from the TLC profiling (Figure 1). The TLC profiling of *Pertulaca oleracea* L. had a concentrate spot compared to that of *Morus* sp. and *Cycas revolutae* Thumb, indicating the presence of high contain of phytoecdysteroid. According to Lafont & Wilson (1996), in most ecdysteroid-containing plants, the level of ecdysteroid are between 0.1%-3% of the dry weight, which is 1000-fold higher than in insect.

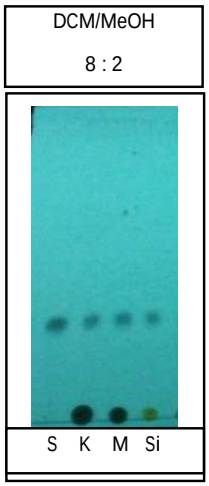

Figure 1. Thin layer choromatography profiling of ecdysteroid isolated from K (*Pertulaca oleracea* L.), M (*Morus* sp.), Si (*Cycas revolutae* Thumb) and S (standard) under UV_{254nm}

Table 1. Percentage of phytoecdysteroid isolated from *Pertulaca oleracea* L., *Morus* sp., and *Cycas revolutae* Thumb

Plant	Percentage of phytoecdysteroid
Pertulaca oleracea L.	0.43%
Morus sp.	0.22%
Cycas revolut ae Thumb	0.09%
Standard	93%

Effect of Phytoecdysteroid on Molting Period

The molting period of tiger shrimp injected with the three phytoecdysteroids and the standard were presented in Table 2.

Injection of three phytoecdysteroid at the same concentration of 275 μg/mL resulted in different responses on molting period of shrimps. Injection of phytoecdysteroid isolated from *P. oleracea* and *Morus* sp. reduced molting period 4 days faster compared to the negative control. At the similar concentration, phytoecdysteroid isolated from *C. revolutae* could also shorten the molting period into 2 days earlier than the negative control. Furthermore, the positive control at the concentration of 8.6 μg/shrimp stimulated molting 5 days earlier than the negative control. The similar result was reported by Jegla and Costlow (1970), that injection of polyhydroxyecdysteroids caused an earlier molting period of horseshoe crab larvae depending on the dose and time of the injection. Ecdysteroid has been found to be a molt stimulating hormone in many crustacean species including shrimp (Sorach *et al*., 2013).

Phytoecdysteroid isolated from the three plants species also affected the survival rate, growth, and haemocyte number of tiger shrimp as shown in Table 3.

The highest survival rate of 88% was showed by the positive control, followed by phytoecdysteroid isolated from *P. oleracea*, *Morus* sp., and *C. revolutae* with the survival rate of 86%, 75%, and 25%, respectively. The survival rate resulted by the injection of phytoecdysteroid isolated from *Pertulaca oleracea* L. and *Morus* sp. was less lower than that of the positive control but much higher than that of the negative control (43%). The low survival rate obtained at the negative control was suspected that the shrimps were in unhealth condition due to infected WSSV as observed. This is indicated that phytoecdysteroid isolated from these two plants at the concentration of 275 μg/mL (27 μg/shrimp) did not negatively affect the shrimps. In contrast, shrimps injected with phytoecdysteroid isolated from *Cycas revolutae* Thumb at the same concentration demonstrated the lowest survival rate which was only 25%. It was suspected that due to the presence of neurotoxic methabolites in *Cycas revolutae* Thumb (Moawad *et al*., 2010).

In regard to growth, injection of phytoecdysteroid isolated from *Pertulaca oleracea* L. showed a better increase on the growth and haemocyte number of shrimp (Table 3) compared to the positive control. The length, weight and increase of haemocyte number at treatment A and B were 0.8 cm (4.42%) and 15 g (15.90%), 260 x 10^4 cells (31.25%), and 0.5 cm (2.26%), 11 g (10.55%) and 424 x 10^4 cells (67.94%), respectively. Although, the negative control (treatment C) showed the increase of growth in length and weight of 0.3 cm (1.66%) and 8 g (6.89%), respectively, it exhibited the haemocyte number from 240 x 10^4 cells/mL to 200 x 10^4 cells/mL. The presence of increasing THC level in haemolymph was correlated to the health of the shrimp (Chithambaran & David, 2014).

CONCLUSION

The pytoecdysteroid isolated as 20-hydroxyecdysone from the three plants at the con-

Table 2. The effect of phytoecdysteroid isolated from *Pertulaca oleracea* L., *Morus* sp., and *Cycas revolutae* Thumb, the commercial ecdysteroid, and negative control (without hormone injection) on molting period

Treatment	Concentration (μg/shrimp)	The day of molting	Percentage of molting
A (*Pertulaca oleracea* L.)	27.5	3-7 (n = 10)	60%; 40%
B (*Morus* sp.)	27.5	3-8 (n = 10)	50%; 50%
C (*Cycas revolut ae* Thumb)	27.5	5-8 (n = 10)	40% ; 60%
D (Positive control)	8.6	2-7 (n = 10)	60% ; 40%
E (Negative control)	0	7-14 (n = 10)	40% ; 60%

Table 3. Effect of pyhtoecdysteroid isolated from the selective plants on survival rate, growth and haemocyte number of shrimp

Treatment	Survival rate (%)	Growth		Haemocyte Increase
		Length increase (cm)	Weight increase (g)	x 10^4 (cell/mL)
A (*P. oleracea*)	86	0.8 (4.42 %), n=8	15 (15.90 %), n=8	260 ± 10, n=8
B (*Morus* sp.)	75	0.5 (2.26 %), n=7	11 (10.55 %), n=7	424 ± 8, n=7
C (*C. revolutae* Thumb)	25	0.4 (2.16 %), n=2	9 (8.73 %), n=2	164 ± 4, n=2
D (Positive control)	88	0.7 (3.32 %), n=8	14 (12.33 %), n=8	316 ± 8, n=8
E (Negative control)	43	0.3 (1.66 %), n=4	8 (6.89 %), n=4	40 ± 6, n=4

centration of 27.5 μg/shrimp could reduce the molting period of shrimp. It was able to shorten molting period of shrimp, 4 and 5 days earlier compared to the negative control.Application of phytoecdysteroid at this concentration also affect the survival rate and growth. From the three phytoecdysteroid source, *Portulaca oleracea* L gave the highest survival rate (86 %), followed by *Morus* sp. and *Cycas revolutae* Thumb with the survival rate value of 75% and 25%, respectively. Compared to the positive control (88%), the survival rate showed by the phytoecdysteroid was lower meanwhile, compared to the negative control (43%) was higher. The similar comparison to the positive and negative control was also displayed by the growth and survival rate of shrimp. The increase of length and weight of *Portulaca oleracea* L, *Morus* sp. and *Cycas revolutae* were 0.8 cm (4.42%) and 15 g (15.90%), 0.5 cm (2.26%) dan 11 g (10.55%) and 0.4 cm (2.16%) and 14 g (12.33%), respectively. Overall, *Portulaca oleracea* was the best ecdysteroid source in accelerating molting, resulted in the highest survival rate, growth and total haemocyte count in tiger shrimp.

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