

Effect of carica papaya leaf extract toward hatching and survival rate of Nile tilapia (*Osteochilus hasselti*) larvae

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Abstract. One of the problems in Nile tilapia (*Osteochilus hasselti*) harvesting is the unfulfilled availability of seed that is caused by low hatchability of eggs and larvae survival as well as high larvae abnormalities due to attacks by microorganisms such as fungi and bacteria. Papaya leaf has antifungal and antibacterial bioactive compounds. This study aimed to determine the effect of papaya leaf extract on egg hatchability, larvae abnormalities, and larvae survival. This study used an experimental method using Completely Randomized Design (CRD) with 4 treatments and 4 repetitions. The treatment doses of the papaya leaf extract that were tested were control (A), 0.75 mg/L (B), 1 mg/L (C), and 1.25 mg/L (D). The results showed that the addition of papaya leaf extract had a significant effect ($P < 0.05$) on egg hatchability, larvae abnormalities, and survival rate of *Osteochilus hasselti* larvae. The hatching rate results for each treatment were 76.50% (A), 79.50% (B), 83.50% (C), and 87.25% (D). The results of larvae abnormalities showed 9.16% (A), 7.24% (B), 5.09% (C), and 4.30% (D). Meanwhile, the results of larvae survival showed 73.87% (A), 76.13% (B), 77.25% (C), and 80.55% (D). Treatment D (1.25 mg/L) was the best treatment in this study, resulting in egg hatchability of 87.25%, larvae abnormality of 4.30%, and larvae survival of 80.55%.

1 Background

Nile tilapia (*Osteochilus hasselti*) is one of the freshwater fish commodities that has great potential to be developed into a superior product for aquaculture. One of the problems in cultivating *Osteochilus hasselti* is the inadequate availability of eggs and larvae. This is because *Osteochilus hasselti* eggs often do not hatch well [1]. Internal and external factors might cause failure to hatch the fish eggs. Internal factors include hormones, egg yolk volume, and mechanical work from the larva's activity from the egg's enzymatic work. Meanwhile, external factors include inappropriate media, mechanical disturbances such as shocks, low water quality, the presence of unfertilized eggs, and attacks by microorganisms such as fungi and bacteria [2-4]. One effort to increase egg hatchability is to prevent microorganism attacks by adding chemicals, such as Methylene blue, Malachite green, NaCl, or $KMnO_4$ [4, 5].

The use of synthetic chemicals can cause resistance to pathogenic microbes and cause environmental pollution [6]. Therefore, natural ingredients derived from plants are needed which are antifungal and antibacterial. So far, plant extracts used as antifungal and

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antibacterial ingredients in fish eggs include papaya leaves [7], betel leaves [8], pineapple leaves [9], cherry leaves [10], ketapang leaves [11], and many others.

Papaya leaves (*Carica papaya* L.) contain natural compounds such as alkaloids, triterpenoids, steroids, flavonoids, saponins, tannins, and quinones which have antifungal and antibacterial activity [12, 13]. Apart from that, papaya leaves also contain the papain enzyme which has a proteolytic activity that can be used as an immunostimulant, can maintain egg health, stimulate faster embryo growth, and increase fish immunity both at the larval and parent stages [12-15].

The use of papaya leaf extract in the field of cultivation has been carried out in several studies, including research on milkfish (*Chanos chanos* Forsk.) eggs which was produced the highest hatchability up to 89.94% with the lowest abnormality as much as 56% with 4 mL papaya leaf extract [15]. Meanwhile, research on gourami (*Sphaerichthys gourami*) fish eggs produced the highest hatchability up to 96% with the highest larvae survival up to 97% with 3000 ppm papaya leaf extract [13]. Then a study on punten carp (*Cyprinus carpio*) eggs produced the highest hatchability up to 89.5% with a survival up to 68% at a papaya leaf extract dose of 1 mg/L [7]. However, the use of papaya leaf extract in hatchery *Osteochilus hasselti* eggs has never been done. Therefore, this research was carried out to see the effect of papaya leaf extract in supporting the successful hatching of *Osteochilus hasselti* eggs.

2 Material and Method

This research was conducted at Fish Seed Centre Krueng Batee, Kuala Batee District, Southwest Aceh Regency, Aceh Province, Indonesia. The experimental method used Completely Randomized Design (CRD) with 4 treatments and 4 repetitions. The treatment concentration of the papaya leaf extract were control (A), 0.75 mg/L (B), 1 mg/L (C), and 1.25 mg/L (D) [7]. The papaya leaf was taken from Banda Aceh City, Aceh Province, Indonesia. The fresh papaya leaf was dried at room temperature for 5 days. The dried papaya leaf was then smashed into powder. As much as 200 g of dried papaya leaf powder was extracted using maceration with 800 mL of 70% ethanol for 3 days at room temperature. The macerate was then filtered and the filtrate was purified using a rotary evaporator [16]. The papaya leaf extract then was screened to identify phytochemical compounds that consisted of alkaloid, saponin, flavonoid, steroid, polyphenols, glycoside, tannin, quinone, and triterpenoid [17]. Next, the research continued with spawning Nilem fish (*Osteochilus hasselti*) using a semi-artificial method. The mature female *Osteochilus hasselti* was injected with 0.5 mL/Kg Salmon Gonadotropin Releasing Hormone (SGnRH) and domperidone and 0.3 mL/Kg for the male, then incubated for 9 hours [18]. Each 100 fertilized *Osteochilus hasselti* eggs were immersed with 4 treatments of papaya leaf extract for 20 minutes [7], and then transferred to the aquarium with an aerator as a hatching container. During the hatching process, random eggs of *Osteochilus hasselti* were collected to observe the embryogenesis process (cleavage, morulation, blastulation, gastrulation, organogenesis, and hatching) under a microscope every 2 hours [8]. Then calculated the fertilization and hatching rates using the following formula [19]:

$$\text{Fertilization Rate (\%)} = \frac{\text{Number of Fertilized Eggs}}{\text{Total Number of Fertilized Eggs}} \times 100 \quad (1)$$

$$\text{Hatching Rate (\%)} = \frac{\text{Total Number of Hatched Eggs}}{\text{Total Number of Fertilized Eggs}} \times 100 \quad (2)$$

Eggs that have hatched into larvae will be kept for 14 days in the aquarium. During the rearing period *Osteochilus hasselti* larvae will be fed with artemia. The larvae are ready to be fed with artemia after they are 34 days old, 5 times during the day every 2 hours [20]. At

the end of rearing *Osteochilus hasselti* larvae, a calculation will be carried out to determine the survival and abnormalities rate of the larvae using the following formula [19, 21]:

$$\text{Survival Rate (\%)} = \frac{\text{Total Number of Larvae} - \text{Total Number of Dead Larvae}}{\text{Total Number of Larvae}} \times 100 \quad (3)$$

$$\text{Abnormalities Rate (\%)} = \frac{\text{Total Number of Abnormal Larvae}}{\text{Total Number of Hatched Larvae}} \times 100 \quad (4)$$

The results of hatching, survival and abnormalities rate are continued to be analyzed using Analysis of Variance (ANOVA).

3 Results and Discussion

3.1 Phytochemical Screening

Based on the phytochemical screening, the papaya leaf extract in this research contained alkaloid, saponin, flavonoid, steroid, polyphenols, glycoside as shown in Table 1. These results could be different from other papaya leaf extracts from different resources. As phytochemical results of papaya leaf extract that was taken in Malang Regency showed it contained alkaloid, triterpenoid, steroid, flavonoid, saponin, and tannin [12]. Meanwhile in this research, it was negative results for tannin, triterpenoid, and quinone.

Table 1. Phytochemical results of papaya leaf extract.

Phytochemical Compounds	Results
Alkaloid	Positive
Saponin	Positive
Flavonoid	Positive
Steroid	Positive
Polyphenols	Positive
Glycoside	Positive
Tannin	Negative
Quinone	Negative
Triterpenoid	Negative

3.2 Embryogenesis Observation

The fertilization rate (FR) in this research was obtained 76%. The fertilization rate of Nilem fish (*Osteochilus hasselti*) eggs can reach 92.588.50%. Several factors that can affect the fertilization rate in fish are egg quality (egg maturity level), male sperm quality, environment or water quality including dissolved oxygen, pH, and temperature, as well as effect of human handling such as hormone injection dose. Fertilized *Osteochilus hasselti* eggs have

clear and transparent characteristics, while unfertilized eggs are cloudy, milky white [2], as shown in Figure 1.



Fig. 1. Fertilized (left) and Unfertilized (right) Egg of Nilem Fish *Osteochilus hasselti*

Observation of the embryogenesis phase of *Osteochilus hasselti* eggs lasted 204 hours. The embryonic development phase begins right after fertilization occurs. The embryogenesis phase consists of cell division (cleavage), morula, blastula, gastrula, and organogenesis, hatching into larvae. These observations were made by taking egg samples and then observed them under a microscope with a magnification [2]. On examination under a microscope, it was found that the development of the egg had entered the morula phase. This shows that the cleavage phase has passed and cannot be observed under a microscope, because this phase has taken place during the egg transfer process from the hatchery to the egg hatching laboratory.

Based on observations, 19 hours after the fertilized, it was found that some of the *Osteochilus hasselti* eggs had hatched into larvae in treatments D, C, and B, while in treatment A (control) there was no eggs had yet hatched. This shows that papaya leaf extract can speed up the hatching process of *Osteochilus hasselti* eggs [15, 20]. Based on the results of phytochemical tests in this study, papaya leaf extract was known contain alkaloid, saponin, flavonoid, steroid, polyphenols, and glycoside. Those compounds might be giving some affection toward the hatching process. Such as saponin, it can degrade the membrane or wall of egg cells so that the larvae in the eggs will emerge quickly. This saponin can damage the egg cell membrane so that there is a change in the structure of the egg cell wall which causes the fluid in the egg cell to come out [20]. However, on the other hand, high concentration of saponin also can cause the egg to die when the cells in the egg are not yet fully formed and not ready to hatch. Apart from that, the proteolytic enzyme content in papaya leaves can also speed up egg hatching. The proteolytic enzymes in papaya leaves work well, thereby encouraging faster embryo growth [15]. After 23 hours, the eggs were completely hatched in all treatments. Generally, *Osteochilus hasselti* eggs will hatch within 1-24 hours [23].

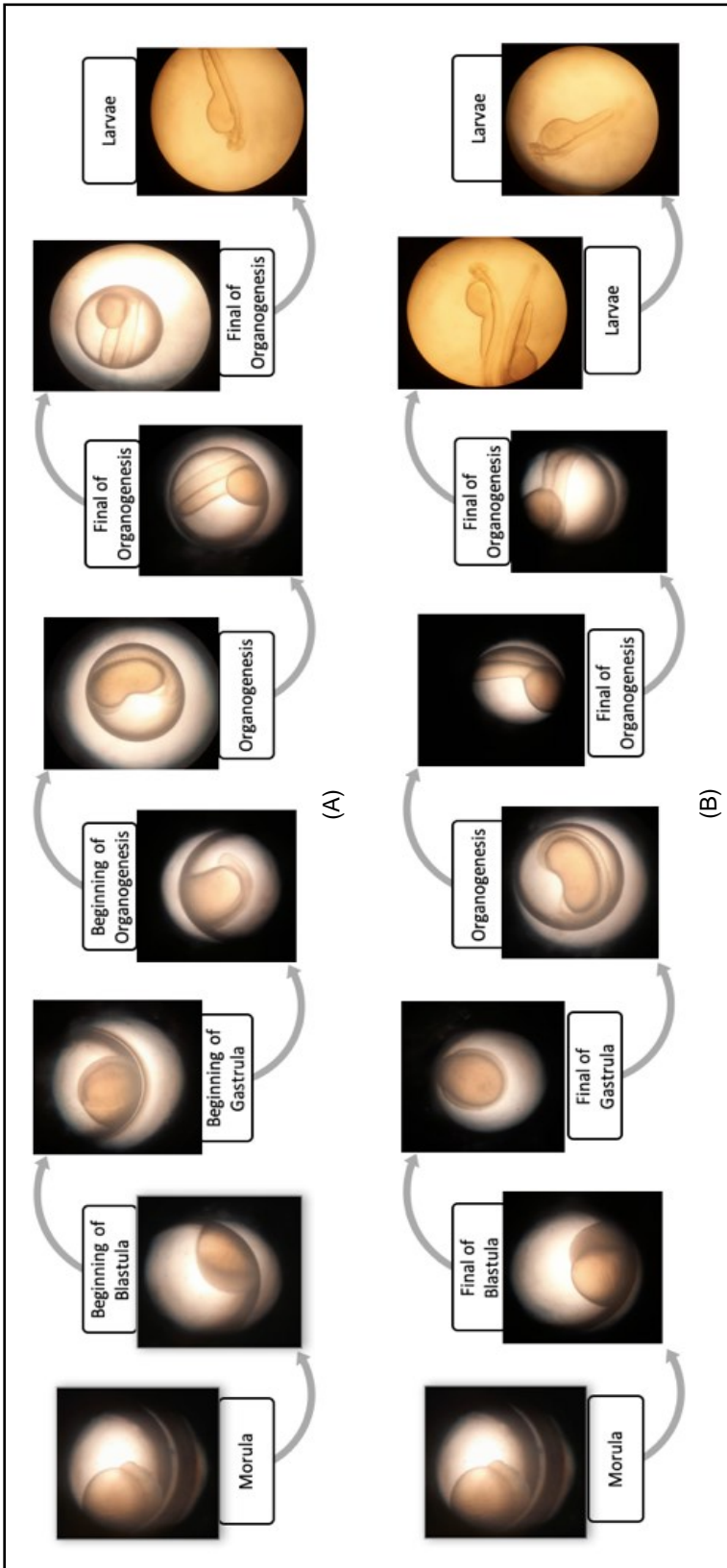


Fig. 2. Observation of Embryogenesis Phase. (A) Egg in Treatment A, (B) Egg in Treatment D.

3.3 Results of Hatching Rate, Larvae Abnormalities and Survival Rate

Hatchability is the rate at which fish eggs hatch. Observation of the hatchability of *Osteochilus hasselti* eggs begins when the eggs first hatch. The egg can be said to have hatched if the chorionic membrane breaks due to the movement of the larva's tail and body [18]. Based on the Homogeneity (HSD) test on egg hatchability, there are significant differences between each treatment (0.50), which can be seen in Table 2

Table 2. Results of hatching rate, larvae abnormalities, and survival rate of *Osteochilus hasselti*

Treatments	Hatching Rate (%)	Larvae Abnormalities Rate (%)	Survival Rate (%)
A (Control)	76,50 ± 1,29 ^a	9,16 ± 1,18	73,87 ± 2,44 ^a
B (0.75 mg/L)	79,50 ± 1,29 ^b	7,24 ± 1,28	76,13 ± 2,86 ^b
C (1 mg/L)	83,50 ± 1,29 ^c	5,09 ± 1,15	77,25 ± 1,95 ^b
D (1.25 mg/L)	87,25 ± 2,24 ^d	4,30 ± 1,14	80,55 ± 2,62 ^c

Note: Different superscript letters in the table above indicate a significantly different effect between treatments and the ± sign indicates the standard deviation.

The highest hatching rate of *Osteochilus hasselti* eggs was found in treatment D (1.25 mg/L) with 87.25% as the result. Meanwhile, the lowest hatching rate was obtained in treatment A without additional of papaya leaf extract, which showed 76.50%. It showed that papaya leaf extract could be able to prevent the eggs from being attacked by microorganisms such as fungi and bacteria which caused the eggs not to hatch because papaya leaf extract contains active compounds that has antifungal and antibacterial activity, such as flavonoid, saponin, tannin, quinone, steroid, triterpenoid, and alkaloid.

Other factors that influence the hatchability of eggs are fertilized eggs, inappropriate media, mechanical disturbances such as shaking mechanical work from the activity of the larva itself and enzymatic work produced by the egg, light intensity, salinity, hormones produced by the pituitary, and thyroid plays a role in metamorphosis, and egg yolk volume [2, 3]. Besides, immotile sperm and poor quality of spermatozoa and eggs related to the embryo development [24]. From the observation, the eggs that did not hatch were do undeveloped egg cell nucleus shown in the following Figure 3



Fig. 3. The Unhatched Egg of *Osteochilus hasselti*

Based on the results of the Duncan test ($P < 0.05$) on larval abnormality, treatment D did not show significantly different results from treatment C but was significantly different from treatment B and A. The overall result of the Duncan test for larval abnormalities can be seen in Table 2. Larval abnormalities in treatments D, C, and B which were given papaya leaf extract were lower compared to treatment A that was without any papaya leaf extract addition. This is thought to be because treatment without papaya leaf extract caused the eggs to be attacked by disease. Eggs infected with the disease will produce unhealthy and abnormal larvae. Many larvae produced from unhealthy eggs have abnormal body conditions in the form of bent bodies, incomplete body organs, and short. As part from that, the proteolytic enzyme content in papaya leaf can also stimulate faster embryo growth and help the embryo escape from the chorion layer during the hatching process, thereby reducing abnormalities in the larva [15]. The results of the observations showed that abnormal larvae were visible in the shape of the spine and tailbone which were bent as in the following Figure 4

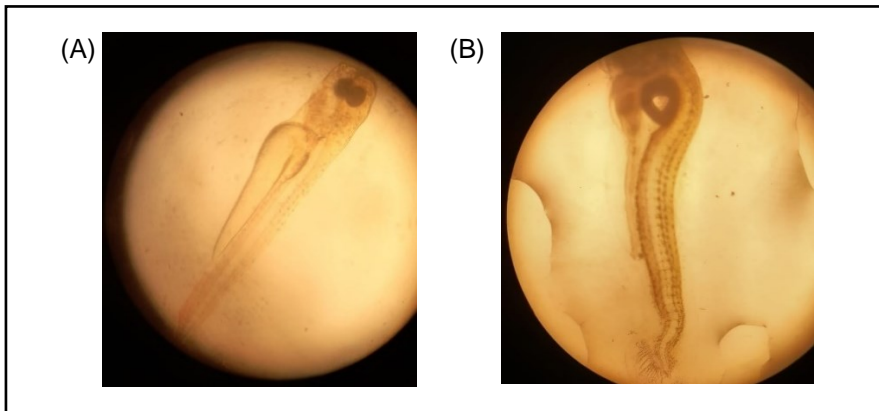


Fig. 4. Normal (A) and Abnormal Larvae (B) of *Osteichilus hasselti*.

Based on the Honestly Significant Difference (BNT) test ($P < 0.05$) on larval survival rate, treatment D showed different results to treatments C, B, and A. The results of the Honestly Significant Difference Test (BNT) on overall larval survival rate were seen in Table 2. Giving papaya leaf extract to *Osteichilus hasselti* eggs had an effect on larval survival because in treatments D, B, and C the level of larval survival was better compared to treatment A (control) which was without addition of papaya leaf extract. This could be because the leaf extract contains flavonoid compounds which can function as immunostimulants. Flavonoid compounds have the potential to work as immunostimulants and work on lymphokines produced by cells so that they will stimulate phagocytic cells to carry out a phagocytic response in forming the body's immune system against fish larvae [16].

In addition, larval survival was lower in treatment A (control) compared to treatments B, C, and D, because treatment A (control) had a high level of larval abnormality rate compared to other treatments. Abnormal larvae usually cannot survive long and will die. Abnormal larvae will spend a lot of energy obtained from food to improve body condition and will not be able to grow properly [26]. This condition causes the abnormal larvae to not survive long so the larvae will die [27].

4 Conclusion

Based on the research and ANOVA test, addition of papaya leaf extract had a significant effect ($P < 0.05$) on hatching, abnormalities, and survival rate of Nile tilapia (*Osteochilus hasselti*) larvae. Treatment D (1.25 mg/L) was the best treatment that produced the highest hatchability, as much as 87.25%, the lowest larvae abnormality, as much as 4.30%, and the highest larvae survival rate, as much as 80.55%.

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