Preliminary study of dietary *Muntingia calabura* leaf on the hematology status of *Clarias* sp

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Abstract. *Muntingia calabura* has antibacterial, antioxidant, and anti-inflammation properties including the leaf and fruit, but has not been explored its effects as feed additive on the hematology status of fish. This study was conducted to determine the effect of different dose of *M. calabura* as a feed additive on the hematology profile of *Clarias* sp. The research design used in this study was a complete randomized design with five treatments and four replications. Five types of diet were prepared by mixing dried powder *M. calabura* leaf with commercial feed manually, using egg white as a binder. The doses of *M. calabura* in feed were 0 g kg\(^{-1}\) (KN), 5 g kg\(^{-1}\) (M1), 10 g kg\(^{-1}\) (M2), 20 g kg\(^{-1}\) (M3), 40 g kg\(^{-1}\) (M4). The fish was fed three times a day for 14 days. The results showed there was a change in the total red blood cells (RBC), total white blood cells (WBC), haemoglobin (Hb), and hematocrit (Hc) levels during treatments. In the end of the observation, the highest WBC were in treatment M2 (169 ± 21.378 x 10\(^7\) cells mL\(^{-1}\)), while the highest RBC was in treatment M1 (365 ± 118.216 x 10\(^7\) cells mL\(^{-1}\)). The highest Hb and Hc level were occurred in treatment M3 (8.600 ± 0.265 g dL\(^{-1}\)) and treatment M4 (65.333 ± 9.609 %), respectively.

1. Introduction

Disease outbreak in aquaculture caused by the imbalanced interactions between pathogens and the environment. The quality of the host (fish) have been contributed to the security of disease includes immunity level, nutritional status, genetic factors, age, and strain. Various methods have been developed to improve the quality and sustainability of aquaculture production, both preventive and curative activity. Some of the prevention methods uses are vaccines [1], probiotics [2], prebiotics [3], and immunostimulants from medicinal plant extracts [4]. Some researchers have reported that the application of the vaccine in aquaculture provides good protection against disease, but its application is relatively expensive and very specific to certain pathogens [5]. The efficacy of providing probiotics was affected by the availability of indigestible fibres (prebiotics), the ability of attachment, and colonization in the digestive tract of organism target [6]. Meanwhile, the efficacy of prebiotics is highly dependent to support its complement (probiotics) performance in the digestive tract [3].

Disease control in aquaculture can be carried out by improving the immune system of fish through prophylactic action by administrating the immunostimulants. Immunostimulants are natural or chemical substances that stimulate the immune system, whether specific (vaccine or antigen) or non-specific immunity [7]. The use of natural ingredients is one of the potential preventive measures to be developed because it has a wide spectrum of activities, relatively low cost, and environmentally friendly [8]. Various studies have successfully reported that the use of medicinal plants increase the...
immune system, show antimicrobial, anti-stress properties, and are proven to be able to improve the immune system, growth, feed efficiency, and nutrient digestibility, both in fish [8, 4] and crustacean groups [9, 10].

*Muntingia calabura* or “Jamaican cherry” or in Indonesia known as Kersen is a type of native plant to South Mexico, South America, Central America, Trinidad, and St. Vincent. This plant also grows widely in warm regions such as India, and Southeast Asia such as Malaysia, Indonesia, and Philippines [11]. In Indonesia, this plant is classified as a garden plant or found as a wild plant. This type of plant has great potential as a medicinal plant but has not been used optimally. The leaves of *M. calabura* are known to have phytochemical components such as glycosides, flavonoids, tannins, flobatanins, terpenoids [12], sterols, and saponins [11]. The fruits have anti-inflammatory, and antioxidant activity, while the leaves are reported to have anti-inflammatory activity [13], antibacterial [14], and anti-proliferative, and antioxidant [15]. Testing of antibacterial activity showed that the leaf extract from *M. calabura* was able to inhibit the growth of a number of bacteria such as *Corynebacterium diphtheria*, *Staphylococcus aureus* (ATCC 25923), *S. epidermis*, *Bacillus cereus*, *Proteus vulgaris*, *Kosuria rhizophila*, *Shigella flexneri*, *Escherichia coli* (O 157), *Salmonella typhii*, and the pathogenic bacteria *Aeromonas hydrophila* in fish [16]. Buhian et al. [11] also reported that *M. calabura* leaf and stem extracts were shown to inhibit the growth of *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *C. albicans* (fungi).

The various potentials possessed by *M. calabura* plants have not received much attention, and further scientific exploration. The active compounds and properties possessed by *M. calabura* are very potential to be developed as a pharmaceutical ingredient in aquaculture to increase the immune response of fish. Application of *M. calabura* leaf especially for *Clarias* sp. cultivation has not been studied scientifically. Therefore, this study was conducted as a first step to determine the effect of different dose of *M. calabura* as feed additive on the hematological profile of *Clarias* sp.

2. Methods and Materials

2.1. Materials

The leaves of the Jamaican cherry (*M. calabura*) used in this study were collected from their natural habitat around Bangka District, Bangka Belitung Island. The catfish used is a fish with a weight of 12.348 ± 2.300 g and a length of 12.820 ± 0.691 cm, originating from commercial fish hatcheries around Bangka District, Bangka Belitung Island. Meanwhile, the type of fish feed used in this study was commercial feed with 30% protein content.

2.2. Culture condition and experimental design

Before the start of the feeding trial, experimental fishes were acclimatized for two weeks in the rearing tanks and fed twice a day. The experimental design was a completely randomized design with five treatments (Table 1) and four replications.

| Table 1. Experimental treatment of different dose of *M. calabura* |
|---------------------------------------------------------------|
| **Treatments** | **Descriptions** |
| Control | Fed by commercial feed without the addition of *M. calabura* leaf |
| M1 | Fed by commercial feed with the addition of *M. calabura* leaf 5 g kg⁻¹ |
| M2 | Fed by commercial feed with the addition of *M. calabura* leaf 10 g kg⁻¹ |
| M3 | Fed by commercial feed with the addition of *M. calabura* leaf 20 g kg⁻¹ |
| M4 | Fed by commercial feed with the addition of *M. calabura* leaf 40 g kg⁻¹ |

For feeding experiments, fishes were stocked at tank (60×35×30 cm³) with stocking density of 10 fishes/tank. The tank was covered with black plastic cover to reduce the light intensity, and on the top was covered with mesh to prevent fish jumping out. The fish were starved for one day before feeding trials, and then weighed to get the initial weight. Feeding experiment was conducted for 14 days in a continually aerated system. Fishes were fed three times a day by satiation feeding method. Water
quality was maintained stable through feeding period. Residual feeds and fish feces that accumulates in the tank were removed by siphoning and water exchange.

2.3. *M. calabura* leaf powder and diets preparation

The leaves used in this study were dark green, not brownish, and not yellowish leaves with a length of 4-6 cm and a width of 3-4 cm. *M. calabura* leaves were separated from the impurities such as stems and fruits. The leaves were washed in running water and dried by oven at 45°C for 48 hours. Dried leaves were crushed into small particles using grinder and then sieved with mesh size of 60. The resulting powder was stored in a dark-airtight container before use.

Five type of diet were prepared by mixing commercial feed with 30% protein content and *M. calabura* leaf powder. Doses of *M. calabura* leaf powder were 5 g Kg\(^{-1}\), 10 g Kg\(^{-1}\), 20 g Kg\(^{-1}\), dan 40 g Kg\(^{-1}\) respectively for each diet. A total of 2% (v/w) of egg white binder was added with 6% (v/w) water and then mixed homogeneously. Then, *M. calabura* leaf flour was added into the previously homogeneous mixture. Finally, the commercial feed was added and mixed until blended. Afterwards, the mixed diet was air-dried and stored at 4°C before use.

2.4. Measurement of haematology parameters

The haematological profile observed consist of total erythrocytes (red blood cells/RBC), total leucocytes (white blood cells/WBC), haematocrit (Ht) levels, and haemoglobin (Hb) levels. Prior to the observation, fish blood was taken first using a 1 mL syringe filled with anticoagulant (Na-citrate 3,8% w/v), and then placed in 1.5 mL microtube. RBC and WBC were measured following Blaxhall and Daisley [17] by using haemocytometer at 400 times magnification on both grids’ sides. For RBC, blood was pipetted by using blood-diluting pipettes up to a scale of 1. Then, Hayem's solution then was added up to a scale of 101 (erythrocytes, proportion of dilution 1:100). Meanwhile for WBC, blood was pipetted up to a scale of 0.5, then Turk’s solution was added up to a scale of 11 (leucocytes, proportion of dilution 1:10). After that, move the pipettes in a figure-8 motion a few times for 3-5 minutes until the solution and blood mixed. The first two drops of the blood solution in the pipette were discarded, then the blood was dripped into a Neubauer-type haemocytometer and covered with cover glass. The RBC were count by using light microscope at 400 x magnification.

Hb levels were measured using a Sahli Haemometer [18]. The blood sample was pipetted using a Sahli pipette up to a scale of 20 mm\(^3\). Then, prepare Hb meter tube with 0,1 N HCL until scale of 10 (red). The blood sample was put into the opal glass plate comparator, stirred gently, and lefted for 3-5 minutes. Some drops of distilled water were added gradually into the mixture until the colour change into the same colour of two-coloured rods in Hb meter indicator. The Hb level was defined by aligning the volume of mixture with the line scale g% (yellow) which indicates the amount of Hb per 100 mL of blood. Haematocrit levels reflect the number of blood cells in blood fluids. Haematocrit levels were determined by comparing the length of sedimented erythrocytes to the total volume of blood in a capillary tube for micro haematocrit [17]. The blood sample were introduced into the tube to about 50 mm depth. The bottom of tube was sealed with crystoseal wax and left in the vertical position for 1h before being read. The tubes must be kept absolutely vertical otherwise the rate of sedimentation would be affected.

2.5. Data analysis

Data were tabulated by using Ms. Excel 365 and presented as mean and deviation by using descriptive statistics.

3. Results and discussion

3.1. Results

The WBC and RBC were increased during the feeding trial (Figure 1 and 2). The highest WBC on the 7\(^{th}\) and 14\(^{th}\) day of feeding trial were found in treatment M2, i.e. 168.850 ± 41.434 x 10\(^7\) cells mL\(^{-1}\), and 169.000 ± 21.378 x 10\(^7\) cells mL\(^{-1}\) respectively, followed by treatment M1, M4, M3, and control...
Meanwhile, the RBC showed a different result. On 7th day of feeding, treatment M1 has the highest RBC, i.e. $345.073 \pm 116.843 \times 10^7$ cells mL$^{-1}$ followed by treatments M3, M2, M4, and control. Whereas, on the 14th day of feeding, the highest RBC also found in treatment M1 ($365 \pm 118.216 \times 10^7$ cells mL$^{-1}$) but followed by treatment M2, M3, M4, and control (KN).
Haematocrit (Hc) levels during feeding trial were showed in Figure 4. Hc level in some treatments i.e. treatment M1 and M4 tend to be increase for two weeks feeding trial. On the 7th day of feeding period, the highest Hc levels were found in treatment M4 (55.192 ± 23.607%), but in treatments KN, M2, and M3, Hc level showed a slight decrease. On the 14th day of trial, all treatments showed an increasing of Hc levels except for control which tend to be stable during experiment. M1 and M4 had the highest value among the treatments i.e. 65.333 ± 9.609% and 65.333 ± 10.017%, respectively.

Figure 4. Haematocrit (Hc) level of Clarias sp. during feeding period

3.2. Discussion
The use of plants or plant parts (leaves, stems, roots, seeds, flowers) has been widely used for disease prevention in aquaculture as a substitute for chemicals. The use of plant materials has several advantages such as easy preparation, relatively inexpensive when compared to chemicals, minimal side effects to organisms and the environment. Several studies have reported the effect of supplementation of plant materials on the immune response and growth of aquatic organisms. Natural ingredients from some plants have been reported to be able to increase the immune system of fish, have antimicrobial properties, stimulate appetite and act as anti-stress agents [8]. Hematological parameter has been used to evaluate the immune response and physiological status of the animal to the environment stressors [19, 20]. Their changes depend on the fish species, age, nutrition, and diet, the cycle of sexual maturity, and health condition [21,22,23].

The medical herbs utilization in aquaculture has potential as antibiotics alternative, and as immunoprophylactic [4]. Immunostimulation is one of promising tools in aquaculture where treatment by injections are difficult, labor intensive and stressful to the fish and where using of antibiotics repeated poses a problem of developing drug resistant strains of pathogens [8]. Thus, oral administration in this study has been chosen as useful alternative method for mass administration to fish of all sizes. Vidal et al. [7] reported that increasing the immune system through dietary supplementation could activate the molecules and cells immune system (lymphocytes, plasma cells, granulocytes, and macrophages) in mucosal tissue of the intestine. Later, Lazado and Caipang [24] call it as gut-associated lymphoid tissue (GALT) which is found in the digestive tract of the test fish.

In this present study, WBC and RBC tend to be increase during the rearing period (Figures 1 and 2). The number of WBC and RBC in the fish fed with diet containing different levels of M. calabura leaf have showed a higher value than the control. Harikrishnan et al. [22] also reported a significant increase in the number of leucocytes in goldfish supplemented with mixed dietary herbal compared to controls. The study stated that fish supplemented with 400 and 800 mg Kg⁻¹ of mixed dietary herbal (Azadirachta indica, Ocimum sanctum, and Curcuma longa) showed faster recovery after the challenge test with Aeromonas hydrophila. Thanikachalam et al. [25] reported that African catfish (Clarias gariepinus) fed with garlic skin flour for 20 days significantly increased the number of leukocytes and erythrocytes compared to controls. The increase in erythrocytes is thought to be an
effect of immunostimulant administration. Nya and Austin [26] and Sahu et al. [27] also found an increase in erythrocytes in Labeo rohita and rainbow trout fish (Oncorhynchus mykiss) which were fed by diet supplemented garlic. Bahrami et al. [28] found a different result that dietary wood betony Stachys lavandulifolia for 8 weeks on different levels to Cyprinus carpio have no no significant differences in hemoglobin, hematocrit, and white blood cell (WBC) counts, while red blood cells (RBC) counts showed significant declining trend by increasing the level of the plant extract both control and treatments.

Other studies have reported that supplementation of probiotic and mixed diet (a mixture of probiotics and herbs) could increase the WBC, RBC, and haemoglobin levels at week 6 of maintenance in Oplegnathus fasciatus infected by Edwardsiella tarda [29]. The increase in the amount of WBC reflects the health status of the fish, where leukocytes are kind of blood cells that plays a role in fish immunity. According to Vidal et al. [7] the main components in the nonspecific immune system in fish includes leukocytes consist of lymphocytes, macrophages, and granulocyte cells. Whyte [30] explains that granulocyte and macrophage cells play a role in producing bioactive components in the process of pathogen destruction. Meanwhile, Vidal et al. [7] reported that the process of eliminating these pathogens will initiate other cellular processes such as oxygen radical production, inflammation, and activation of the adaptive immune system.

The increasing of WBC seems to have correlate with composition of M. calabura phytochemical in experimental diet, such as flavonoid and saponin. Flavonoid can be a bio catalyst to produce leukocytes and stimulate leukocytes as nonspecific cellular immunity. The increasing of RBC in this study also related to physiological stress caused by some phytochemical composition of herb in fish diet. Flavonoid has a positive effect of reducing RBC hemolysis, by protecting biological membranes of RBC from free radical which induces oxidative damage [31]. Some investigators reported that the antioxidants present in the plant extract might trigger erythropoiesis and antioxidant activity [32,33]. Roy and Munshi [34] found that there was an increasing in the RBC, haemoglobin, and packed cell of Anabas testudineus after exposure to 5 mg/L of saponin for 24 h. Meanwhile, saponin has been reported to have a toxic effect at high level on fish [35]. It was observed that pure saponin at high levels caused severe stress and mortality to tiger shrimps, following exposure to 200 mg L⁻¹ [36].

In this present study, haemoglobin levels during trial (Figure 3) tend to be slightly decrease. The content of haemoglobin (Hb) in the blood plays an important role as an element of oxygen transport to body tissues. Misra et al. [37] reported that it is scientifically acceptable that under stressful condition, there will be an increase in releasing of immature RBC from head kidney as a haematopoietic tissue, which can cause elevation in haemoglobin concentration in blood. Hematocrit levels show the ratio of the number of red blood cells to blood volume. The hematocrit levels of treatment M1 and M4 on the 7th day of maintenance showed higher values among treatments. On the 2nd weeks of trial, the haematocrits levels also showed an increasing value than the control. This increase indicates a good fish health status where the increase is closely related to an increase in the number of erythrocytes in the body. Study by Talpur and Ikhwanuddin [38] found that the RBC, WBC, haematocrits, and hemoglobin level of Lates calcarifer fed by different levels of garlic (Allium sativum) showed no difference following garlic treatments, but show enhancement compare to control (without garlic supplementation). Later, Talpur and Ikhwanuddin [39] declare that dietary Azadirachta indica significantly improve RBC, WBC, and haematocrit both pre and post challenge test with Vibrio harveyi. Then, Talpur et al. [40] reported that RBC, WBC, haemoglobin, and haematocrits were significantly higher in L. calcarifer fed by ginger as feed additive compared to controls including pre- and post-challenge with V. harveyi.

4. Conclusion
Dietary M. calabura in different dose could affect the haematological profile of Clarias sp. The number of leucocytes, erythrocytes were increase during observation. Meanwhile, levels of haematocrit and haemoglobin were slightly decrease during observation.
5. References
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