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RESPONSE IMMUNITY OF GOLDFISH (*Carassius auratus*) INFECTED WITH *Aeromonas hydrophila* BACTERIA AND POST-TREATMENT WITH PROPOLIS SOLUTION

M. Riswan[1*], Iesje Lukistyowati[2], Henni Syawal[2], Ronal Kurniawan[2,4], Rianti Putri[3], Mega Novia Putri[2]
[1]Department of Marine Science, Faculty of Marine and Fisheries, Universitas Syiah Kuala Banda Aceh 23111 Indonesia
[2]Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Riau, Pekanbaru, 28293 Indonesia
[3]Department of Marine Science, Faculty of Fisheries and Marine, Universitas Riau, Pekanbaru, 28293 Indonesia
[4]Center for Peatland and Disaster Studies, Universitas Riau, Pekanbaru, 28293 Indonesia
*m.riswan@usk.ac.id

ABSTRACT

This study aims to determine the immunity response of *Carassius auratus* infected with *Aeromonas hydrophila* bacteria and post-treatment with propolis solution by measuring total leukocytes and phagocytosis index. The method used is experimental by applying a completely randomized design (CRD) with five treatments and three replications. The treatments were Kn (not infected with *A. hydrophila* and not treated with propolis), Kp (infected with *A. hydrophila* but not treated with propolis), while fish infected with *A. hydrophila* were treated with propolis doses P1 (700 ppm), P2 (800 ppm), and P3 (900 ppm). Treatment was done by injecting 0.1 ml of propolis into fish infected with *A. hydrophila* intramuscularly. The test material was 150 fish of 8-10 cm in size. The results showed that propolis significantly gave an immune response to the *C. auratus* infected with *A. hydrophila* ($p<0.05$). The 800 ppm propolis dose was the most effective, as indicated by a total leukocyte count of 3.70×10^4 cells/mm^3, a phagocytosis index value of 28.33%, and a survival rate of 83.33%. This study highlights that propolis has potential as a natural immunostimulatory agent in enhancing non-specific defense mechanisms in *C. auratus* infected with pathogenic bacteria.

Keywords: Leukocytes, Phagocytosis, Immunity, *Carassius auratus*, *Aeromonas hydrophila*

1. INTRODUCTION

Goldfish (*Carassius auratus*) is a type of freshwater ornamental fish widely cultivated because of its high economic value. However, intensive culture systems can lead to an increased risk of disease attack, one of which is by the pathogenic bacterium *Aeromonas hydrophila*. This bacterium is known as the main cause of Motile Aeromonas Septicemia (MAS) disease that causes symptoms such as ulceration, abdominal swelling, and mass mortality in fish[1-2]. When an infection occurs, the fish's innate immune system will respond through increased leukocyte activity and phagocytosis, which are the main components of non-specific defense. Total leukocytes reflect the immunological status of the organism, while the phagocytosis index indicates how active phagocytic cells, such as neutrophils and monocytes, are in engulfing and destroying pathogenic microorganisms[3-4].

The use of antibiotics in the treatment of bacterial infections in aquaculture has been widely questioned due to side effects, such as antimicrobial resistance and accumulation of chemical residues in the

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environment. Therefore, alternative approaches utilizing natural materials such as propolis are gaining attention. Propolis, a resinous substance collected by honeybees from various plants, is known to have antibacterial and antioxidant properties and can boost the immune system of fish[5].

Some studies have shown that propolis in feed can increase the number of leukocytes, strengthen phagocytic activity, and increase fish resistance to *A. hydrophila* infection[6]. Especially in comet fish, propolis as an immunostimulant has increased phagocytic ability and accelerated post-infection recovery[1,7]. However, few studies have comprehensively examined changes in hematological parameters such as total leukocytes and phagocytosis index in comet fish after infection and propolis treatment. Therefore, conducting this study to reveal the potential of propolis as a natural immunotherapeutic agent through an immunological hematology approach in goldfish is important.

2. RESEARCH METHOD

Time and Place

This research was conducted from January to April 2023 at the Laboratory of Fish Parasites and Diseases, Faculty of Fisheries and Marine Sciences, Universitas Riau, Pekanbaru.

Method

The method used is a complete random design experimental method (CRD) with five treatments and three repetitions. The treatments carried out in this study are:

Kn : Fish not infected with *A. hydrophila* and without propolis treatment.
Kp : Fish infected with *A. hydrophila* and without propolis treatment.
P1 : Fish infected with *A. hydrophila* and treated with propolis solution at a dose of 700 ppm.
P2 : Fish infected with *A. hydrophila* and treated with propolis solution at 800 ppm.
P3 : Fish infected with *A. hydrophila* and treated with propolis solution at a dose of 900 ppm.

Procedures

Preparation of Propolis Solution

The doses used are as follows: a) A dose of 700 ppm for 2 mL requires: 140 µl propolis + 1860 µl aquabides. b) Dose of 800 ppm for 2 mL requires: 160 µl propolis + 1840 µl aquabides. c) Dose of 900 ppm for 2 mL requires: 180 µl propolis + 1820 µl aquabides. The propolis solution that has been mixed is then ready for use.

Infection of Test Fish with *A. hydrophila* Bacteria

Inoculants from TSA media were aseptically transferred to GSP media, then incubated at 28ºC for 18-24 hours. After incubation, pure isolates were obtained, cultured on TSA media, and incubated. The colonies were then re-inoculated in TSB media and incubated in an incubator for 18-24 hours. Before bacterial infection, bacterial dilution was carried out to obtain a bacterial density of 10^8 CFU/mL. Immersing fish in a fiber tub measuring 60 x 50 x 35 cm^3, given *A. hydrophila* bacteria. The bacterial suspension is 6.6 L dissolved in a fiber tub with a 10^8 CFU/mL density.

Treatment with Propolis Solution

Treatment was carried out by injecting the test fish intramuscularly using a syringe with a 45º slope with different doses, namely doses of 700 ppm, 800 ppm, and 900 ppm. The fish injection process must be under aseptic conditions. Fish treated are put into aquariums to be maintained and given aeration, then continued maintenance until day 14 post-infection. During maintenance, the fish were fed at satiation three times a day.

Collecting Fish Blood

Blood collection of test fish was carried out three times, namely at the beginning of maintenance before being treated, the second at 10 hours post-

infection, and the third on day 14 post-treatment. Blood was taken from three test fish for each treatment.

Total Leukocyte Observation

Calculating the total leukocyte count begins by drawing the blood sample into a leukocyte pipette up to the 0.5 mark. Subsequently, Turk's solution is added until it reaches the 11 mark. The contents are mixed thoroughly by agitating the pipette in a figure-eight pattern for approximately five minutes. Following homogenization, two drops of the blood sample were discarded to eliminate air bubbles. The remaining sample was then placed onto a hemocytometer and covered with a cover glass. Microscopic observation was carried out at 400x magnification. The total leukocyte count was determined by examining four large squares of the hemocytometer, using the appropriate calculation formula[8]:

∑leukocytes =∑total of leukocytes x 50 cells/mm^3

Phagocytosis Index Observation

The procedure for determining the phagocytosis index refers to Siwicki et al.[9], namely, a total of 50 µL of blood sample was transferred into a microtube, followed by the addition of 50 µL of a *Staphylococcus aureus* bacterial suspension at a concentration of 10^7 cells/mL. The mixture was then thoroughly homogenized and incubated for 20 minutes under controlled conditions. Furthermore, 5 µl was taken to make blood screw preparations on a glass object and then dried. After fixation in 95% methanol solution for 5 minutes, the dried slides were immersed in Giemsa solution for 15-20 minutes. Subsequently, the slides were rinsed with running water to eliminate excess stain and then air-dried. Once dry, the preparations were examined under a microscope. The percentage of phagocytic cells was determined by counting up to 100 cells and identifying those that had engulfed bacteria. The calculation was performed using the following method:

$$\text{Phagocytosis Index} = \frac{\sum \text{phagocytic cells}}{100} \; x \; 100\%$$

Survival Rate

According to Effendi[10], the survival rate was determined using the formula:

$$SR = \frac{Nt}{No} x \; 100\%$$

SR = Survival rate (%)
Nt = The number of fish that remained alive after the study
No = The number of fish that were alive at the start of the experiment

Data analysis

Data from hematological measurements were collected and tabulated in the Table. Next, it was analyzed statistically using the SPSS version 26 application and One-Way ANOVA. If the analysis results showed an influence, a further test was carried out using Student Newman Keuls (SNK).

3. RESULT AND DISCUSSION

Total Leukocytes

Total leukocyte counts were conducted to see changes in total leukocytes of fish infected with *A. hydrophila* and post-treatment with propolis solution. The mean total leukocytes can be seen in Table 1.

Table 1. Total leukocytes of Goldfish (*C. auratus*)

Observation	Treatment	Total Leukocytes (×10^4 cell/mm^3)
10 hours fish infected with *A. hydrophila*		7.21 ± 1.12
Day 14 post-propolis treatment	Kn	3.58 ± 0.90^b
	Kp	7.20 ± 1.28^d
	P1	3.93 ± 0.56^c
	P2	3.70 ± 0.73^b
	P3	3.35 ± 0.85^a

Description: Superscript on the same line indicates there is an influence between treatments (P<0.05)

Based on Table 1, total leukocytes increased after 10 hours of *A. hydrophila* infection to 7.21×10^4 cells/mm^3. Total leukocytes of fish after *A. hydrophila* infection indicate the body's resistance to foreign bodies, namely *A. hydrophila* that infects the fish body, resulting in an immune response characterized by an increase in total leukocytes. Kurniawan et al.[11] found that the increase in total leukocytes in fish is a defense response of the fish body against existing antigens. Total leukocytes changed after treatment with propolis solution on day 14 post-treatment, ranging from 3.35-72.0× 10^4 cells/mm^3. The decrease in leukocyte count occurs because leukocyte cells become active and migrate out of the blood vessels towards the infected tissue area[12].

The total leukocytes in the Kp treatment were almost the same as the value at 10 hours post-infection, indicating that without propolis treatment, *A. hydrophila* infection continued to trigger an immune response characterized by sustained leukocytosis. This confirms that *A. hydrophila* is pathogenic and causes systemic infections that do not subside without intervention. The Kn treatment showed significantly lower total leukocytes, reflecting the normal condition of healthy fish without infection. This value corresponds to the normal leukocyte range in freshwater fish, as Pattipeiluhu et al.[13] reported, which is about 2.9-4.1×10⁴ cells/mm³.

All three propolis treatments (P1, P2, P3) showed a decrease in total leukocytes compared to Kp. This decrease indicates that propolis effectively relieves *A. hydrophila* infection, thus reducing the need for an excessive immune response. Treatments P2 and P3 experienced total leukocytes close to normal conditions because the fish are trying to restore body conditions to their normal conditions, but also due to the influence of active compounds in propolis that can reduce total leukocytes. The effectiveness of propolis in normalizing leukocyte counts has been proven in various studies, including in goldfish and Tilapia, which showed increased resistance to *A. hydrophila* after propolis supplementation[4]. Propolis contains bioactive compounds such as flavonoids, phenolics, and caffeic acid esters known to have anti-inflammatory, antibacterial, and immunomodulatory properties[14].

Phagocytosis Index

Phagocytosis refers to the mechanism by which phagocytic cells, such as monocytes and neutrophils, engulf and remove microorganisms or foreign particles from the body. Calculation of the phagocytosis index is done to see the ability of leukocyte cells to eat foreign objects, especially pathogenic bacterial attacks on fish infected with *A. hydrophila,* and post-treatment with propolis solution. The percentage of phagocytosis index can be seen in Table 2.

Table 2. Percentage of phagocytosis index

Observation	Treatment	Phagocytosis Index (%)
10 hours fish infected with *A. hydrophila*		36.66 ± 1.54
Day 14 post-propolis treatment	Kn	28.66 ± 1.15^b
	Kp	36.66 ± 0.57^c
	P1	24.66 ± 1.52^a
	P2	28.33 ± 1.52^b
	P3	27.66 ± 1.15^b

Description: Superscript on the same line indicates there is an influence between treatments ($P<0.05$)

Based on Table 2, after 10 hours, the fish infected with *A. hydrophila* showed a relatively high phagocytosis index of 36.66%, which indicates an active innate immune response in fish. Phagocytosis is one of the main defense mechanisms in the fish immune system, which involves the activity of macrophages and neutrophils to

engulf and destroy pathogens. Pereira et al.[15], bacterial infections such as *A. hydrophila* can trigger an increase in phagocytic activity as an initial response to control the spread of pathogens in the fish body. This increase occurs due to the recognition of pathogen-associated molecular patterns (PAMPs) by receptors on immune cells, which triggers the production of proinflammatory cytokines and the activation of phagocytes. The phagocytosis index changed after treatment with propolis solution on day 14 post-treatment, ranging from 24.66-36.66%.

The treatment Kp remained high at 36.66%, equivalent to the value at the start of infection. This suggests that without propolis treatment, the phagocytic response remained active, possibly as an attempt by the body to continue fighting the unresolved infection. In contrast, the Kn treatment showed a decrease in the phagocytosis index to 28.66%, which can be interpreted as a normal condition in healthy uninfected fish, as supported by the research of Biller & Takahashi[16], healthy fish tend to have lower phagocytic activity, due to the absence of pathogen stimulation. P2 and P3 treatments showed phagocytosis index conditions close to normal Kn, indicating the physiological immune system recovery process. This suggests that propolis can modulate phagocytic activity to be more controllable, by suppressing excessive inflammatory reactions while still maintaining the effectiveness of immunity.

The flavonoids, phenolic acids, and caffeic esters in propolis play a role in immunomodulatory and anti-inflammatory activities that support the efficiency of the phagocytic response[5]. Edrees et al.[7] found that propolis can balance the gene expression of proinflammatory cytokines, thus not only suppressing the clinical symptoms of infection and stabilizing the function of phagocytic cells.

Survival Rate

Fish survival can be used to indicate whether the propolis solution affects fish health or does not affect fish healing. Observations on the survival of fish during the study can be seen in Figure 1.

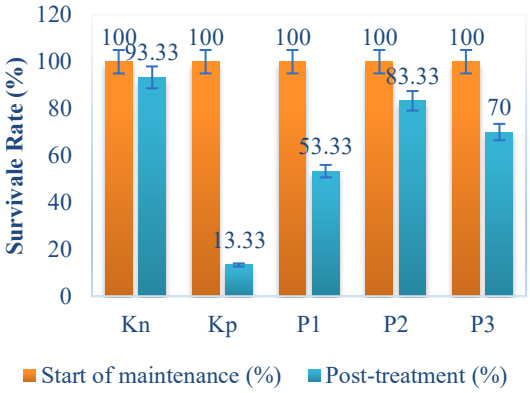

Figure 1. Survival rate

Based on Figure 1, all treatments showed an initial survival rate of 100%, indicating the fish's healthy and adaptive condition at the beginning of rearing. However, significant differences emerged after the infection and treatment period. The Kp treatment, which was infected with *A. hydrophila* without treatment, showed a sharp decrease of 13.33%. This indicates that *A. hydrophila* bacteria continue to grow to produce more severe clinical symptoms and cause fish to be stressed and die easily.

Souza et al.[17] said that bacterial infection is one of the causes of free radicals, resulting in mortality in fish. The Kn treatment found the highest survival percentage, which amounted to 93.33%. The Kn treatment had fish that died due to changing environmental conditions, so some fish were weak; therefore, the survival rate in the Kn treatment was not 100%.

The next highest survival rate was followed by the P2 treatment, which amounted to 83.34%. The high survival rate in P2 is due to the presence of active compounds in propolis that can increase the immune system, so that the immune system of fish, when infected with *A. hydrophila* bacteria, is in a strong condition and can maintain its survival. The effectiveness of propolis in increasing survival can be attributed to the content of active compounds such as flavonoids, phenolic acids, and terpenoids that have antibacterial

and immunostimulating properties. These substances can inhibit the growth of pathogenic bacteria, repair damaged tissues, and strengthen the fish's immune system[18]. Edrees et al.[7] found that propolis can increase the immune response and prolong the survival of infected fish significantly compared to the treatment without propolis.

4. CONCLUSION

Propolis solution can provide an immune response to goldfish infected with *A. hydrophila* bacteria. The dose of 800 ppm propolis solution is the best, as seen from the total leukocytes of 3.70×10^4 cells/mm^3, phagocytosis index of 28.33%, and survival rate of 83.33%.

REFERENCES

1. Lu, H., Tao, T.L., Liu, Y.L., Jia, F., Cui, Y.F., & Shan, X.F. Comparative Evaluation of Selected Adjuvants for Possible Application of *Aeromonas caviae* Inactivated Vaccine in Crucian Carp (*Carassius auratus*). *Aquaculture International*, 2025; 33(5): 1-20
2. Rashidian, G., Boldaji, J.T., Rainis, S., Prokić, M.D., & Faggio, C. Oregano (*Origanum vulgare*) Extract Enhances Zebrafish (*Danio rerio*) Growth Performance, Serum, and Mucus Innate Immune Responses, and Resistance Against *Aeromonas hydrophila* Challenge. *Animals*, 2021; 11(2): 299
3. El Alem, M.M., Hamed, T.A., & Mohamed, D.T. Pathological and Biochemical Studies on some Antimicrobials in *Clarias gariepinus* Fish Infected with *Aeromonas hydrophila*. *Zagazig Veterinary Journal*, 2017; 45(2): 143-155.
4. Hassanien, H.A., Alrashada, Y.N., Abbas, A.O., & Abdelwahab, A.M. Dietary Propolis Complementation Relieves the Physiological and Growth Deterioration Induced by *Flavobacterium columnare* Infection in Juveniles of Common Carp (*Cyprinus carpio*). *Plos one*, 2023; 18(10): e0292976.
5. Orsi, R.O., Santos, V.G., Pezzato, L.E., Carvalho, P.L.D., Teixeira, C.P., Freitas, J., ... & Barros, M.M. Activity of Brazilian Propolis Against *Aeromonas hydrophila* and its Effect on Nile Tilapia Growth, Hematological and Non-Specific Immune Response under Bacterial Infection. *Anais da Academia Brasileira de Ciências*, 2017; 89: 1785-1799
6. Yonar, M.E., Yonar, S.M., & Silici, S. Protective Effect of Propolis against Oxidative Stress and Immunosuppression Induced by Oxytetracycline in Rainbow Trout (*Oncorhynchus mykiss*, W.). *Fish & Shellfish Immunology*, 2011; 31(2): 318-325.
7. Edrees, A., Abdel-Daim, A.S., Shaban, N.S., Shehata, O., & Ibrahim, R.E. Dietary Intervention of Propolis and/or Turmeric Boosted Growth, Hematology, Biochemical Profile, and Antioxidant-Immune Responses and their Associated Gene Expression in Nile tilapia (*Oreochromis niloticus*) Challenged with *Edwardsiella tarda*. *Aquaculture International*, 2025; 33(1): 46
8. Blaxhall, P.C., & Daisley, K.W. Routine Haematological Methods for Use with Fish Blood. *Journal of Fish Biology*, 1973; 5(6): 771-781.
9. Siwicki, A.K., Anderson, D.P., & Waluga, J. *Fish Disease Diagnosis and Prevention Methods*. Blackwell Publishing, Inc. 1993.
10. Effendi, H. *Telaah Kualitas Air bagi Pengelolaan Sumber Daya dan Lingkungan Perairan*. Kanisius. Yogyakarta. 2002; 257pp
11. Kurniawan, R., Putri, M.N., Riswan, M., Wahyuni, S., & Mursawal, A. Immunostimulant Effect of *Chaetomorpha* sp in Tilapia Infected with *Aeromonas hydrophila*. *Aceh Journal of Animal Science*, 2025; 10(2): 64-69
12. Chen, H., & Luo, D. Application of Haematology Parameters for Health Management in Fish Farms. *Reviews in Aquaculture*, 2023; 15(2): 704-737
13. Pattipeilohy, C.E., Suprayudi, M.A., Setiawati, M., & Ekasari, J. Evaluation of Protein Sparing Effect in Nile tilapia *Oreochromis niloticus* Fed with Organic Selenium Supplemented Diet. *Jurnal Akuakultur Indonesia*, 2020; 19(1): 84-94

14. Alishahi, M., Tollabi, M., & Ghorbanpour, M. Comparison of the Adjuvant Effect of Propolis and Freund on the Efficacy of *Aeromonas hydrophila* Vaccine in Common Carp (*Cyprinus carpio*). *Iranian Journal of Fisheries Sciences*, 2019; 18(3): 428-444.
15. Pereira, C., Duarte, J., Costa, P., Braz, M., & Almeida, A. Bacteriophages in the Control of *Aeromonas* sp. in Aquaculture Systems: An Integrative View. *Antibiotics*, 2022; 11(2): 163
16. Biller, J.D., & Takahashi, L.S. Oxidative Stress and Fish Immune System: Phagocytosis and Leukocyte Respiratory Burst Activity. *Anais da Academia Brasileira de Ciências*, 90: 3403-3414.
17. Souza, C.D.F., Baldissera, M.D., Verdi, C.M., Santos, R.C., Da Rocha, M.I.U., da Veiga, M.L., ... & Baldisserotto, B. Oxidative Stress and Antioxidant Responses in Nile tilapia *Oreochromis niloticus* Experimentally Infected by *Providencia rettgeri*. *Microbial Pathogenesis*, 2019; 131: 164-169
18. El-Asely, A.M., Abbass, A.A., & Austin, B. Honey bee Pollen Improves Growth, Immunity, and Protection of Nile tilapia (*Oreochromis niloticus*) against Infection with *Aeromonas hydrophila*. *Fish & Shellfish Immunology*, 2017; 40(2): 500-506