Histopathology of *Cyprinus carpio* tissue infected by *Aeromonas hydrophila* in profilactical therapy *Pothos tener* Wall

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Abstract. *Photos tener* Wall is a semi-aquatic plant that lives in the Bantimurung waterfall, South Sulawesi. The aims of study to determine the effectiveness of *P. tener* Wall in prophylactic therapy against *Aeromonas hydrophila* infection in *Cyprinus carpio*. The treatment procedures were: a) Soaking the live *P. tener* Wall in aquarium at a dose of 15 gr, 30 gr, 60 gr wet weight, b) Provision of *P. tener* Wall through feed at a dose of 1%, 2% and 4%, c) mixed treatment of 30 gr *P. tener* Wall live and 4% *P. tener* Wall in feed. Positive control (d) *C. carpio* given oxytetracyline antibiotics and negative control (e) *C. carpio* without *P. tener* Wall treatment and without antibiotics. Histological observations of liver, kidney and lymph were carried out: a) Before prophylactic therapy (as a normal condition), b) 30th day of prophylactic therapy c) 7th day after *A. hydrophila* infection. The results of histological observations showed that *C. carpio* in normal condition. Treatment with oxytetracyline antibiotics showed a lot of tissue damage, fatty degeneration, haemorrhage, and no necrosis. Negative controls included more necrosis, fatty degeneration, tissue damage, haemorrhage, hyperplasia. The *P. tener* wall treatment had little of tissue damage compared to positive and negative controls. Conclusion Prophylactic therapy with *P. tener* Wall can emphasize and reduce tissue damage caused by *A. hydrophila* attack rather than antibiotic treatment.

1. Introduction
Koi is one of popular freshwater ornamental fish which has great potential to be developed and becomes an attraction for ornamental fish hobbyists in the world. However, the Koi development and trade has obstacles in fulfilling the market demand. The main obstacle is an *Aeromonas hydrophila* bacteria infection, which causes 80-100% mortality known as Motile *Aeromonas* Septicemia (MAS) disease [1]. Moreover, Hossain et al. (2014) [2] stated that this disease caused mortality in different fish, mainly in carp, Nile tilapia, rainbow trout, brown trout, eel, perch, catfish, and salmon. Disease caused by these bacteria is increasingly resistant along with high frequency of antibiotic use in the culture [3]. This bacteria pathogen can strongly attach fish immune system and cause infectious disease. Similarly to human, the body immune system stimulation can protect fish from infectious disease.

In the latest decade, studies about fish body immune system improvement through herbal treatments have been performed [4]. Furthermore, herbal plants are also utilized as an immune stimulant and an
antiphatogenic [5]. A safe phyto-pharmaceutical for water environment and human is P. tener Wall. According to Nugraha\textit{et al.} (2019), based on the phytochemical screening, \textit{P. tener} Wall has flavonoid, phenol, and glycoside compounds. Based on this condition, this study performed an effectiveness test of \textit{Pothos tener} Wall to prevent from \textit{Aeromonas hydrophila} infection in Koi (\textit{Cyprinus carpio}) as \textit{Pothos tener} Wall plant effect on fish disease prevention has not been performed, therefore it requires a further test about the effectiveness of this plant freshly or mixed with feed to prevent \textit{Aeromonas hydrophila} infection in Koi.

2. Methods

2.1. Treatment
The test fish used Koi obtained from Research Center Institute for Ornamental Fish, Depok, Indonesia, with the length of 7-9 cm and stocking density of 10 fish per aquarium at a size of $60 \times 30 \times 40$ cm$^3$ and 30 L water volume. The test plant used \textit{Pothos tener} Wall.

Treatments
A) Koi immersed with fresh \textit{P. tener} Wall during rearing period and fed with a commercial feed. Koi immersion with fresh \textit{P. tener} Wall during rearing period fed with a commercial feed. Doses were (A1 = 15 g), (A2 = 30 g), (A3 = 60 g) wet weight with three replications and control (-) without \textit{P. tener} Wall treatment.
B) \textit{P. tener} Wall simplicia supplementation in feed during rearing period. Feed was made by mixing the simplicia with commercial feed based on the doses used. Doses were (B1 = 1\%), (B2 = 2\%), and (B3 = 4\%) with three replications and control (-) without \textit{P. tener} Wall treatment.
C) Combination treatment based on the treatment dose from A and B treatments. The best doses were (A2 = 30 g) and (B3 = 4\%) with three replications, control (+) using 5 g/kg feed oxytetracycline, and control (-) without plant treatments.

2.2. \textit{A. hydrophila} characterization
Bacteria used were characterized following Cowan (1974) [6] method and performed a KIT API 20NE test by reading the results through the API WEB software (http://apiweb.biomeriux.com/strip/3).

2.3. Bacteria culture preparation
The characterized \textit{A. hydrophila} bacteria were cultured on a tryptic soy agar (TSA) and Rimler Shotts (RS) media, and then incubated at 30°C for 24 hours.

2.4. Prophylaxis period and \textit{A. hydrophila} infection
For 30 days, test fish were treated with treatments and doses applied. Fish were fasted on the 31st day of rearing period and fish were infected with \textit{A. hydrophila} on 32nd day of rearing period. A 0.1 mL of 105 CFU/mL bacteria was intramuscularly injected on fish distributed in each treatment, except fish in negative control which were injected with 0.1 mL Phosphate buffer saline (PBS) solution. Observation was performed for 7 days

2.5. Liver, gill, and kidney histological observation
Koi histological observation was performed on 30th day of prophylaxis period and 7th of \textit{A. hydrophila} post-infection. Test fish were initially euthanized and dissected from anus to dorsal stomach, which was opened using a tweezers to take liver, spleen, and kidney. These three organs were moved to different sampling bottles filled with 4\% formalin and prepared for histological samples. The histology preparation was performed following the methods developed by the Laboratory of Aquatic Organism Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University. Staining was performed using a hematoxyline-eosine (HE).
2.6. Histopathological sample preparation

2.6.1. Fixation. Liver, gill, and kidney that were observed their tissues, were taken throughout fish dissection. These tissues were fixated in 10% neutral buffered neutral formaline (BNF) solution for 24 hours.

2.6.2. Dehydration. The fixated organs were moved in 24x24x5 mm casette SS Base Mould and dehydrated using stratified alcohols: 70%, 80%, 85%, 90%, 95%, followed by immersing the samples in Ethanol absolute twice for 2 hours.

2.6.3. Clearing. This was performed by dipping the samples in xylene solution for 30 minutes. Xylene solution was changed three times for 1 hour.

2.6.4. Impregnation. Organs were moved to liquid paraffin three times for 30 minutes.

2.6.5. Embedding. Organs were moved in different blocks heated on a hot plate and filled with liquid paraffin. Samples were stood until the paraffin hardened.

2.6.6. Sectioning. The hardened paraffin block was cut with microtome and the cutting results were dipped in warm water at 50ºC until the tissue expanded on object glass and the tissue was dried.

2.6.7. Clearing. The embedded tissue on object glass was moved in xylene and repeated twice for 5 minutes, then immersed in absolute ethanol for 1 minute twice and 95% alcohol for 1 minute, rinsed with aquadest four times.

2.6.8. Staining. Staining with hematoxyline solution was performed for 10 minutes and rinsed with flowing water for 10 minutes. Tissue was then immersed in eosine solution for 2 minutes and rinsed with flowing water, the dipped in 95% alcohol twice and immersed in absolute ethanol for 2 minutes three times.

2.6.9. Observation. Liver, spleen, and kidney of Koi tissues were observed under a light microscope (Olympus CX23) with 100x and 400x magnifications.

3. Results
Koi tissue histology in normal condition was similar to the control negative treatment (without test plant) on 30th day of prophylaxis period.

3.1. Fresh P. tener wall treatment

3.1.1. Koi histology after rearing period (30th day of prophylaxis period).
Liver, kidney, and spleen histological conditions on initial rearing period are presented in Figure 1.
Figure 1. Koi organ histology in A treatment on 30th day of prophylaxis period. 
Note: A1 = 15 g wet weight of P. tener Wall, A2 = 30 g wet weight of P. tener Wall, A3 = 60 g wet weight of P. tener Wall. (N) hepatocytes with peripheral nuclei, (SM) connective tissue and smooth muscle cells, (H) cross section of hepatic bile duct, (C) simple columnar epithelium, (Kb) Browman’s capsule, (Td) distal tubules, (H) haemopoetic tissue, (G) glomerulus, (C) connective tissue, (E) squamous epithelium, (B) invest the spleen numbers of blood sinuses.

3.1.2. Koi histology on 7th day of A. hydrophila post-infection
The histological conditions of liver, kidney, and spleen in Koi on 7th day of A. hydrophila post-infection are presented in Figure 2.
Figure 2. Koi organ histology on 7th day of A. hydrophyla post-infection.
(Note: (K-) without fresh P. tener Wall, A1 = 15 g wet weight of P. tener Wall, A2 = 30 g wet weight of P. tener Wall, A3 = 60 g wet weight of P. tener Wall. (HE) hemorrhage, (N) necrosis, (HS) hyperplasia, (K) congestion, (H) haemosiderosis.

3.2. Treatments with test plant simplicia supplementation in feed

3.2.1. Koi histology after rearing period (30th day of prophylaxis period)
The histological conditions of liver, kidney, and spleen in Koi after rearing period 30th day of prophylaxis period are presented below (Figure 3).
Figure 3. Koi organ histology in B treatment, 30th day of prophylaxis period.
Note: K+) without P. tener Wall in feed, B1 = 1% simplicia of P. tener Wall in feed, B2 = 2% simplicia of P. tener Wall in feed, B3 = 4% simplicia of P. tener Wall in feed. (N) hepatocytes with peripheral nuclei, (H) haemopoetic tissue, (Kb) Browman’s capsule, (Td) distal tubules, (E) squamous Epithelium, (B) invest the spleen numbers of blood sinuses, (C) connective tissue.

3.2.2. Koi histology on 7th day of A. hydrophila post-infection
The histological conditions of liver, kidney, and spleen in Koi on 7th day of A. hydrophila post-infection are presented in the following figure.
Figure 4. Koi organ histology in B treatment on 7th day of A. hydrophylla post-infection. K-) without P. tener Wall in feed, B1 = 1% P. tener Wall simplicia in feed, B2 = 2% P. tener Wall simplicia in feed, B3 = 4% P. tener Wall simplicia in feed. (N) necrosis, (HS) hyperplasia, (DL) fatty degeneration, (K) congestion, (HE) Hemorrhage, (H) haemosiderosis.

3.3. A and B treatment combination

3.3.1. Koi histology after rearing period (30th day of prophylaxis period)
The C treatment was the best treatment compared to A (30 g fresh P. tener Wall) and B (4% P. tener Wall simplicia in feed) treatments with positive control using 5 g/kg feed oxytetracycline antibiotic and negative control using no test plant.
Figure 5. Koi organ histology in C treatment, 30th day of prophylaxis period.
Note: K(-) = without test plant treatment, K(+) = 5 gr/kg feed oxytetracylin, C = A2 and B3 treatment combination. (N) hepatocytes with peripheral nuclei, (Kb) Browman’s capsule, (G) glomerulus, (E) squamous Epithelium, (H) haemopoetic tissue, (B) invest the spleen numbers of blood sinuses, (C) connective tissue.

3.3.2. Koi histology on 7th day of A. hydrophila post-infection
The organ histology of liver, kidney, and spleen in Koi on 7th day of A. hydrophilla infection can be shown in Figure 6.
Figure 6. Koi organ histology in C treatment on 7th day of A. hydrophylla post-infection. Note: K (-) = without test plant treatment, K (+) = 5 g/kg feed oxytetracylin, C = A2 (30 g fresh P. tener Wall) and B3 (4% P. tener Wall simplicia in feed) treatment combination. (N) necrosis, (H) haemosiderosis, (HE) hemorrhage.

4. Discussions

4.1. Tissue histological condition in Koi on 30th day of prophylaxis period

Liver tissue condition for 30 days during the prophylaxis period in each treatment was still in normal condition. This condition was marked by the existence of hepatocytes and rounded nuclei found in all treatments. Specifically, connective tissue, soft muscle cells, bile duct, and simple epithelia were also found in A2 treatment. This result was also similar to Yilin et al. (2012), who stated that hepatocytes had large single nuclei with rounded shape that were clearly found in the cell body center or closed to the sinusoid membrane, moreover bile duct was also found in liver, which was formed from several cubical epithelial cells and distinct basal lamina that separated epithelial periphery connective tissue. The lateral surface of epithelial cells is enclosed with tight joints, which turns into inter-columnar epithelial cells surrounded by thicker connective tissue. In general, the normal liver histological appearance is indicated by the presence of hepatocytes located between sinusoid and bile duct. Liver tissue necrosis indicates that all membranes are lyses, resulting in sinusoidal-like nuclei and unclear hepatocytes as mixed together with other cells [7].

Spleen tissue in all treatments showed a normal condition on 30th days of prophylaxis period. This condition was marked by the observation results on spleen tissue of Koi, which had spleen tissue covered with squamous epithelia, connective tissue, and abundant blood sinusoid in the connective tissue. This condition followed Camila et al. (2017), who stated that spleen was surrounded by a capsule which showed simple squamous epithelia and thin connective tissue in all species, as irregular trabeculae occurred that expanded into parenchyma [8].

Kidney tissue histology on 30th day of prophylaxis period in each treatment showed normal condition based on distal tubules, Browman's capsule, and hematopoietic tissue. In C treatment with 5 g/kg oxytetracycline supplementation, A2 and B2 treatments were found to have glomeruli. The observation results showed that the kidney structure observed were found to be normal kidney tissue marked by the existence of Browman's capsule surrounding glomerulus. This condition was similar to Bruno et al. (2013) who stated that glomerulus in normal condition was covered by Browman's capsule forming a bowl. The glomerular capillaries are coated with porous endothelial cells at 100 nm diameter located on the basal membrane with outer visceral epithelia (podocytes). The koi kidney had distal...
contortus tubullus characterized by a short tubullus containing one layer of cubical epithelial cells with cilia and rounded nucleus [9].

4.2. Tissue histological condition on 7th day of A. hydrophila post-infection

The histological condition on 7th day of A. hydrophila post-infection showed hemorrhage, hyperplasia, fatty liver degeneration, congestion, haemasyderosis, and necrosis. In liver tissue in the A treatment, liver damage was less severe than the negative control treatment. Necrosis damages were only found in A1 and A3 treatments, while A2 treatment was only found hemorrhage damage. In B2 and B3 treatments, hemorrhage were found in both treatments, while in B1 treatment, hemorrhage and fatty degeneration were found in this treatment. Liver in C treatment had hemorrhage, while positive control treatment (antibiotic treatment) only had liver necrosis. The most tissue damage was abundantly occurred in the negative control treatment (without test plant treatment), while the A and B treatments only had necrosis and hyperplasia.

The observation results on kidney tissue after fish infected with A. hydrophila in the A treatment showed less severe damage than the negative control treatment (without test plant treatment). Necrosis in fish liver can be caused by several factors, namely, virus, fungi, bacteria, and parasites that disrupt blood transportation, affecting the blood supply to a certain tissue. All of these disruptions can cause cell damage or necrosis [10].

Damages found in kidney tissue in the A1 and A2 treatments showed hemorrhage and congestion in the A3 treatment. The B1 and B2 treatments also showed necrosis, while the B3 treatment only showed hemorrhage damage. In the C and positive treatments, hemorrhage was found in both treatments. The most severe damage was found in the negative control, which showed necrosis, hemorrhage, and congestion. Damages found in kidney tissue occurred was thought due to the toxin availability from A. hydrophila which caused a severe damage such as necrosis. Kidney damage such as necrosis caused by bacteria was also found by Ibrahim et al. (2011), who stated that the dermatotoxin and hemolysine produced by Edwardsiella tarda could cause oedema in fish liver and kidney, and severe necrosis in kidney [11]. Liver damage due to bacterial infection was also found by Maftuch et al. (2018), who stated that necrosis and hyaline degeneration in Koi kidney caused by the Myxobolus sp. infection showed an opaque nucleus, resulting in an unclear membrane. The myxosporean infection in kidney tissue causes several damages, such as glomerulus disruption, hemorrhage, leucocytic infiltration as the main histopathological effect [12].

The spleen histological condition in Koi after A. hydrophila infection showed haemosiderosis in all treatments. In the positive and negative control treatments, abundant haemosiderosis damage was occurred marked by brighter color of the tissue sample. Haemosiderosis is a pathological condition due to the haemosiderin deposition. This condition was also found in David and Kartheek (2015), who stated that the consequence of sodium cyanide exposure could cause haemosiderosis in Cyprinus carpio spleen. Haemosiderin deposition can cause erythrocyte damages, resulting in irregular movement of haemoglobin in erythrocytes [13].

Based on the spleen histological observation, haemosiderosis occurred in the positive and negative control treatments showed the most severe damage compared to other treatments with P. tener Wall plant. This condition was thought due to P. Tener Wall plant contains several phytochemical compounds. Nugraha et al. (2019) mentioned that P. Tener Wall contained flavonoids, phenols, and glycosides [14]. According to Citarasu (2010), flavonoids are polyphenol group that can damage protoplasma through cell-wall lysing mechanism and terminating bacteria protein and DNA synthesis, which can inhibit the bacterial activity [4].

5. Conclusion

Prophylactic therapy with P. tener Wall can emphasize and reduce tissue damage caused by A. hydrophila attack rather than antibiotic treatment.
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