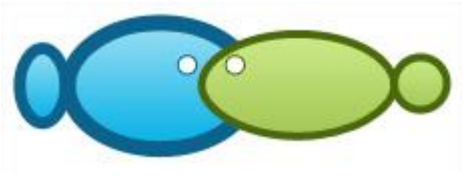

Anti-inflammatory effects of *Holothuria scabra* extract on *Pangasianodon hypophthalmus* tissues infected with *Aeromonas hydrophila*

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Abstract. The coastal environment has an abundance of organisms that provide various primary and secondary metabolites via their biological activities. Some reviews have noted that *Holothuria scabra* possesses anti-inflammatory properties as a natural drug against serious diseases. The study aimed to observe the *H. scabra* extract's anti-inflammatory effect on preventing the *Pangasianodon hypophthalmus* tissue's damage following the *Aeromonas hydrophila* infection. *H. scabra* extract at several dosages, 0 mg L^{-1} (T0), 50 mg L^{-1} (T1), 100 mg L^{-1} (T2), and 150 mg L^{-1} (T3), was introduced to *P. hypophthalmus* tank before the challenge test with *A. hydrophila*. Furthermore, histopathology changes were measured, engaging the gill and spleen. The results revealed that the bathing method applied to *P. hypophthalmus* tissue using *H. scabra* extract concentration at 100 mg L^{-1} was the optimum dosage for protecting the spleen and gill against *A. hydrophila*, compared with the other treatments.
Key Words: alteration, fish tissue, histopathology, sea cucumber.

Introduction. Many freshwater fish species can be a profitable commodity on the market. *Pangasianodon hypophthalmus* also has a considerable economic value (Andriawan et al 2019; Singh & Lakra 2012). In Indonesia, *P. hypophthalmus* production significantly raised by 38%, compared to the previous years (Ramadhan et al 2016). Furthermore, the increase of *P. hypophthalmus* cultivation leads to a negative impact on fish health statuses such as immunosuppression, water-quality deterioration (Inendino et al 2005) and infectious diseases (Afrianto & Liviawaty 1992; Griffiths et al 2010). Bacterial infections are most often generating a problem, in the fish cultivation and even ornamental fishes culture, which leads to mortality (Nahar et al 2016; Sarker & Faruk 2016). Some pathogens trigger various profound impacts to fish, including dropsy, inflammation, mouth fungus (Austin & Austin 2012; Banu 1996).

Aeromonas hydrophila, the most common freshwater bacteria, is one of the most contagious pathogens of the freshwater fish, amphibians, even causing diarrheal disease in humans (Shotts 1990; Simmons & Gibson 2012). For instance, *A. hydrophila* leads to a high mortality in various fishes, such as *Pangasius* sp. (Nahar et al 2016), *Heteropneustes fossilis* (Rashid et al 2008), *Cyprinus carpio* (Harikrishnan et al 2003), *Oreochromis niloticus* (Pachanawan et al 2008), *Labeo rohita* (Giri et al 2015), and *Oreochromis aureus* (AlYahya et al 2018). According to Abdelhamed et al (2019) and Nahar et al (2016), *A. hydrophila* is considered as an agent for motile *Aeromonas* septicemia (MAS) disease, which causes septicemia and hemorrhage to the diverse fish organs. In a casestudy, the Transmission Electron Microscopy (TEM) showed *Ictalurus punctatus* gill and spleen destruction by *A. hydrophila* after 48 h incubation (Abdelhamed et al 2017). Moreover, in a similar study, gill, liver, spleen and kidney tissue breakdown of mandarin fish were also identified after being infected by *A. hydrophila* (Chen et al

2018). A recent study found that *A. hydrophila* was identified infecting *P. hypophthalmus* tissue, including kidney, liver and muscle (Nahar et al 2016).

Many antibiotics for preventing and combating are still applied to control and deal with infectious diseases in aquaculture, but they generate many adverse effects on both fish and the environment (Laith & Najiah 2013). In the fish farm, antibiotics were supplemented into the feed or directly introduced into the water (Rico et al 2013; Wang et al 2015). However, Qiu et al (2020) argued that antibiotics have side effects on the environment and aquatic organisms. According to Costanzo et al (2005), antibiotics reduce water denitrification activity by bacteria, that is considered a severe impact on the aquatic environment. Meanwhile, in aquatic organisms, it increases bacterial resistance (Depaola et al 1995), reduces fish immune capacities (Samanidou & Evaggelopoulou 2007) and increases the pharmacological effects on the aquatic biota (David et al 2017; Rand-Weaver et al 2013; Xie et al 2015). Some studies have recently tried to replace chemical substances in aquaculture with something more natural (El Asely et al 2020; Elumalai et al 2021; Stratev et al 2018), for example, the application of *Litsea cubeba* and *Euphorbia hirta* for *Cyprinus carpio*, to face *A. hydrophila* invasions (Nguyen et al 2016; Pratheepa & Sukumaran 2014).

Our study investigated the natural ingredients from *Holothuria scabra* that might replace antibiotics, based on previous studies. Sea cucumber (*H. scabra*), belonging to the class Holothuroidea, is a marine animal with the potential as a functional food, due to its nutrition properties (Kareh et al 2018; Pangestuti & Arifin 2018; Pangkey et al 2012). Moreover, *H. scabra* also has biological substances, particularly triterpene glycoside, sulfated polysaccharides, phenolic compounds, pigments and saponin (Kamyab et al 2020). Due to these secondary metabolites, sea cucumber has been used as a natural drug for treating several diseases, such as tumors, arthritis, high blood pressure, fungal infection, pain, and muscular disorders (Hashim 2007; Ibrahim et al 2018; Kiew & Don 2012). Besides, sea cucumber has been considered as a drug for wound healing in several treated organisms, compared to untreated organisms (Ibrahim et al 2018).

Histopathology is a dominant disease diagnostic tool. It is generally employed as biomarker in interpreting the contaminants threat to the fish health, both in the lab and even in field studies (Camargo & Martinez 2007). Therefore, this study aimed to examine histopathology, mortality rate and clinical symptoms of *P. hypophthalmus* and its tolerance to *A. hydrophila*, after the immersion into a *H. scabra* extract.

Material and Method

Fish and H. scabra extraction. *P. hypophthalmus* preparation and *H. scabra* extraction referred to our previous research (Andriawan et al 2019): the fish was cultivated in a tank with water recirculation and was fed with commercial fish feed. Meanwhile, crude extract of *H. scabra* was isolated using methanol and n-butanol. Finally, the extract was evaporated using a vacuum evaporator machine.

Experimental design. *H. scabra* extract was applied through the immersion method with double booster, at days 0 and 7. The bathing used various dosages of *H. scabra* extract: 0, 50, 100 and 150 ppm (T0, T1, T2, and T3, respectively) for 1 h. Next, the fish sample was challenged with *A. hydrophila* (10^8 CFU ml^{-1}) for 24 h, until fish became pale, with an unbalanced swimming and often staying in the surface area. Eventually, the tissue collections were conducted at 144 h post-challenge for examining the histopathological changes in the spleen and gill.

Preparation of histological slides. Histopathology methods followed the studies of Hossain et al (2007) and Paul & Mukti (2017), with modification, including the preparation and observation under a microscope. The slide preparation followed several steps, such as: the fixation using the saline solution (0.75% NaCl) and the direct fixation in 10% formalin; the dehydrationusing alcohol concentrations of 70, 80, 90, 96 and 100% and clearing in a xylene solution for 10 min; the impregnation with paraffin; the trimming and sectioning, using paraffinand a Microtome; the deparaffinization

(incubated at 5 to 6ºC above the melting point of paraffin, 60ºC, for 20 to 60 min) and affixing using anadhesive; the cleaning and rehydration using xilol solution and the staining using hematoxylin and eosin; the mounting of the slides with a DPX medium and labeling.

Histopathology analysis. The study of histopathology followed Nurin & Maftuch (2018). A score was allocated based on Table 1, where the percentage of destruction per field area was calculated based on the total of injured tissue using the formula:

$$\text{Damage percentage} = (\text{Damaged cells} / \text{Total analyzed cells}) \times 100$$

Table 1

Percentage of scoring

Score	*Damage percentage (%)*
1	0-5
2	6-25
3	26-50
4	>50

Statistical analysis. ANOVA (One-way analysis of variance) was employed to test the differences among groups. Multiple correlations (Duncan test) were employed to measure significant changes among the treatments using SPSS (version 17, USA). Data were presented as the mean ± SD, $P<0.05$ was considered significant.

Results

Gill histopathology. This study's objective was to determine the resistance of pangasius fingerlings to *A. hydrophilla* challenge, after dipping into a *H. scabra* extract and by examining the histopathological differences between healthy and infected fish. The present study recorded severe histopathological changes in pangasius fish gills, due to the exposure to *A. hydrophila* (Figure 1). The healthy gill has the primary gill lamella (PL) with a central axis (CA) and the secondary gill lamellae (SL) on both sides, separated by the interlamellar region (ILR). The histopathological investigation showed many damages, appeared post-challenge with *A. hydrophila*, compared with healthy *P. hypophthalmus* gills (Figure 1B).

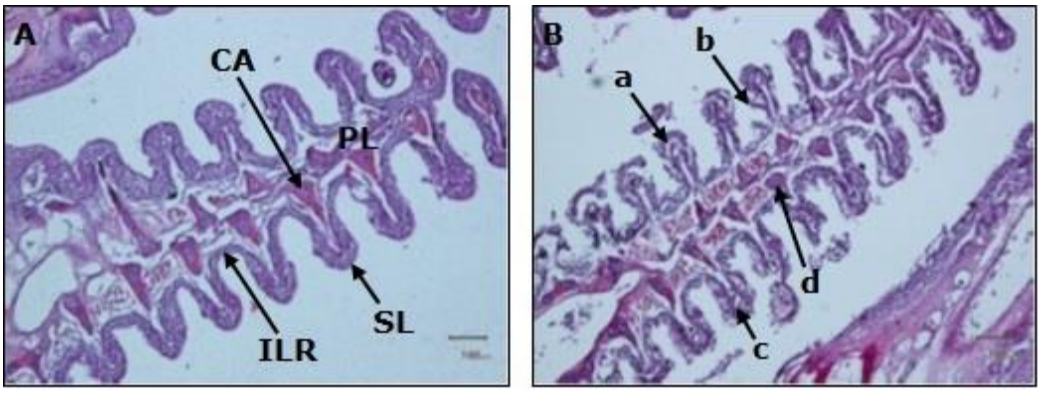

Figure 1. Comparison of robust and infected *Pangasianodon hypophthalmus* gills observed under a microscope: healthy gill (A) and infected gill by *Aeromonas hydrophila* (B). A: central axis (CA), primary lamella (PL), secondary lamella (SL), and interlamellar region (ILR); B: necrosis (a), epithelial lifting (b), epithelial hyperplasia (c) and congestion (d).

Initially, the observation concerned the health gill tissue and the identification of anomalies in the infected gill tissue, such as: edema, necrosis, epithelial lifting, epithelial hyperplasia and congestion, in *A. hydrophila* post-challenged *P. hypophthalmus* gills.

Figure 1 shows that *A. hydrophila* was the pathogen agent causing the damage of *P. hypophthalmus* gills. Consequently, the application of *H. scabra* extract was intended to prevent and even recover *P. hypophthalmus* from gill inflammation, after a *A. hydrophila* infection. The *S. scabra* extract was formulated in several dosages, including 0, 50, 100 and 150 mg L^{-1} (T0, T1, T2, and T3, respectively). The gill inflammation was recorded during the study (Figure 2).

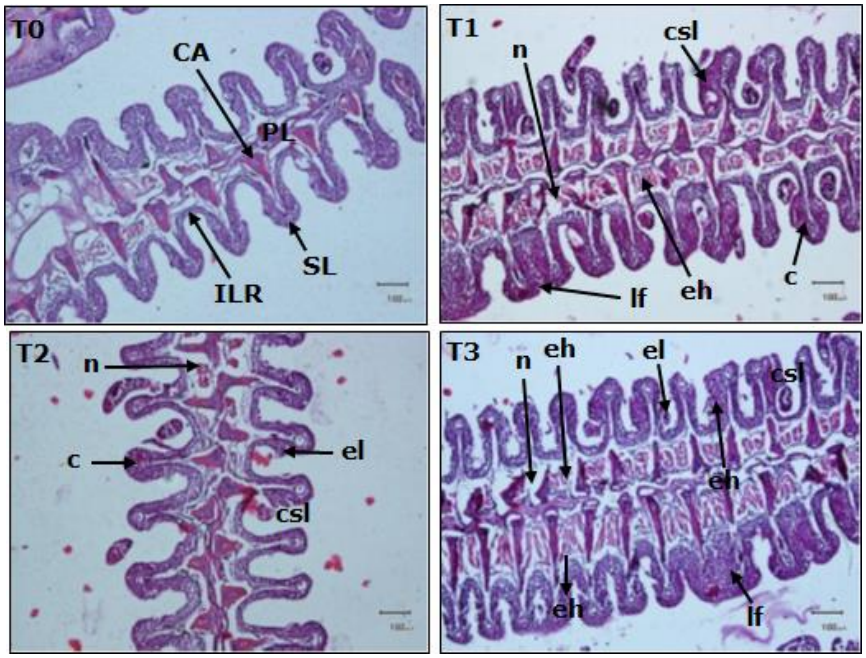

Figure 2. Observation (100× magnification) of the *Pangasianodon hypophthalmus* gill histopathology for various concentrations (T0) 0 mg L^{-1}, (T1) 50 mg L^{-1}, (T2) 100 mg L^{-1} and (T3) 150 mg L^{-1} of *Holothuria scabra* extract; the main observed parts were were the central axis (CA), primary lamella (PL), secondary lamella (SL) and interlamellar region (ILR); the identified anomalies were: necrosis (n), epithelial lifting (el), epithelial hyperplasia (eh), curling of secondary lamellae (csl) and congestion (c).

As displayed in Figure 2, there were differences between treatments, in the histopathology of *P. hypophthalmus* gill, suggesting that the *H. scabra* extract could prevent tissue damage caused by *A. hydrophila* infection. All treatments showed better tissue appearance than the control group. The structural details of the *P. hypophthalmus* gill tissue, for each treatment, are shown in Figure 2. In the gill tissues of *P. hypophthalmus* exposed to *H. scabra* extracts at concentrations of 50, 100 and 150 mg L^{-1}, pathologies like necrosis, epithelial lifting, epithelial hyperplasia, curling of secondary lamellae and congestion were identified post-infection with *A. hydrophilla*.

Statistically, the present study showed a significant difference ($P<0.05$) between each treatment and the control group (Table 2). The T2 revealed the best results in preventing gill tissue damage such as edema, necrosis, congestion, epithelial hyperplasia and epithelial hyperplasia (1.5±0.31, 1.5±0.11, 1.4±0.20, 1.8±0.45, and 1.6±0.49, respectively), followed by T1 and T3. The T2 revealed the *H. scabra* extract's optimum dosage in preventing the *A. hydrophila* infection, identified due to fewer lesions (Figure 3) and to a lower scoring (Table 2) compared to others.

Besides, our result revealed that the highest dosage of *H. scabra* extract (T3) showed no significant difference ($P>0.05$) from T1 and the alterations were almost as bad as in the control group. We assumed that a dose increase of the *H. scabra* extract acted like a toxin to the gill tissue of *P. hypophthalmus.* Based on this finding, it was suggested that a concentration of 100 mg L^{-1} of *H. scabra* extract would be recommendable for preventing *A. hydrophila* infection, wihout toxicity for the gill tissue of *P. hypophthalmus.*

Table 2

The scoring of *Pangasianodon hypophthalmus* gill histopathology post *Aeromonas hydrophila* infection

Treatment	*Histological alterations*				
	Oedema	*Necrosis*	*Congestion*	*Epithelial lifting*	*Epithelial hyperplasia*
T0 (Negative control/without infection)	1.0 ± 0.14^{a}	1.1 ± 0.14^{a}	1.0 ± 0.00^{a}	1.2 ± 0.05^{a}	1.1 ± 0.17^{a}
T0 (Positive control)	3.5 ± 0.23^{d}	3.2 ± 0.40^{c}	3.5 ± 0.23^{d}	3.0 ± 0.10^{c}	3.7 ± 0.49^{c}
T1 (50 mg L^{-1})	2.4 ± 0.35^{c}	2.7 ± 0.26^{b}	2.9 ± 0.23^{c}	3.0 ± 0.20^{c}	2.0 ± 0.26^{b}
T2 (100 mg L^{-1})	1.5 ± 0.31^{d}	1.5 ± 0.11^{a}	1.4 ± 0.20^{b}	1.8 ± 0.45^{b}	1.6 ± 0.49^{ab}
T3 (150 mg L^{-1})	2.7 ± 0.23^{c}	2.9 ± 0.30^{bc}	3.0 ± 0.20^{c}	2.7 ± 0.17^{c}	3.2 ± 0.53^{c}

Spleen histopathology. Our results revealed that the spleen has a capsule and a short trabecula, separated into a red and white pulp, as in other fish. The present study revealed histopathological alterations in the splenic section of fish exposed to *A. hydrophila*, with a relatively low number of fish lesions (Figure 3). The lesions were found in the spleen tissue infected by *A. hydrophila*, while no lesions were identified in the control fish (Figure 3A). All observations were performed under a 100X magnification of the microscope in order to examine the injuries after the infection and to compare the infected tissue with healthy *P. hypophthalmus* spleen tissue.

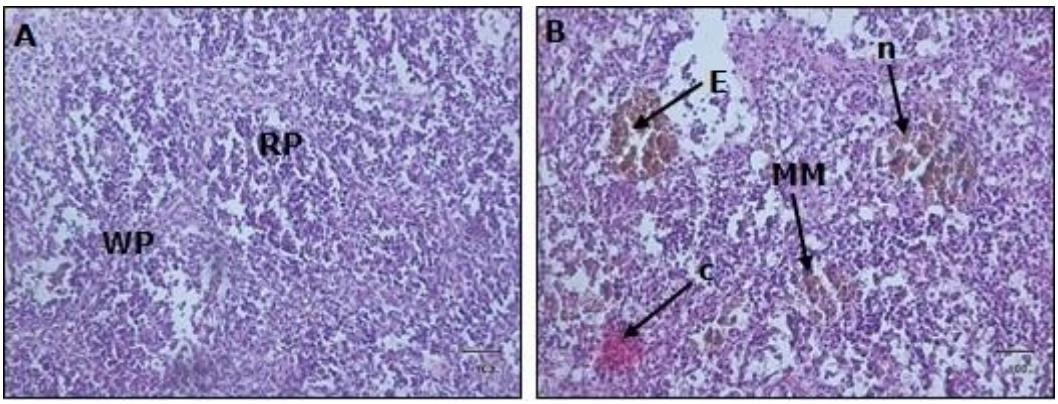

Figure 3. A comparison (100× magnification) of robust and infected *Pangasianodon hypophthalmus* spleen was observed under a microscope, healthy spleen (A) and infected spleen by *Aeromonas hydrophila* (B). A: Red Pulp (RP), White Pulp (WP); B: melanomacrophage centers (MMC), ellipsoids (E), necrosis (n), and congestion (c).

Microscopically, our result revealed that there were differences between the control spleen tissue and the infected spleen tissue. The initial comparison between the healthy *P. hypophthalmus* spleen tissue and the infected spleen tissue identified alterations like hemorrhage, necrosis and congestion. Figure 3B reveals immune reactions in melanomacrophage centers (MMC) and ellipsoids (E), after *A. hydrophila* infection: the ellipsoids were detected in *A. hydrophila* post-challenged *P. hypophthalmus* spleens (Figure 3B), with bathing treatment.

All treatment indicated melanomacrophage centers (MMC), ellipsoids (E), necrosis (n), congestion (c) and hemorrhage (h) post-infection presence (Figure 4).

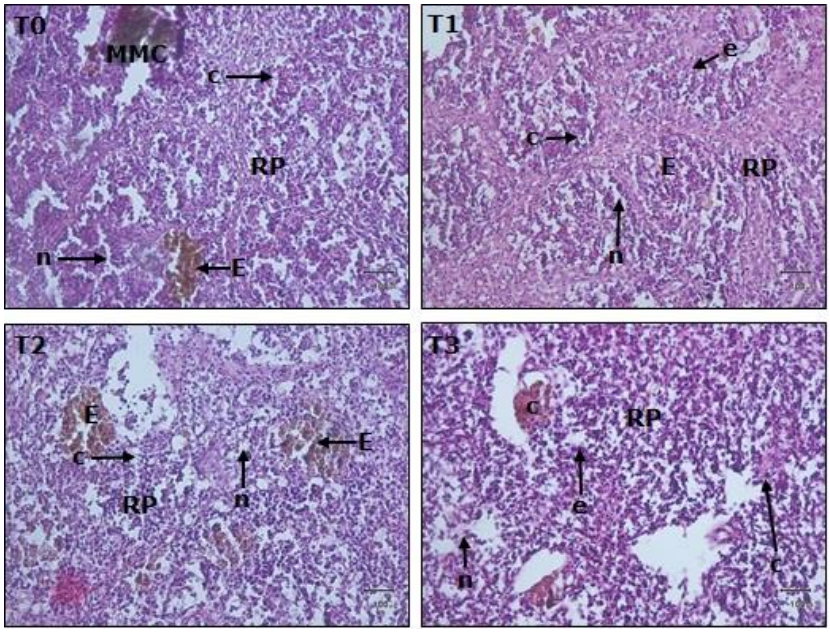

Figure 4. *Pangasianodon hypophthalmus* spleen histopathology (100× magnification) for various concentrations: (T0) 0 mg L^{-1}, (T1) 50 mg L^{-1}, (T2) 100 mg L^{-1} and (T3) 150 mg L^{-1} of *Holothuria scabra* extract; identified elements: red pulp (RP), melanomacrophage centers (MMC), ellipsoids (E), necrosis (n), congestion (c), and hemorrhage (e).

Differences in the histopathology of *P. hypophthalmus* spleen were identified between the treatments, suggesting that *H. scabra* extract could prevent tissue damage caused by *A. hydrophila* invasion. All treatments showed a significant difference ($p<0.05$) compared with the control group. The scoring of the hemorrhage (h), necrosis (n) and congestion (c) types of lesions in all treatments could be seen in Table 3, with T2 showing the best results among the treatments. A concentration of 100 mg L^{-1} of *H. scabra* worked effectively for the tissue protection against *A. hydrophila,* with the scores: 1.53±0.23 (hemorrhage), 2.07±0.31 (necrosis) and 1.47±0.23 (congestion), followed by the *H. scabra* concentration of 50 mg L^{-1}, when comparing the infected and uninfected groups.

Table 3

The scoring of *Pangasianodon hypophthalmus* spleen histopathology post *Aeromonas hydrophila* infection

Treatment	*Histological alterations*		
	Hemorrhage	*Necrosis*	*Congestion*
T0 (Negative control/without infection)	$1.20±0.23^{a}$	$1.07±0.16^{a}$	$1.27±0.23^{a}$
T0 (Positive control)	$3.33±0.15^{d}$	$3.73±0.16^{e}$	$3.73±0.16^{c}$
T1 (50 mg L^{-1})	$2.67±0.16^{c}$	$2.53±0.23^{c}$	$3.00±0.20^{b}$
T2 (100 mg L^{-1})	$1.53±0.23^{b}$	$2.07±0.31^{b}$	$1.47±0.23^{a}$
T3 (150 mg L^{-1})	$3.07±0.20^{d}$	$3.00±0.20^{d}$	$3.40±0.20^{c}$

Discussion. *Holothuria* sp. possesses high concentrations of triterpenoid saponins, that are the essence of their chemical defense for healing their body against a predator or exogenous agent (Bahrami et al 2018; Wang et al 2014; Zhao et al 2018). According to Ceesay et al (2019), saponins and triterpenoids could be extracted by several solvents, especially methanol. In our previous study, sea cucumber's glycoside triterpene was obtained with BuOH (butanol) using the conventional method (Andriawan et al 2019) and were successfully detected by LC (liquid chromatography) and MS (mass spectrometry) analysis tools (Grauso et al 2019).

Histopathological examination of gill and spleen tissues was essential for providing the initial disease information and for understanding the stress responses. The gills, a

vital organ, plays an indispensable role in the respiration process (Wegner 2011), osmoregulation system (Malakpour et al 2018), excretion of nitrogenous waste (Rodela 2013), acid-base balance (Perry & Gilmour 2006) and is even engaged in the defence system (Adinarayana et al 2017). Gill histopathology alterations were observed by this study in *A. hydrophila* post-challenged tissues, such as edema, necrosis, epithelial lifting, epithelial hyperplasia and congestion (Table 3). According to several reviews, *A. hydrophilla* causes gills tissue damage such as hyperplasia, fusion of gill lamellae and congestion in *O. niloticus* (El Deen et al 2014), channel catfish (Zhang et al 2016), *Clarias gariepinus* (Sellegounder et al 2018) and goldfish (Harikrishnan et al 2008). A previous study found that *A. hydrophila* infection caused hyperplasia and leukocytic infiltration in catfish' gills (Abdelhamed et al 2017). The treatments with *H. scabra* extract at diverse concentrations were intended to prevent the gill damage in tissues post-challenged with *A. hydrophilla.* Our results revealed that all treatments could protect and reduce lesion on gill post-challenge *A. hydrophilla* compared with the control group ($p<0.05$).

Besides the gill properties, the spleen is a primary peripheral lymphoid unit that plays an essential role in the antigens trapping (Agius & Roberts 2003; David & Kartheek 2015). The spleen plays a vital role in lymphocytes and macrophages production that serve as immune defense agents (Sales et al 2017). Spleen includes red pulp and white pulp, with structural differences, such as the linking system of sinusoid capillaries and the splenic cords. It mainly contains lymphoid cells surrounding arterial vessels' melano-macrophage centers (Duggina et al 2015). Many histopathological studies had been conducted on diverse freshwater fish, especially related to the infection with *A. hydrophila* pathogen (Hamid et al 2018).

Evaluations of *H. scabra* anti-inflammatory properties based on fish histopathology were missing. The present study assumed that the extract of *H. scabra* plays an essential role in reducing inflammation and lesions after the infection *A. hydrophilla.* The study of Sroyraya et al (2017) states that the sea cucumber possesses an anti-inflammatory effect on MDA-MB-231 human breast cancer cells. Moreover, triterpene glycoside plays the role of an immunity booster, protects nerve tissue and reduces pain or lesion (Kareh et al 2018). A review by Agra et al (2015) examined the healing properties of the triterpene forms and derivatives. Other studies on triterpene healing agents focused on the epithelization and high tissue tensile in pigs (Shukla et al 1999), on the inflammatory mediators in rats (Ngo et al 2013) and on the the splitting strength increase in the granulation tissue of the rat (Sharath et al 2010). According to Aminin (2019), millimolar and micromolar concentrations of sea cucumber glycosides showed cytolytic, hemolytic, antifungal and other biological activities of membranotropic action.

Interestingly, the higher the dosage of *H. scabra* extracts, the worse the observed histopathology results of gill and spleen tissues (Table 2 and 3). The triterpenoid glycosides show positive effects as an immunostimulant, but are also highly toxic to the fish's respiratory epithelia (Francis et al 2002). According to Dos Santos et al (2018) study, a dosage of at least 750 µg mL^{-1} of *Himatanthus drasticus* extract containing triterpenoid caused necrosis on the gill tissue of the zebrafish, *Danio rerio*.

Conclusions. The present study found that *A. hdrophila* could cause several tissue alterations, including melanomacrophage centers (MMC), necrosis (n), congestion (c), and hemorrhage (h) of *P. hypophthalmus* gill and spleen. However, the results demonstrated that *H. scabra* extracts worked well for reducing the lesions, which the T2 (100 mg L^{-1} of *H. scabra* extracts) was the best treatment. Therefore, this study highly suggests that the extract could be applied to real aquaculture to combat infectious diseases, particularly bacteria.

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Conflict of interest. The authors declare no conflict of interest.

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