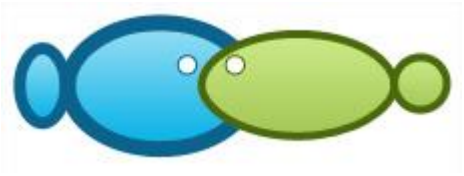

Bacteria *Aeromonas hydropilla*-induced disease treatment in catfish (*Clarias* sp.) culture, with a combination of honey and asthma plant *Euphorbia hirta*

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Abstract. Kefa forest honey and *Euphorbia hirta* plants have a proven antibacterial effect on pathogenic bacteria in fish. For an optimal use in fish, honey must be combined with *E. hirta*, which contains more active ingredients. This study aimed to determine the ability of the honey and asthma plant *E. hirta* combination to cure the catfish infected with *Aeromonas hydropilla* by normalizing the hematology and increasing the survival rate. It is an experimental study using a Complete Randomized Design. The experimental treatments were a mixture of honey and *E. hirta* at different concentrations, 2:1 of honey-*E. hirta*, 1:1 and 1:2 of honey-*E. hirta*, positive control, and negative control. Each treatment has 3 replications. The hematological data (erythrocyte, hematocrit, hemoglobin, and leucocyte) and the fish survival were analyzed using ANOVA. Results showed that honey-*E. hirta* combination could cure the catfish infected with *A. hydropilla* by increasing the erythrocyte level from 1,800,000 cells mm^{-3} (infected condition) to 2,833,333 cells mm^{-3} (after treatment), the hemoglobin from 9.37 g dL^{-1} to 12.97 g dL^{-1}, and decreasing the leucocyte level from 34,870 cells mm^{-3} (infected condition) to 28,467 cells mm^{-3} (after treatment).

Key Words: infection, treatment, hematology, asthma plant, survival.

Introduction. Honey has long been used as an effective antimicrobial and antioxidant for thousands of years (Cokcetin et al 2016). Honey is used for skin lesions, burns, inflammation, bacterial infection, influenza, cough, and various infectious diseases (Albaridi 2019). The use of honey has decreased since the antibiotics were found (Combarros-Fuertes 2020), but with the increase of the microorganism's resistance to the antibiotics, the use of honey as an antimicrobial has started being scientifically considered (Nolan et al 2019). Research findings has proved the efficacy of honey as an antibacterial, to inhibit Gram-negative and Gram-positive bacteria, and even those resistant to many drugs, categorized as broad spectrum bacteria (Laallam et al 2015). As an antibacterial, honey does not cause bacterial resistance (Maddocks & Jenkins 2013).

The antibacterial activity of honey against pathogenic bacteria in fish has been shown by previous scientists. Ramalivhana et al (2014) found that South Africa-originated honey has antibacterial activity against *Aeromonas hydropilla*. Stratev et al (2015) also reported that Rapa honey and Royal jelly from Bulgaria have a potential as antibacterial against *A. hydropilla*. Similar information is also reported for ant honey and stone honey from Semau island, East Nusa Tenggara Province, against *A. hyropilla* and *Vibrio alginolitycus* (Salosso 2019a,2019b). Moreover, Salosso (2019c) found an in vitro antibacterial activity of Kefa-originated forest honey against *A. hydropilla* with an inhibition zone of 12 mm. This honey type is also able to cure the *A. hydropilla*-infected carp (Salosso et al 2020).

The use of honey as an antibacterial in fish through immersion method still has constraints, since it needs a high amount of honey. To reduce the use of a high amount

of honey in fish treatment through immersion, other natural material that also has antibacterial activity against *A. hydrophilla* needs to be combined (Assidqi et al 2012; Salosso & Jasmanindar 2014). This study examines the active compounds in honey, asthma plant *Euphorbia hirta*, and their combination, the best honey-*E. hirta* ratio for *A. hydropilla*-infected catfish through hematological observations, and the catfish survivorship.

Material and Method

Honey and asthma plant E. hirta collection. This study utilized Kefa forest honey from Northern Central Timor regency, East Nusa Tenggara Province, collected traditionally in the dry season. The asthma plant *E. hirta* was collected around Liliba village, Kupang Juga. The plant was cleansed, wind-dried, chopped into 2-3 cm long pieces, and blended to a coarse powder. The powder was then boiled at a concentration of 3% (3 g in 100 mL of distilled water), left for 6 hours, filtered, and prepared for further testing. The pure kefa honey was diluted in distilled water at a concentration of 50%.

Phytochemical test of honey and E. hirta. The phytochemical examination was carried out for honey, *E. hirta*, and their combination. The chemical compounds included alkaloid using the Culvenor-Fiztgerald method, saponin with foam test, phenol with the addition of $FeCl_3$, flavonoid with the addition of HCl and Mg powder, terpenoid and steroid with Lieberman-Burchard method, and tannin with the addition of $FeCl_3$ in the hot water extract.

Experimental tank preparation and acclimatization. There were 2 types of culture tanks, 12 50 x 30 x 30 cm^3 aquaria, and a 10L treatment tank. Each tank was filled with 30 L of water at the density of 6 ind $tank^{-1}$. The catfish specimens, at a size range of 10-12 cm long, were taken from the Laboratory of the Faculty of Animal Husbandry, Marine, and Fisheries, Nusa Cendana University. The catfish were acclimated for 7 days in the aerated culture tank. During acclimation, the fish were fed with pellets twice a day (morning and afternoon) and siphoned daily to remove the feces and uneaten feed.

Aeromonas hydropilla infection. The catfish were infected with bacteria *A. hydropilla* at the density of 10^6 cells mL^{-1} on the tail base, with as much as 0.1 mL ind^{-1}. The infecting process occurred until the fish showed the infection symptoms, such as a change in the body color, especially on the injection mark, then the treatment was accomplished.

Aeromonas hydropilla infected catfish. After the catfish had shown the bacterial infection symptom, the treatment was done using honey and *E. hirta* combination at different ratios as desired treatments. The infected fish were immersed in a treatment tank. The immersion was accomplished in 1.5-2 min, then returned to the culture tank. The immersion was done for 10 days, while blood observation was conducted on day 12.

Hematological examination. The catfish blood was taken at the fore part of the caudal fin using a 1 ml syringe rinsed with Na-citrate 3.8%. The hematological observations, such as hematocrit, hemoglobin, and leucocyte, followed the method of Susandi et al (2017). The catfish hematological examinations were done before, after infection, and after treatment application, while the survivorship was observed at the end of the study.

Method and research design. The study is experimental using a Complete Randomized Design, with one independent variable (honey-asthma plant ratio). The treatments of honey-*E. hirta* ratio studied through the immersion method were A= 2:1 (more honey than *E. hirta*), B= 1:1 (honey and *E. hirta* are in similar amounts), and 1:2 (less honey than *E. hirta*), negative control (healthy catfish), and positive control (infected catfish), with 3 replications each. The hematological data and the fish survivorship were analyzed using ANOVA.

Results and Discussion

Phytochemical content. Table 1 shows the active compounds contained in honey, *E. hirta,* and a mixture of them.

Table 1

Active compounds of honey, *Euphorbia hirta,* and their combination

Sample	*Phytochemical analysis*						
	Alkaloid	*Flavonoid*	*Saponin*	*Terpenoid*	*Steroid*	*Phenol*	*Tannin*
Honey (M) 50%	++	+	++	+++	-	+	+
Asthma plant (PK) 3%	+++	+	+	++	-	+++	+++
Combined honey 50% and *E. hirta* 3%	+++	+	++	+++	-	++	++

+++ = very strong; ++ = moderate; + = low; - = absent.

All samples have similar active compounds, but at different concentrations (Table 1). The active compounds in the individual or combined forms make them have antibacterial activity with different mechanisms. The antibacterial mechanism of alkaloids is by disrupting the peptidoglycan components in the cell wall layer and causing cell mortality (Ajizah 2004; Ningsih et al 2016). Phenols and their derivatives, such as flavonoids and tannins, are antibacterial, by disrupting the bacterium's cell membrane. The hydroxyl groups of flavonoids and tannins can interact with the protein of the bacterial cell membrane through hydrogen binding so that the protein loses its function (Cowan 1999). Furthermore, according to Bucekova et al (2019), polyphenols are usually responsible for destroying free radicals and inhibiting oxidation.

The antibacterial mechanism of saponins is to disturb the stability of the bacterial cell membrane causing damage to the cell membrane and eventually resulting in cell lysis (Kurniawan & Aryana 2015). The antibacterial mechanism of terpenoids occurs through cell membrane destruction by lipolytic compounds (Cowan 1999). Terpenoids can react with porine (transmembrane protein) on the external membrane of the bacterial cell wall, form a strong polymer bond, disrupt the porcine, and reduce the permeability of the bacterial cell wall, which then kills the bacteria. The honey-*E. hirta* combination could increase the active compounds, especially phenol and its derivatives contained in low amounts in honey. The limited amount of polyphenols in honey (Combarros-Fuertes et al 2020) cannot give singly an antibacterial effect. The antibacterial effect of honey is a synergic effect of phenol and other compounds, such as hydrogen peroxide. Thus, the combination of honey and *E. hirta* could increase the antibacterial activity due to the presence of the active compounds of *E. hirta*.

Hematology of healthy fish and infected fish, and after treatment

Erythrocyte. Mean erythrocytes of healthy, infected and post-treatment specimens of catfish are presented in Figure 1, which shows that the mean number of erythrocytes in the healthy fish is 2.833.333 cells mm^{-3}, and when the catfish are infected with the bacteria, the erythrocyte level falls down to 1,800,000 cells mm^{-3}. After treated with the mixture of honey and *E. hirta*, the erythrocyte level rises again, except in the untreated catfish (positive control), in which the erythrocyte level continuously declines to 1.733.333 cells mm^{-3}. However, there is no difference of effect between treatments ($P>0.05$); all treatments could increase the erythrocyte level of the catfish.

Furthermore, the erythrocyte level of healthy catfish depends ona series of factors. The present study found the erythrocyte level of 2,833,333 cells mm^{-3}, lower than that reported by Sukenda et al (2008), 4,210,000 cells mm^{-3}, but higher than that found by Cerlina et al (2021), 2,460,000 cells mm^{-3}. According to Witeska et al (2021) and Ejraei et al (2015), the environmental conditions, species, age, and nutrition could influence the erythrocyte level.

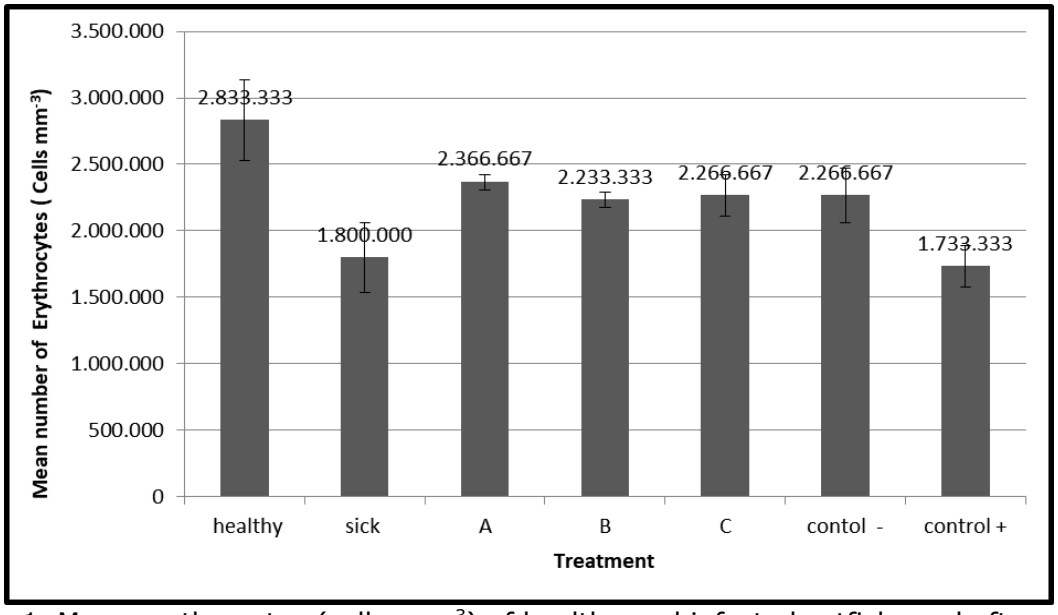

Figure 1. Mean erythrocytes (cells mm^{-3}) of healthy and infected catfish, and after treatment with honey and *Euphorbia hirta* combination. A=soaking in 2:1 of honey-*Euphorbia hirta*; B=soaking in 1 honey:1 *Euphorbia hirta*; C=soaking in 1 honey:2 *Euphorbia hirta.*

A high number of erythrocytes in healthy fish could result from suitable environmental conditions. A sufficient number of erythrocytes also ensures enough oxygen for cells. On the other hand, the bacteria-infected fish suffer from a declined number of erythrocytes due to the virulence of the bacteria. According to Straved & Odeyemi (2017), bacteria produce a number of virulence factors, particularly hemolysin and aerolysin, that trigger the disease. The hemolysin enzyme dissolved in the blood can break down the erythrocytes and remove the hemoglobin so that there will be much blood coming out through the wound of the infected body surface (Triyaningsih et al 2014). It causes the declined erythrocytes in the fish infected with *A. hydropilla.*

Hemoglobin. Mean hemoglobin in the healthy catfish was 12.97 gr dL^{-1}, while in *A. hydropilla*-infected catfish, it declined to 9.37 gr dL^{-1} (Figure 2). Declined hemoglobin occurred in the infected catfish and the positive control treatment, down to 8.76 g dL^{-1}. All catfish at treatments A, B, and C could increase the hemoglobin level, no different effects were found between treatments A, B, and C ($P>0.05$), indicating that all combinations of honey-*E. hirta* result in the same effect on the hemoglobin level of the catfish. Hemoglobin in the erythrocytes works as oxygen binding, so that it highly determines the fish metabolism ability (Dewantoro 2019). Furthermore, low hemoglobin in fish, according to Palmi et al (2019), makes the oxygen content in the blood fall, followed by a low metabolic rate, and yielding a low energy. This condition causes the fish to lose their appetite as one of the behavioral symptoms of the infected fish (Stoskopf 1993).

The *A. hydropilla*-infected catfish treated with honey-*E. hirta* combination had increased hemoglobin due to no more lysis of the erythrocytes caused by the toxin produced by *A. hydropilla*. Therefore, the erythrocytes increase, followed by increased hemoglobin, indicating that the combination of honey-*E. hirta* has an antibacterial activity against *A. hydropilla*. The antibacterial activity of honey can occur through several mechanisms, such as high glucose, high acidity, the presence of an antibacterial organic matter, and the presence of radical compound hydrogen peroxide (H_2O_2) capable of killing the pathogenic microorganisms (Nadhilla 2014; Carina et al 2014; Johnston et al 2018; Nolan 2019). The antibacterial activity of honey synergically works with that of *E. hirta*, which possesses an antibacterial activity due to the presence of alkaloids, flavonoids, saponins, phenols, terpenoids, and tannins (Table 1).

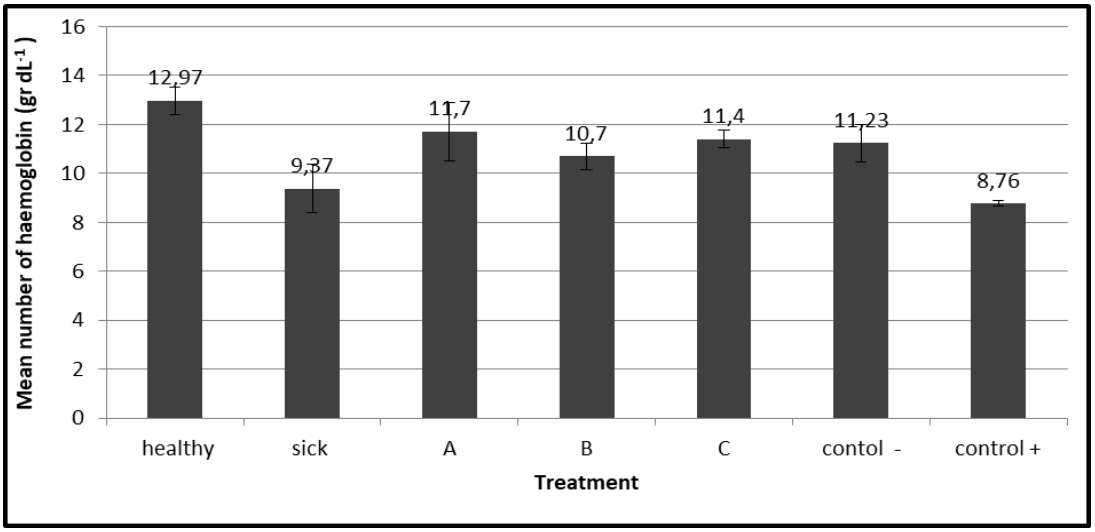

Figure 2. Mean hemoglobin (gr dL^{-1}) of healthy catfish, infected fish, and after treatment with honey-*Euphorbia hirta* combination. A=soaking in 2:1 of honey- *Euphorbia hirta*; B=soaking in 1 honey:1 *Euphorbia hirta*; C=soaking in 1:2 of honey- *Euphorbia hirta.*

Leucocytes. Changes in leucocyte levels of the infected catfish, healthy fish, and treated fish are presented in Figure 3.

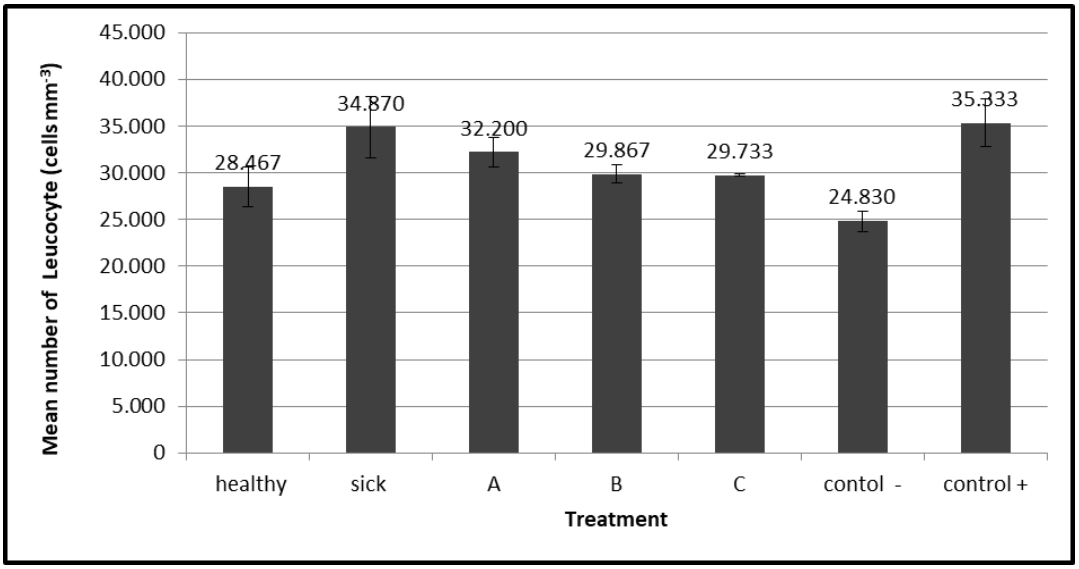

Figure 3. Mean leucocyte (cells mm^{-3}) of healthy catfish, infected catfish, and after treatment with honey-*Euphorbia hirta* combination. A=soaking in 2:1 of honey-*Euphorbia hirta*; B=soaking in 1 honey:1 *Euphorbia hirta*; C=soaking in 1:2 of honey- *Euphorbia hirta.*

Leucocyte is cell blood playing important role in fish's defense system against pathogenic infections. It could be seen in the catfish infected with *A. hydropilla* that the leucocytes increase from 28,467 cells mm^{-3} to 35,870 cells mm^{-3} (Figure 1). Rosidah et al (2019a) also found an increased leucocyte level from 70,020 cells mm^{-3} to 103,300 cells mm^{-3} in the Sangkuriang catfish infected with *A. hydropilla*. A similar condition is also reported by Salosso et al (2020) for carp and Nile tilapia infected with *Flavobacterium columnare* (Sebastiao et al 2011).

All treatments yield leucocyte level decline even though it is still higher than that in healthy fish. No significant differences were found in effects between treatments ($P>0.05$), indicating that all treatment combinations of honey-*E. hirta* could give the same effect on the leucocyte level of the catfish. Thus, the application of honey-*E. hirta*

combination can kill the bacteria *A. hydropilla* and cure the catfish infected with *A. hydropilla*. The application of honey-*E. hirta* combination can help the fish defense system against bacterial infection.

The antioxidant activity of *E. hirta* and honey has been reported by Jeba et al (2018) and Rosidah et al (2019a), respectively. Honey administered in feeding at the dose of 200 mL kg^{-1} can increase the immune system of Koi carp against bacteria *A. hydropilla*. Fuandila et al (2019) have also proved the immunostimulant ability of honey in vannamei shrimp against *Vibrio parahaemoyliticus*. The immunostimulant effect of ethanol-extracted propolis of bee *Apis mellifera* from Brazil has been shown in Nile tilapia infected with *A. hydropilla* (Orsi et al 2017).

Catfish survivorship. Figure 4 demonstrates that the catfish have 100% survivorship at all treatments of honey-E. hirta combination. The mortality occurs only in the infected catfish without honey-*E. hirta* treatment application. High survivorship of the catfish after the combined honey-*E. hirta* application indicates that this material combination could heal the catfish infected with *A. hydropilla*. The healing ability of the combined honey-*E. hirta* application to the catfish infected with bacteria could occur through the antibacterial, antioxidant, and immunostimulant mechanisms of the material. *E. hirta* and honey have antibacterial, antiinflammatory, and antioxidant activity (Gufta et al 2018; Dewi et al 2017). Both materials are synergetic in healing the catfish.

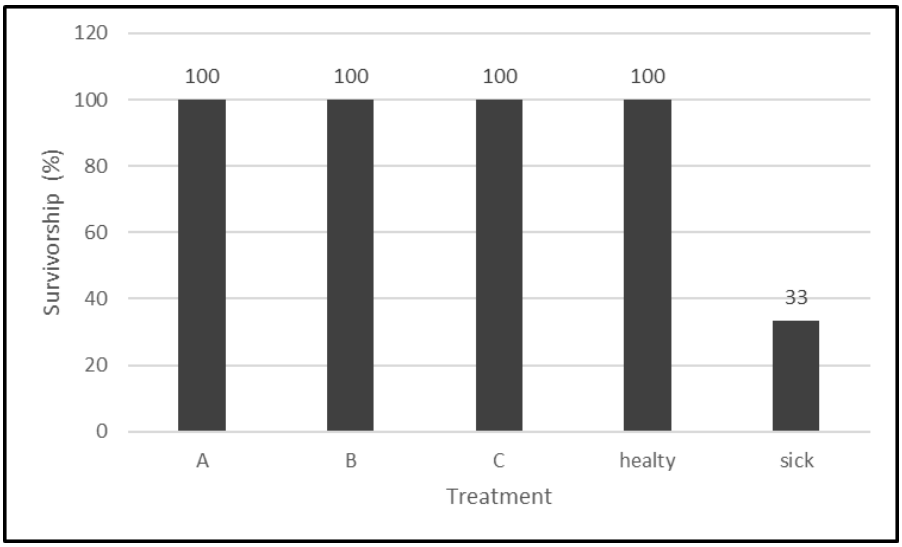

Figure 4. Mean catfish survivorship at the end of the study. A=soaking in a 2:1 solution of honey-*Euphorbia hirta*; B=soaking in a solution of 1 honey:1 *Euphorbia hirta*; C=soaking in a solution of 1:2 of honey-*Euphorbia hirta*.

High mortality of the catfish occurred in *A. hydropilla*-infected fish with no treatment application. According to Orsi et al (2017), *A. hydropilla* is one of the bacteria that often infect cultured freshwater fish and can result in mortality up to 100% (Rosidah et al 2019b). Lukistyowati & Kurniasi (2011) added that *A. hydropilla* often causes disease outbreaks with a mortality rate of 80-100% in a short period, 1-2 weeks. The symptoms of carp infected with MAS disease are changes in skin color, skin lesions, hemorrhage, and bruises or muscle ulcers (Susandi et al 2017; Laith & Najlah 2013).

Conclusions. The combination of honey (50%) and *E. hirta* (3%) could cure the catfish infected with *A. hydropilla* by normalizing the catfish hematology. Healthy catfish had a high erythrocyte, hematocrit, and hemoglobin, but fell down in the infected catfish. The leucocyte was low in healthy catfish but rose in the infected catfish. After treatment with honey and *E. hirta* combination, all the hematological parameters returned to the normal level. Compared to the control treatment, all treatments with honey-*E. hirta* improved the survival and cured the catfish infected with *A. hydropilla*.

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

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Received: 03 December 2022. Accepted: 14 March 2023. Published online: 27 March 2023.
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How to cite this article:
Salosso Y., Ressie J. D., Ridwan, Foes Y. W., Pasaribu W., 2023 Bacteria *Aeromonas hydropilla*-induced disease treatment in catfish (*Clarias* sp.) culture, with a combination of honey and asthma plant *Euphorbia hirta*. AACL Bioflux 16(2):878-886.