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Screening of some Indonesian medicinal plant extracts for anti quorum sensing activity to prevent *Aeromonas hydrophila* infection in *Oreochromis niloticus*

ARTINI PANGASTUTI♥, SITI LUSI ARUM SARI, AGUNG BUDIHARJO, SARI TRIYANA FITRI, PUJI SAYEKTI, SYARAFINA RATNA PUTRI

Microbe Biomaterials Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: artini_p@staff.uns.ac.id

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Abstract. *Pangastuti A, Sari SLA, Budiharjo A, Fitri ST, Sayekti P, Putri SR. 2021. Screening of some Indonesian medicinal plant extracts for anti quorum sensing activity to prevent* Aeromonas hydrophila *infection in* Oreochromis niloticus. *Biodiversitas 22: 3517-3522. Aeromonas hydrophila* disease is a threat to Indonesia's aquaculture and fishing industries because of its results in mass deaths. Efforts to treat and prevent *A. hydrophila* infection in fish have so far been limited to the use of antibiotics. The use of these antibiotics can result in bacterial resistance as well as issues related to fish quality and environment. Another option is to target the bacterial quorum sensing (QS) system, a bacterial intercellular communication system that uses signal molecules, regulates the expression of *A. hydrophila* virulence factors such as exoprotease enzymes, elastase, and biofilms. The expression of *A. hydrophila* virulence factor can be inhibited by using QS inhibitor compounds, preventing infection from starting. The purpose of this study was to screen some Indonesian medicinal plant extracts for anti-QS activity and then use the selected extract to prevent *A. hydrophila* infection in Tilapia fish (*Oreochromis niloticus*). With the administration of extract, QS inhibition was observed *in vitro* based on the production of violacein in *Chromobacterium violaceum*; enzyme exoprotease, elastase, and the formation biofilm in *A. hydrophila*. Tilapia fishes were fed with extract supplementation and then infected with *A. hydrophila* in an *in vivo* infection challenge test. These test fishes were divided into six treatment groups given a different concentration of extract in feed (Healthy fish with a concentration of 0 g/100 g of feed as control; infected fish with a concentration of 0 g/100 g of feed; infected fish with a concentration of 0.2 g/100 g of feed; infected fish with a concentration 0.4 g/100 g of feed; infected fish with a concentration of 0.6 g/100 g of feed; infected fish with a concentration of 0.8 g/100 g of feed). Fish were reared for 2 weeks with the frequency of feeding 3 times a day. The variables observed included fish survival rate, behavior (swimming, agility, movement, and frequency of operculum opening), and morphology of the test fish. Data analysis of virulence factors production, fish survival rate, fish appetite, and frequency of operculum opening was carried out using the ANOVA variant test, continued with a 5% DMRT test. Others were analyzed descriptively. Of all the extracts studied, 4 extracts showed inhibition of the production of the violaceum pigment in *C. violaceum*. *Muntingia calabura* leaves extract was chosen for further testing due to its ease of availability and safety. The extract was significantly decreased the production of *A. hydrophila* caseinolytic and elastolytic protease, but had no effect on biofilm formation. In vivo challenge test with *A. hydrophila* showed better survival rate and fish condition in fish groups that were supplemented with *M. calabura* extract.

Keywords: *Aeromonas hydrophila*, *Muntingia calabura*, quorum sensing, tilapia fish

INTRODUCTION

Aeromonas hydrophila is an opportunistic pathogen that infects fishes when their immune systems weaken due to stress and environmental changes, lead to mass mortality in the culture of freshwater fish. So far, antibiotic treatment has been used to treat *A. hydrophila* infection. Inappropriate use of antibiotics results in bacterial resistance against them and rendering treatment ineffective. Antibiotic use also poses a problem for export products due to the requirement that they should be free from any antibiotic residues.

The expression of virulence factors that determine *A. hydrophila* pathogenicity is regulated by a system known as quorum sensing (QS) (Rasmussen-Ivey et al. 2016). This system allows bacteria to communicate with one another by secreting signaling molecules that regulate gene expression in *A. hydrophila*, including the production of virulence factors and biofilm formation. N-acyl homoserine lactone (AHL) is the signaling molecule that is released into the environment. When the number of bacteria increases, the AHL molecules return to the cell and activate the expression of specific genes. Inhibiting the QS system may be an option for controlling *A. hydrophila* infection in Tilapia fishes (*Oreochromis niloticus*). If the bacteria's communication system is disrupted, the bacteria will not produce virulence factors. Bacteria can still live in fish without causing disease symptoms. Because bacterial growth is not disrupted, this type of virulence inhibition prevents bacteria from developing resistance (Haque et al. 2018). Furthermore, QS-inhibitory compounds are not expected to eliminate beneficial bacteria existent in the host.

Several compounds of plant origin are known to have inhibitory activity against *A. hydrophila* QS systems, such as Naringin (Srinivasan et al. 2020), Rosmarinic acid (Devi

et al. 2016), and curcumin (Mangoudehi et al. 2020). Giving extracts, not pure compounds, as supplement to prevent bacterial infection can reduce capital costs in the aquaculture industry. Extracts can be mixed in fish feed, more practical at aquaculture scale than immersion methods, minimizing the possibility of leaching of active ingredients. Indonesia has a huge diversity in plant kingdom that has been a source of medicine for many years. This study was aimed to screen the anti QS activity of some Indonesian medicinal plant extracts, explore the effect of selected extract on the virulence factors production in *A. hydrophila,* and the application of selected extract to reduce mortality of fish infected with *A. hydrophila*.

MATERIALS AND METHODS

Plant materials and extraction

The Indonesian medicinal plant used was clove flowers (*Syzygium aromaticum*), red onion tubers (*Allium cepa*), senggani leaves (*Melastoma candidum*), Talok leaves (*Muntingia calabura*), pandan leaves (*Pandanus amaryllifolius*), radish leaves (*Raphanus sativus*), Tea leaves (*Camellia sinensis*), lidah buaya (*Aloe vera*), temu hitam (*Curcuma aeruginosa*) rhizome, and strawberry (*Fragaria annanasa*). These materials came from Tawangmangu in the Karanganyar District, Central Java, Indonesia were materials used as herbal medicine in local community.

Two kilograms of fresh materials were oven-dried for two days. Dried materials were ground and mixed with 96 percent ethanol or ethyl acetate (100g dry wt/L) before being vacuum filtered with filter paper to remove particulate matter. The filtrate was then concentrated in a rotary evaporator and stored in an amber bottle in the freezer until use.

Bacterial strains and growth media

Chromobacterium violaceum wild type obtained from R&D PT Charoen Phokphand Indonesia and *Aeromonas hydrophila* wild type, was isolated from infected fish, obtained from *Balai Laboratorium Kesehatan,* Yogyakarta were used in present study.

All bacterial cells were maintained on Luria-Bertani broth (LB) agar plates and in LB liquid for overnight cultures. The composition of LB medium was 1% peptone, 0.5% Yeast extract, 0.5% NaCl and additional 1.5% agar for LB agar plates. AB minimum media supplemented with glucose and casein amino acids (20% w/v) was used for elastase assays.

Anti QS assay

The well diffusion method was used to conduct qualitative testing of quorum sensing inhibition (Adonizio et al. 2006). *C. violaceum* was cultured in LB broth medium overnight. A total of 20 mL of sterile LB agar was poured into a sterile Petri dish, followed by 100µL overnight culture of *C. violaceum*. The Petri dish was slowly shaken to distribute the bacterial suspension evenly and then stand until it solidified. The solid media was perforated with a cork borer of diameter 6 mm, forming a well on the disc. The extracts were then dissolved in 2% DMSO and 20 µL of the extract solution was placed in a well. Various concentration of extract was tested (200, 400, 600, 800, 1000 µg/mL). Ethanol, ethyl acetate, and DMSO were used as negative control. Plates were incubated overnight at 30 °C, and QS inhibition was detected by a ring of colorless, but viable, cells around the disk. Measurements were made from the outer edge of the disks to the edge of the zones of anti-QS inhibition. Tetracyclin 10 µg per well was included to differentiate between antibiotic effect and anti-QS activity. One extract was selected for further assays based on activity and availability of the material.

Inhibition of *A. hydrophila* virulence factors production

Culture conditions were as follows for all assays except biofilm formation. *A. hydrophila* cultures were grown overnight in LB at 37 °C with constant shaking. The cultures were then diluted 100-fold into AB or LB media and grown to an OD600 of 1.7 (early stationary phase). The culture was then divided into 10 mL aliquots and 1 mL of fresh media containing crude plant (or media and DMSO as control) was added to a final concentration of 1 mg/ml extract. Cultures were recovered when they were in the late stationary phase (approximately 12 hours after addition). Centrifugation at 10,000 x g for 10 minutes separated cells from growth media for subsequent assay (Adonizio et al. 2008).

Caseinolytic activity assay

For 10 minutes, 0.5 mL culture supernatant was incubated with 2.5 mL casein solution (0.65% w/v) in phosphate-buffered saline (pH 7) at 37 ºC. The undigested protein was then precipitated by adding 2.5 mL of 10% w/v trichloroacetic acid. For 30 minutes, the completed reaction mixture was incubated at 37°C. Centrifugation at 10,000 rpm for 10 minutes at 4 ºC was used to separate the precipitates from supernatant. The supernatant was then mixed with 2.5 mL of 0.5 M sodium carbonate and 0.5 mL of 10% v/v Folin–Ciocalteau reagent, then incubated at 37ºC for 20 minutes. The absorbance was measured at 660 nm. Under the assay conditions, one unit of enzyme activity (U/mL) is defined as the amount of enzyme liberating 1 µg of tyrosine/min/mL.

Elastolytic activity assay

Elastin Congo red (ECR; Sigma, St. Louis, MO) was used to determine the elastolytic activity of AB culture supernatants. A 100 µL aliquot was added to 900 µL of ECR solution in buffer (20 mg ECR, 100 mM Tris, 1 mM CaCl2, pH 7.5). This was then shaken for 3 hours at 37 °C. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. Activity was expressed as OD495 per µg protein.

Biofilm formation assay

The effect of plant extracts on the attachment phase of biofilm formation was measured using the PVC biofilm formation assay (O'Toole 2011). Briefly, overnight

cultures of *A. hydrophila* were resuspended in fresh AB medium in the presence and absence of plant extracts. After a 10 hour incubation period at 30°C, biofilms were visualized in the microtiter plates by staining with a crystal violet solution. Next, plates were rinsed to remove planktonic cells, and surface-attached cells were then quantified by solubilizing the dye in ethanol and measuring OD650.

Growth curves

The effect of plant extracts on cell proliferation was determined by monitoring *A. hydrophila* growth curve. Briefly, an overnight culture (LB) of *A. hydrophila* was diluted 100-fold into 1 liter LB media. OD600 was monitored at 60-minute intervals until an OD600 of ~1.7 (approximately 8 hours). The culture was then divided into 28 mL aliquots, to which 2 mL of LB (controls) or 2 mL concentrated extract were added. The final extract concentration was 1 mg/mL. Cultures with added extract were normalized to the control OD600 of 1.7 at this time to account for plant pigmentation. Optical density was monitored at 1.5-hour intervals until a final time point of 24 hours. All OD600 measurements were verified at 1/10 dilution for greater accuracy.

Assay of total protein

Protein concentration was measured by the method of Bradford (Gotham et al. 1988) using Bovine Serum Albumin (BSA) as the standard. Raw data from all enzymatic assays were normalized to total protein concentration.

Tilapia feed formulation

Tilapia fish feed was mashed and given 0.1% CMC then stirred until smooth. Each 100 g of feed was mixed with the extract in the form of a paste to a concentration of 0, 0.4; 0.6; 0.8 g/100g of feed. The feed was reshaped and dried in the oven, then stored in an airtight container until used.

Tilapia fish rearing

Fifty Tilapia seeds with a 7-10 cm size were acclimatized for 14 days in the aquarium (40x40x40 cm). Fish were fed 0.4 g/fish for one feeding 3 times a day. After acclimatization, fish fasted for 24 hours. Overnight culture of *A. hydrophila* was adjusted to 5×10^7 CFU total cells in 200 µL PBS introduced by intraperitoneal injection, control fish were injected with 200 µL sterile PBS (Barger et al. 2020) then returned to aquarium until fully recovered. Then, 6 Tilapia/aquarium were added for each treatment group (0, 0.4; 0.6; 0.8 g extract supplementation/100g of feed). Extract supplemented feed was given 3 times a day, 0.4 g per administration. Fishes were reared for 14 days. During the rearing, parameters observed include: fish survival rate (FSR), response, behavior, morphology, and environment parameters (pH, temperature, and Dissolved Oxygen). The effect of the concentration extract on the average number of fish living at the end of the study was evaluated by regression analyses.

RESULTS AND DISCUSSION

Anti-QS activity is frequently found in plants that have been used as traditional medicine since ancient times (Bouyahya 2017). Several mechanisms of QS system inhibition have been identified up to this point (Asfour 2018). Based on their interactions with the QS system, inhibitory compounds can be degraders, antagonists, or competitors. A furanone from the algae Delisea pulchra, which is a competitor of QS signal and prevents the activation of regulatory proteins in some bacteria, was the first compound reported to inhibit QS (Hentzer et al. 2002). Anti-QS compounds were also detected in plants and studied for many applications (Quecan et al. 2019; Mok et al. 2020; Kumar & Arthanareeswaran 2019). Several natural compounds that interact with the QS system have also been identified using the in silico approach (Ahmad et al. 2017; Satari et al. 2021). However, references to the specific inhibition of the QS system in *A. hydrophila* are still very limited.

Table 2. Attenuation of *Aeromonas hydrophila* virulence factors by *Muntingia calabura* extract

Activity	Control (DMSO)	Extract	% change
Caseinolytic activity (OD280/hours/µg protein)	0.572[a]	0.416[b]	-27.3
Elastase activity (ΔOD495/µg protein)	0.594[a]	0.429[b]	-27.8
Biofilm (OD650)	2.201[a]	2.202[a]	Not significant

Note: Numbers followed by the same letter (in the same row) are not significantly different in Duncan's test at the 5% test level

Table 1. Anti quorum sensing (QS) activity of plant extracts on *C. violaceum*

Extracts	Solvent	Diameter of quorum sensing inhibition zone (mm)				
		200 µg/mL	400 µg/mL	600 µg/mL	800 µg/mL	1000 µg/mL
Melastoma candidum leaves	Etanol	8.32	8.76	9.24	9.84	10.46
	Ethyl acetate	2.72	3.23	3.86	4.42	4.72
Muntingia calabura leaves	Ethanol	0	0	2.73	3.42	4.15
	Ethyl acetate	0	0	2.23	2.77	3.32
Curcuma aeruginosa rhizome	Etil asetat	0	0	2.32	2.34	2.53
Fragaria annanasa	Ethanol	0	0	2.23	2.43	2.48
DMSO	0					
Ethanol	0					
Ethyl acetate	0					

Four medicinal plant extracts were found to have anti-QS activity using ethanol or ethyl acetate solvent (Table 1). *M. candidum* leaves had the highest activity in both ethanolic and ethyl acetate extracts. *Melastoma* species are rich in tannins of the hydrolyzable type, mainly di- and trimers, with reported bactericidal and antiviral activities (Zheng et al. 2020). Several other studies using plants with high tannin levels also showed anti-quorum sensing activity (Shukla and Bhathena 2016; Deryabin & Tolmacheva 2015). Flavonoids are another type of compound found in abundance in *M. candidum.* Several studies have shown that flavonoids have anti-QS activity (Manner & Fallarero 2018; Bodede et al. 2018), which were thought to be mediated by a QS receptor allosteric inhibition mechanism (Paczkowski et al. 2017). This finding indicated that this plant has the potential to be studied further as an anti-QS compound. *M. candidum*, on the other hand, is a relatively rare plant, limiting its widespread use as an anti-infection agent in aquaculture.

Muntingia calabura) leaves also demonstrated anti-QS activity, at a lower level than *M. candidum*. Leaves extract of *M. calabura* contains common compounds responsible for anti QS activity, namely flavonoids, polyphenols, flavonols, steroids, terpenoids, alkaloids, and tannins (Putri and Fatmawati 2018; Sari et al. 2020), had inhibitory activity on several microbes and also antioxidant activity. *M. calabura* leaves are available in large quantities and are not seasonal so they are more likely to be used in aquaculture for infection prevention, therefore, we chose this extract for further assays.

Ethanolic extract of *M. calabura* leaves (1000 µg/mL) did not affect the growth of *A. hydrophila* (Figure 1.). Growth measurements were taken to back up the findings of the virulence factor production assay. If the addition of ethanol extract of *M. calabura* leaves did not affect bacterial growth, then the change in virulence factor production was caused by a disruption in the QS mechanism rather than a disruption in bacterial cell growth. This is important because an inhibition to growth may lead to antimicrobial resistance while inhibition of virulence is not.

Table 2 shows the effect of *M calabura* leaves ethanolic extract on *A. hydrophila* virulence factors. The addition of extract to the culture led to 27.3% and 27.8% reduction in the caseinolytic and elastolytic activity of *A. hydrophila* respectively. Most strains of *A. hydrophila* secrete two proteases into the culture medium: a thermostable metalloprotease and a temperature-labile serine protease (Cascón et al. 2000). Exoprotease, especially elastolytic protease, correlates with the establishment of infection and is important to the initiation and progression of disease (Li et al. 2019). Cascon et al. (2000) showed that *A. hydrophila* virulence required the presence of elastase in a rainbow trout (*Oncorhynchus mykiss*), indicated by the LD_{50} value which was 100 times higher in the elastase-deficient mutant than in the wildtype. During i*n vivo* challenge in the channel catfish, *A. Hydrophila* pathogenicity in deficient mutants with a lack of protein secretion and activity of enzyme/toxin, including elastolytic activity, was attenuated completely, while, virulence recovered in the complemented mutant (Barger et al. 2020). The QS system regulates exoprotease expression in *A. hydrophila*, then inhibition of the QS system might prevent the emergence of *A. hydrophila* infection in fish.

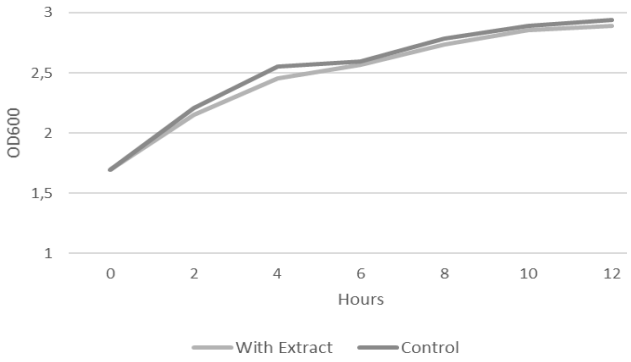

Figure 1. Effect of *Muntingia calabura* extract on growth of *Aeromonas hydrophila*

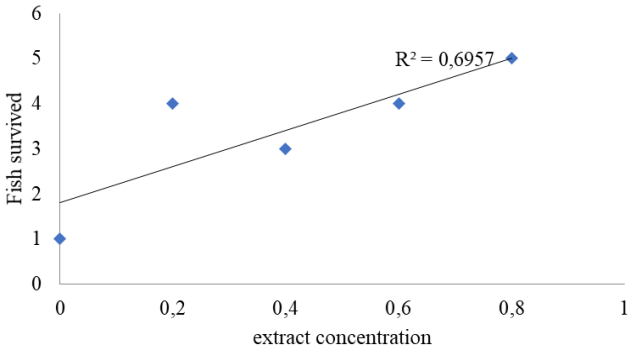

Figure 2. The effect of extract concentration on the average number of fish that lived at the end of the study

Table 3. The effect of the *Muntingia calabura* supplementation on the survival rate

Treatment	Fish survival rate (%)
Control	50b
0 g extract	16.67a
0.2 g extract	66.67c
0.4 g extract	50b
0.6 g extract	66.67c
0.8 g extract	83.30d

Note: Numbers followed by the same letter are not significantly different in Duncan's test at the 5% test level

However, the addition of extract did not have a significant effect on biofilm formation. Although, intercellular communication is important for biofilm development and formation, the QS system only indirectly regulates biofilm formation by regulating the expression of genes encoding intercellular signaling proteins and initiating the formation of polysaccharides (Passos da Silva et al. 2017), suggested that biofilm differentiation in *A. hydrophila* was less susceptible than exoprotease production to antagonism against QS signal. Inhibiting the QS system does not necessarily impede biofilm formation as well. Our result was contrary to Pratiwi et al. (2018), which discovered antibiofilm activity of *M. calabura* extract in *Pseudomonas aeruginosa*, suggesting differences in the regulation of biofilm formation in different bacterial species.

Regression analysis of the effect of extract concentration on the average number of fish that lived at the end of the study (Figure 2.) showed a coefficient of determination of 0.6957, meaning that 69.57% of the variation in the number of fish that survived at the end of the study was caused by variations in the concentration of the extract. Extract concentration had a significant effect on the survival of fish, as presented in Table 3. Supplementation of as little as 0.2 g extract/ 100 g feed was sufficient to protect the fish against *A. hydrophila* infection, but 0.8 g extract had the highest survival rate level among the other treatment groups. The flavonoid compound in the extract might be responsible for this result. Devi et al. (2016) reported that rosmarinic acid can reduce the expression of several virulence genes in *A. hydrophila*, and treatment with this compound in Zebrafish infected with *A. hydrophila* showed an increase in survival rate. Naringin has also been investigated for protection of fish against infection (Srinivasan et al. 2020).

The survival rate of Tilapia which was supplemented with the extract of *M. calabura* leaves in the feed exceeded the survival rate of the non-infected fish. This indicated another effect of the ethanolic extract of *M. calabura* on fish body functions and protection from infection. *M. calabura* leaves contain compounds that have antioxidant activity such as phenolic compounds. Giving ethanol extract of *M. calabura* leaves could prevent infection of pathogenic bacteria in fish and increase the fish's body resistance to free radicals and environmental conditions. The compound in the extract could have immunostimulant effect too.

The effect of extract supplementation on fish behavior and response was also observed. Infection of *A. hydrophila* disrupts digestive tract of fish, causing decrease in appetite and eventually death, which seemed to occur in the group not given the extract. However, fish appetite increased along with the increase in extract concentration in the feed. The group of fish given the extract also had good sensibility to stimuli, swim upright with active movement, similar to the control group. A healthy fish normally swim upright, fast and active because both sides of its body function properly.

The extract in the feed also had a significant effect on the fish breathing behavior. Bacterial infection increases the respiratory rate at the beginning of exposure and gradually decreases until it reaches the death phase. This is because at the beginning the gills are damaged, the fish try to maintain oxygen intake by breathing more often to enter more oxygen. In fish infected with *A. hydrophila*, the gills are also covered with more mucus and the bacteria can damage the tissue until the gills are torn. This abnormal structure causes anomalies in fish respiration. Subsequently, a gradual decrease in breathing frequency can occur due to the reduced energy of the fish to carry out the respiratory process. However, the frequency of operculum opening and closing in Tilapia supplemented with extract were normal around 128-135 times per minute, the same as in the uninfected group.

Morphological and topographical observations of fish that died during rearing confirmed that fish mortality in treatment groups was due to bacterial infection. Fish mortality in control group, on the other hand, did not show signs of bacterial infection. The control fish had a bright body color and there were no lesions and excess mucus on their bodies. The digestive tract is intact and there was no swelling in the constituent organs. Dead fish that were not supplemented with extract, which has the lowest survival rate, had lesions on their bodies. Other pathological changes observed in treatment group include pale body color, presence of mucus, and damaged gill conditions. The degree of organs damage as a characteristic of *A. hydrophila* bacterial infection decreased with increasing concentration in the feed, suggesting the protective effect of *M. calabura* leaves extract. These findings are consistent with Srinivasan et al. (2018), who demonstrated the protective effect of naringin against pathological changes in vital organs that followed *A. hydrophila* infection in Zebra fish.

In conclusion, 0.8 g *M. calabura* leaves extract per 100 g of Tilapia feed was the optimal concentration that could prevent *A. hydrophila* infection, providing the best fish survival rate value, morphology, topography, response, and behavior of the fish. In summary, the anti-QS potential of medicinal plants may be as important as the antibacterial effect to combat the disease in fish aquaculture, without the risk of developing antimicrobial resistance. In this study, we proved *M. calabura* leaves to have anti-QS activity and could attenuate the bacterial virulence factor which is important in initiating the infection process. Therefore, the use of *M. calabura* extract to prevent *A. hydrophila* infection is expected to solve disease problems in aquaculture.

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