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Antibacterial Potency of Mangrove Plant (*Avicennia marina*) to Control *Aeromonas hydrophila* Infection in Gouramy (*Osphronemus gouramy* Lac.)

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ABSTRACT

Gouramy (*Osphronemus gourami* Lac.) is a freshwater fish in Indonesia that can be infected by *Aeromonas hydrophila*, leading to motile Aeromonas septicemia (MAS). This study aimed to evaluate the antibacterial potential of bioactive compounds from the *Avicennia marina* mangrove plant to control *A. hydrophila* infections in gouramy. Using a completely randomized design, the researchers tested four concentrations of *A. marina* leaf extracts (0g L–1, 0.2g L–1, 0.3g L–1, and 0.4g L–1) with four replicates. The leaves were extracted using methanol, and infected fish were soaked in the extracts for 60 minutes, with recovery monitored over 14 days. The study assessed disease symptoms, recovery, and survival rates, analyzing survival data with ANOVA and Duncan's test. Symptoms of MAS included skin depigmentation, ulcers, hemorrhaging, abdominal dropsy, fin erosion, and exophthalmia. Recovery began on days 7-8 for the treated groups, while control fish showed decreased appetite and ongoing symptoms. Statistical analysis revealed that *A. marina* extract significantly improved survival rates (P<0.05), with the most effective concentration being 0.2g L–1. The findings suggest that *A. marina* leaves could serve as a natural resource for developing anti-*A. hydrophila* treatments.

INTRODUCTION

Gouramy (*Osphronemus gouramy* Lac.) is one of main commodities of freshwater fish farming in Indonesia. Gouramy production has continued to increase year after another. **The Statistic Center Bureau (2022)** reported that carp production reached 234,904 tons in 2017 and increased to 875,594 tons in 2018. It is often that gouramy cultivation must cope with red spot disease or motile Aeromonas septicemia (MAS) caused by *Aeromonas* sp. The *Aeromonas* genus has many pathogenic species, such as *A. caviae*, *A. veronii*, *A. salmonicida*, *A. jandaei*, *A. sobria*, and *A. hydrophila* that potentially cause diseases **(Menanteau-Ledouble *et al*., 2016; Abd El -Salam *et al*., 2017; Dong *et al*., 2017; Emeish *et al*., 2018; Peatman *et al*., 2018; Mulia *et al*., 2020)**. The attack of *A. hydrophila* was recorded to the African catfish (*Clarias gariepinus*), walking catfish (*Clarias* sp.), tilapia (*Oreochromis niloticus*), gouramy (*O. gouramy*), eel (*Anguilla japonica*), koi (*Anabus testudineus*), common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), and rainbow trout (*Oncorhynchus mykiss*) **(Mulia, 2007; Yi *et al*., 2103; Catagay & Şen, 2014; Borty *et al*., 2016; Soltani *et al*., 2016; Abd El Tawab *et al*., 2017; Rozi *et al*., 2018a; Wamala *et al*., 2018; Mulia *et al*., 2021)**.

Common efforts to control MAS disease included the use of drugs and synthetic antibiotics treatments. However, inappropriate antibiotics were used on field, in terms of timing, dosage, or target, which has potential cause of bacterial resistance **(Serwecińska, 2020; Bombaywala *et al*., 2021)**. Therefore, it is important to search for alternative uses of natural materials having antibacterial compounds. Mangrove plant such as *Avicennia marina* can be an option. Its extract has different antimicrobial activity depending on its part and solvent used to prepare the extract **(Okla *et al*., 2021)**. Methanol extract of *A. marina* leaves indicates higher antibacterial activity against *Staphylococcus aureus* **(Dhayanithi *et al*., 2021)**. *A. marina* seed extract also has antibacterial activity against other bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* **(Naidu *et al*., 2019)**. A research results by **Okla *et al*. (2021)** showed chloroform extract of *A. marina* root having antibacterial activity against *S. aureus* and *E. coli*; ethanol extract of *A. marina* root showed antibacterial activity against *P. aeruginosa*, *Bacillus subtilis*, *S. aureus*, and *E. coli*; ethyl acetate extract of *A. marina* leaves showed antibacterial activity against *S. aureus* and *E. coli*; and ethanol extract of *A. marina* fruit also has antifungal activity against *Aspergillus fumigatus* and *Candida albicans*. The results of previous studies also reported that methanol extract of *A. marina* leaves had potential as an antibacterial against *A. hydrophila* tested *in vitro* because *A. marina* contains natural bactericidal compounds, such as flavonoids, alkaloids, terpenoids, and tannins **(Mulia *et al*., 2018)**. Thus, the objective of this study was to determine the antibacterial potential of *A. marina* mangrove plants to control *A. hydrophila* infection in gouramy.

MATERIALS AND METHODS

Ethical approval

The management, conditions, and procedures of the experiment in this study were approved by the Ethical Clearance Commission of Universitas Gadjah Mada (approval # certificate: 00137/04/LPPTI/|/201).

Research design

This study used experimental method with a completely randomized design (CRD), consisting of 4 treatments and 4 replications, including E0: without extract addition (control), E1: 0.2g L^{-1}, E2: 0.3g L^{-1}, and E3: 0.4g L^{-1}.

Preparation of *Avicennia marina* leaf extract

Avicennia marina leaves from Karang Talun village, Cilacap, Central Java, were taken in April 2022. *A. marina* was identified based on the study of **Cronquist (1981)**. The leaves were for their active components by thin layer chromatography (TLC) containing alkaloids, flavonoids, terpenoids, and tannins. *A. marina* leaf extract was prepared by maceration method using methanol solvent. *A. marina* leaves were weighed before being cut into small pieces and were put in the oven at 60°C for 4 × 24h. After reaching a dry state, the leaves of *A. marina* were crushed and sifted to become powder. 100g of simplicia was dissolved in 500mL methanol (80%) and incubated for 2 × 24h **(Mulia *et al*., 2018)**. Simplicia solution was stirred to ensure the optimal dissolution of the secondary metabolites. The macerated solution was filtered using filter paper to obtain a filtrate. It was then evaporated using a rotary evaporator until the solvent was evaporated. Additionally, water bath was used to produce thick extract.

Increasing virulence of *Aeromonas hydrophila* bacteria

This study used isolates *A. hydrophila* GPl-04 collected in the microbiology laboratory. To increase virulence of the bacteria, reinfection, and re-isolation of the isolate from gouramy were carried out first **(Mulia, 2012)**. Gouramy fish were obtained from the Sidabowa Fish Seed Center, Patikraja District, Banyumas Regency, measuring 9-13cm long. The fish were correctly handled and by the code of ethics for animal handling.

Aeromonas hydrophila pathogenicity test to determine the lethal dose (LD_{50})

The isolate of *A. hydrophila* GPl-04 was grown on GSP medium and incubated at 30°C for 24h. A single loop of *A. hydrophila* GPl-04 from GSP medium was transferred to 10mL tryptic soy broth (TSB) liquid medium (Merck) and was incubated at 30°C for 24h. The bacterial suspension in the TSB culture was diluted in stages, starting from 10^{-1} to 10^{-5} in PBS solution pH 7.4. The pathogenicity test was carried out by injecting 0.1mL of *A. hydrophila* GPl-04 suspension with dilution densities of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} at 10 gouramy per bucket and 2 repeating times. Observations were made for 7 days.

Aeromonas hydrophila infection in gouramy

Bacterial suspension of LD_{50} dose, as much as 0.1mL/ fish, was injected intramuscularly. Betadine was smeared on the back of the injection point to prevent infection. Then, fish were returned to the rearing containers.

A. marina leaf extract treatment

After being infected with *A. hydrophila*, gouramy individual was left for 2 days to let development of disease signs to appear. Next, the gouramy sample was treated with 60 minutes-soaked *A. marina* leaf extract based on the dose. The gouramy recovery process was observed within the 14 days after treatment.

Research parameters

The main research parameters included the development of disease signs, recovery process, and survival rate. Supporting research parameters cconsisted of water quality parameters such as temperature, pH, and levels of dissolved oxygen.

Data analysis

Survival rate data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test (DMRT) at 5% test level, while the development of disease signs and fish recovery processes, as well as supporting parameter data, were given with descriptive qualitative analysis.

RESULTS

Development signs of fish disease

Gouramy infected with *A. hydrophila* showed external signs of MAS disease on day 1–2 (Table 1). The signs appeared on day 1 was the decreasing appetite indicated by feed settled on the bottom of the test container. In addition, fish showed frequent swimming to the surface and slow movements. It also began to experience skin depigmentation. The injection wound on the back showed white ulcer (Fig. 1). On day 2, more specific external signs appeared such as widening ulcer from 0.1 × 0.1 to 1.2 ×1.4cm, hemorrhagic on body parts, abdominal dropsy, erosion fins, and exophthalmia.

Table 1. Development of external signs of disease in gouramy infected with *A. hydrophila*

Days	External development signs of MAS disease
1	Skin depigmentation, white ulcers on injection wounds, decreasing appetite, tendency movement to water surface
2	Widening ulcer on the wound, hemorrhagic on the body, abdominal dropsy, erosion fins, exophthalmia, weakness and frequent movement to surface

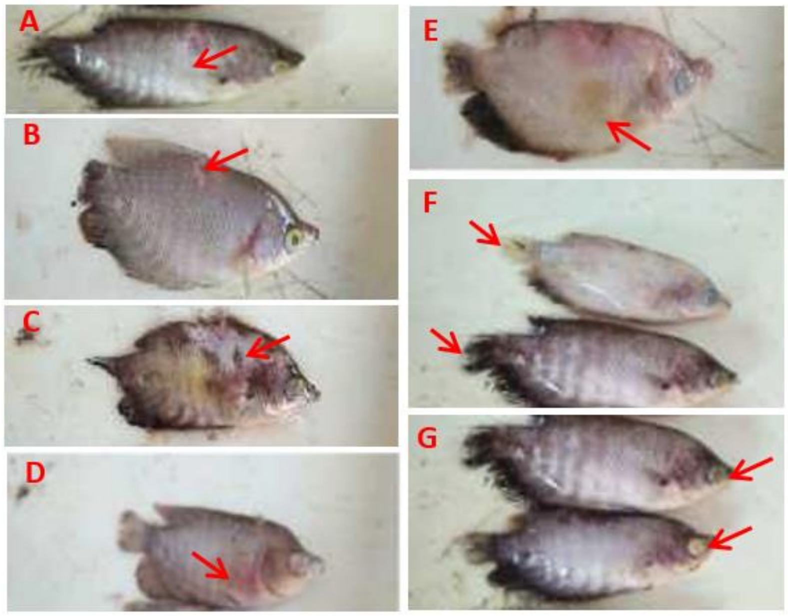

Fig. 1. Clinical signs of gouramy infected by *A. hydrophila* showing: **A.** Skin depigmentation; **B.** White ulcers on injection wounds; **C.** Widening ulcer on the wound; **D.** Hemorrhagic on the body; **E.** Abdominal dropsy; **F.** Erosion fins; and **G.** Exophthalmia

Recovery process of gouramy after treatment with *A. marina* leaf extract

The recovery process was observed over 14 days after the gouramy fish were treated with *A. marina* leaf extract. In this stage, a healing process of gouramy previously experiencing signs of MAS disease after given *A. hydrophila* infection was expected. The recovery process in control fish (E0) was not clearly visible during observations, although in fact all living things can recover naturally. On day 1-2, fish showed good appetite in general and still showed active movement, although clinical signs such as hemorrhagic and ulcers on the dorsal of the injection point, as well as white ulcers, still appeared. On day 3-4, the ulcer was widened instead of healed, exophthalmia even appeared. In addition, the fish appetite began to decrease. On the following day, fish showed slow movements and frequent movement to water surface, hyperemia on the body, hemorrhagic on gills, as well as flaky fins. At the end of observation, the fish appetite was still low with frequent slow movements toward water surface, having white nodules on the back, with still visible ulcers. Within the observation, fish mortality occurred until the end of the study.

Unlikely with the control fish (E0), there was a recovery process of gouramy treated with *A. marina* leaf extract (E1-E3). In E1 (*A. marina* leaf extract 0.2 g L^{-1}), the recovery process began on day 7-8 as marked by smaller ulcers and some fish looking healed with increasing appetite and active movements. On day 9-10, ulcer and hemorrhagic recovered, having good feeding response, active movement, and at the end of the study looking normal with higher appetite and agile swimming.

Similar to E1, the recovery process of E2 (*A. marina* leaf extract 0.3g L^{-1}) occurred on day 7-8 as marked with shrinking ulcer, good appetite response, and active movements. On day 9-10, ulcers and hemorrhagic were healed, with good response to feed and active movements. On day 11-14, ulcer and hemorrhagic were not visible, fish looking normal and healthy with normal eyes, better appetite, and agile swimming.

Recovery process of E3 (*A. marina* leaf extract 0.4g L^{-1}) was faster compared to E1 and E2, on day 5-6. It was marked by smaller ulcers, increasing appetite, and active movements. On day 9-10, a small part of ulcer on the injection point was still visible, but wound was starting to be covered, with the fish giving better response to food and moving normally. On day 11-14, ulcer and hemorrhagic were not visible and fish body was healthy showing no wounds, besides normal fins, good appetite, and active movement and agile were markedly observed.

Survival rate of gouramy

The results of *A. marina* leaf extract treatment were observed based on the survival rate. Gouramy treated with *A. marina* leaf extract (E1-E3) showed survival rate of 30-50% that was significantly different ($P<0.05$) from the control (E0), which was 12.50% (Fig. 2). Between treatments of *A. marina* leaf extract, the results had insignificant difference ($P>0.05$).

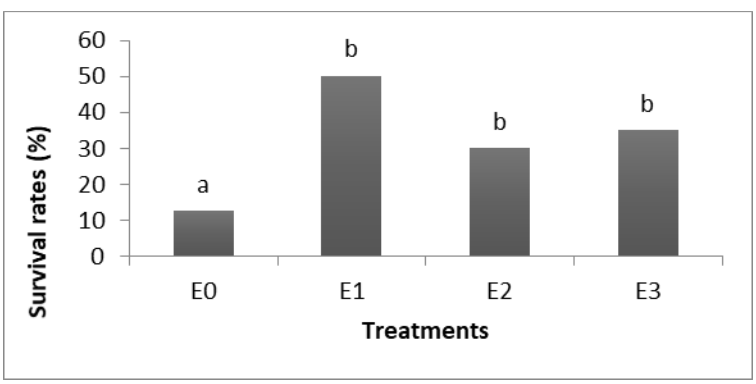

Fig. 2. Survival rate of gouramy

Water quality

Water quality parameters were always well maintained to remain optimum for gouramy to live in. The results showed ranges of water temperature, pH, and dissolved oxygen of 26-27°C, 7.2-7.6 and 5.4-9.2ppm, respectively (Table 2). The study's results showed no significant difference ($P>0.05$) in water quality parameter values between treatments.

Table 2. Water quality parameters

Treatment	Water quality parameters		
	Temperature(0C)	Acidity (pH)	Dissolved oxygen (ppm)
E0	26-27	7.2-7.6	6.0-9.2
E1	26-27	7.2-7.5	5.8-8.8
E2	26-27	7.2-7.6	6.0-8.7
E3	26-27	7.2-7.6	5.4-9.2

DISCUSSION

Gouramy infected with *A. hydrophila* showed clinical signs of MAS disease such as skin depigmentation, ulcers on injection wounds, hemorrhagic on the body, abdominal dropsy, eroded and black fins, exophthalmia, and yellow eyes, decreasing appetite, tendency movement to water surface, and weakness. Signs of MAS disease were almost uniform in all gouramy infected with *A. hydrophila*, and similar signs were reported by **Rozi *et al*. (2018a)**. In gouramy, with infection of *A. hydrophila*, showed clinical signs of ulcers, hemorrhagic, abscesses, and exophthalmia **(Rozi *et al*., 2018a)**. Similar signs also occurred on other types of freshwater fish infected with *A. hydrophila*. In the tilapia (*O. niloticus*), clinical signs caused by *A. hydrophila* included hemorrhagic eyes, deep skin external ulcers, severe hemorrhage on ventral aspect and on pectoral fins **(El-Son *et al*., 2019)**, whereas in the African catfish (*C. gariepinus*), signs included skin discoloration, erosive skin lesions, hyperemic spots, ulcers on the body, septicemic hemorrhage, erythrema fins, fin rot, and dorsal fins erosion **(Anyanwu *et al*., 2015; Mulia *et al*., 2022)**.

Gouramy infected with *A. hydrophila* experienced a decreasing feed response. It was suspected to experience stress due to injection that could decrease appetite. Such condition weakened the fish body resistance and made it susceptible to disease. Additionally, fish showed tendency of slow and weak movement and swimming toward the water surface. Similar condition occurred in a research by **Febrianti *et al*. (2021)**. Disease malignancy was influenced by the type and virulence of bacteria, host physiological conditions, as well as stress and genetic resistance levels **(Yengkhom *et al*., 2019)**. The appearing clinical signs were also assumed to be due to the role of various toxins produced by *A. hydrophila* **(Al-Fatlawy & Al-Ammar, 2013)**.

The clinical signs of MAS disease are suspected to be caused by *A. hydrophila* since it can produce various toxins **(Silva *et al*., 2012; Doan, 2013)**. Some virulence and pathogenicity factors were related to the exotoxins produced by *A. hydrophila*, such as protease, hemolysin, elastase, lipase, cytotoxin, enterotoxin, gelatinase, caseinase, lechitinase, and leucocidin. Proteases play a role in fighting host defenses and disease development **(Bond *et al*., 2019)**. Hemolysin is a toxin produced by *A. hydrophila* as hemolysin gene is detected **(Rozi *et al.*, 2018b)**. It can lyse red blood cells causing hemorrhagic on the surface of fish skin **(Huys *et al*., 2002; Li *et al*., 2013)**.

The impact of treating gouramy infected with *A. hydrophila* by using *A. marina* leaf extract was shown on the body recovery process. In general, it began to appear on day 7 - 8. On the other hand, it went to be very slow and unclear in the control fish. Even at the end of this study, there were still clinical signs such as visible ulcers on the body, white nodules appearing on the back of injection point, aligned with a decreasing appetite, and slow movements. It was suspected that natural recovery process for gouramy was quite slow. In fact, diverse conditions were shown by the African catfish infected with the same bacteria where recovery process on the control fish began to appear on day 7-8 and until the end of the study; although clinical signs of infection were still visible, the healing process continued **(Mulia *et al*., 2022)**. Different types of fish are also related to the fish durability and immune system **(Smith *et al*., 2019)**.

The recovery process at E1-E3 was indicated by shrinking ulcers, good response to food, and active movement. On day 9 -10, it could be concluded that gouramy infected with MAS disease had been recovered which was marked by healing signs such as shrinking ulcers and recovering hemorrhagic, increasing appetite and active movement. Complete recovery in each treatment was visible on day 11-14 as marked by the disappearing ulcer, invisible hemorrhagic, normal eyes, and normal fins.

The recovery process occurred in gouramy was presumably due to the addition of *A. marina* leaf extract. Previous studies reported that *A. marina* leaf extract contained several secondary metabolite compounds, including flavonoids, alkaloids, terpenoids, and tannins **(Mulia *et al*., 2018)**. This result coincides with those previously reported **(Rahman *et al*., 2020; Okla *et al*., 2021; Hassan *et al*., 2022)**. *A. marina* leaf extract indicates an antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *P. fluorescens*, and *Vibrio alginolyticus* **(Danata & Yamindago, 2014; Saravanan & Radhakrishnan 2016; Hassan *et al*., 2022)**.

Antibacterial compounds contained in *A. marina* leaf extract are assumed to inhibit bacterial growth. Flavonoids can inhibit the synthesis of nucleic acids, damage the cytoplasmic membrane, and result in the attenuation of the pathogenicity **(Wu *et al*., 2008; Xie *et al*., 2015)**. Alkaloid compounds play a role in interfering with the preparation of peptidoglycan in the bacterial cell wall, so that the cell wall layer is not completely formed, and eventually the bacteria die **(Cushnie *et al*., 2014)**.

Terpenoids can inhibit action of enzymes in cells, change the permeability of the cytoplasmic membrane, damage the bacterial cell wall, and cause lysis **(Yang *et al*., 2020)**. Tannin compounds are believed to inhibit the formation of bacterial cells by inhibiting the reverse transcriptase and DNA topoisomerase enzymes. In addition, tannins can deactivate bacterial cell adhesion, deactivate enzymes, and interfere with the transport of protein layers in cells. Tannins inhibit the absorption of sugars and amino acids, thereby limiting bacterial growth, disrupting cell metabolism, and resulting in the destruction of bacterial cells **(Theisen *et al*., 2014; Pandey & Negi, 2018; Belhaoues *et al*., 2020)**.

Treatment using the extract of *A. marina* leaf showed effect on survival rate which reached 30-50% for E1-E3. This rate was higher than that of the control (E0) by 12.5%. Such low survival rate in control fish was due to fish being untreated with *A. hydrophila* following the infection. This was unlikely in the case of fish treated with *A. marina* leaf extract that contained antibacterial compounds and assumed to have the role in inhibiting the growth of *A. hydrophila* and healing infected fish. Similar findings were suggested by previous study using *A. marina* leaf extract to treat the African catfish infected with *A. hydrophila* **(Mulia *et al*., 2022)**.

The results showed that E1-E3 treatments did not give significant difference. Giving 0.2-0.4g L^{-1} extract showed relatively similar effect on the survival rate. Thus, the use of *A. marina* extract with at 0.2, 0.3, and 0.4g L^{-1} concentrations was useful as natural medicine to treat gouramy infected with MAS disease. Based on these results, the concentration of 0.2g L–1 is the minimum concentration tested though having the same ability as the concentration above it, namely 0.3 to 0.4g L–1. These results show that the 0.2g L–1 concentration is more effective and efficient in treating gouramy infected with *A. hydrophila*. The use of natural ingredients drugs is an effective solution to cope with the problem of bacterial diseases in fish **(Dhayanithi *et al*., 2013; Mulia *et al*., 2022)**.

In this research, water quality parameters were maintained within normal range which included water temperature (26-27°C), dissolved oxygen (5.4-9.2ppm) and water pH (7.2-7.6). The optimal water temperature and dissolved oxygen for gouramy cultivation are 25-30^{0}C **(Saparinto & Susiana, 2013)** and 4-6ppm, respectively. Gouramy fish have a pH tolerance of 5-9, while notably the optimal pH is 7 **(Fitriadi *et al*., 2014)**.

CONCLUSION

The extract of *A. marina* mangrove leaves demonstrated potential as an antibacterial agent for managing *A. hydrophila* infections in gouramy, with an effective dose of 0.2g L–1. These leaves are promising as a natural resource for developing compounds to combat *A. hydrophila*.

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