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Antibacterial activity of mangrove plant extract of *Rhizophora apiculata* in inhibiting the growth of various strains of *Aeromonas hydrophila*

DINI SISWANI MULIA[1,♥], SUCI DWI RAHAYU[1], AMAN SUYADI[2], IKHSAN MUJAHID[3], ALIM ISNANSETYO[4]

[1]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Purwokerto. Jl. KH. Ahmad Dahlan, Purwokerto Utara, Banyumas 53182, Central Java, Indonesia. Tel.: +62-281-636751, Fax.: +62-281-637239, ♥email: dinisiswanimulia@ump.ac.id
[2]Department of Agrotechnology, Faculty of Agriculture and Fisheries, Universitas Muhammadiyah Purwokerto. Jl. KH. Ahmad Dahlan, Purwokerto Utara, Banyumas 53182, Central Java, Indonesia
[3]Department of Medical Laboratory Technology, Faculty of Health Sciences, Universitas Muhammadiyah Purwokerto. Jl. Letjen Soepardjo Roestam 229, Purwokerto, Banyumas 53181, Central Java, Indonesia
[4]Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

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Abstract. *Mulia DS, Rahayu SD, Suyadi A, Mujahid I, Isnansetyo A. 2023. Antibacterial activity of mangrove plant extract of* Rhizophora apiculata *in inhibiting the growth of various strains of* Aeromonas hydrophila. *Biodiversitas 24: 4803-4810.* Aeromoniasis is a bacterial disease caused by *Aeromonas hydrophila*. Synthetic antibiotics are often used to treat this disease. However, using antibiotics for too long with excessive doses can cause side effects for the environment, fish, and other aquatic biota. One of the natural ingredients with the potential as an antimicrobial is the mangrove plant *Rhizophora apiculata*. This study aims to determine the antimicrobial activity of mangrove plant extract *R. apiculata* in inhibiting the growth of various strains of *A. hydrophila*. The study used a completely randomized design experimental method with a factorial pattern with 3 factors (plant part, bacterial strain, and extract concentration) with 48 treatments and 3 replications. Methanol (polar) and n-hexane (non-polar) were used as extract solvents. The parts of the plant used are leaves and stems. The bacterial strains used were GPl-04, GB-01, GK-01, GJ-01, GL-01, and GL-02. The concentration of mangrove extract used was 0, 10, 20, and 30%. The inhibition zone diameter measured the antimicrobial activity using a paper disc (Kirby Bauer method). The data analysis used was the non-parametric Kruskall-Wallis test and qualitative descriptive. The results showed that *R. apiculata* methanol extract's leaves and stems contain bioactive compounds of flavonoids, alkaloids, and tannins, while the n-hexane extract contains terpenoids. Leaves methanol extract *R. apiculata* exhibited better than stems, namely the inhibition zone diameter of 2.87 mm, at a concentration of 20% against the GJ-01 bacterial strain, while the methanol extract of stem *R. apiculata* showed the best antimicrobial activity, namely the diameter of the inhibition zone of 2.10 mm, at a concentration of 30% against the bacterial strain GL-02. Leaves n-hexane extract *R. apiculata* had the best antimicrobial activity at a concentration of 10% against the bacterial strain GL-01, with an inhibition zone diameter of 2 mm. The n-hexane extract of stems *R. apiculata* showed antimicrobial activity at a 30% concentration of 1.23 mm in the same strain. Mangroves plant *R. apiculata* have bioactive compounds that can be used as natural bactericides to control *A. hydrophila* bacterial disease.

Keywords: *Aeromonas hydrophila*, antimicrobial, methanol, n-hexane, *Rhizophora apiculata*

INTRODUCTION

Aeromoniasis or red-sore diseases are bacterial diseases that mostly attack freshwater fish. This disease is caused by species from the genus Aeromonas, one of which is *Aeromonas hydrophila* (Lopamudra et al. 2020; Monir et al. 2020; Zhang et al. 2020; Mulia et al. 2022a). *A. hydrophila* is a rod-shaped, Gram-negative, motile, chemo-organoheterotrophic, facultative anaerobic, oxidase-positive, catalase positive, fermentative, non-spore, and opportunistic (Mulia et al. 2011; Stratev and Odeyemi 2016; Abeyta et al. 2019; Pessoa et al. 2019; El-Sharaby et al. 2021; Semwal et al. 2023).

Aeromonas hydrophila can cause epidemics of diseases with high mortality rates (80-100%) in a short period (1-2 weeks) if not treated quickly and appropriately (Mulia et al. 2011; Anyanwu et al. 2015). The clinical signs that arise due to infection with *A. hydrophila*, namely hemorrhagic, hyperemia, necrosis, erosion, ulcer, abscess, gastroenteritis, abdominal dropsy, abdominal ascites, exophthalmia, depigmentation of the skin, hemorrhagic and congested liver, kidney, and spleen (Anyanwu et al. 2015; Hamid et al. 2017; Emeish et al. 2018; Mulia et al. 2021; Semwal et al. 2023).

Bacterial disease control can be done in two ways: prevention and treatment. Prevention of bacterial diseases can be conducted by vaccination (Siriyappagouder et al. 2014; Gong et al. 2015; Kaur et al. 2021; Mulia et al. 2022b; Rahman et al. 2022) or immunostimulants and probiotics (Newaj-Fyzul and Austin 2014; Isnansetyo et al. 2016; Sherif and Mahfouz 2019; Andayani et al. 2020; Krishnan and Raja 2021) with significant results. Fish disease treatment can be carried out by administering drugs and antibiotics.

Synthetic antibiotics and chemicals are often used to control bacterial diseases in the field. Still, prolonged use with excessive doses can have negative impacts: toxicity, death of non-target biota, resistant bacteria, antibiotic residues, environmental pollution, and public health (Harikrishnan et al. 2020; Serwecińska 2020; Bombaywala et al. 2021). Previous studies reported that *A. hydrophila* was resistant to methicillin, rifampicin (Vivekanandhan et al. 2002), ampicillin, and erythromycin (Mulia et al. 2021). Therefore, it is necessary to look for active compounds from natural ingredients with the potential as antibacterial agents and can replace synthetic antibiotics so that fish farming activities can be carried out sustainably. Plants are a source of secondary metabolite compounds with the potential as a natural antibiotics source (Gorlenko et al. 2020). These antibacterial activities could be derived from phytochemicals or bioactive substances, including polyphenols (Hong et al. 2011; Sulaiman et al. 2011; Xie et al. 2015; Lubaina et al. 2019; Vittaya et al. 2022).

Phytochemical constituents from a tropical coastal plant (*Diospyros maritima*) is to be potential antibacterial is to be potential antibacterial (Isnansetyo et al. 2022). Ethanol extracts from banana, cassava, and pineapple peel waste have active compounds of flavonoids, alkaloids, terpenoids, tannins, and saponins and can inhibit the growth of *A. hydrophila* (Mulia et al. 2023a). Other plants that have the potential to have antibacterial compounds are mangrove plants. Mangrove plant extract *Avicennia marina* contains bioactive compounds: flavonoids, alkaloids, terpenoids, and tannins, and inhibits the growth of *A. hydrophila* (Mulia et al. 2018; 2022a). In another study, *A. marina* inhibits the growth of *Staphylococcus aureus* and *Vibrio alginolyticus* (Danata and Yamindago 2014). *Excoecaria agallocha* has antibacterial compounds of alkaloids, flavonoids, terpenoids, and tannins and can inhibit the growth of *A. hydrophila* (Mulia et al. 2023b). The extract of *E. agallocha* inhibits the growth of *Escherichia coli* and *S. aureus* bacteria (Prihanto et al. 2011; Puspitasari 2017). Other types of mangrove plants, namely *Rhizophora* sp., also produce active compounds. *Rhizophora apiculata* contains tannins, saponins, flavonoids, steroids, and terpenoids and inhibits the growth of *A. hydrophila*, *Pseudomonas aeruginosa*, and *S. aureus* (Syawal et al. 2020). Vittaya et al. (2022) reported that *R. apiculata* inhibited the growth of *A. hydrophila*, *Streptococcus agalactiae, Vibrio harveyi*, and *V. parahaemolyticus*. Extract of *R. apiculata* inhibits growth of *E. coli, Salmonella typhi, Proteus vulgaris*, and *S. aureus* (Arulkumar et al. 2020).

Aeromonas hydrophila consists of various isolates and strains that vary in morphological, biochemical, pathogenicity, and resistance characterization (Janda and Abbott 2010; Mulia et al. 2011; Nagar et al. 2011; Králová et al. 2016; Nayak 2020). Leaves and stems extract *R. apiculata* tested on *A. hydrophila* with several strains at once has not been done. Therefore, this study will test the antimicrobial activity of extract inhibition *R. apiculata* to *A. hydrophila* strains GPl-04, GB-01, GK-01, GJ-01, GL-01, and GL-02. The six strains of *A. hydrophila* have high pathogenicity and have been tested with mangrove plants *A. marina* and *E. agallocha* (Mulia et al. 2018; 2023b). This research was conducted to study the antimicrobial activity of mangrove plant extracts *R. apiculata* in inhibiting the growth of various strains of *A. hydrophila*.

MATERIALS AND METHODS

Experimental design

The study used a completely randomized design experimental method with a factorial pattern with three factors, namely the plant part of *R. apiculata* (leaves and stems), strains *A. hydrophila* (GPl-04, GB-01, GK-01, GJ-01, GL-01, and GL-02), and concentrations of mangrove extracts (0, 10, 20, and 30%) with 48 treatments and 3 replications. The solvent used is methanol (polar) and n-hexane (non-polar). The leaves used are the 3rd to 5th leaves from the shoot, and the stalk is taken from the part near the leaf (Figure 1).

Preparation of mangrove plant extracts *R. apiculata*

Mangrove leaves and stems of *R. apiculata* were washed and then cut into pieces. Each piece of leaf and stem is dried using an oven at 60°C and blended until it becomes a fine powder called simplicia (Figure 2). *R. apiculata* extract was prepared by maceration (Jaberian et al. 2013). As much as 100 g simplicia leaves and stems of mangrove plants *R. apiculata*, each soaked with 500 mL of n-hexane (non-polar) for 2 x 24 hours. After that, it was separated between the n-hexane extract and the dregs. The n-hexane obtained was evaporated using a vacuum evaporator to become a thick extract that would be used for the antibacterial test. At the same time, the dregs were dried and soaked with 500 mL methanol (polar) for 2 x 24 hours. After that, the methanol extract and dregs were separated. The extract obtained was then evaporated from the methanol with a vacuum evaporator to become a thick extract that would then be used for the antibacterial test.

Phytochemical test of mangrove plant extracts *R. apiculata*

Phytochemical test of mangrove plant extracts *R. apiculata* using a color test on a Thin Layer Chromatography plate. This test was conducted to determine the presence of antibacterial bioactive compounds contained in the methanol and n-hexane extracts of leaves and stems of *R. apiculata*.

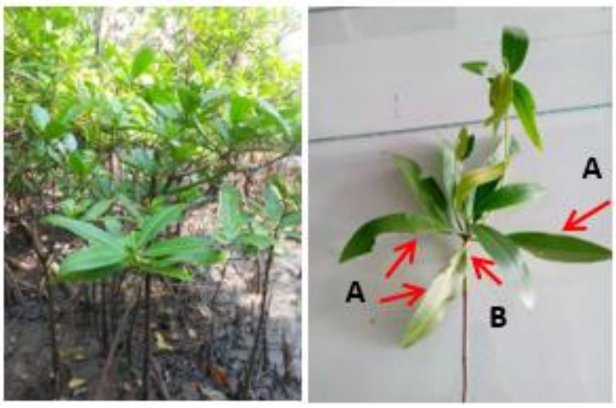

Figure 1. The mangrove plant *Rhizophora apiculata*. A: leaves; B: stem

Figure 2. Raw materials for extracting the mangrove plant *R. apiculata*: A. Stems wet materials; B. Leaves wet materials; C. Stems dry materials; D. Leaves dry materials; E. Leaves simplicia powders; F. Stems simplicia powders

Test the inhibition of mangrove plant extracts *R. apiculata*

Aeromonas hydrophila strain GPl-04, GB-01, GK-01, GJ-01, GL-01, and GL-02 (Table 3) were grown on tryptic soy broth (TSB) medium (Merck, Darmstadt, Germany) and incubated at 37°C for 24 hours. As much as 1 mL of each strain was grown into a petri dish by the method for flat, and then 20 mL of tryptic soy agar (TSA) medium (Merck, Darmstadt, Germany) was poured into the petri dish. Viscous extract *R. apiculata* diluted according to the concentration used, namely diluted with distilled water up for the extract from methanol and 10% DMSO for the extract from n-hexane. Paper discs were sterilized for the inhibition zone test for each strain (GPl-04, GB-01, GK-01, GJ-01, GL-01, and GL-02), and each concentration was dripped with 2 drops of extract. The disc paper for the methanol extract control (0%) was dripped with distilled water, while the n-hexane extract control (0%) was dripped with 10% DMSO. Furthermore, paper discs containing mangrove extracts and solvents were placed on the surface of the TSA medium and incubated at 37°C for 24 hours. After 24 hours, it was observed whether there was a clear zone around the disc paper.

Data analysis

Data from phytochemical test results were analyzed descriptively and qualitatively. Data on the diameter of the inhibition zone were analyzed using the Kruskall-Wallis test, a non-parametric test at a level of 5%.

RESULTS AND DISCUSSION

The phytochemical test results of mangrove plant extracts *R. apiculata*

Phytochemical tests on the mangrove plant *R. apiculata* extracts were carried out to determine the presence of secondary metabolites or bioactive compounds. Different polar solvents are used to detect compounds based on their polarity. According to the reagents, secondary metabolites were detected qualitatively through color changes and precipitation or foam formation (Campagna et al. 2012). The results of the phytochemical test of the methanol extract of the leaves and stems of *R. apiculata* showed the presence of bioactive compounds: flavonoids, alkaloids, and tannins (Table 1). Mangrove plants generally contain phenolic compounds such as phenolic acid, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins (Roby et al. 2013; Malik et al. 2017). The flavonoid phytochemical test showed a positive result marked by the appearance of a yellow color; the alkaloid compound test showed a positive result with the formation of orange-yellow spots; the tannin compound test showed a positive result with the appearance of an orange-brown color, while the terpenoid compound test showed a negative result because no brown spots were formed. The results of this study followed previous studies, that flavonoids were detected marked by changing the color to reddish-yellow (Malik et al. 2017; Syawal et al. 2020), alkaloids were detected by changing the color to orange (Arnida et al. 2021), while tannins were detected by changing the color to brownish yellow (Arnida et al. 2021; Refamurty et al. 2023). Extraction results depend on the extract solvent. Flavonoids, alkaloids, and tannins are polar, so they dissolve easily in the same polar methanol (Mahardika and Roanisca 2019).

Previous research reported that the methanol extract of the leaves *R. apiculata* contains saponins, phenolics, flavonoids, and terpenoids (Vittaya et al. 2022), while the methanol extract with the HPLC test proved to contain flavonoid compounds (Nisar et al. 2019). Laith et al. (2021) did not detect flavonoids in the methanol extract of the leaves *R. apiculata* but found other secondary metabolites: alkaloids, terpenoids, tannins, saponins, and steroids. The difference in the bioactive compounds detected is possibly due to the location of the environment where they grow *R. apiculata*. Differences in bioactive compounds were also detected in *A. marina* (Ananthavalli and Karpagam 2017; Mulia et al. 2018; Rahman et al. 2020) and *E. agallocha* (Prihanto et al. 2011; Mulia et al. 2023b) sampled from different growing locations. In addition, seasonal differences have implications for plant ecophysiological activities. Hastuti et al. (2023) differentiated the tannin content of *R. apiculata* and *A. marina* in different seasons, namely the rainy and dry seasons. Tannin samples were sampled from mangrove stem bark. The results showed that the tannin content of *R. apiculata* during the rainy season ranged from 17.87% to 19.16% lower than in the dry season, which reached 22.55% to 46.59%. However, the tannin content of *A. marina* during the rainy season ranged from 4.14 -6.58% higher than in the dry season, ranging from 2.88-4.91%.

The screening results with phytochemical tests on the n-hexane extract of *R. apiculata* leaves and stems show the presence of terpenoid compounds but not flavonoids, alkaloids, and tannins (Table 2). Previous research reported that the n-hexana extract of *R. apiculata* leaves contains steroid and triterpenoid compounds (Rahayuningsih et al. 2021). The same results were reported by Basyuni et al. (2019); n-hexane extract from five true mangrove leaves does not contain alkaloids, flavonoids, glycosides, saponins, and tannins but only triterpenoids/phytosterols. The difference in the detected secondary metabolites between methanol and n-hexane is due to the difference in the polar level of the solvents. The efficiency and effectiveness of extraction are greatly influenced by the ability of the solvent to diffuse into the cell (Sarker et al. 2006).

Antimicrobial activity of mangrove plant extracts *R. apiculata*

Methanol extract from leaves and stems of *R. apiculata* has antimicrobial activity as indicated by its inhibition of growth *A. hydrophila* (Table 3). The results showed that leaf methanol extract with concentrations of 20% had the highest antimicrobial activity with an inhibition zone diameter of 2.87 mm. Stems methanol extract *R. apiculata* had the best antimicrobial activity at a concentration of 30% against the GL-02 strain of 2.1 mm, but it was not in GPl-04. The results showed slight inconsistency in the test results with different doses on bacterial strains GB-01 and GK-01 and the results of mangrove stem extract on bacterial strains GL-01 and GL-02, allegedly due to the inhomogeneity of the extract results. Apart from that, this condition is thought to be due to the uneven distribution of the bacterial suspension when placed in the petri dish and differences in the diffusion of the active extract ingredients into the medium. The same results were also reported by Zeniusa et al. (2019).

The previous study reported that *R. apiculata* tea extract could inhibit *E. coli* with an inhibition zone diameter of 2 mm, relatively the same as the results of this study. However, the diameter of the inhibition zone was higher for *Salmonella typhi* and *Proteus vulgaris*, namely 5 mm and 7 mm. At the same time, for Gram-positive bacteria, namely *S. aureus*, it was even higher, reaching 15 mm (Arulkumar et al. 2020). *R. apiculata* tea extract contains steroids, tannins, glycosides, sterols, terpenoids, flavonoids, and phenol compounds. Vittaya et al. (2022) reported differences in the content of bioactive compounds and antimicrobial activity between *R. apiculata* and *R. mucronata*. The methanol extract of *R. apiculata* leaves contains saponins, phenolics, flavonoids, and terpenoids, while the methanol extract of *R. mucronata* leaves, apart from containing the same four compounds, also contains anthraquinones and alkaloids. The methanol extract of *R. apiculata* leaves inhibited *A. hydrophila* with a clear zone diameter of 6.63 mm, while *R. mucronata* could inhibit the same bacteria by 10.88 mm. In other types of bacteria, the antimicrobial activity appeared to be higher than that of *A. hydrophila*; the methanol extract of *R. apiculata* leaves inhibited *V. harveyi* with a clear zone diameter of 10.13 mm, while *R. mucronata* could inhibit the same bacteria by 12.19 mm. In this study, the ability of the methanol extract of leaves *R. apiculata* to inhibit *A. hydrophila* was lower than Vittaya et al. (2022). Mangrove leaves are taken during the rainy season, so it is thought to affect the content of the resulting bioactive compounds (Hastuti et al. 2023). The different antimicrobial activity is thought to be caused by the content of bioactive compounds in the mangrove extract, the type of mangrove, the type of extract solvent, the type of bacteria inhibited, and the season when the mangrove plant was harvested.

Table 1. Phytochemical screening performed of methanol extract on parts of *R. apiculata*

Phytochemical compounds	Methanol extract	
	Leaf	Stem
Flavonoids	+	+
Alkaloids	+	+
Terpenoids	-	-
Tannins	+	+

Table 2. Phytochemical screening performed of n-hexane extract on parts of *R. apiculata*

Phytochemical compounds	n-hexane extract	
	Leaf	Stem
Flavonoids	-	-
Alkaloids	-	-
Terpenoids	+	+
Tannins	-	-

Table 3. The antimicrobial activity of methanol extract of *Rhizophora apiculata* against pathogenic bacteria *Aeromonas hydrophila*

Plant parts	Concentration (%)	Zone of inhibition diameter (mm)					
		GPl-04	GB-01	GK-01	GJ-01	GL-01	GL-02
Leaf	0	0	0	0	0	0	0
	10	0.92	0.03	0.97	2.33	0	0
	20	2.73	0	0	2.87	0.22	0
	30	0.57	0.15	0.1	2.87	0.28	0
Stem	0	0	0	0	0	0	0
	10	0	0.17	0.5	1.69	0.2	0.97
	20	0	0	0	1.68	0	0
	30	0	0.08	0.4	1.83	0.23	2.1

Table 4. The antimicrobial activity of n-hexane extract of *Rhizophora apiculata* against pathogenic bacteria *Aeromonas hydrophila*

Plant parts	Concentration (%)	Zone of inhibition diameter (mm)					
		GPl-04	GB-01	GK-01	GJ-01	GL-01	GL-02
Leaf	0	0	0	0	0	0	0
	10	0.17	0	0	0	2	0
	20	0	0	0	0.21	0.88	0
	30	0.73	0	0	0	0.95	0
Stem	0	0	0	0	0	0	0
	10	0	0	0.48	0.18	0.87	0
	20	0	0	0	0	0	0
	30	0	0	0.89	0	1.23	0.9

In this study, the antimicrobial activity of *R. apiculata* is due to the activity of the bioactive compounds contained in it, namely flavonoids, alkaloids, and tannins. Bioactive compounds such as alkaloids, tannins, and terpenoids are antimicrobial agents found in plants (Mulat et al. 2020). Bioactive molecules can inhibit the growth of pathogens (Mandal and Shi 2020). Flavonoids have excellent antibacterial activity (Farhadi et al. 2019). These compounds are phenolic compounds that can act as a protein coagulator. Proteins that experience coagulation will no longer function so they will interfere with the formation of microbial cell walls (Biharee et al. 2020; Donadio et al. 2021). The mechanism of action of flavonoids as antibacterials is divided into three: inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism. Flavonoids can inhibit the synthesis of nucleic acids; these compounds react with DNA, RNA, and proteins, resulting in disruption of the function of these substances and causing total damage to cells (Wu et al. 2013; Biharee et al. 2020; Cowan and Talaro 2021). Flavonoids can also damage the cytoplasmic membrane, change membrane permeability, and suppress cell wall synthesis caused by D-alanine-D-alanine ligase inhibition (Xie et al. 2015).

Alkaloid contains a nitrogenous base group that can react with the amino acids that comprise the bacterial cell wall and DNA. This reaction causes structural changes to the amino acids, leading to genetic changes and bacterial lysis. Alkaloids can also affect the production of peptidoglycan (a cell wall component) in bacteria, causing the formation of imperfect cell wall layers and bacterial death (Cushnie et al. 2014). Tannins can damage cell membranes and induce the formation of complex compounds that attack bacterial enzymes, thereby increasing their toxicity to bacteria (Akiyama et al. 2001). Tannins are thought to contract cell membranes or cell walls, damaging the permeability of bacterial cells and inhibiting bacterial cell growth (Farha et al. 2020).

N-hexane extract from leaves and stems of *R. apiculata* has antimicrobial activity against growth *A. hydrophila* (Table 4). The results showed that the best inhibition was n-hexane leaf extract with a 10% concentration of 2 mm in the GL-01 strain and n-hexane stem extract with a concentration of 30% of 1.23 mm in the same strain. However, concentrations of 10-30% could not inhibit the growth of GPl-04 and GB-01 strains. It is suspected that the two strains are already resistant to the bioactive compounds in the extract. The results showed a slight inconsistency in the test results with different doses of mangrove leaf extract on the bacterial strains GPl-04 and GJ-01 and mangrove stem extract on strains GK-01, GJ-01, and GL-01; this was thought to be due to the inhomogeneity of the extract results, the uneven distribution of the bacterial suspension when placed in the petri dish, and differences in the diffusion of the active extract ingredients into the medium (Zeniusa et al. 2019).

In this research, the n-hexane extract of the mangrove plant *R. apiculata* is already better at inhibiting the growth of *A. hydrophila*. Syawal et al. (2019) reported that the extract of mangrove leaves hexane *R. apiculata* 1000-10,000 ppm can inhibit the growth of *A. hydrophila* with a diameter of 6.15-8.45 mm. The antimicrobial activity of the extract is thought to be due to the activity of the terpenoid compounds. Terpenoids are compounds that have antibacterial effects (Yang et al. 2020). Terpenoids can cause protein denaturation, inhibit enzymatic work, cause leakage of nutrients from inside the cell, change the permeability of the cytoplasmic membrane, damage the bacterial cell wall, and cause lysis (Guimarães et al. 2019).

The results showed that the extract of *R. apiculata* can inhibit the growth of *A. hydrophila*. However, different antimicrobial activities, methanol extract is higher than n-hexane extract, and leaf extract is higher than plant stem extract *R. apiculata*. Differences in antimicrobial activity also occurred in previous studies (Syawal et al. 2020; Laith et al. 2021; Mulia et al. 2023b). Differences in antimicrobial activity are assumed to be due to variations in the source of isolates, frequency, and types of antimicrobial agents (Bengtsson-Palme et al. 2018; Peterson and Kaur 2018). Mangrove plant extract *R. apiculata* has the potential as a natural antibacterial *A. hydrophila* and can be used as an alternative for treating and preventing disease in fish.

Conclusions, the phytochemical screening of the methanol extract of leaves and stems of *R. apiculata* showed the presence of bioactive compounds of flavonoids, alkaloids, and tannins, while the n-hexane extract contained terpenoids. This study has successfully documented the antimicrobial activity of *R. apiculata* against growth *A. hydrophila*. Leaf methanol extract of *R. apiculata* is more sensitive in inhibiting the growth of *A. hydrophila* than other solvents. The results suggest that the mangrove plant *R. apiculata* is a prospective natural resource for developing anti-*A. hydrophila* compounds.

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