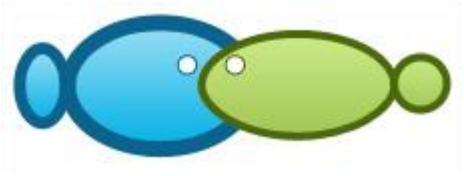

Bioactivity of *Bruguiera gymnorrhiza* leaf extract on fish pathogenic bacteria *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*

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Abstract. *Bruguiera gymnorrhiza* is part of the mangrove community that has numerous benefits for human life, ranging from ecological benefits to being a source of food and medicine. *Aeromonas hydrophila*, *Pseudomonas aeroginosa*, and *Staphylococcus aureus* are pathogenic bacteria for fish and humans, that can be found in a mangrove ecosystem. The study aimed to determine the group of secondary metabolites of *B. gymnorrhiza* leaf extract as bioactive inhibitory compound to the growth of *A. hydrophila*, *P. aeroginosa*, and *S. aureus*. The phytochemical analysis showed that the secondary metabolites contained in the mangrove leaf extract were compounds from alkaloids, flavonoids, tannins, and phenolics groups. *B. gymnorrhiza* extract strongly inhibited the pathogenic bacteria of *P. aeruginosa*. The highest inhibition was at a concentration of 25% (23.13 mm) and the lowest at a concentration of 100% (5.77 mm). The extract also strongly inhibited *A. hydrophila*, where the highest inhibition occurred at a concentration of 12.5% (23.47 mm) and the lowest at a concentration of 100% (14.17 mm). Meanwhile *S. aureus* was only weakly inhibited, with a diameter of inhibition of 1.70-4.43 mm.
Key Words: bioactive compounds, secondary metabolites, alkaloid, flavonoid, tannin, phenolic.

Introduction. *Bruguiera gymnorrhiza* is an important species of the mangrove community. It has been useful to the human activity, especially in coastal areas, as firewood, food and medicinal ingredients, and tourist attraction, and it also has ecological functions. Some mangrove species are used as food, one of which is species *B. gymnorrhiza*. It is reported that this plant is used as a stomach ache medicine by utilizing leaves, flowers and fruit (Mahmud et al 2017). Other researchers also reported that this mangrove extract can suppress the growth of some pathogenic bacteria (Haq et al 2011). *Aeromonas hydrophila* can be found in various places, especially in waters that contain high organic matter. This bacterium is a pathogen, either in humans or animals, especially in fish. It is an opportunistic pathogenic bacteria, Gram negative, can cause fish death in a very short time, at a rate up to 80-100%. These microorganisms are found in both marine and freshwater waters, and are referred to as opportunistic pathogens in hemorrhagic septicemia in fish under stress conditions (Yogananth et al 2009). *P. aeruginosa* causes infections in human blood, lungs, or other parts of the body after surgery. The bacterium is also pathogenic to fish like tilapia. It is gram negative and can occur singly, in pairs or sometimes in short chains (Gajdács et al 2021). These bacteria are oxidase positive, do not ferment lactose and are easily distinguished from lactose-fermenting bacteria, but have multiple pathways for oxidizing glucose. *Staphylococcus aureus* causes a variety of human skin infections and mild to very serious diseases like endocarditis and pneumonia (Pivard et al 2021). This bacterium is also detected in various food-producing animals, including fish, mussel and shrimp. The growth and development of resistant strains of *S. aureus* to some drugs has become a

serious challenge for disease prevention in the community. The medical world is forced to seek and find new drugs for the prevention and treatment of diseases caused by this pathogen. It grows well on various bacteriological media, under aerobic or micro aerobic conditions. Colonies on dense media will be round, smooth, prominent and shiny, forming various golden yellow pigments (Sato et al 2019).

Some plant species, including mangrove communities, synthesize hundreds of chemical compounds which functions for the body's defense system from bacteria, fungi, insects and herbivorous mammals (Simlai et al 2014; Przerwa et al 2020). Many potential bioactive substances have been identified. These substances generally show bioactivity in the form of antifungal (Mahboubi & Mahboubi 2014; Mahboubi & Bidgoli 2009; Manilal et al 2016), antiviral (Bhimba et al 2016), antioxidant (Ertürk et al 2020), and antibacterial (Oulkheir et al 2019; Thiem et al 2010; Ulmursida et al 2017). They were used to combat some gastrointestinal diseases like thypoid, diarrhea, cholera and hemorrhoids (Mahboubi & Mahboubi 2014). Antibacterial applications of this compound have been applied to treat infections in humans, fish and other aquatic animals. This study aimed to determine the group of secondary metabolites of *B. gymnorrhiza* leaf extract and their inhibitory action on the growth of fish pathogenic bacteria *A. hydrophila*, *P. aeroginosa*, and *S. aureus*.

Material and Method

Research method. This research was an experimental study, using a completely randomized design using 4 concentration levels, namely 12.5, 25, 50, and 100% of *B. gymnorrhiza* leaves extract, negative control (distilled water) and positive control, with 3 repetitions. It was conducted from April-June 2021. Leaf samples of *B. gymnorrhiza* were collected from Mengkapan Village, Siak, Indonesia.

Extraction of bioactive substances. Samples of *B. gymnorrhiza* leaves were collected and dried at room temperature for 14 days. The dried leaves were then mashed using a blender, then macerated using 96% ethanol solution with a ratio of 1:5 for 24 hours at 20-30^{o}C room. The samples were filtered using a Whattman No. 42 filter paper into a container to separate the dregs and the filtrate liquid. The first filtered dregs were macerated in the same procedure and repeated again until a transparent liquid was obtained. The filtered solution was evaporated using an evaporator, at a temperature of 60^{o}C, a pressure of 100 mbar, and a rotation of 15 rpm, until the solvent was evaporated and the extract was obtained in the form of a thick solution.

Phytochemical analysis. Phytochemical analysis aims to determine the groups of bioactive substances contained in the leaves of *B. gymnorrhiza,* including saponins, alkaloids, flavonoids, phenols, tannins, steroids and terpenoids. In this study, the analysis was carried out with reference to Bertoli et al (2010) and Menghini et al (2018).

Analysis of saponin. The sample (0.1 g) was removed into a beaker glass then added with 10 mL of hot water and boiled for 5 minutes. The mix then was filtered and the filtrate was put into a test tube, closed, shaken for ±10 seconds, placed on a table for 10-15 minutes and then 1 mL of 2M HCl was added. The test of saponin compounds in the extract was positive if a stable foam was formed.

Analysis alkaloids. A sample (0.05 g) was placed into a test tube and then 2 drops of H_2SO_4 2 N were added and shaken until completely mixed. Then it was poured into 9 wells of a porcelain drip plate. Every 3 wells dripped with Meyer's, Wagner's and, Dragendorff's reagent. The presence of white precipitate in the well where Meyer's reagent was dropped, brown precipitate in Wagner's reagent, and orange precipitate in Dragendorff's reagent is a sign that the sample contains alkaloids.

Analysis of flavonoids. Leaf extract *B. gymnorrhiza* was mixed vigorously with 5 drops of concentrated HCl and Mg, and shaken gently to form a reddish yellow to red layer. The

formation of this color is an indicator of a positive sample containing flavonoid compounds.

Phenol analysis. Initially the leaf extract was place in a test tube, added with 3 drops of FeCl and mixed gently. The formation of a dark blue color after 5-10 minutes means that phenolic substances are present in the compound. The test for the presence of tannins in the extract was carried out by adding a few drops of 10% iron (III) chloride solution to 1 mL of the extract. If a dark blue or greenish black precipitate is formed, the extract is positive for tannins.

Steroid and terpenoid analysis. Firstly, the chloroform liquid was prepared, then poured into the 2 holes of the drip plate and fan until dry, then concentrated anhydrous acetic acid and concentrated H_2SO were added to the 2 holes of the drip plate. The presence of steroid compounds is indicated by a green color and the terpenoids presence is indicated by a purple.

Antibacterial assay. First, a solution of mangrove leaf extract was prepared aseptically to form solutions of 12.5, 25, 50, and 100%. The antibacterial activity assay against pathogenic bacteria *A. hydrophila*, *P. aeruginosa*, and *S. aureus* was carried out by using the Kirby-Bauer paper disc diffusion agar method (Effendi et al 2020). The fresh bacterial isolates (aged 2 days) were harvested from nutrient broth (Oxoid) media, a volume of 50 μL was pipetted and spread using a glass rod on Mueller Hillton Agar (Oxoid). A set of paper disc was dripped with the mangrove extract (concentrations of 12.5, 25, 50, 100%), 10 chloramphenicol solution (as positive control) and distilled water (as negative control). Paper discs were room dried and placed on the inoculated Mueller Hillton Agar and incubated at 22°C overnight. The inhibition zone was examined by measuring the clear zone diameter that surrounds the paper disc, using a caliper. The inhibition level was categorized (Syawal et al 2020; Syawal et al 2021) and the antibacterial assay was repeated 3 times.

Data analysis. The data obtained were analyzed descriptively based on the formation of color, precipitate, and foam. The sensitivity analysis of secondary metabolites to pathogenic bacteria was carried out by measuring the inhibition zone formed.

Results

Phytochemical analysis. The leaf extract of *B. gymnorrhiza* contained several groups of bioactive compounds, namely; alkaloids, flavonoids, tannins and phenolics. Meanwhile, groups of saponins, steroids and terpenoids were not found (Table 1).

Table 1

Bioactive compounds of *Bruguiera gymnorrhiza* leaf extract

Bioactive compound	*Result*	*Mark*
Saponin	-	Negative
Alkaloid	+	Positive
Flavonoid	+	Positive
Tannin	+	Positive
Phenolic	+	Positive
Steroid	-	Negative
Terpenoid	-	Negative

Antibacterial assay. Antibacterial properties of the leaf extract of *B. gymnorrhiza* were indicated by the presence of a clear zone surrounding the 6 mm diameter paper disc. The assay revealed that the mangrove leaf extract inhibited the pathogenic bacteria *S. aureus*, *A. hydrophila* and *P. aeruginosa* (Table 2). The zone of inhibition on the growth of *P. aeruginosa* ranged from 5.77–23.13 mm. The highest inhibition was at a

concentration of 25% (23.13 mm), while the lowest was at a concentration of 100% with (5.77 mm) (Figure 1). For *A. hydrophila*, the extract inhibition zone ranged from 14.50–23.17 mm. The highest inhibition was at 100% concentration (23.17 mm), while the lowest was at 100% concentration (14.50 mm) (Figure 2). The average value of the inhibition zone for the growth of *S. aureus* ranged from 1.70–4.43 mm (Figure 3). The highest inhibition zone was at 50% concentration (4.43 mm), while the lowest occurred at a concentration of 50%, namely 1.70 mm.

Table 2

Antibacterial activity of *Bruguiera gymnorrhiza* leaf extract on the fish pathogenic bacteria *Aeromonas hydrophila, Pseudomonas aeroginosa,* and *Staphylococcus aureus*

Pathogenic bacteria	*Concentration (%)*	*Inhibition zone (mm)*				*Average*
		Sample 1	*Sample 2*	*Sample 3*	*PCEU*	
P. aeruginosa	12.5	16.9	21.3	22.2	29.5	20.13±2.84
	25	17.4	22.3	29.7	21.7	23.13±6.19
	50	26.6	18.2	6.3	22.7	17.03±10.20
	100	4.8	5.1	7.4	8.3	5.77±1.42
A. hydrophila	12.5	20.7	21.1	27.7	31.6	23.17±3.93
	25	22.1	23.5	22.2	31.7	22.60±0.78
	50	21.5	27.9	18.2	31.4	22.53±4.93
	100	15.2	13.1	15.1	31.7	14.47±1.18
S. aureus	12.5	1.5	1.7	2.1	28.8	1.77±0.31
	25	4.6	3.8	3.6	31.6	4.00±0.52
	50	4.2	4.5	4.6	31.2	4.43±0.21
	100	1.2	2.6	1.3	33.7	1.70±0.78

PCEU-positive control experimental unit.

Discussion

Phytochemical analysis. *B. gymnorrhiza* leaf extract has antibacterial secondary metabolites, namely; alkaloids, flavonoids, tannins, and phenolics. Some researchers (Rahman et al 2011) reported that this mangrove extract contains flavonoids, reducing sugars, gums, saponins and tannins. Sur et al (2016) also reported relatively similar analysis results: the identified polyphenols consisted of gallic acid, quercetin, and coumarin. Mahmud et al (2017) mentioned that alkaloids, tannins, flavonoids and phenolic compounds are the most important chemically active constituents of plants.

Plants are regarded as the oldest source of alkaloids, amino acids derivatives and where these compounds are formed as secondary metabolites. These materials own a broad scale of antibacterial properties. Many have important pharmaceutical uses; in very low concentration, they demonstrated significant antibacterial activity on humans and on other animals (Nagappan et a 2011).

As plants' secondary metabolites, flavonoids are found widely in food other parts of plants. The compound owns some bioactive properties such as anti-inflammatory, cardioprotective, anti-viral, anti-diabetic, anti-cancer (Marzouk 2016), antioxidant and anti-aging (Bhimba et al 2016). Flavonoid compounds are polyphenolic compounds that have fifteen carbon atoms which are arranged in the C6C3C6 configuration. This means that the carbon skeleton is composed of two C6 groups (substituted benzene rings) linked by an aliphatic tricarbon chain (Wang et al 2018; Munhoza et al 2014).

Tannins are substances that are found almost in all parts of plants, for instance in leaves, immature fruit, stems, and bark. The tannin content in immature fruit is used as an energy source in the metabolic mechanism in the form of tannin oxidation. Tannins are said to be a source of acid and bitterness in fruit (Kaczmarek 2020; Mahmud et al 2017).

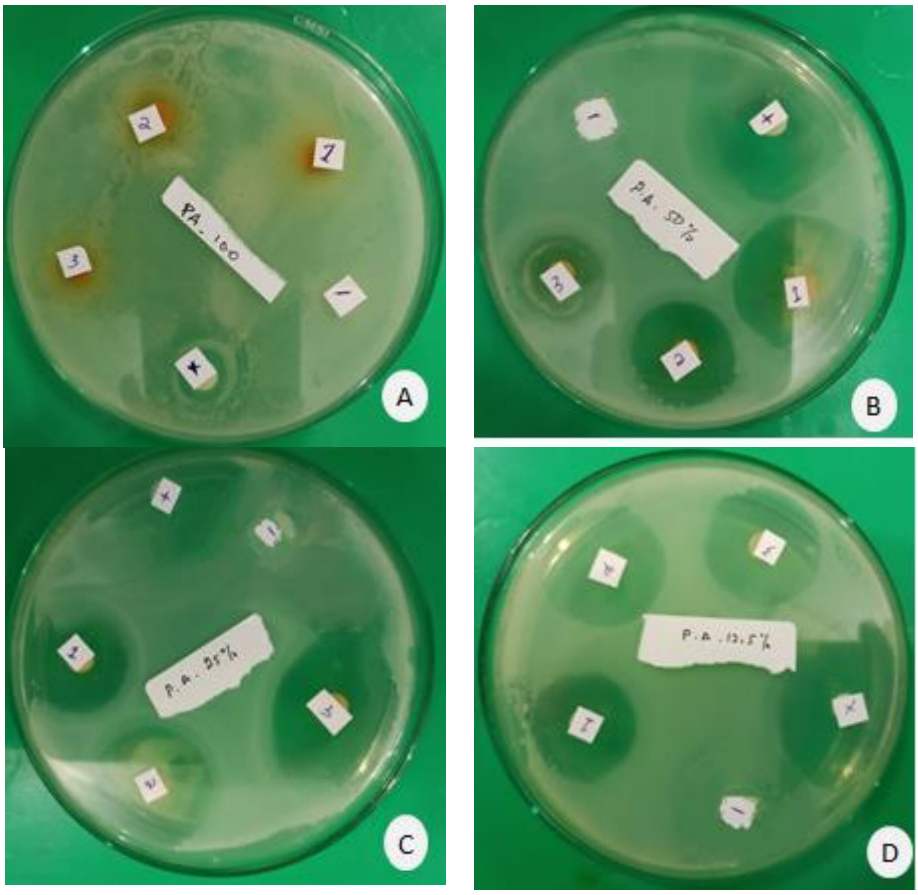

Figure 1. Inhibition zone of *Bruguiera gymnorrhiza* leaf extract on *Pseudomonas aeruginosa*. A (100%), B (50%), C (25%), D (12.5%). 1, 2, 3 = replication. + = positive control (chloramphenicol 12.5%), - = negative control (distilled water).

Antibacterial assay. In this experiment, *B. gymnorrhiza* leaf extract restrains the pathogen *S aureus* at any concentration. However, the inhibition is classified as of weak category (zone <5mm). Some researchers (EUCAST 2012; Poracova et al 2009; Chandrasekaran et al 2009) reported antimicrobial compounds of some mangrove and mangrove associate plants against a strain of *S. aureus* which is resistant to methicillin. Other workers (Kwon et al 2007; Oulkheir et al 2019) also reported similar cases involving this pathogen.

Based on the clear zone formed on the bacteria *A. hydrophila* by the extract of *B. gymnorrhiza*, this was categorized as very strong (>20 mm) (EUCAST 2012). Similar results were also reported also with different types of mangroves (Salosso et al 2020; Syawal et al 2020; Syawal et al 2019,2021).

The inhibition category of *B. gymnorrhiza* extract against *P. aeruginosa* can be categorized as moderate (5-10 mm) to very strong (>20 mm) (EUCAST 2012). Studies on the biological activity of plant extracts against *P. aeruginosa* have also been reported by some other researchers (Mahboubi & Bidgoli 2009; Mahboubi & Mahboubi 2014).

Modes of mechanisms of antimicrobial materials include the lysis of the cell wall, the synthesis of bacteria, the destruction of the bacterial cell membrane integrity, the inhibition of microbial cell protein synthesis, the disruption of microbial cell metabolism, and the inhibition of nucleic acid and protein synthesis. It is reported that alkaloid compounds may hamper the peptidoglycan constituent synthesis in bacterial cells causing the disrupted formation of cell wall layer. Disruption of peptidoglycan synthesis causes imperfect cell formation because it does not contain peptidoglycan and the cell wall only covers the cell membrane, causing cell death (Yuan et al 2021; Salosso et al 2020; Chandrasekaran et al 2009).

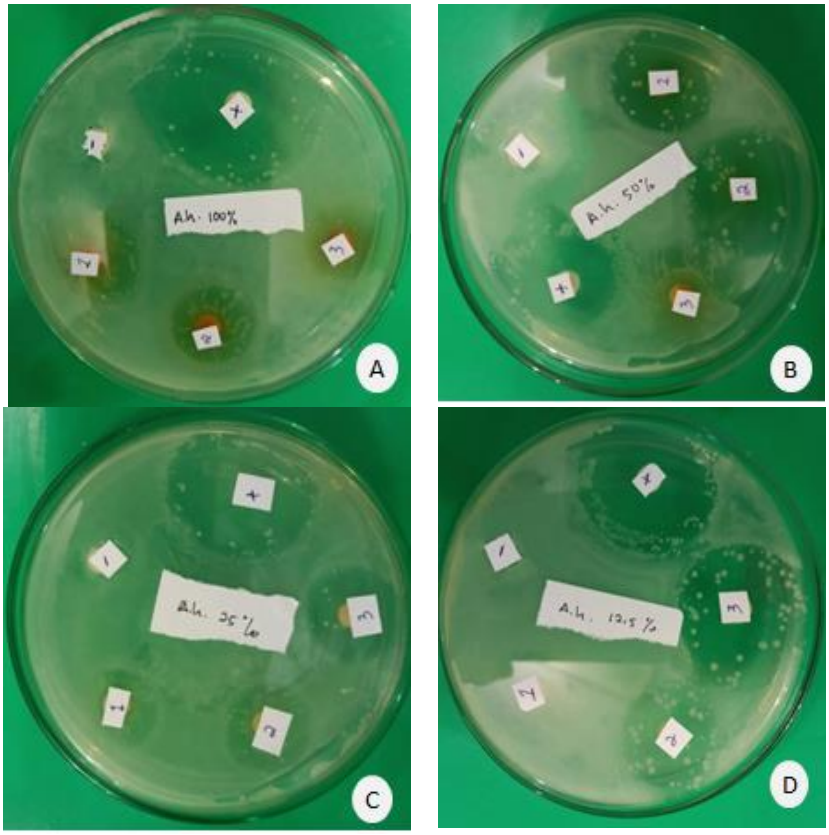

Figure 2. Inhibition zone of *Bruguiera gymnorrhiza* leaf extract on *Aeromonas hydrophila*. A (100%), B (50%), C (25%), D (12.5%). 1, 2, 3 = replication. + = positive control (chloramphenicol 12.5%), - = negative control (distilled water).

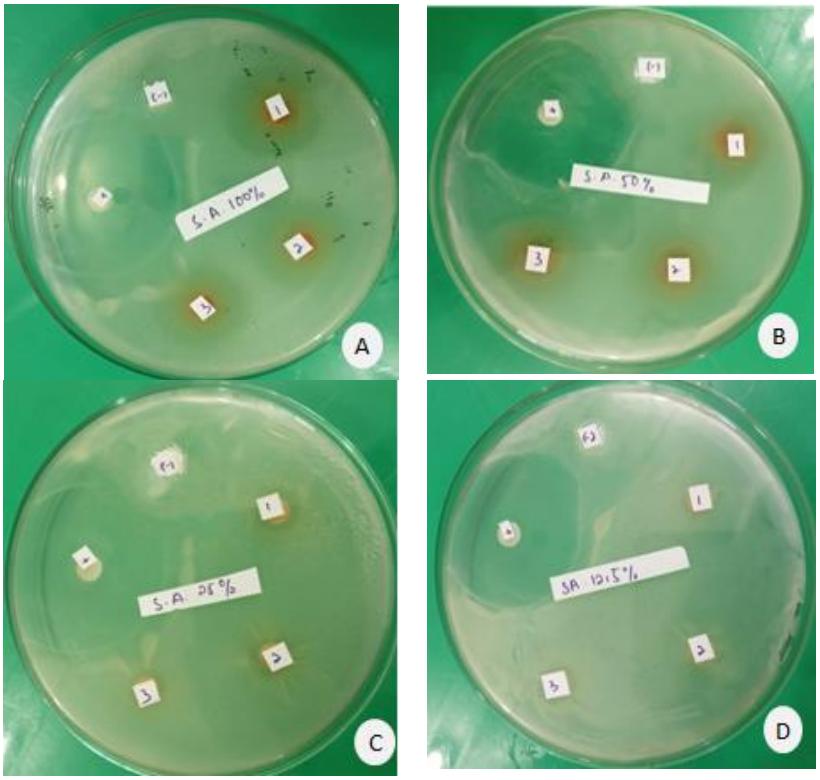

Figure 3. Inhibition zone of *Bruguiera gymnorrhiza* leaf extract on *Staphylococcus aureus*. A (100%), B (50%), C (25%), D (12.5%). 1, 2, 3 = replication. + = positive control (chloramphenicol 12.5%), - = negative control (distilled water).

Flavonoids will inhibit energy metabolism in bacteria, so that it can inhibit oxygen respiration which then the bacteria will lose permeability of the cell wall, microsomes and lysosomes as a result of bacterial DNA and flavonoids interaction (Nagappan et al 2011; Kanakis et al 2005; Thiem et al 2010). Compounds from the flavonoid and triterpenoid groups have greater inhibitory level on the thin cell wall bacteria like *S. aureus*. The cell wall of *S. aureus* contains two main components, namely peptidoglycan and teichoic acid. The compounds of the flavonoid group are polar and those of the triterpenoid group are nonpolar, but have a hydroxy group so that they have polar properties. The polar nature causes the triterpenoid and flavonoid group compounds to penetrate the polar peptidoglycan layer easier than the nonpolar lipid layer. As a result, the inhibitory effect on Gram-positive bacteria is greater than the inhibitory effect on Gram-negative bacteria (Homhual et al 2006).

Tannin compounds can precipitate proteins without changing their physical and chemical properties. Tannin compounds inhibit bacterial cells after the cells are first lysed by flavonoids. Cells that have undergone lysis will be easily penetrated by tannins. As a result, the cells will grow stunted and some of them even die (Kaczmarek 2020; Mahmud et al 2017). Tannin is also a complex phenolic compound that contains tannic acid which acts as an antiseptic. Therefore, these secondary metabolites are often used for medical needs (Ulmursida et al 2017).

Conclusions. The phytochemical analysis showed that the secondary metabolites contained in the extract were compounds of the groups: alkaloids, flavonoids, tannins, and phenolics. *B. gymnorrhiza* extract strongly inhibited the pathogenic bacteria *P. aeruginosa*. The highest inhibition was at a concentration of 25% (23.13 mm) and the lowest was at a concentration of 100% (5.77 mm). This extract also strongly inhibited *A. hydrophila*, where the highest inhibition occurred at a concentration of 12.5% (23.47 mm) and the lowest at a concentration of 100% (14.17 mm). Meanwhile, *S. aureus* was only weakly inhibited, with a diameter of 1.70-4.43 mm.

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

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