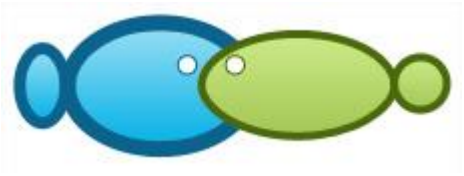

Xylocarpus granatum leaf extract inhibits *Aeromonas hydrophilla, Staphylococcus aureus,* and *Pseudomonas aeruginosa*

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Abstract. *Xylocarpus granatum,* a mangrove species, is used by the community for several needs. *Aeromonas hydrophilla*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* are pathogenic bacteria for humans and fish. This study aimed to analyze the bioactive substances in the leaf extract of *X. granatum* and their inhibition properties against these three pathogenic bacteria. Samples were collected from Mengkapan Village, Siak, Riau, Indonesia. Leaf samples were washed, cut into thin slices, dried, finely blended, and macerated with 96% ethanol solution for 24 h. The solution was filtered and evaporated until a crude extract was obtained and analyzed for its bioactive compound content. Antibacterial assay was carried out by using the paper diffusion agar method. The mangrove leaf extract contains secondary metabolites: saponins, flavonoids, steroids, and tannins. This extract inhibited the growth of pathogenic bacteria *A. hydrophilla* and *S. aureus* with weak inhibition category (diameters of 0.6-2.2 mm and 0.78-2.23 mm, respectively), and with moderate category against *P. aeruginosa* (4.15-8.7 mm). Inhibition of *P. aeruginosa* requires further research, for example by carrying out an antibacterial assay of purified secondary metabolites of this mangrove leaf.
Key Words: antibacterial assay, bioactive compound, marine environment, pathogenic bacteria, phytochemical analysis.

Introduction. *Xylaocarpus granatum* grows along river banks, which are still affected by tides. These mangroves are also found along tropical beaches. The bark of this plant is used in traditional medicine, for example, as a medicine for dysentery, diarrhea, stomach ache, febrifuge, and so on. The ashes of this plant are mixed with sulfur and coconut oil and applied as an ointment for itching. The fruit is used as a medicine for elephantiasis (Alamgir et al 2007; Dey et al 2021).

Aeromonas hydrophila can be found in various places, living in marine and fresh waters, water habitats, foods, and domestic animals (raw meat, poultry, fish, and shellfish), especially in places containing high organic matter. It is an opportunistic fish pathogen, Gram-negative, and can cause fish death in a very short time up to 80-100% through hemorrhagic septicemia. The bacterium has the potential to be a foodborne pathogen, associated with clinical cases of illness (Daskalov 2006).

Staphylococcus aureus is an aggressive human pathogen that is responsible for a variety of diseases from minor skin infections to life-threatening conditions such as bacteremia, pneumonia, and endocarditis. The emergence of multidrug resistance in *S. aureus* is a huge public health problem. There is an urgent need for additional and alternative therapeutic targets for infections caused by this bacterium. The pathogen grows well on various bacteriological media under aerobic or microaerobic conditions. It is reported that the bacterium grows rapidly at 37ºC, with the best pigment formation at room temperature (20-35ºC). Colonies on solid media will be round, smooth, prominent, and shiny, forming various golden-yellow pigments (Rasigade & Vandenesch 2014).

Pseudomonas aeruginosa is a rod-shaped, motile, and about 0.6x2 µm in size bacteria. It is a major cause of illness and death in humans with immunosuppressive and chronic conditions, and infections in these patients are difficult to treat due to some antibiotic resistance mechanisms and the organism's propensity to form multicellular biofilms. The pathogen belongs to the group of Gram-negative bacteria and can appear singly, in pairs, or sometimes in short chains. They are found in low numbers in a wide variety of environments including soil and water and almost any human and animal-impacted environment. The bacteria are also known as a pathogen for tilapia that can cause infection in wounds (Ali et al 2021).

Mangrove communities and some other plants have been widely recognized as containing bioactive compounds. They include compounds such as alkaloids, saponins, tannins, flavonoids, terpenoids, and glycosides (Bhimba et al 2010; Effendi et al 2022b; Effendi et al 2023a). These substances generally show bioactivity in the form of antifungal (Yang et al 2012; Manilal et al 2016;), antiviral (Wang et al 2018), antioxidant (Simlai et al 2014; Sudirman et al 2014), and antibacterial activities (Ulmursida et al 2017; Audah et al 2020).

Antibacterial applications of these compounds have been used to treat infections in fish and other aquatic animals (Effendi et al 2022a; Effendi et al 2023b). Findings suggest that *Xylocarpus* contains various important minerals and phytochemicals, where flavonoids, terpenes and terpenoids are the most prominent (Lakshmi & Gupta, 2007; Das et al 2015). Isolated compounds from this plant possess diverse biological activities, including anti-inflammatory, anti-microbial, antineoplastic, anti-diarrheal, insecticidal, antifeedant, neuropharmacological (e.g., central nervous system depressant), anti-atherosclerotic, and lipid-lowering activities (Islam et al 2020). *X. granatum* extract had the potential to inhibit *Vibrio harveyi* and *Saprolegnia* sp., reducing infection and improving the survival of shrimp. Leaf extracts of *X. granatum* have antimicrobial activities preventing pathogenic infections in tiger shrimp (*Penaeus monodon*) (Saptiani et al 2019). From the description and background above, we hypothesized that the leaf extract of *X. granatum* has bioactive substances that can inhibit the growth of some pathogenic bacteria. This study aimed to analyze the bioactive content and antibacterial activity of *X. granatum* leaf extract on the growth of fish pathogenic bacteria *A. hydrophilla*, *S. aureus*, and *P. aeruginosa*.

Material and Method

Place and research design. Mangrove leaves of *X. granatum* samples were collected from Mengkapan Village, Siak, Indonesia, in February 2022. This research was an experimental study, using a completely randomized design (CRD) with two factors, namely the concentration of leaf extract (A) and the pathogenic bacteria (B). The mangrove leave extracts were diluted into sterile distilled water to obtain concentration of 12.5 (A1), 25 (A2), 50 (A3), and 100% (A4), positive experimental control (PEC; 10% chloramphenicol or A5) and negative experimental control unit (NEC; water or A6). The pathogenic bacteria are *A. hydrophilla* (B1)*, S. aureus* (B2)*,* and *P. aeruginosa* (B3).

Extract preparation. Fresh *X. granatum* leaves weighing up to 5 kg were thoroughly cleaned before being sliced into thin strips and dried. The drying procedure was completed in an air-conditioned space. The dried leaves were macerated in a 96% ethanol solution for 24 h. Wahattman No. 42 filter paper was used to filter the samples into a container, separating the filtrate liquid from the dregs. The same process was applied to the initial filtered dregs and repeated until a translucent liquid was produced. A rotating vacuum evaporator was used to evaporate the filtered solution at 60°C, 100 mbar pressure, and 15 rpm, until the solvent evaporated and the desired form of extract was achieved.

Phytochemical analysis. The goal of the phytochemical analysis was to identify the many classes of bioactive compounds, such as saponins, alkaloids, flavonoids, phenols, tannins, steroids, and terpenoids present in the leaves of *X. granatum*. In this study, the analysis was conducted using Bertoli et al (2010) and Menghini et al (2018) as

references. A more detailed analysis procedure is presented as follows.

Alkaloids analysis. A sample (0.05 g) and two drops of H_2SO_4 2 N were added to a test tube and thoroughly mixed after shaking. A porcelain drip plate with nine wells was then used to contain it. Meyer's, Wagner's, and Dragendorff's reagent were drip-fed into each of the three wells. A clue that the sample includes alkaloids is the appearance 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.

Saponin analysis. The sample (0.1 g) of dried extract was weighed and placed in a beaker glass. 10 mL of boiling water was then added, and the sample was boiled for 5 min. The mixture was then filtered, and the filtrate was added to a test tube, sealed, and agitated for approximately 10 seconds. It was then left on a table for 10 to 15 min, after which 1 mL of 2M HCl was added. The presence of saponin compounds in the extract was positive if a stable foam was formed.

Phenol analysis. The leaf extract was first placed in a test tube with 3 drops of FeCl and was gently stirred. After 5–10 min, the chemical develops a dark blue hue, which indicates the presence of phenolic compounds. A few drops of a 10% iron (III) chloride solution were added to 1 mL of the extract to conduct the test for the presence of tannins. A dark blue or greenish black precipitate indicates the presence of tannins in the extract.

Flavonoids analysis. Leaf extract from *X. granatum* 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.

Steroid and terpenoid analysis. The prepared chloroform liquid was put into the drip plate's two places and allowed to dry with a fan. Next, the drip plate's two places were filled with concentrated acetic acid and H_2SO. The presence of terpenoids is shown by a purple color, whereas the presence of steroid compounds is indicated by a green color.

Antibacterial assay. The Kirby-Bauer paper disc diffusion agar method was used to conduct an antibacterial experiment against pathogenic bacteria (*A. hydrophilla, S. aureus*, and *P. aeruginosa*) (EUCAST 2012; Effendi et al 2020). Aseptically, by diluting with sterile distilled water, a set of solutions was prepared from mangrove leaf extract with 12.5, 25, 50, and 100%. Using a loop needle, the three bacterial isolates were inoculated into NA medium (Oxoid) and incubated for 48 h. NB (Oxoid) medium was inoculated with the bacteria and incubated overnight. A volume of 50 µL was pipetted and spread using a glass rod on Mueller Hillton Agar (Oxoid). A set of paper discs 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^{o}C overnight. The inhibition zone was calculated by measuring the diameter of the clear zone around the paper disc using a caliper. The inhibition level was categorized by referring to Syawal et al (2020), Syawal et al (2021), and EUCAST (2012) and the antibacterial assay was repeated 3 times.

Results

Phytochemical analysis. The results of the phytochemical analysis showed that the content of bioactive compounds included alkaloids, flavonoids, tannins, and steroid groups (Table 1).

Table 1

The results of the phytochemical analysis of the leaf extract of *Xylocarpus granatum*

No	*Bioactive compounds*	*Result*	*Indication*
1	Alcaloid	+	Red to brown and forms a white precipitate Brownish red and forms a brown precipitate
2	Saponin	-	No foam is formed
3	Tanin	+	Dark green color
4	Flavonoid	+	Reddish yellow to red
5	Steroid	+	Green color
6	Terpenoid	-	No color change to purple

Antibacterial assay. Inhibition properties of *X. granatum* leaf extract against *A. hydrophilla*, *S. aureus*, and *P. aeruginosa* bacteria can be viewed in Figures 1, 2, and 3. *X. granatum* extract can inhibit the growth of *A. hydrophilla* even though categorized as weak (<5 mm). The highest and lowest inhibition occurred at concentrations of 100% and 12.5%, respectively. The extract also inhibited *S. aureus* bacteria in the weak category. The inhibition was noted more clearly against *P. aeruginosa* (Table 2). The highest and lowest inhibition zones occurred at concentrations of 100% and 12.5%, respectively, and were categorized as medium (5-10 mm).

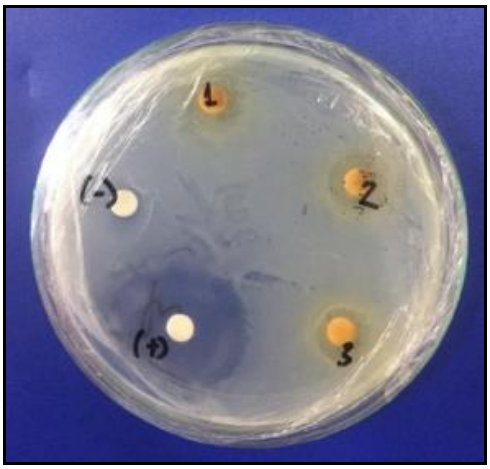

Figure 1. Inhibition zone of *Xylocarpus granatum* leaf extract against *Aeromonas hydrophilla*.

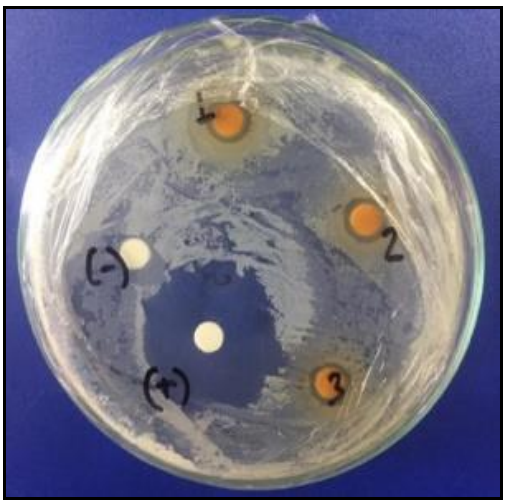

Figure 2. Inhibition zone of *Xylocarpus granatum* leaf extract against *Staphylococcus aureus*.

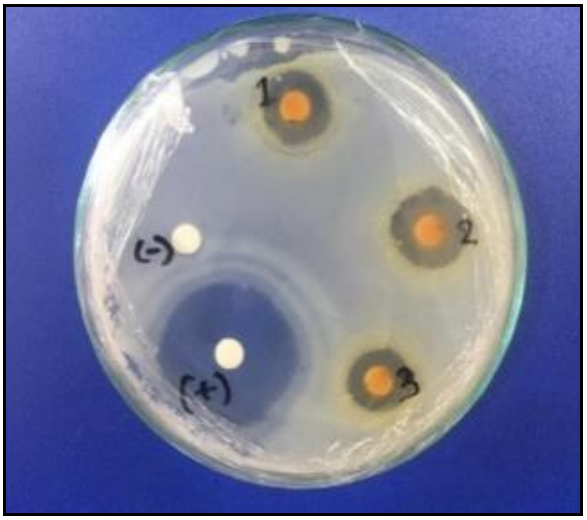

Figure 3. Inhibition zone of *Xylocarpus granatum* leaf extract against *Pseudomonas aeruginosa*.

Table 2

Inhibition of pathogenic bacteria by *Xylocarpus granatum* extract

No	Pathogenic bacteria	Concentration (%)	Inhibition zone (mm)			Average±SD (mm)
			R-1	R-2	R-3	
1	*Aeromonas hydrophilla*	12.5 (A_1B_1)	1	0.4	0.6	0.67±0.3
		25 (A_2B_1)	1.1	1.6	1.45	1.38±0.25
		50 (A_3B_1)	1.7	1.9	0.9	1.5±0.52
		100 (A_4B_1)	3.1	1.6	1.9	2.2±0.79
		PEC (A_5B_1)	22.8	22	22.9	22.56±0.49
		NEC (A_6B_1)	0	0	0	0±0
2	*Staphylococcus aureus*	12.5 (A_1B_1)	1.15	0.55	0.65	0.78±0.32
		25 (A_2B_1)	1.1	1.6	1.55	1.41±0.27
		50 (A_3B_1)	2.4	2.55	2.55	2.5±0.08
		100 (A_4B_1)	3.45	1.55	1.7	2.23±1.05
		PEC (A_5B_1)	20.7	19.8	21.3	20.6±0.75
		NEC (A_6B_1)	0	0	0	0±0
3	*Pseudomonas aeruginosa*	12.5 (A_1B_1)	4.55	4.2	3.7	4.15±0.42
		25 (A_2B_1)	5.4	6	5.55	5.65±0.31
		50 (A_3B_1)	6.6	6.7	6.4	6.56±0.15
		100 (A_4B_1)	8.6	9.15	8.35	8.7±0.41
		PEC (A_5B_1)	24.4	24.6	29.4	26.13±2.83
		NEC (A_6B_1)	0	0	0	0±0

Note: R - replication; PEC - positive experimental control; NEC - negative experimental control; SD - standard deviation.

Discussion. The results of the phytochemical analysis indicated that the general secondary metabolite groups contained in the leaf extract of *X. granatum* were alkaloids, flavonoids, tannins, and steroids. The presence of antibacterial bioactive compounds in *X. granatum* has been empirically proven in coastal communities. This mangrove is used as a traditional herbal medicine (Shahid-Ud-Daula & Basher 2009). Usually, different parts of this plant are used for different purposes. It is mostly used for the treatment of malaria, cholera, fever, diarrhea, dyslipidemia, inflammation, dysentery, and others (Lakshmi & Gupta 2007). The results found in this research were not much different from those of other researchers (Das et al 2019) who reported that the secondary metabolite compounds found in *X. granatum* mangrove leaves were triterpenoids, steroids, saponins, and tannins. Other researchers also reported that various types of secondary

metabolites were extracted from various plant parts, such as leaves, bark, fruit, and flowers, for example, limonoid compounds, phragmalins, limonoid-based alkaloids, mexicanolides, protolimonoids, compounds such as flavonols, lactones, ethanol extracts, methanol extracts, and alkaloids (Kokpol et al 1996; Cheng et al 2009; Du et al 2020).

The results of the antibacterial activity assay showed the presence of antibacterial compounds in the leaf extract of *X. granatum* that could inhibit the growth of *A. hydrophilla*, *S. aureus*, and *P. aeruginosa* bacteria. However, this inhibition is classified as weak and moderate. Research on antibacterial assays against these three species of microbial pathogens by bioactive substances from plants in coastal ecosystems has been widely carried out (Syawal et al 2020; Syawal et al 2021). Some groups of alkaloid compounds, flavonoids, tannins, and steroids have been reported. The mechanism of action of these active substances is by inhibiting cell wall synthesis, inhibiting cell membrane function, and inhibiting protein synthesis. Previous researchers (Cushnie et al 2014; Pérez et al 2016; Dias et al 2021) explained that antimicrobial substances such as alkaloids and flavonoids are thought to inhibit the work of bacterial enzymes, resulting in disruption of metabolism or the death of bacterial cells. The results of the phytochemical analysis of *X. granatum* leaf extract proved that the bioactive compounds include steroids, flavonoids, saponins, and tannins.

The inhibition of several bacterial pathogens is not too surprising. *In vitro* screening of organic solvent extracts of *X. granatum* showed specific activity in inhibiting the growth of six virulent strains of bacteria pathogenic to fish: *Edwardsiella tarda, Vibrio alginolyticus, Pseudomonas fluorescens, P. aeruginosa,* and *A. hydrophila* (Choudhury et al 2005). Traditionally, this plant is widely used to treat several diseases because of its chemical content. Alkaloid compounds can interfere with the peptidoglycan constituent components in bacterial cells so that the cell wall layer is not fully formed. Disruption of peptidoglycan synthesis causes imperfect cell formation, eventually causing cell death (Dey et al 2021).

Tannins are substances widely distributed in plants. The tannin content in immature fruits is used as a source of energy in the metabolic process in the form of oxidation (Li et al 2011). Tannins are said to be a source of acid and bitterness in fruit. The mechanism of action of tannins as an antibacterial agent is by causing bacterial cells to lyse. This happens because tannins have a target on the polypeptide wall of the bacterial cell wall, causing issues in its formation, after which the bacterial cell will die (Farha et al 2020). Tannins also can inactivate bacterial enzymes and interfere with the passage of proteins in the inner layer of cells (Cheng et al 2009; Hilmi et al 2021).

Flavonoids are secondary metabolites of polyphenols, widely found in plants and food, and have various bioactive effects including anti-viral, anti-inflammatory (Wang et al 2018), cardioprotective, anti-diabetic, anti-inflammatory and anti-cancer (Marzouk 2016), anti-aging, antioxidant (Munhoza et al 2014), and other properties. Flavonoid compounds are polyphenolic compounds that have 15 carbon atoms arranged in a C6-C3-C6 configuration, meaning that the carbon skeleton consists of two C6 groups (substituted benzene rings) connected by a three-carbon aliphatic chain (Dias et al 2021).

The limonoid, xyloccensin K, has been isolated from the seeds of *X. granatum*, along with a mixture of steroids and long-chain fatty acids and alcohols (Yin et al 2007). X-ray crystallography has shown that xyloccensin K is similar to some previously reported limonoids, but contains tetrahydrofuran subunits with oxygen linkages from C-3 to C-8 (Roy et al 2006). The way steroids act as antimicrobials is related to lipid membranes and sensitivity to steroid components that cause leakage in bacterial liposomes (Kokpol et al 1996; Wangensteena et al 2006).

Terpenoids are natural products whose structure is divided into several isoprene units. Therefore, these compounds are also called isoprenoids (C_5H_8). Terpenes are hydrocarbon compounds found in all plants, animals, insects, and marine animals (Wu et al 2008). Terpenoids act as medicinal compounds for diabetes, menstrual disorders, snake bites, skin disorders, liver damage, and malaria (Wangensteena et al 2006; Lakshmi et al 2010).

Conclusions. The secondary metabolite bioactive substances contained in the *X. granatum* mangrove leaf extract are alkaloids, tannins, flavonoids, steroids, and terpenoids. The extract has the potential to inhibit the growth of pathogenic bacteria *A. hydrophilla* and *S. aureus* with a weak effect, with inhibitory diameters of 0.6-2.2 mm and 0.78-2.23 mm, respectively. As for *P aeruginosa*, the inhibition was in the moderate category, where the diameter of the inhibition ranged from 4.15-8.7 mm.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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