Screening of bioactive compounds and antibacterial activity of *avicennia marina* leaf extract against pathogenic bacteria

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Abstract. High levels of marine pollution are thought to trigger the growth of pathogenic bacteria and cause disease in farmed fish. One of the natural antibiotic alternatives to inhibit the growth of pathogenic bacteria is *Avicennia marina* leaf extract. *A. marina* leaves extract is thought to produce bioactive compounds, antibacterial and stimulate the growth of endophytic bacteria. The study aimed to screen bioactive compounds and antibacterial activity of *A. marina* leaf extract against pathogenic bacteria. This survey research was conducted from March to May 2023. A fermentation process of mangrove leaves was carried out for 14 days, and the extract containing bioactive compounds was tested. Antibacterial test of *A. marina* leaf fermentation extract was conducted against *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Vibrio alginolyticus*. The results showed that bioactive compounds from fermented *A. marina* leaves were identified, namely phenolics, saponists and terpenoids. Total colonies of endophytic bacteria ranged from 4.9 x 10^4 – 1.0 x 10^7 CFU/gram. The antibacterial test results of *A. marina* leaf extract were able to inhibit the growth of *P. aeruginosa* with an inhibitory diameter of 7.5-11 mm (strong category), while *A. hydrophila* and *V. alginolyticus* with an inhibitory power of 2-5 mm (weak category).

1 Introduction

The sea water of Dumai is one of the coastal areas, located on the east coast of Sumatra's island, support for various community activities (anthropogenic). It is thought to produce pollutants (domestic and industrial waste), including organic and inorganic materials. The waters of Dumai also gained high pressure due to infrastructure, settlement, landscaping, agriculture, fisheries, and industrial development. The pollution directly or indirectly can cause a decrease in the quality of sea water, environmental damage and affect the life of marine biota [1].

High levels of marine pollution can cause obstacles in the development of fish farming businesses in mangrove forest areas. This is thought to trigger the growth of pathogenic bacteria that cause disease in marine fish. For example, vibriosis caused by *Vibrio alginolyticus* bacteria, red spot disease caused by *Pseudomonas aeruginosa* bacteria, dropsy

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disease caused by *Aeromonas hydrophila* bacteria and enteroaggregateive and anteroinvasive diseases caused by *Escherichia coli* bacteria. The excessive use of synthetic antibiotics for the treatment of diseases in marine life creates resistance to pathogenic bacteria, produces antibiotic residues in aquaculture products and threatens fish health.

The inhibitory power of bacteria is a compound that can kill or barrier the growth of pathogenic bacteria. This is to kill pathogenic bacteria (microbicidal) or inhibit the bacterial growth (microbiostatic). The results showed that the highest diameter of the clear zone by treating amoxicillate concentration (antibiotic chloramphenicol) against pathogenic bacteria compared to other concentrations. Bioactive substances can kill pathogenic bacteria (microbicidal) or inhibit growth (microbiostatic) [2].

Phytochemical tests were carried out to detect secondary metabolites of plants based on their class. Phytochemical test is an approach method used in determining the presence of secondary metabolite compounds in plants. Classes of secondary metabolites are determined qualitatively using several phytochemical test reagents [3]. Various species of mangrove contain secondary metabolites, such as alkaloids, flavonoids, steroids, terpenoids, saponins and others. The secondary metabolite compounds are bioactive substances which make plants appropriate for medicinal use [4], [5].

The use of mangrove extracts has not been optimal to inhibit the growth of pathogenic bacteria and the pathogenic bacteria still has a strong pathogenicity. This study is looking for a new natural antibiotic alternative by using fermented mangrove leaves of *Avicennia marina* to control the growth of pathogenic bacteria. Fermentation of mangrove leaves is thought to produce bioactive compounds and can stimulate the growth of endophytic bacteria. Natural materials obtained from fermented mangrove leaves are expected to be used as an alternative to natural antibiotics. Therefore, this study is looking for a new compound that is effective and safe to treat infectious diseases by pathogenic bacteria in marine fish farming businesses, without causing adverse side effects.

According to [6], endophytic bacteria have the ability to systematically colonize plant tissues and develop mutually symbiotic relationships with host plants, resulting endophytic bacteria as efficcient biocontrol agents. [7] that endophytic bacteria are able to produce secondary metabolite compounds such as antibiotics, anticancer compounds, antifungals, antivirals and act as insecticidal agents.

The purpose of the study was to analyze bioactive compounds and antibacterial bioactivity of fermented extracts of *A. marina* leaves against the growth of pathogenic bacteria. The benefit of research was to obtain information about fermented extracts of *A. marina* leaves to overcome pathogenic bacterial infections as a natural antibiotics.

2 Materials and Methods

The research was conducted survey methods *A. marina* leaves were obtained from the mangrove forest of Pangkalan Sesai Village, Dumai City. The study was conducted from March – May 2023. Microbiological analysis of samples was performed in the Marine, Faculty of Fisheries and Marine, University of Riau, Indonesia.

Preparation of fermentation process of the leaves was as follows: a) *A. marina* leaves (1.5 kg) was added with given water (3 l) and brown sugar (375 g); b) All ingredients was blended until smooth; c) Putted the delicate ingreidients into the container and tightly closed; d) A fermentation process was carried out for 14 days (Figure 1); and e) The extract containing bioactive compounds was examined using maceration method. Isolation of endophytic bacteria was a) *A. marina* leaf samples was rinsed with aquades; b) Soaked in 70% ethanol for 2 minutes; c) Soaked in sodium hypochlorite for 1 minutes, rised aqueously; d) The sample was mashed by 10 g, poured sterile sea water to a volume of 100 ml; and e) Cultured

on sea water complex agar media. Count total colonies of endophytic bacteria and biochemical characteristics was tested [8], [9], [10].

Test the inhibitory power of pathogenic bacteria (P. *aeruginosa, A. hydrophilla*, *V. alginolyticus*) against fermented extracts of *A. marina* leaves using the Kirby-Baeuer method with a disc diameter of 6 mm [11]. Analysis of bioactive compounds was carried out on fermented extracts of *A. marina* leaves [12].

Fig 1. The fermentation process of *A. marina* mangrove leaves.

3 Results and Discussion

3.1. Characteristic of Mangrove Forests

Mangrove forests have distinctive forest characteristics, growing on freshwater and seawater flooded substrates, along coasts, river estuaries and peat swamps. Mangrove forests in Pangkalan Sesai Subdistrict, Dumai City can grow well and have been used as mangrove forest conservation areas. The marine ecosystem in Pangkalan Sesai Village has a unique attraction because it has a very important function ecologically; economic, social used as a source of income for local communities; It is located on the border between land and sea, coastal areas and around the river mouths that are influenced by tides. The influence of tides can provide a supply of nutrients for marine waters in Pangkalan Sesai Village, while river water containing mineral materials can also provide a source of nutrients in the sediment.

Around the beach, the waters in Pangkalan Sesai Village is overgrown by mangrove vegetation along with flora and fauna. These waters are strongly influenced by the nature of the marine climate, where the rainy season falls from September to February. The dry period that starts from March to August. As an ecosystem and natural resources, the use of marine waters can be directed to improve community welfare and continue to be sustainable. Some of the results of measuring the quality parameters of the marine environment in Pangkalan Sesai Village can be seen in Table 1.

Based on Table 1, it can be seen that the quality of marine waters in Pangkalan Sesai Village still supports the mangrove growth, namely temperatures ranging from 28-30.3 ^{o}C; pH ranged from 5.3-5.7; salinity 30 ppt; the transparency of the seawater ranged from 48.3-59 cm; The current speed ranged from 0.12-0.16 m/s and the water depth ranged from 0.73-1.25 m. The environmental quality parameters of these marine waters are still below the pollution threshold.

The mangrove forest area in Pangkalan Sesai Village is part of the Dumai sea water area which is directly related to the waters of the Strait of Malacca, located in the Rupat Strait, estuarial waters (Sungai Mesjid Estuary, Dumai River).

Table 1. The average measurement of marine water quality parameters in Pangkalan Sesai Village.

Research Station	Test	Water Quality Parameters					
		Temperature (°C)	pH	Salinity (ppt)	Transparancy (cm)	Current speed (m/st)	Depth (m)
I (Dumai Estuary)	1	27	5	30	55	0.07	0.65
	2	29	6	30	47	0.11	0.72
	3	28	5	30	43	0.19	0.83
	Average	**28**	**5.3**	**30**	**48.3**	**0.12**	**0.73**
II (Bandar Bakau)	1	31	5	30	62	0.18	0.87
	2	30	5	30	44	0.19	0.92
	3	30	6	30	69	0.12	0.85
	Average	**30.3**	**5.3**	**30**	**58.3**	**0.16**	**0.88**
III (TPI)	1	29	6	30	63	0.18	1.23
	2	30	6	30	67	0.13	1.46
	3	31	5	30	47	0.14	1.06
	Total	**30**	**5.7**	**30**	**59**	**0.15**	**1.25**

These marine waters have high accessibility for shipping both domestically and internationally, are influenced by anthropogenic activities from the mainland and receive input from various types of domestic and industrial waste. [13] The development of an area will have an impact on the surrounding environment. If not planned properly, there will be a decrease in water quality due to an increase in waste discharges that are harmful to humans and biota in the waters.

3.2. Screening of Bioactive Compounds

Biaoctive compounds are essential compounds (karatenoids, omega-3) and non-essential compounds (vitamins, polyphenols) found in nature. Screening analysis of bioactive compounds performed on fermented leaves of *A. marina* mangroves can be seen in Table 2.

Table 2. Screening of bioactive compounds from fermentation extract of *A. marina* leaves.

No.	Bioactive Compounds	*A. Marina* leaves
1	Alkaloid	-
2	Steroid	-
3	Phenolic	+
4	Flavonoid	-
5	Saponin	+
6	Terpenoid	+

Notes: + : positive (identified); - : negative (not identified)

Based on Table 2, it can be seen that the test results of bioactive compounds in fermentation of *A. marina* mangrove leaves have antibacterial substances. The test results of bioactive compounds in the fermentation of *A. marina* mangrove leaves can be identified phenolic compounds, saponins and terpenoids, while for alkaloids, steroids and flavonoids compounds are not identified.

Bioactive compounds have been widely used as supplements and medicinal ingredients [14]; build the immune system and increase body stamina [15]; source of antioxidants, antibiotics, antibacterial and anticancer [16]. Bioactive compounds are widely contained in the body of animals and plants such as mangrove leaves of *R. apuculata* and *A. marina* [17].

3.3. Isolates and Characteristics of Endophytic Bacteria

Several samples of *A. marina* leaves have been taken from the mangrove forest ecosystem in Pangkalan Sesai Village. Mangroves have potential as traditional medicinal materials, the presence of endophytic bacteria in mangrove plant tissue does not harm the host.This is done to examine some endophytic bacteria found in mangrove leaf tissue. Endophytic bacteria have a mutualism symbiotic relationship to help produce bioactive compounds in mangrove leaves. Endophytic bacteria grow in plant tissue and can produce a number of secondary metabolites (bioactive). [7] states that isolates endophytic bacteria founded in *A. marina* capable of producing amylase, protease, cellulase and galatinase enzymes. For more details can be seen in Table 3.

Table 3. Average total colonies of endophytic bacteria of A. marina leaves.

Samples	Average total cell of endophytic bacteria (CFU/ml)
EB3	4.9×10^4
EB4	1.0×10^5
EB5	$1.2\ 10^6$
EB6	1.0×10^7

Based on Table 3 it can be seen that the total endophytic bacteria from *A. marina* mangrove leaves ranged from 4.9 x 104 – 1.0 x 107 CFU/ml. The presence of endophytic bacteria still supports to establish a symbiotic relationship of mutualism and live on the leaf tissue of *A. marina* mangroves. [18] Endophytic microorganisms are microscopic living organisms (bacteria and fungi) that live in plant tissues (xylem and phloem) leaves, roots, fruits and stems. Mangrove plants are a rich source of endophytic bacteria. [19] *Avicennia* sp. leaf extract It can be used to discover new bioactive natural products and used as a potential source to control pathogenic bacteria.

Endophytic bacteria are one type of microorganism that has a high abundance in plant tissues.Endophytic bacterial isolates from *A. marina* leaves have been identified based on biochemical tests of colonies grown on agar media as shown in Table 4.

Tabel 4. Results of biochemical test of endophytic bacteria from *A. marina* leaves.

Isolate Code	Biochemical Test of Endophytic Bacteria						
	Gram Staining	Cell Shape	Catalase	Motility	Sulfit	SCA	TSIA
EB3.1	+	Bacill	-	+	-	-	-
EB3.2	+	Bacill	+	+	-	-	-
EB3.2	+	Bacill	-	+	-	-	-
EB3.2	+	Coccus	-	+	-	-	-
EB3.2	+	Coccus	-	+	-	-	-
EB3.1	+	Basil	-	+	-	-	-
EB3.1	-	Coccus	-	+	-	-	-
EB1.3	+	Coccus	-	+	-	-	-
EB1.3	-	Coccus	+	+	-	-	-
EB4.3	+	Coccus	-	+	-	-	-
EB4.2	+	Coccus	-	+	-	-	-
EB4.2	+	Bacill	-	+	-	-	-
EB4.2	+	Coccus	-	+	-	-	-
EB3.3	+	Bacill	-	+	-	-	-
EB3.3	+	Coccus	-	+	-	-	-
EB3.3	+	Bacill	-	+	-	-	-
EB3.1	+	Coccus	-	+	-	-	-

Based on Table 4, it can be seen that there are 17 isolates that have been identified as endophytic bacteria, namely 15 isolates of endophytic bacteria, most of which belong to the Gram positive bacteria group and 2 isolates belong to the Gram negative group. According to the classification, bacteria are divided into 2, namely Gram positive bacteria and Gram negative bacteria. Gram positive bacteria are able to absorb purple, while Gram negative bacteria are able to absorb red or orange. Gram negative bacteria produce a reddish color because at the time of staining, the pores of the bacterial layer will enlarge which contains a lot of lipids. This causes violet crystals to dissolve easily when washing with alcohol. [20] Gram negative bacteria have a more complex cell wall structure than Gram positive bacteria so that they can survive in extreme environmental conditions.

The bochemical test of Gram staining is an effective criterion for the classification of microorganisms, showing basic and complex differences in bacterial cells i.e. cell wall structure [21]. Endophytic bacteria are generally motile (mobile) and there are 2 isolates that produce gas bubbles (catalase), namely endophytic bacteria are able to produce the enzyme catalase. In addition, there are 17 isolates that are unable to produce sulfites, no sulfide-reducing bacteria and are unable to ferment glucose.

3.4. Antibacterial Bioactivity Test Against Pathogenic Bacteria

Antibacterial activity test against pathogenic bacteria can be known by the formation of a clear zone around the disc paper (diameter 6 mm) and measuring the diameter with a caliper. Test of antibacterial activity of *A. marina* mangrove leaf fermentation against pathogenic bacteria *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Vibrio alginoliticus*. The antibacterial test results can be seen in Table 5, Figure 1 and Figure 2.

Table 5. Test of antibacterial activity of fermented extract of A. marina leaves against pathogenic bacteria.

No.	Isolat Code	Uji antibakteri terhadap bakteri pathogen (mm)		
		Pseudomonas aeruginosa	***Aeromonas hydrophila***	***Vibrio alginolyticus***
1	EB3.1	7	0	0
2	EB3.2	4	0	2
3	EB3.2	10,5 *	4	0
4	EB3.1	7,5 *	4	0
5	EB4.2	3	0	0
6	EB4.2	11 *	3,5	0
7	EB4.2	0	4	0
8	EB3.3	4,5	4,5	2
9	EB3.3	5	3	5
10	EB3.1	9 *	0	0

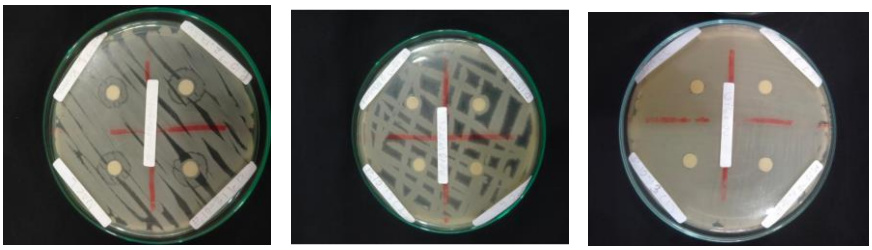
Notes: Disc diameter 6 mm
Diameter > 6 mm: There is an inhibitory power of bacteria

Based on Table 5, it can be seen that antibacterial tests from fermented extracts of *A. marina* mangrove leaves was able to inhibit the growth of pathogenic bacteria *P. aeruginosa* with a very strong category, namely the diameter of the inhibitory power ranging from 7.5 – 11 mm. Antibacterial tests of fermented extracts of *A. marina* leaves have not been able to inhibit the growth of pathogenic bacteria *A. hyrophilla* and *V. alginolyticus* with a weak category, namely inhibitory power ranging from 2 – 5 mm.

According to [22], antimicrobials that affect cell wall formation/cell membrane permeability work as bactericides and protein synthesis work as bacteriostatics. [23]

Antibacterial clear zone criteria are very strong criteria (inhibitory power > 20 mm), strong criteria (inhibitory power 10-20 mm), medium criteria (inhibitory capacity 5-10 mm) and weak criteria (resistency from 4-5 mm).

The results showed that there was a clear inhibitory zone around the disc, meaning that fermented extracts of *A. marina* leaves were able to inhibit the growth of pathogenic bacteria, especially in *P. aeruginosa* bacteria (strong category). The strength of bioactive compounds (such as phenolic, saponin and terpenid) from fermented extracts of *A. marina* leaves shows that the wider the diameter of the inhibitory zone, the stronger the bioactive compounds inhibit the growth of pathogenic bacteria.



P. aeruginosa Bacteria *A. hydrophilla* Bacteria *V. alginolyticus* Bacteria

Fig. 2. Results of inhibitory power of *A. marina* leaf fermentation extract.

4 Conclusion

The test results of bioactive compounds in the fermentation of *A. marina* mangrove leaves can be identified phenolic compounds, saponins and terpenoids, while for alkaloids, steroids and flavonoids compounds are not identified. The total endophytic bacteria from *A. marina* mangrove leaves ranged from 4.9 x 10^4 – 1.0 x 10^7 CFU/ml. The fermented extracts of *A. marina* mangrove leaves was able to inhibit the growth of pathogenic bacteria *P. aeruginosa* with a very strong category, namely the diameter of the inhibitory power ranging from 7.5 – 11 mm. Bacterial isolates found in this research need to be identified molecurlaly.

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