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Original Article

Antibacterial Activity of Coastal Plants and Marine Sponges from Kei Island Indonesia against Bacterial Fish Pathogens

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ABSTRACT

Objective: The objective of this study was to investigate the antibacterial activity of coastal plants and marine sponges extracts against fish bacterial pathogens. **Methods**: Samples were extracted by maceration and the extracts were examined for their antibacterial activities against *Streptococcus* sp. BJ0509, *Staphylococcus aureus* ATCC 6538, *Aeromonas hydrophila* BA03 and *Vibrio parahaemolyticus* 29S by means of paper disc diffusion method. Active extracts were partitioned and purified by column chromatography. The purified substance was tested for minimum inhibitory concentration (MIC) against seven bacterial fish pathogens namely *Streptococcus* sp., *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *Photobacterium damselae*, *Aeromonas hydrophila* and *A. dhakensis*. **Results:** The highest antibacterial activity against all bacteria used in the assay was demonstrated by OKA 6, a bark extract sample of a coastal plant, *Diospyros maritima*. It showed a diameter of inhibition zones against *Streptococcus* sp. BJ0509, *S. aureus* ATCC 6538, *A. hydrophila* BA03 and *V. parahaemolyticus* 29S of 19, 33, 18, and 18 mm, respectively. The column chromatography fraction of OKA 6 inhibited the growth of *S. aureus* ATCC 6538 with MIC of 3.125 µg/mL. The MIC of this fraction against seven bacterial fish pathogens ranged < 0.098 to 3.125 µg/mL. The antibacterial activity of partially purified substance obtained from column chromatography fractionation of OKA 6 was higher than those of oxytetracycline and kanamycin. **Conclusions:** This result indicates that antibacterial activity of the partially purified substance is potentially higher than those of the commercial antibiotics tested. It further indicates that OKA 6 extract from *D. maritima* can serve as a promising resource for the development of therapeutic agents against bacterial infections in aquaculture.

Key words: Antibacterial activity, Secondary metabolite, Coastal plant, Marine sponge, Fish pathogen, Bacteria

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INTRODUCTION

Coastal plants and sponges are marine resources that have been widely known as producers of bioactive compounds.[1-3] They are rich sources of alkaloids, saponins, tannins, flavonoids, terpenoids, and glycosides[1,4] many of which exhibit various bioactivities including anticancer,[5] antifungal,[2,3,6] antiviral,[4] antioxidant[7-9] and antibacterial activities.[2,4,6,8,10,11] The application of antibacterial compounds from coastal plants and sponges are not only limited in medication of disease caused by human pathogenic bacteria[12,13] but also have been applied to overcome the problem of fish bacterial infections.[2,12]

Fish infection by pathogenic bacteria would cause a serious economic loss in aquaculture as it causes a negative impact either on the growth and survival rates of fishes.[14-18] A common practice to overcome the problem of bacterial diseases in fish is by administering antibiotics.[19,20] However, long-term uses of antibiotics and improper doses have caused bacterial resistance.[20-22] Therefore, a search for new compounds is important in overcoming the problem of bacterial infections in aquaculture.[3,6,13,23]

Indonesia is known as an archipelagic country with mega biodiversity. Kei Islands, one of the 117 islands in Southeast Maluku Region, Maluku Province, possess a great potency of its coastal and marine resources. To the best of our knowledge, research on the antibacterial substances from coastal plants and sponges from Kei Islands has never been conducted. When we screened coastal plants and sponges from this island for anti-tuberculosis activity, we also found several extracts active against fish pathogenic bacteria. The purposes of this study were to extract secondary metabolites from the coastal plants and sponges, to evaluate antibacterial activity, and to partially purify the potential substances.

MATERIALS AND METHODS

Sample collection

Samples were collected in May 2017 at Ohoi Kelanit (S-5°39'18,34" E 132°40'48,32) and Ohoi Letman (S -5°34'43,67" E 132°43'20,18), Kei Islands, Southeast Maluku Regency, Maluku. Freshly-collected specimen of sponges and part of coastal plants including barks, leaves, fruits, and twigs were immediately transported to the laboratory. A total of 32 samples were washed with water to remove

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adhering soil particle and salts, air-dried, chopped into small pieces and ground coarsely into a powder in a mechanical grinder.

Preparation of crude extracts

An amount of 20 g powdered samples were extracted using maceration method with 80 mL solvents for 24 h and the process was repeated three times. Two types of solvent were used, ethanol and ethyl acetate. The soluble parts of extracts were filtered using Whatman paper, evaporated using rotary evaporator (Heidolph) at 40°C, and air-dried in shade for 3 days. The crude extracts were stored at 4°C prior to use.

Bacterial strains and media

Eight bacteria were used in the antibacterial activity assay, namely *Streptococcus* sp. BJ0509 and *Staphylococcus aureus* ATCC 6538, as representatives of Gram-positive bacteria, *Aeromonas hydrophila* BA03, *A. dhakensis* SB01, *Vibrio parahaemolyticus* 29S, *V. alginolyticus* GD22, *V. harveyi* GD38 and *Photobacterium damselae* SB25 as representatives of Gram negative bacteria. All isolates were bacterial collections of Laboratory of Fish Health Management, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, except *S. aureus* ATCC 6538. All bacteria were stored in TSB (Oxoid, UK) medium containing 20% (v/v) glycerol, except *Vibrio* spp. and *P. damselae* SB25, which were stored in Zobell broth medium,[24] and stored frozen at -80°C.

Antibacterial activity assay

The extracted samples from previous step were screened for their antibacterial activity. Antibacterial activity assay was carried out against *Streptococcus* sp. BJ0509, *Staphylococcus aureus* ATCC 6538, *Aeromonas hydrophila* BA03, and *Vibrio parahaemolyticus* 29S, using the paper disc diffusion method on double layer agar.[25] The bacterial inoculums were prepared in TSB (Oxoid, Japan), except *V. parahaemolyticus* 29S *was* in Zobell broth. Before inoculation, the cell density was estimated based on McFarland standard using spectrophotometer (Apel, Japan) at λ 625 nm. Inoculum of 10^6 cells/mL were transferred into TSB (Oxoid, UK) added with 0.7% agar for *Streptococcus* sp. BJ0509, *S. aureus* ATCC 6538, and *A. hydrophila* BA03; and Zobell medium added with 0.7% agar for *Vibrio* spp. and *Photobacterium damselae* SB25. Then, the mixtures were poured into TSA (Oxoid, UK) or Zobell agar medium. Sterile paper disks (ɵ 8 mm, Advantec, Tokyo) were dripped with 50 µL of extracts, placed on the surface of inoculated agar, and incubated at 30°C for 24 h. Antibacterial activity was measured based on the diameter of the inhibition zone.

Partition of crude extract

The crude extract showing the highest antibacterial activity was continued for partition. The crude extract in a powder form was dissolved in 50 ml ethanol (96%) and added with distilled water by 1:1 (v/v) and partitioned with 50 ml chloroform:distilled water (1:1, v/v) in a separating funnel. The mixture was shaken slowly until two layers were formed. The bottom layer was collected as the chloroform fraction and the process was repeated three times. Chloroform fraction was evaporated under reduced pressure and dried. Its antibacterial activity was confirmed by the paper disc diffusion assay as described previously and stored at 4°C prior to purification.

Column chromatography

One gram of the chloroform fraction was dissolved in 1 mL n-Hexane: Chloroform: Ethanol (7:2:1) and purified using silica gel column chromatography (column diameter of 3 cm and length of 30 cm). Silica gel F_{254} (60; particle size: 0.065-0.200 mm) (Merck, Germany). The sample was dissolved with 96% ethanol, and then poured carefully into the chromatography column. The chloroform fraction of 1 mL was applied into the column, and eluted subsequently by solvent combination of n-hexane:chloroform:ethanol (7:2:1 and 4:5:1) and ethanol (100%). The column fractions were tested for antibacterial activity.

Measurement of MIC and MBC of column fraction

The fraction obtained from the column chromatography showing the highest antibacterial activity was determined for its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC was evaluated against *Streptococcus* sp. BJ0509, *Staphylococcus aureus* ATCC 6538, *Aeromonas hydrophila* BA03, *A. dhakensis* SB01, *Vibrio parahaemolyticus* 29S, *V. alginolyticus* GD22, *V. harveyi* GD38 and *Photobacterium damselae* SB25 using microdilution method.[26] While the measurement of MBC were performed against *Streptococcus* sp. BJ0509 and *V. parahaemolyticus* 29S. Known antibiotics, Oxytetracycline (Sigma, Japan) and Kanamycin (Wako, Japan), were used as positive controls. Fraction was tested at concentrations starting from 0.098 µg/mL to 50 µg/mL. A total of 100 µL (bacterial suspension 10 µL, 2 strength of Mueller Hinton Broth (MHB) 50 µL and samples or antibiotics 40 µL) were mixed in each well of 96-well microplate and incubated for 24 h at 30°C. MBC was tested by inoculating 20 µL of test samples from 96-well microplate on Mueller Hinton (Conda, India) Agar (MHA) plate and incubated for another 24 h at 30°C.

RESULTS AND DISCUSSION

Screening of antibacterial activity from coastal plants and sponges

This paper describes the antibacterial activity of several coastal plants and marine sponges. The results of the primary screening for antibacterial activities from 63 crude extracts showed that only three samples (OKA 6, OLS 7, and OLS 8) (Table 1) exhibited promising antibacterial activities. OKA 6, sample extract with the highest antibacterial activity, was bark of coastal plant that collected from Ohoi Kelanit (S -5°39'18,34" E 132°40'48,32). The leaves are shiny dark green and egg-shaped with rounded tips (Figure 1). The outer surface of bark of the plant was dark (black).

From a total of 63 extracts, there were 8% of crude extracts showing high antibacterial activity with inhibition zones diameter more 19 mm against *V. parahaemolyticus* 29S and 2% against *S. aureus* ATCC 6538 (Figure 2). The second and the third highest antibacterial activities were shown by the ethyl acetate extracts of two different sponges, encoded OLS 7 and OLS 8, but the highest antibacterial activity was shown by the ethanol extract of the bark obtained from a coastal plant, OKA 6, with 33 mm in diameter of inhibition zone against *S. aureus* ATCC 6538, 19 mm against *Streptococcus* sp. BJ0509, 18 mm against *A. hydrophila* BA03 and 18 mm against *V. parahaemolyticus* 29S (Table 1). The OKA 6 plant was identified to be *Diaspyros maritima*.

Table 1: Diameter of inhibition zone (mm) of active crude extracts of coastal plants and sponges (1000 µg/disk) from a total of 63 extracts against bacterial test.

Bacteria	Plant (Bark)	Sponges	
	Ethanol extract of OKA 6	Ethyl acetate extract of OLS 7	Ethyl acetate extract of OLS 8
Streptococcus sp. BJ0509	19	11	12
S. aureus ATCC 6538	33	18	18
A. hydrophila BA03	18	9	11
V. parahaemolyticus 29S	18	13	0

Diameter of paper disc (ɵ) = 8 mm

Figure 1: The plant of OKA 6.

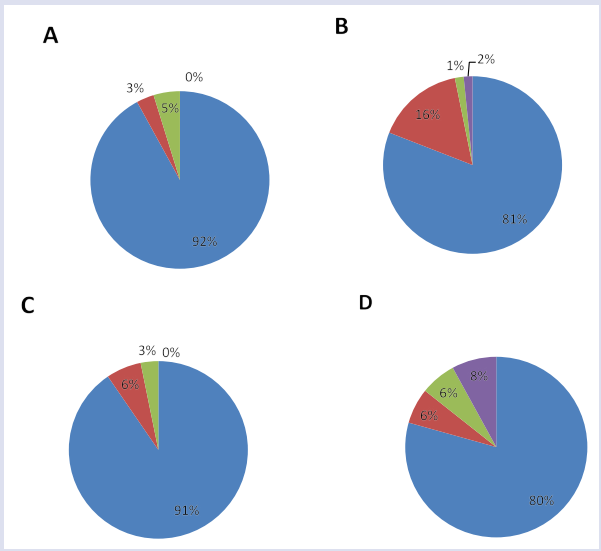

Figure 2: The distribution of antibacterial activity of coastal plants and marine sponges crude extracts against (A) *Streptococcus* sp. BJ0509, (B) *S. aureus* ATCC 6538, (C) *A. hydrophila* BA03, and (D) *V. parahaemolyticus* 29S.

The antibacterial activities of bark extracts from the coastal plants had been reported previously. Arivuselvan *et al.* evaluated the antibacterial activity of bark extract of coastal plants.[27] Bark extract of *Ceriops tagal* inhibited *V. parahaemolyticus* and *S. aureus* with the MIC of 125 and 75 µg/mL, and *Pemphis acidula* inhibited *V. parahaemolyticus* and *S. aureus* with the MIC of 150 and 150 µg/mL, respectively. In comparison to our study, bark extract of OKA 6 has a lower MIC than *Criops tagal* and *Pemphis acidula.* Another studies also showed that barks from coastal plant have an antimicrobial activity, such as *Sonneratia apetala* and *S. caseolaris.*[28]

Fractionation of the highest active extract, OKA 6

As the ethanol extract of OKA 6 exhibited highest antibacterial activity, this extract was proceeded to further fractionation by partition and open column chromatography. The fractionation was carried out by partition and open column chromatography. Partition step resulted the chloroform fraction with high antibacterial activity against all tested bacteria. As shown in Table 2, the antibacterial activity of this fraction was comparable to a positive control, oxytetracycline. The diameter of inhibition zone produced by OKA 6 chloroform fraction at 1,000 µg/disk was up to 38 mm against *Streptococcus* sp. BJ0509, meanwhile the positive control only exhibited 26 mm of inhibition zone against the same bacterial strain at 100 µg/disk.

The further purification of OKA 6 chloroform fraction was conducted by column chromatography eluted with three solvent combinations of n-hexane:chloroform:ethanol (7:2:1 and 4:5:1). The results of antibacterial activity assay of the OKA 6 fractions indicated that the active compound responsible for the antibacterial activities were eluted in the fraction number 4 to 11 (Figure 3).

MIC and MBC

The active fraction obtained from column chromatography showed antibacterial activity with MIC of 3.125 µg/mL against *Streptococcus* sp. BJ0509 and 1.563 µg/mL against *V. parahaemolyticus* 29S, whereas the MICs of two antibiotics, oxytetracycline and kanamycin, were 6.25 µg/mL and 6.25 µg/mL against *Streptococcus* sp. BJ0509; and 6.25 µg/mL and 6.25 µg/mL against *V. parahaemolyticus* 29S, respectively (Table 3). The MBCs of the fraction were 12.5 µg/mL against *Streptococcus* sp. BJ0509 and 6.25 µg/mL against *V. parahaemolyticus* 29S, whereas the MBCs of two antibiotics, oxytetracycline and kanamycin, were 25 µg/mL and > 50 µg/mL against *Streptococcus* sp. BJ0509; and 25 µg/mL and > 50 µg/mL *V. parahaemolyticus* 29S, respectively (Table 3). The column chromatography fraction of OKA 6 against six other bacterial fish pathogens of *Vibrio* spp, *Aeromonas* spp and *P. damselae* SB25 were in the range of < 0.098 to 0.391 µg/ml. The MICs and MBCs exhibited that OKA 6 fraction from column chromatography were lower than two commercial antibiotics, oxytetraciline and kanamycin indicating that the fraction was more potent than those of two commercial antibiotics.

A number of research reported the MIC of bark extract from several plants. The MIC of chloroform extract of *Sonneratia caseolaris* bark

Table 2: Antibacterial activity of OKA 6 chloroform fraction.

No.	Bacteria	Diameter of inhibition zone (mm)		
		1000 µg/disk	500 µg/disk	Positive Control*
1	*A. hydrophila* BA03	24	19	10
2	*Streptococcus* sp. BJ0509	38	26	26
3	*S. aureus* ATCC 6538	32	30	21
4	*V. parahaemolyticus* 29S	27	22	12

*Control = oxytetracycline (100 µg/disk)

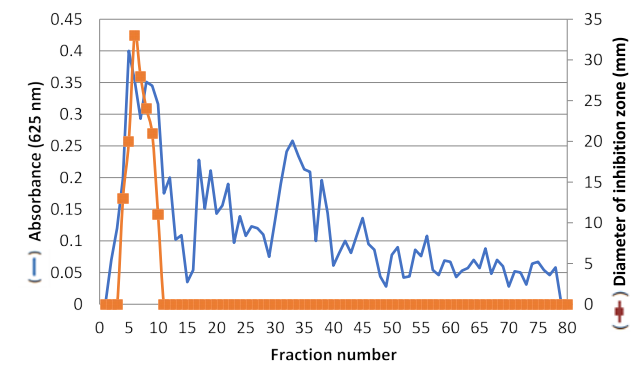

Figure 3: The Absorbance and antibacterial activity of column chromatography fractions of OKA 6 against *S. aureus*. ATCC 6538.

Table 3: MICs and MBCs of active compound.

Bacterial	MIC (µg/mL)			MBC (µg/mL)		
	OKA 6-F7	OTC	Kan	OKA 6-F7	OTC	Kan
Streptococcus sp. BJ0509	3.125	6.25	6.25	12.5	25	>50
S. aureus ATCC 6538	3.125	1.563	12.5			
V. parahaemolyticus 29S	1.563	6.25	6.25	6.25	25	>50
V. alginolyticus GD22	<0.391	0.781	6.25			
V. harveyi GD38	<0.098	3.125	0.391			
P. damselae SB25	<0.391	3.125	6.25			
A. hydrophila BA03	<0.391	3.125	3.125			
A. dhakensis SB01	<0.098	>25	1.563			

OKA 6-F7 = column chromatography active fraction of OKA 6
OTC = oxytetracyline
Kan = kanamycin

was 7.81 mg/mL against *Bacillus coagulans*[7] and the ethanolic extract of *Pempis acidula* bark was 190 µg/mL against *Vibrio parahaemolyticus*.[27] This MIC was much higher than MIC of OKA 6 fraction. Stem bark of *Croton floribundus, Cariniana legalis* and *Myrcia velutina* inhibited growth of *Flavobacterium columnare* dan *Aeromonas hydrophila* with MIC of 93.75 µg/mL to 375 µg/mL.[29] In contrast, MICs of *Ficus ovata* bark ethanol extract against *S. aureus* and *E. coli* has been reported to be > 624 µg/mL.[30] Our work indicated that the bark extract of a plant OKA 6 was a potential resource for antibacterial agent against bacterial infections in aquaculture. The further attempt are required to scale up the purification of the active compound, to elucidate the chemical structure, to determine the cytotoxicity and therapeutics effectiveness of the compound against fish bacterial pathogens.

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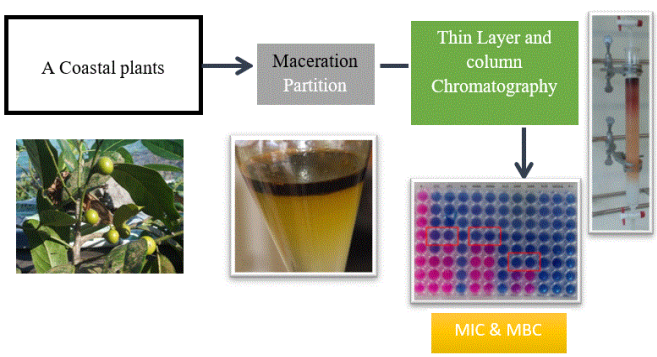
GRAPHICAL ABSTRACT



SUMMARY

- Three from a total of 32 coastal plants and marine sponges samples exhibited high antibacterial activity.
- The highest antibacterial activity was shown by the ethanol extract of the bark obtained from a coastal plant, *Diospyros maritima*.
- Antibacterial activity of purified substance from *D. maritima* was higher than commercial antibiotics against fish bacterial pathogens.

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Scopus Link: https://www.scopus.com/authid/detail.uri?authorId=6507970139
Google Scholar Link: https://scholar.google.co.id/citations?user=3fZJEFcAAAAJ&hl=en

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