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 295S 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. algirinolyticus, 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 295S 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

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 glycosides4-11 many of which exhibit various bioactivities including antitumor,1-3 antifungal,4-11 antiviral,7-9 and antibacterial activities.2,4-6,10,11 The application of antibacterial compounds from coastal plants and sponges are not only limited in medication of disease caused by human pathogenic bacteria2,11 but also have been applied to overcome the problem of fish bacterial infections.2,11

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.12-18 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.3,6,11,13

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 barns, leaves, fruits, and twigs were immediately transported to the laboratory. A total of 32 samples were washed with water to remove...
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, 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. The bacterial inoculums were prepared in TSB (Oxoid, Japan), except *V. parahaemolyticus* 29S in Zobell broth. Before inoculation, the cell density was estimated based on McFarland standard using spectrophotometer (Apel, Japan) at λ 625 nm. Inoculum of 10⁶ cells/mL were transferred into TSA (Oxoid, UK) with 0.7% agar for *Streptococcus* sp. BJ0509, *S. aureus* ATCC 6538, and *A. hydrophila* BA03; and Zobell medium 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 (8 mm, Advantec, Tokyo) were drilled 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 F₂₅₄ (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. While the measurement of MBC was 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 (o) = 8 mm
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 Ceriops 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 V. parahaemolyticus 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).

**Table 2: Antibacterial activity of OKA 6 chloroform fraction.**

| No. | Bacteria                     | 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)
was 7.81 mg/mL against *Bacillus coagulans* and the ethanolic extract of *Pennisetum setaceum* bark was 190 µg/mL against *Vibrio parahaemolyticus*. This MIC was much higher than MIC of OKA 6 fraction. Stem bark of *fraxinus griffithii* 2018:9:1-37.  

| Bacterial          | MIC (µg/mL) OKA 6-F7 | OTC | Kan | MIC (µg/mL) 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| 12.5                 | 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 = oxytetracycline Kan = kanamycin

ACKNOWLEDGEMENT

This research was supported financially by the Global Alliance for TB Drug Development (TB ALLIANCE), 40 Wall Street, 24th floor, New York, NY, USA. Special thanks to Drs. Wiyono, M.Si (a faculty member of Faculty of Forestry Universitas Gadjah Mada) for his help to identify the OKA 6 plant.

REFERENCES

1. Edu EAB, Edwin-Wosu NL, Udensi OU. Evaluation of bioactive compounds in mangroves: A panacea towards exploiting and optimizing mangrove resources. Journal of Natural Sciences Research. 2016:5(23):1-8.
2. Manial A, Tsalla T, Zerdo Z, Ameya G, Merdekios B, John SE. Evaluating the structure, to determine the cytotoxicity and therapeutics effectiveness of the compound against fish bacterial pathogens. 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.

11. Ulmurisida A, Ambariyanto A, Trianto A. Antibacterial activity of mangrove avocado marina leaves extract against Virgibacillus marismortui and Micrococcus luteus bacteria. AAIC Bioflux. 2017;10(2):372-80.
12. Narendran R, Kandasamy K. Biocatalysis and agricultural biotechnology antimicrobial activity of crude extracts from mangrove-derived trichoderma species against human and fish pathogens. Biocatalysis and Agricultural Biotechnology. 2016:6:189-94.
13. Saad S, Taher M, Susandi D, Garaleh H, Nurul A, Rahim A. Antimicrobial activity of mangrove plant (*Lumnitzera littorea*). Asian Pacific Journal of Tropical Medicine. 2011;4(7):523-5.
14. Aml MNA, Zam-Maad S. Streptococcus in Tilapia (Oreochromis niloticus): A review. Pertanika Journal of Tropical Agricultural Science. 2011;34(2):195-206.
15. Dong HT, Techanakitarman C, Sindadtikphu T, Thenprompoon W, Taeprasop S, et al. Aeromonas Jrandaei and Aeromonas Veronii caused disease and mortality in nile tilapia, Oreochromis niloticus. IL Journal of Fish Diseases. 2017:1-9.
16. Frans I, Michels CW, Bossier P, Willems KA, Lievens B, Rediers H. Vibrio Anguillarum as a fish pathogen: Virulence factors, diagnosis and prevention. Journal of Fish Diseases. 2011;34(9):634-61.
17. Pidgeon JW, Phillip HK. Major Bacterial diseases in aquaculture and their vaccine development. Cab Reviews. 2012;14(8):1-16.
18. Zhao Xi, Han T, Ren ST, Ma YM, Li H, Peng XX. L-proline increases survival of nile tilapia infected by Streptococcus agalactiae in higher water temperature. Fish and Shellfish Immunology. 2015;44(1):33-42.
19. Deforidis T, Gorgoleos P, Bossier P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Currnet Opinion in Microbiology. 2011;14(1):251-8.
20. Santos L, Fernando R. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. International Journal of Antimicrobial Agents 2018;52(2):136-43.
21. Food and Agriculture Organization of the United Nations (FAO). Antimicrobial Resistance (AMR) in Aquaculture. 2017.
22. Grenn P, Valeria A, Barra A. Ecological effects of antibiotics on natural ecosystems: A review. Microchemical Journal. 2018;136:25-39.
23. Turker H, Birinci A. Screening for antibacterial activity of some turkish plants against fish pathogens: A possible alternative in the treatment of bacterial infections. Biotechnology and Biotechnological Equipment. 2015;29(2):281-8.
24. Isansetoyo A, Kamei Y. MC21-1A, A bactericidal antibiotic produced by a new marine against Methicillin-resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2003;47(1):480-8.
25. Isansetoyo A, Kamei Y. Direct antagonistic method for screening anti- Methicillin-Resistant Staphylococcus aureus (MRSA) substance-producing marine bacteria. Biota. 2008;10:141-5.
26. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard-8th ed. CLSI document M07-A8. Wayne, PA:2009.
27. Arvuskau L, Silambarasan D, Govindan T, Kathiresan K. Antibacterial activity of mangrove leaf and bark extracts against human pathogens. Adv Bio Res. 2011;5(8):251-4.
28. Banerjee D, Chakraborti S, Hazra AK, Banerjee S, Ray J, Mukerjee B. Antibacterial Activity and total phenolic of some mangrove in sandbar. Afr J Biotechnol. 2008;7(6):805-10.
29. Castro SBR, Leal CAG, Freire FR, Carvalho DA, Oliveira DF, Figueiredo HCP. Antibacterial activity of plant extracts from brazil against fish. Brazilian Journal of Microbiology. 2008;39:756-60.
30. Kuete V, Nana F, Ngameni B, Tsafack A, Keumedjio F, Tchaleu B. Antimicrobial activity of the crude extract, fractions and compounds from stem bark of Ficus ovata (Moraceae). Ethnopharmacol J. 2009;124:556-61.
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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Cite this article: Hamdillah A,Isnansetyo A, Istiqomah I, Puspita ID, Handayani DP, Kaneko T. Antibacterial Activity of Coastal Plants and Marine Sponges from Kei Island Indonesia against Bacterial Fish Pathogen. Pharmacog J. 2019;11(4):812-7.