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Evaluation of Antibacterial Activities of Secondary Metabolites Produced by Aaptos suberiptoides associated bacteria

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Abstract: *A study had been conducted for antibacterial and cytotoxic activities against secondary metabolite of Artemisia saline resulted from the association between sponge Aaptos suberiptoides and bacteria. The experimental study was conducted by isolating sponge Aaptos suberitoides-associated bacteria, which were then extracted using n-hexane, ethylacetate and methanol as solvents. The bacteria were isolated with pour plate method and the resulting secondary metabolites were brought under antibacterial assay for their activities against the bacteria Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillusniger and cytotoxic activities against A. saline. Conclusion. All extracts showed weak antibacterial activities with inhibiting diameter ranging from 6.32 ±0.27 – 9.95 ± 0.09. An assay for A saline showed that ethyl-acetate extracts were more toxic than methanolic extracts and n-hexane, with an LC_{50} value of 21,93µg/mL, 65.02 µg/mL, and 178.22µg/mL, respectively.*

Key words: *antibacterial, Cytotoxic, secondary metabolite, Aaptos suberiptoides,*

I. Introduction

Sponge is a biota component that makes up coral reef. It has bioactive potential that has not been fully explored. The marine biota has active compounds with higher percentages compared to land-based plants. The compounds are largely derived from Demospongiea class, particularly from the clusters of Dictyoceratida and Dendroceratida, Haplosclerida, Halichondrida, Poeciloscleride, and from Calcarea(1) class. Marine sponges serve as hosts for various kinds of microbe, such as bacteria. It is because sponges could protect the microbes against predators by producing chemical compounds. The chemical compounds produced by the sponges make the microbes living in the sponges produce specific secondary metabolites. The specific secondary metabolites are expressed in the form of microbial response against environmental conditions.(2)

Structures of sponge Aaptos suberitoides consists of osculi-pherous globular lobes with brownish orange color and coarse surface. The sponge may turn black when it is immersed into alcohol solution.(3) Sponge Aaptos suberitoides is known to produce an alkaloid bioactive compounds called aaptamin. Aaptamin contains 1H-benzo(de)-1,6-napthyridine and has cytotoxic properties. 1Hbenzo (de)-1,6-napthyridine is an important compound for it can inhibit CDK-Cyclin Complex activities. CDK-Cyclin Complex is a protein complex that plays essential role in abnormal cell proliferation that leads to cancer.(4) There are several potential drawbacks for future studies on sponge-symbiotic microorganisms and related natural products. First, identification and isolation of symbiotic microorganisms producing bioactive natural products area crucial step for the future culture and production of these metabolites.(5) The study aims at determining antibacterial and cyctotoxic activities of secondary metabolites against A Saline resulted by sponge Aaptos suberiptoides associated bacteria.

II. Materials and method

Sampling and isolation of sponge-associated bacteria

Colonies of sponge were collected from Pecaron Bay, Situbondo, East Java, Indonesia (Figure 1) by scuba diving. Upon collection, sponge colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA). The tissues were rinsed with sterile seawater and then homogenized with blender. The homogenized tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates.(6)

Microorganisms

The microbial strains are identified strains and were obtained from Department of Microbiology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia. The bacterial strains studied are Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillusniger

Antibacterial Assay

The antibacterial activity of crude extract was evaluated by agar disk diffusion method for aqueous extract and agar well diffusion for solvent extract using Mueller Hinton Agar No. 2 medium for the assay. The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (25 ml) and incubated at room temperature on a rotary shaker. Then 0.2 ml of inoculum (inoculum size was 108cells/ml as per McFarland standard) was inoculated into the molten Mueller Hinton agar media and after proper homogenization it was poured into 100 mm petri dishes (Hi-Media). For the agar disk diffusion, the test compound was introduced onto the disk (7 mm) (Hi- Media) and then allowed to dry. Thereafter, the disk was impregnated on the seeded agar plate. For the agar well diffusion, a well was made in the seeded plates with the help of a cup-borer (8.5 mm). The test compound was introduced into the well and all the plates were incubated at 37 ¡C for 24 h. The experiment was performed 3 times under strict aseptic conditions. Microbial growth was determined by measuring the diameter of the zone of inhibition.[7]

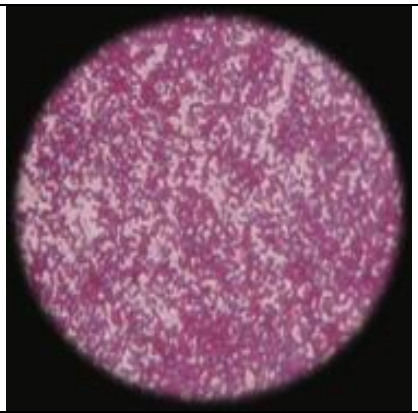
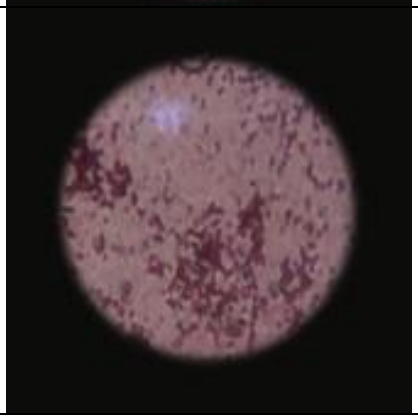
Brine Shrimp Lethality Assay

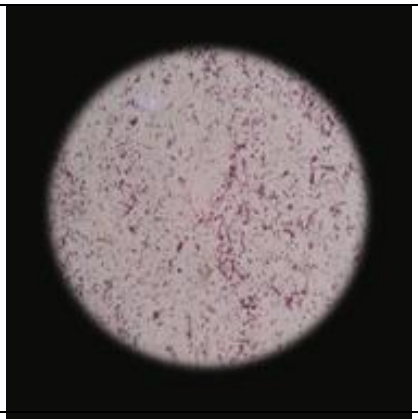
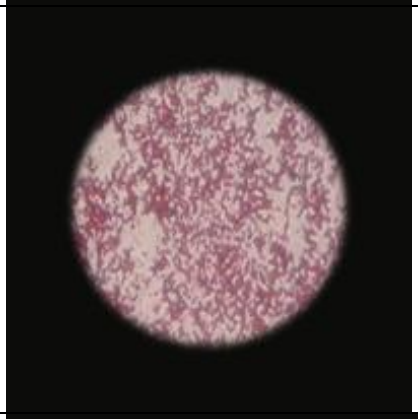
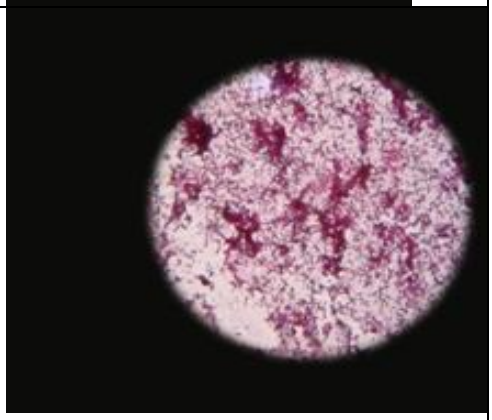
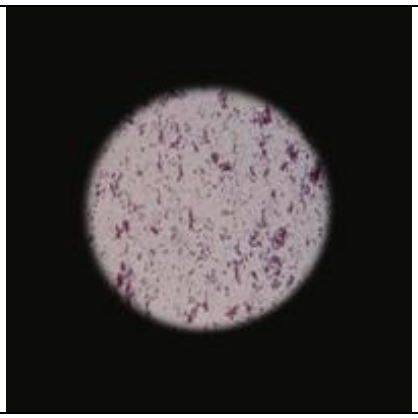
Brine shrump eggs (Artemiasalina) were incubated at 27 ± 1 C in a conical shaped vessel (1L), containing saline solution under constant aeration and an incandescent lamp for 24 h.After hatching, active brine shrimp larvae (nauplii) free from egg shell were collected with a pipette. Ten nauplii were introduced into vial containing 5 ml saline solution and graded concentrations (ranging from 1 to 1000 µg/ml) of the tested extracts. The experiments were conducted along with control contain 5 ml saline solution, 0.1 ml DMSO and 10 nauplii. After 24 h, the number of affected or dead shrimps was counted at each concentration of the extracts. The data were analyzed.[8]

III. Results

Microscopic observation of Aaptos suberitoides-associated bacteria isolated from sponge Aaptos suberitoides produced 6 (six) isolates. All Aaptos suberitoides-associated bacteria resulted from microscopic observation was subjected to gram staining, as presented in Table 1.

Table 1. Results of sponge Aaptos suberiptoides-associated isolation.

No. of Sample	Morphological description of the bacteria	Picture
1a.	Color : red Shape : Bacillus Type : Clustered Gram : negative	
1b.	Color : red Shape : bacillus Type : grape stem Gram : negative	

1c.	Color : red Shape : bacillus Type : Diffuse Gram : positive	
1d.	Color : red Shape : bacillus Type : clustered Gram : negative	
1e.	Color : red Shape : bacillus Type : clustered Gram : negative	
1f.	Color : red Shape : diffuse Type : clustered Gram : positive	

Isolation of Aaptos suberiptoides derived bacterial produced 6 bacterial isolates that are classified into gram positive and gram negative isolates. Partition was conducted upon supernatant resulting from Aaptos suberitoides fermentation by using solvents n-hexane-ethylacetate (1:1). Resulting n-hexane phase was isolated using isolating funnel and then aired to produce dried n-hexane extracts. The residue was then partitioned using

acetyl-acetate (1:1) using isolating funnel, subsequently evaporated to produce dry ethyl-acetate extracts. Each extract was assayed for antimicrobial and cytotoxic activities against A. Saline, as presented in Table 2 and Table 3.

Table 2. Results of antimicrobial assay for secondary metabolites of sponge A. Suberiptoides-associated bacteria

Tested Microbes	Diameter of inhibition area (mm)			
	Before extraction	After extraction		
		n-hexane	ethylacetate	methanol
S. aureus	7.42±0.24	8.39±0.08	9.83±0.49	9.75±0.06
E. coli	7.52±0.08	9.95±0.09	9.55±0.04	9.18±0.04
C. albicans	6.44±0.08	7.27±0.08	8.44±0.07	7.94±0.04
A. niger	NA	6.42±0.14	6.31±0.27	6.70±0.07
Tetracycline	28.30±0.15	26.35±0.21	26.96±0.13	27.14±0.06
Nystatine	26.15±0.25	25.66±0.19	25.60±0.60	25.68±0.08

Table 3. Results of toxicity assay for shrimp larvae of A. Saline derived from sponge A. Suberiptoides-associated bacteria, as seen with LC_{50} value (µg/mL)

Before extraction	After extraction		
	n-hexane	ethylacetate	methanol
437.04±13.41	178.22±12.41	21.93±8.31	65.02±9.21

Table 3 presents results of toxicity assay for shrimp larvae of A. Saline derived from sponge A. Suberiptoides-associated bacteria. The table shows that LC_{50} values after extraction were more toxic than it was before extraction. This may be because after extraction, secondary metabolite compounds were more concentrated based on the solvent's polarity used. Based on the LC_{50} values, ethylacetate extract was found to be stronger (21.93±8.31µg/mL) than methanolic extract (65.02±9.21µg/mL) and n-hexane extract (178.22±13.41). A solution or secondary metabolite is said to be highly toxic with $LC_{50} \leq 30$µg/ml, toxic with LC_{50} 30µg/ml – 1000 µg/ml, and not toxic with LC_{50}> 1000 µg/ml. [8] Based on the criteria, it can be concluded that both n-hexane and methanolic extracts are toxic while ethylacetate extract is highly toxic.

IV. Discussion

After-extraction antimicrobial activities using solvents with different polarities showed that diameters of inhibition area were greater than before-extraction antimicrobial activities. It may be because extraction is a pre-screening phase for secondary metabolite compounds based on solvent's polarity. Based on the study, inhibition areas for tetracyclic and nystatin were significantly greater than test samples both before extraction and after extraction. It was because tetracyclic and nystatin are single antibiotics, while the samples used were composite secondary metabolite compounds.

Another study had been conducted on sponge Aaptossp collected from North Java and filtered for antibacterial activities against MDR strain. Three of 64 tested bacterial isolates showed that two isolates (SPA1 and SPA5) were active against resistant Escherichia coli strain and one isolate (SPA21) was active against resistant Proteus sp. The active isolates were Halomonas aquamarina, Alpha proteobacterium, and Pseudoalteromonas luteviolacea. [9] The results of the study were different from those of other studies, probably because of different sampling areas.

A study conducted by Shaari et al.(2009) showed that sponge Aaptos sp fom Malaysia contains 3-(phenethylamino)demethyl(oxy)aaptamine and 3-(isopentylamino)demethyl(oxy) Aaptamine compounds that are active against some cancerous cells. The study had provided opportunities to obtain aaptamine compounds and their sponge-Aaptos sp-associated bacterial metabolites. Further study will isolate secondary metabolites from ethylacetate and methanolic extracts with an LC_{50} value less than that of n-hexane extract.[10]

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