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Original Article

ANTIBACTERIAL AND ANTICANCER ACTIVITIES OF MARINE BACTERIAL EXTRACTS AND DETECTION OF GENES FOR BIOACTIVE COMPOUNDS SYNTHESIS

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ABSTRACT

Objective: This study was aimed to determine the antimicrobial, toxicity, and anticancer activities of ethyl acetate extracts from sponge-associated bacteria and to detect the presence of PKS and NRPS genes.

Methods: Crude extracts of sponge-associated bacteria HAA-01, HAL-13, and HAL-74 were used as samples for toxicity test using Brine Shrimp Lethality Test (BSLT). The Minimum Inhibitory Concentration (MIC) of bacterial extracts were tested using agar diffusion method. Cytotoxicity test was carried out by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)method against HeLa cell line. To determine the presence of keto synthase (KS) domain of polyketide synthase (PKS) and adenylation (A) domain of nonribosomal peptide synthetase (NRPS) genes were carried out using Polymerase Chain Reaction (PCR). DNA of PCR products were purified and sequenced. The DNA sequences were analyzed by the Blast program, and phylogenetic tree analysis was constructed using MEGA5 program with neighbor-joining method.

Results: The research results indicated that the best value of MIC of bacterial extracts against five bacteria such as *Escherichia coli*, Enteropathogenic *E. coli* (EPEC), *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* were 0.1 mg/ml. The half maximal lethal concentration (LC_{50}) value of HAL-74, HAA-01 and HAL-13 bacterial metabolite extracts against the brine shrimp *Artemia salina* were 378 µg/ml, 463.028 µg/ml and 444.796 µg/ml, respectively. The bacterial metabolite extract has cytotoxicity effect on HeLa cell line with the best half maximal inhibitory concentration (IC_{50}) value was 132.877 µg/ml as performed by extract of HAL-74. Genetic analysis showed that HAL-74 and HAA-01 genomes contained genes that encoded the KS domain of PKS gene and A domain of NRPS gene, while HAL-13 genome encoded the KS domain of PKS gene, which played a role in the synthesis of bioactive compounds.

Conclusion: Bacterial metabolite extracts of HAA-01, HAL-13, and HAL-74 had antimicrobial, toxicity against *A. salina*, and anticancer against HeLa cell line activities. The best toxicity and anticancer activity were performed by HAL-74 crude extract with LC_{50} and IC_{50} were 378 µg/ml and 132.877 µg/ml, respectively. HAA-01 and HAL-74 genomes contained PKS and NRPS genes while HAL-13 genome contained PKS gene.

Keywords: Antibacterial, Metabolite, Sponge-associated bacteria, Anticancer, PKS, NRPS.

INTRODUCTION

Infectious diseases and cancer are important to a health problem. Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. Each year infectious diseases kill 3.5 million people [1]. Cancer is a generic term for a large group of diseases that can affect any part of the body. Cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012 [2]. Therefore the search for novel therapeutic agents continue, and the need still remains to uncover the initial structural lead that interact with therapeutic targets. Natural products give a good chance for the discovering an effective medication of the remaining untreatable diseases.

Marine sponges are known to produce a wide array of bioactive secondary metabolites with a wide variety of biological activities, such as antibacterial, antifungal and anticancer. To date, more than 12.000 substances have been isolated from marine habitats many of which are bioactive [3] Since 33% of the marine secondary metabolites originate from sponges [4]. The limitation of sponge biomass is the main factor for isolating the large scale of bioactive compounds. In the case of bryostatin, 13.000 kg of the *Bryozoans neritina* had to be harvested in order to obtain only 18 g of bryostatin for clinical trials [3]. Therefore, marine microorganisms which associated with marine sponges became one of the alternative ways to solve that problem. Microbial associates can comprise as much as 40% of sponge tissue volume with densities in excess of 10^9 microbial cells per mm^3 of sponge tissue [5]. Marine microorganisms have contributed to the majority of bioactive compounds. They can produce the same metabolite compounds as their host [6].

The exploration of bioactive compounds has been carried out in Indonesia. Three bacterial isolates coded as HAL-74, HAA-01 and HAL-13 have been isolated from marine sponge *Haliclona* sp. at Waigeo Island, Raja Ampat District, West Papua Province [7]. The crude extract of HAL-74, HAA-01 and HAL-13 isolates have broad-spectrum antimicrobial activity [8]. This study was aimed to determine the antimicrobial, toxicity, and anticancer activities of ethyl acetate extracts from sponge-associated bacteria and to detect the presence of PKS and NRPS genes. Determination of MIC and cytotoxic assay of the bacterial extract were important to find out the antibacterial and anticancer potency of the extract. Detection of KS domain of PKS and A domain of NRPS genes were important for identifying these bacteria as well as ensuring their capability in synthesizing the bioactive compounds.

MATERIALS AND METHODS

Extraction of metabolite crude extract from bacteria

Each of those three bacterial isolates (HAA-01, HAL-13, or HAL-74) was cultured in 1 L Sea Water Complete (SWC) media (bacto peptone 5 g, yeast extract 1 g, glycerol 3 ml, seawater 750 ml and distilled water 250 ml) and incubated in shaker incubator at 100 rpm for 3 d at 30 °C. Then the bacterial culture was added with 1 L of ethyl acetate and stirred for 2 h. After that the ethyl acetate was evaporated, and the bacterial extracts were stored below 5 °C until used [9].

Determination of MIC

The MIC of bacterial extracts was tested using agar diffusion method. Bacterial extracts dissolved in ethyl acetate, then were applied

carefully into 6 mm paper disks (Whatman) with various concentration (1 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.3 mg/ml, 0.2 mg/ml and 0.1 mg/ml). After that the disks were dried up using an oven at 40°C overnight, then were sterilized under UV light for 2 h. and put into agar plate that has been seeded with 1% (v/v) of microbial test strains (concentration $1x10^6$ CFU/ml, OD_{620} 0.45). The plate was incubated at 40°C for 3 h to optimize the diffusion of bacterial extract into the media. This assay was carried out in triplicate. The diameters of inhibition zones were measured in millimeter after incubation for 24 h at 37 °C. Control disks soaked with ethyl acetate solvent were used as negative control. Each sample was used in triplicate for the determination of MIC. The lowest concentration required to arrest the growth of bacteria was regarded as MIC [10].

Evaluation of the cytotoxicity activity

The cytotoxic effect of the extracts was evaluated according to BSLT method. Twenty *A. salina* larvae were placed in each vial containing 4 ml sea water with different concentrations of bacterial extract (1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml dan 0 µg/ml). The vials were maintained at room temperature for 24 h under the light, and surviving larvae were counted. Each sample was repeated three times. The percent deaths at each concentration and control were determined using formula:

$$\% \text{ deaths} = \frac{\text{counts deaths test} - \text{counts deaths control}}{\text{control}} \text{ x } 100\%$$

LC_{50} were determined from the 24 h counts using the probit analysis method. This assay was carried out in triplicate. In the case where data transformed into a straight line by means of a logit transformation, LC_{50} was derived from the best fit line obtained by linear regression analysis [11].

Anticancer activity assay

A cytotoxicity property of bacterial extracts was carried out by MTT method against HeLa cell lines. Hela cells line were grown in Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin. 100 µl of cell suspension was seeded into 96-well plates at a plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of extracts. 100 µl of cell suspension was seeded into 96-well plates at plating density of 5 x 10^3 cells/well and incubated to allow for cell attachment at 37° C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of extracts. The bacterial extract was solubilized in dimethyl sulfoxide (DMSO) and diluted in serum-free medium. After 24 h, 100 µl of the medium containing the extracts at various concentrations (0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml) was added and incubated at 37 °C, 5 % CO_2, 95 % air and 100% relative humidity for 48 h. Triplicate was maintained, and the medium containing without extracts were served as control. After 48 h, 100 µl of MTT in phosphate buffered saline (PBS) was added to each well and incubated at 37° C for 4 h. The medium with MTT was flicked off, and the formed formazan crystals were solubilized in 100 µl of isopropanol and then measured the absorbance at 570 nm using enzyme-linked immunosorbent assay (ELISA) plate reader. Each sample was repeated three times. The % cell inhibition was determine using the following formula:

$$\% \text{ cell inhibition} = 1 - \frac{\text{OD of sample}}{\text{OD of control}} \text{ x } 100\%$$

This assay was carried out in triplicate. The IC_{50} value was determined from % Cell inhibition and concentration curve [12].

DNA extraction and amplification of KS and A domains

DNA extraction was carried out by Cetyltrimethyl Ammonium Bromide (CTAB) method [13]. KS domain of PKS and A domain of NRPS genes from those three isolates were amplified using PCR (LA Taq with GC Buffer, TAKARA-Japan) with primers for KS domain (f: 5-GCSATG GAYCCSCARCARCGSVT-3); (r: 5-GTSCCSGTSCCRT-GSSCYTCSAC-3) and for A domain (f: 5-AARDSIGGIGSIGSITAYBICC-3); (r: 5-CKRWAICCICKIAIYTTIAYYTG-3) [14]. The PCR condition of KS and A domain were carried out in three steps such as initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 min, and post PCR at 72 °C for 10 min. The PCR products were sequenced and then compared to the database of National Center for Biotechnology Information (NCBI) using the Blast X program. Construction of phylogenetic tree was carried out using Molecular Evolutionary Genetics Analysis (MEGA) 5 program with neighbor-joining method [15].

RESULTS

Determination of MIC

The reference strains, MIC varied from 0.1 mg/ml to 1 mg/ml. The HAL-74 and HAA-01 metabolite extracts have a broad spectrum of action on the reference isolated strains. It was observed a variation of MIC from 0.1 mg/ml to 0.2 mg/ml, whereas HAL-13 metabolite extract was 1.0 mg/ml, on *E. coli*, EPEC, *P. aeruginosa*, *S. aureus* and *B. subtilis*. These results indicate that metabolite extracts of HAL-74 and HAA-01 had better antibacterial activity than metabolite extract of HAL-13 (table 1).

Table 1: Minimum Inhibitory concentration of bacterial extracts against five bacterial test strains

Test organism	MIC (mg/ml)		
	HAL-74	HAA-01	HAL-13
Escherichia coli	0.1	0.1	1.0
EPEC	0.1	0.2	1.0
Pseudomonas aeruginosa	0.1	0.2	1.0
Staphylococcus aureus	0.2	0.1	1.0
Bacillus subtilis	0.1	0.1	1.0

n=3

Cytotoxic assay

Bioactivity study of the marine sponge bacterial extracts was conducted using BSLT employing 48 h *A. salina.* The degree of lethality was directly proportional to the concentration of the extracts treated. The cytotoxic activity is best performed by HAL-74 isolate with an average of LC_{50}, 378±6.07 µg/ml followed by HAL-13 isolate (LC_{50}, 444.796±23.33 µg/ml) and HAA-01 isolate (LC_{50}, 463.082±13.82 µg/ml) (fig. 1).

Based on the cytotoxic assay, HAL-74 extract is the most active extract among others (IC_{50}, 132.877±22.82 µg/ml) followed by HAL-13 isolate (IC_{50}, 307.776±57.09 µg/ml) and HAA-01 isolate (IC_{50}, 457.641±28.41 µg/ml) (fig. 2).

DNA fragments of the bacterial isolates encoding KS and A domain were successfully amplified using PCR. The HAL-74 and HAA-01 bacterial isolates expressed DNA fragment encoding KS and A domain, whereas HAL-13 only expressed DNA fragment encoding KS domain. The result of amplification of DNA fragment encoding KS and A domain consisted of 700 bp and 1000 bp, respectively (fig. 3).

Bioinformatics analysis

Bioinformatics sequences analysis of DNA fragment encoding KS domain from bacterial isolates using Blast X program showed that HAL-74, HAA-01 and HAL-13 isolates have a similarity with polyketide synthase type I PKS from *B. subtilis*, with similarity level of 99%, 96% and 98% respectively. Sequences analysis of DNA

fragment encoding A domain showed that HAL-74 isolate has a similarity level of 96% with NRPS from *Comamonas* sp. POUX and HAA-01 isolate have a similarity level of 96% with Peptide synthetase *B. subtilis* BSN5 (table 2, table 3).

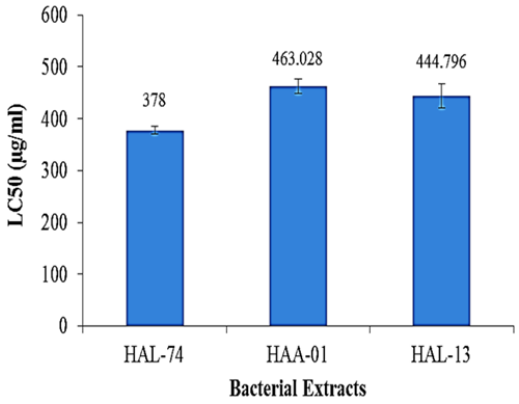

Fig. 1: Cytotoxic of bacterial extracts of HAL-74,HAA-01 and HAL-1on *Artemia salina*(n=3)

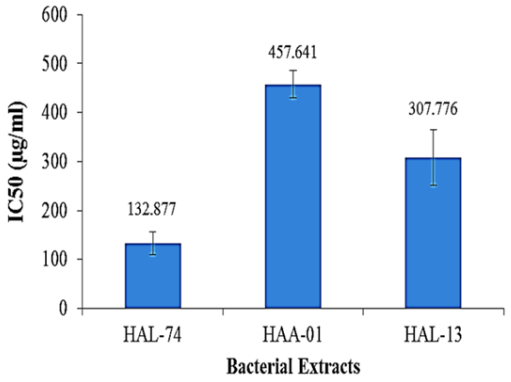

Fig. 2: Cytotoxic of IC_{50} values of HAL-74, HA-01, and HAL-13 extraction HeLa cells line *in vitro* (n=3)Amplification of KS and A domain

Phylogenetic analysis of amino acid sequences of KS domain showed that HAL-74, HAA-01 and HAL-13 isolates closely related to *B. subtilis* strains (fig 4). Further phylogenetic analysis of amino acid sequences of A domain showed that HAL-74 and HAA-01 isolates closely related to *Comamonas* sp. POUX and *B. Subtilis* BSN5, respectively (fig. 5).

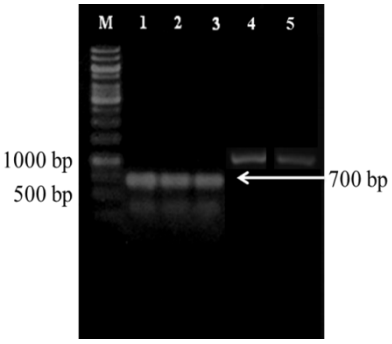

Fig. 3: DNA fragments encoding KS Domain 1 = HAL-13, 2 = HAL-74, 3 = HAA-01 and A Domain 4 = HAL-74, 5 = HAA-01 in the electrophoretic agarose gel. M = Marker 1 kb

DISCUSSION

Minimum inhibitory concentration was evaluated to study the efficacy of the antibiotics effectivity against the bacterial pathogens. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of microorganisms. MIC is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. A MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism.

Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents [16]. Antibacterial activity of HAL-74 and HAA-01 metabolite extracts was categorized moderate activities while extracts of the HAL-13 metabolite extract has relatively weak activity. The antimicrobial activity of extracts was good activity if the MIC value below 0.1 mg/ml, was moderate activity if the MIC values of 0.1-0.5 mg/ml, and was weak activity if the value of MIC 0.5-1 mg/ml and was not active if the MIC value more than 1 mg/ml [17].

The toxicities of metabolite extracts from sponge-associated bacteria against brine shrimps indicated the presence of potent cytotoxic and probably antitumor components of these extract. The crude extract was toxic (active) if it has an LC_{50} value less than 1000 µg/ml while non-toxic (inactive) if it was greater than 1000 µg/ml. Toxicity study using this method was reported to be comparable to cell culture so that it can be associated with anticancer activities [11]. Cytotoxicity assay is an appropriate method for screening new substances within a short time in order to determine cytotoxicity on cancer cells. Bacterial metabolite extracts caused cytotoxicity in a concentration-dependent manner. Bacterial extracts showed anti-proliferative activity on Hela cells. The results demonstrated treatment bacterial extract with high concentration showed that the growth rates of the cells were more decreased than of low concentration.

Table 2: Bioinformatics sequences analysis of DNA fragment encoding KS domain using BlastX program

Bacterial isolates	Similarity	Identity (%)	*E-value*	Accession number
HAL-74	Polyketide synthase *B. subtilis*BE19	99	1e-154	WP_003245841.1
HAA-01	Polyketide synthase *B. subtilis* BZ1	96	7e-128	WP_019712691.1
HAL-13	Polyketide synthase *B. subtilis* BG4	98	4e-153	WP_032721576.1

Table 3: Bioinformatics sequences analysis of DNA fragment encoding A domain using BlastX program

Bacterial Isolates	Similarity	Identity (%)	*e-value*	Accession number
HAL-74	NRPS *Comamonas* sp. POUX	96	0.0	AHG59387.1
HAA-01	Peptide synthetase *B. subtilis* BSN5	99	0.0	WP_029317185.1

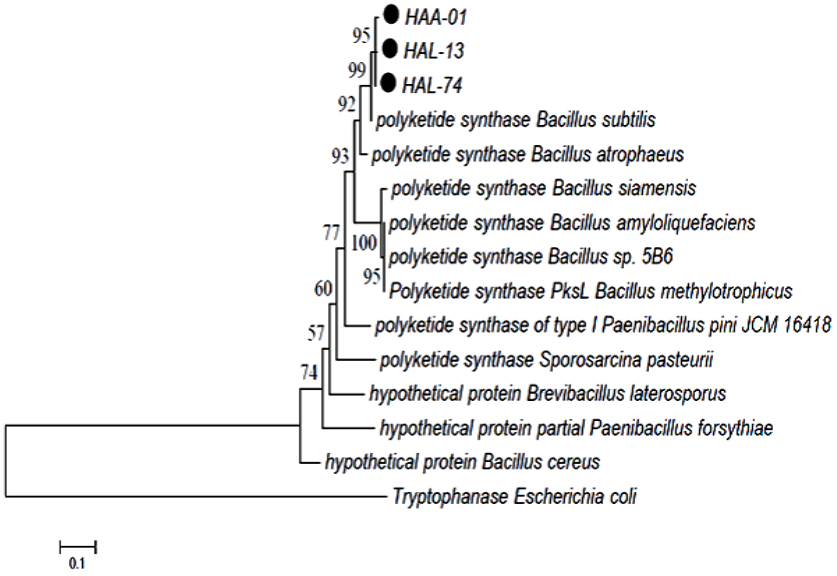

Fig. 4: Phylogenetic tree of HAL-74, HAA-01 and HAL-13 isolatesto the reference strains with based on amino acid sequences of KS domain. Numbers at the nodes indicated the levels of bootstrap support with based on 1000 re-sampled data sets. The scale bar indicated 0.1 substitutions per nucleotide position

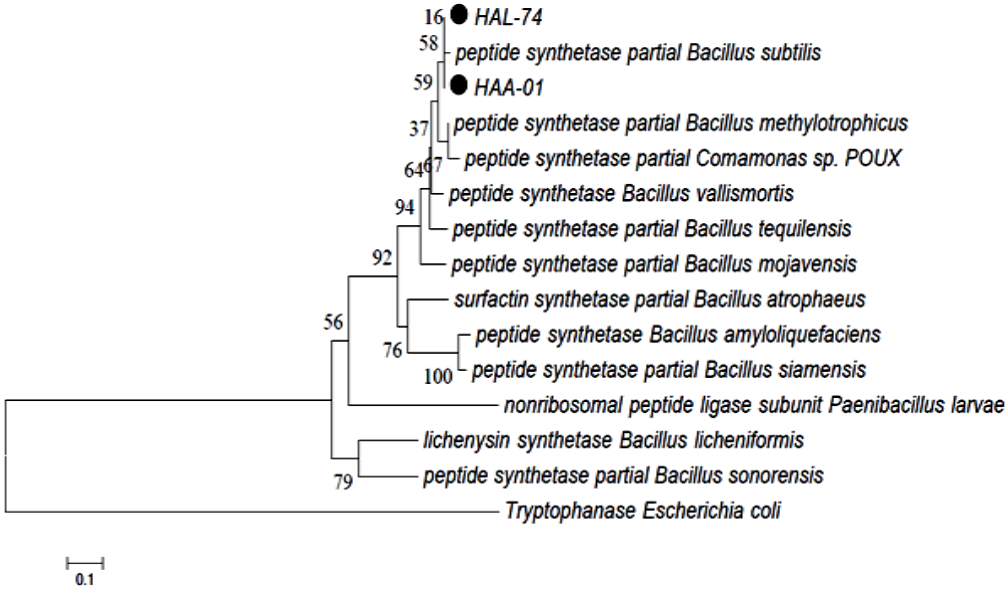

Fig. 5: Phylogenetic tree of HAL-74 and HAA-01 isolates to the reference strains with based on amino acid sequences of A domain. Numbers at the nodes indicate the levels of bootstrap support with based on 1000 re-sampled data sets. The scale bar indicates 0.1 substitutions per nucleotide position

Most of the bioactive compounds produced by marine bacteria did not getting loose from the involvement of two multifunctional enzymes PKS and NRPS. These two multifunctional enzymes mostly involved in the biosynthesis of bioactive compounds. The simplest functional PKS module consists of a KS, an acyltransferase (AT), an acyl carrier protein (ACP) and a thioesterase (TE) domain [14]. Besides that, the simplest NRPS module consists of an A, a thiolation (T), a peptidyl carrier protein (PCP) and a condensation (C) domain [18]. The common feature of complex PKS gene is keto synthase (KS) domain that usually present in each module and exhibits the highest degree of conservation among all domains [19]. Likewise, A domain become the most conserved domain of NRPS gene compared to the others [14]. Therefore, the KS and A domain are especially well suited for phylogenetic analyses of PKS and NRPS gene diversity.

The presence of both KS and A domain in the cluster of PKS and NRPS genes at HAL-74 and HAA-01 isolates indicated that these isolates had high potency in generating many kinds of bioactive compounds. Besides that, we also assumed that they can establish the hybrid of PKS-NRPS genes. The modular PKS and NRPS have been involved in natural product synthesis in many microorganisms [20]. Interestingly, the existence of these hybrid PKS-NRPS systems will enlarge the variation of each module in forming an immense variety of bioactive compounds. Many kinds of natural products are formed through the combination of PKS-NRPS hybrid systems such as bleomycin (BLM), a family of anticancer antibiotics produced by *Streptomyces verticillus* and generated from BLM mega synthetase that consist of 10 NRPS modules and 1 PKS module [21]. In the marine environment, members of the genus *Bacillus* are known for their production of metabolites with antimicrobial, antifungal or generally cytotoxic property. They were regularly isolated from invertebrates and thus display a high potential in the search for new antimicrobial substances [22]. Many antibiotics including cyclic peptides, cyclic lipopeptides, and novel thiopeptides have also been reported from this strain [23].

CONCLUSION

Marine bacterial extracts of HAA-01, HAL-13, and HAL-74 had antimicrobial, toxicity against *A. salina*, and anticancer against HeLa cell line activities. The best toxicity and anticancer activity were performed by HAL-74 crude extract with LC_{50} and IC_{50} were 378 μg/ml and 132.877 μg/ml, respectively. The genome of HAA-01 and HAL-74 contained PKS and NRPS genes while HAL-13 genome contained PKS gene.

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CONFLICT OF INTERESTS

Declared none

REFERENCES

1. World Health Organization; 2014. Infectious Diseases Health Topic. Available from: http://www.who.int/ topics/ infectious_ diseases/en/index.html. [Last accessed on 15 Aug 2014].
2. World Health Organization; 2014. Cancer Health Topic. Available from: http://www.who.int/cancer/en/index.html. [Last accessed on 15 Aug 2014].
3. Taylor MW, Radax R, Steger D, Wagner M. Spons associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 2007;71:295-347.
4. Imhoff JF, Stohr R. Sponge-associated bacteria: general overview and special aspects of bacteria associated with *Halichondria panicea*. Prog Mol Subcell Biol 2003;37:35-57.
5. Hoffmann F, Larsen O, Thiel V, Rapp HT, Pape T, Michelish W, *et al*. An anaerobic world in sponges. J Geomicrobiol 2005;22:1-10.
6. Proksch P, Edrada RA, Ebel R. Drugs from sea current status and microbiological implications. Appl Microbiol Biotechnol 2002;59:125-34.
7. Tokasaya P. Sponge-associated bacteria producing antimicrobial compounds and their genetic diversity analysis [thesis]. Bogor (ID): Institut Pertanian Bogor; 2010.
8. Banoet Y. Aktivitas senyawa bioaktif antimikrob dari bakteri yang berasosiasi dengan spons *Haliclona* sp. dan telaah genetiknya [thesis]. Bogor (ID): Institut Pertanian Bogor; 2011.
9. Muller WEG, Grebenjuk VA, Pennec G, Schroder H, Brummer F, Hentschel U, *et al*. Sustainable production of bioactive compounds by sponges-cell culture and gene cluster approach: a review. Mar Biotechnol 2004;6:105-17.
10. Sudirman LI. Partial purification of antimicrobal compounds isolated from mycelia of tropical *Lentinus cladopus* LC4. Hayati J Biosci 2010;17:63-7.
11. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active constituent. J Med Plants Res 1982;45:31-4.
12. Thakur AN, Thakur NL, Indap MM, Pandit RA, Datar VV, Muller WEG. The antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria. Mar Biotechnol 2005;7:245-52.
13. Sambrook W, Russel DW. Molecular cloning: a laboratory manual. 3rd Ed. New York: Gold Spring Harbor Laboratory; 2001. p. 362.
14. Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF, Hutchinson CR. Metagenomic analysis reveals diverse polyketide synthase gene cluster in microorganisms associated with the marine sponge *Discodermia dissoluta*. Appl Environ Microbiol 2005;71:4840-9.
15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5 molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
16. Wahi AK, Singh A, Singh AK. Determination of minimum inhibitory concentration (MIC) of some novel triazole. Int J Res Pharm Chem 2011;4:1108-14.
17. Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV, Filho BPD. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz 2002;97:35-47.
18. Schwarzer D, Finking R, Marahiel MA. Nonribosomal peptides: from genes to products. Nat Prod Rep 2003;20:275-87.
19. Kim TK, Fuerst JA. The diversity of polyketide synthase genes from bacteria associated with the marine sponge *Pseudoceratina clavata*: culture-dependent and culture-independent approaches. Environ Microbiol 2006;8:1460-70.
20. Zhao J, Yang N, Zeng R. Phylogenetic analysis of type I polyketide synthase and nonribosomal peptide synthetase genes in antarctic sediment. Extremophiles 2008;12:97-105.
21. Shen B, Du L, Sanchez C, Edwards DJ, Chen M, Murrell JM. The biosynthetic gene cluster for the anticancer drug bleomycin from *Streptomyces verticillus* ATCC15003 as a model for hybrid peptide-polyketide natural product biosynthesis. J Ind Microbiol Biotechnol 2001;27:378-85.
22. Muscholl-Silberhorn A, Thiel V, Imhoff JF. Abundance and bioactivity of cultured sponge-associated bacteria from the mediterranean sea. Microb Ecol 2008;55:94-106.
23. Nagai K, Kamigiri K, Arao N, Suzumura K, Kawano Y, Yamaoka M, *et al*. YM-266183 and YM-266184, novel thiopeptide antibiotics produced by *Bacillus cereus* isolated from a marine sponge. J Antibiot 2003;56:123-8.