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In vitro cytotoxic activity assay of bacteria extract derived marine sponge *Haliclona fascigera* toward Hela, WiDr, T47D, and Vero cell line

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ABSTRACT

Symbiotic association between marine sponge and microorganism was a promising chance in the discovery of lead compound of anticancer. This association was probably concluded that symbiotic microorganism would contain the same secondary metabolites with the host. In this continuation research, we had isolated symbiotic bacteria from a marine sponge and tested for cytotoxic activity. Twenty-six isolates of bacteria derived from marine sponge *Haliclona fascigera* were isolated from Setan Island, West Sumatra, Indonesia. Screening of cytotoxic activity by BSLT method and MTT assay was conducted toward 21 ethyl acetate extracts of symbiotic bacteria with weight >50 mg. One bacterial extract with code H2N was very toxic according to BSLT test, while 18 isolates were toxic with LC_{50} ranging from 31.17 to 283.38 ppm. All of the bacterial extracts did not show a good percentage of viability (>50%) against Hela, WiDr, T47D, dan Vero cell line in MTT assay. However, bacterial extract with code H2N have shown potential cytotoxic compared to other extracts. As per the phytochemical study, this extract probably contained terpenoid group. Based on biochemical examination this bacterium, H2N, was identified as *Bacillus* sp.3.

INTRODUCTION

Cancer was one of the problems in the worldwide that need to overcome. Based on WHO statistic report (2007), about 40 million people were estimated died due to non-communicable disease (WHO, 2007). One of them leading to cancer and assumed about 8.8 million deaths (22%) in 2015. During period 2000–2015, the mortality rate by cancer cause was stagnant.

The research and development of anticancer drugs was the largest market area in the pharmaceutical industry and over 200 anticancer compound was in a various stage in a clinical trial (Moffat *et al*., 2014; Xu and Wenwei, 2016). Nevertheless, some problems are still obstacles. The adverse effect, resistance in a primary and a secondary line of cancer treatment, the persistence of cancer stem cell, and many others still limit the ability of an anticancer drug to stabilize, minimize the metastate, and cure the cancer disease (Widmer *et al*., 2014). In the discovery of lead compound of the anticancer drug, the small amount of compound, a minimum effect to a cancer cell *in vivo*, long term in clinical trial still the most factor that faced by a researcher.

Indonesian is the big archipelago state in the world, located in the tropic region, comprised 17,000 islands with many different types of habitat led to the megadiverse fauna and flora (Bruyn *et al*., 2014; Lohman *et al*., 2011). The marine sponge was available in abundance amount. *Haliclona fascigera* sponge that grows in West Sumatra Indonesia had been known as a potential source for various extraordinary activity (Handayani *et al*., 2015a; 2015b; 2016a; 2016b; 2018).

The sponge was a host for growing many symbiotic microorganisms. Association between endophyte microbe with the host could be as symbiotic mutualism until pathogenic relation (Strobel and Daisy, 2003). The different microbe can be feed sources, pathogen/parasite, or mutualism symbiotic for sponge otherwise bacteria have the essential contribution

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toward the self-defense of host by secreting antibiotic and other bioactive substances. Especially, a sessile marine organism, such sponge, was expected to have a tight connection to host defenses mechanism by producing a bioactive compound to protect from predators (Taylor *et al*., 2007). This fact was the fundamental reason by way of a symbiotic association between sponge and bacteria will be the promising source in exploring and finding many attractive bioactive compounds without damage the marine ecosystem on a large scale.

MATERIAL AND METHODS

Sponge material

Marine sponge sample (200 g) of *H. fascigera* was collected from Setan Island, South Coast of West Sumatra, Indonesia at depth 13 m. The surface of the sponge was rinsed by sterile seawater and kept in icebox and then transferred to the laboratory.

Isolation of marine-derived bacteria from sponge H. fascigera

Isolation of symbiotic bacteria methods was conducted by the same method that had to describe previously (Handayani *et al*., 2015a). The rinsed sponge was cut into small pieces and wrecked down by mortar and stamper. The marine sponge was weighed as much as 10 gram and dispersed into 100 ml sterile seawater to make serial dilution up to 10^{-6}. One milliliter from each dilution poured to sterile Nutrient Agar (NA) medium and incubated at 37°C–38°C for 24 hours. The different shape and colors of colonies assumed as pure bacteria isolates and separate by scratch method into new sterile NA medium.

Identification of bacteria isolate

Symbiotic bacteria were identified by macroscopic, microscopic, and biochemical examination. Shape and color colonies in NA medium after incubated overnight and Gram staining was observed to know the group of bacteria. Some biochemical test was conducted, such as TSIA, H2S, oxidation and fermentation, catalase, nitrate, indole, Methyl Red-Voges Proskauer, carbohydrate fermentation (lactose, Glucose, sucrose, and mannitol), citrate, urease, motility, and gelatin, and result test was compared with manual book of the identification bacteria (Capuccino and Sherman, 2001; Case and Johnson, 1984).

Cultivation of isolated bacteria

The pure of bacteria derived sponge was cultivated in Nutrient Broth (NB) medium. Cultivation was started with inoculum starter. One loop of each bacterium was inoculated into 50 ml of NB medium and incubated in an incubator shaker at 37°C speed 150 rpm for 24 hours. The starter inoculum inoculated into 500 ml of NB medium aseptically and incubation at the same temperature and speed overnight.

Isolation and extraction of the secondary metabolite from bacteria isolate

The liquid culture extracted with ethyl acetate solvent in ratio 1:1 for 3–5 days by triplicate. The extract was sonicated for 10 minutes with the sonicator. Ethyl acetate fraction was separated from the medium culture using a separating funnel and filtered by Whattmann no 2. The ethyl acetate solvent was evaporated in vacuo by rotary evaporator and obtained the dried extract of symbiotic bacteria. Each dried extract was weighed and furthermore, the weight >50 mg was continued to cytotoxic activity test.

Screening of cytotoxic activity

BSLT method

The Brine Shrimp Lethality Test method, shrimp nauplii *Artemia salina* Leach used as an indicator. Thirty milligrams from each ethyl acetate extract of symbiotic bacteria was solved in 3 ml ethyl acetate (10^3 ppm) as a working solution. Three variations of testing concentration (1,000, 100, and 10 ppm) were made by taken 500, 50, and 5 µl from working solution. The dried extract of bacteria was dissolved in 50 µl dimethyl sulfoxide. Ten shrimp nauplii were put in a vial that contained extract, DMSO, and sterile seawater and volume add into 5 ml with seawater. The died nauplii were counted after 24 hours. LC_{50} was calculated using the curve method by probit table. The BSLT was repeated for triplicate.

MTT assay

MTT assay was carried out with procedure experimental in Handayani *et al*. (2018) using symbiotic bacteria extract of marine sponge *H. fascigera* as a sample (Handayani *et al*., 2015a). The formula for the calculated % viability based on MTT Assay result was:

$$\% \text{ viability} = A1 - A0/A2 - A0 \times 100\%$$

where $A0$ = blank absorbance
$A1$ = absorbance of test cell + sample
$A2$ = control absorbance (cell + 10% DMSO)

Phytochemical screening

The crude extract of symbiotic bacteria derived marine sponge *H. fascigera* were screened for the presence of terpene, steroids, alkaloids, and phenolic using the standard qualitative procedure. Anisaldehyde, concentrated sulfuric acid, dragendorff, and $FeCl_3$ were used as the thin layer chromatography spray reagents.

RESULT AND DISCUSSION

More than 15,000 species of sponges that spread around the world have been known as a potential organism. Exploration of a bioactive compound from sponge for producing an active substance in the pharmaceutical industry mainly as an anticancer drug was a promising opportunity (Thomas *et al*., 2010). This condition certainly triggers the possibility of massive sponge exploitation. The exploration of microorganism that lives in sponge tissues or known as symbiont microbe was used as the best solution (Webster and Taylor, 2012).

The microbial symbionts are a microorganism that lives on the host tissue at a certain period, able to live by forming colonies on the host tissues and reach 40% of totally sponge tissue by the density of 10^9 bacteria cell per mm of sponge tissue (Hoffman *et al*., 2005). The Mayer's study (2008) showed that symbiotic mutualism occurred between a sponge and a number of bacteria and algae, where the sponge provides support and protection for its symbionts and the symbionts provide food for

the sponge (Mayers *et al*., 2014). The biochemical interaction that occurs between the sponge and the symbiotic bacteria allow the symbiotic bacteria to produce the same bioactive substance as its host. So, some types of bacteria that are symbiotic with sponges are thought to produce bioactive compounds (Mearns-Spragg *et al*., 1998).

The purification of sponge *H. fascigera* were obtained 26 of pure isolates, nine bacteria were identified as *Bacillus* sp., four bacteria were *Corynebacterium* sp., eight bacteria were *Micrococcus* sp., and two other bacteria were *Staphylococcus aureus* based on identification result that had published before (Handayani *et al*., 2015a).

The amount of ethyl acetate extract on 550 ml cultivation in NB medium was range 0.044 to 0.204 g. Some factor such as the ability of bacteria in produce secondary metabolite was affected the extract's amount. The product ability of secondary metabolite was usually depended on a stationary phase on bacteria's growth, which the total amount of bacteria that grow and died was constant.

The screening of cytotoxic activity was only conducted toward 19 bacteria isolates which extract weight >50 mg. The test could not be done in the least amount due to it would make a difficulty in the variation of concentration. In BSLT test using shrimp nauplii, *A. salina* Leach as an indicator showed that all of the ethyl acetate extracts of bacteria had LC_{50} value <1,000 ppm. One extract (code H2N) was very cytotoxic while 18 other extracts were toxic in LC_{50} range 31.17 to 283.38 ppm (Fig. 1). The concordance with Mayer (1982), the plant and animal's extract was classified toxic if it has LC_{50} value 30–1,000 ppm and categorized as very toxic when LC_{50}value <30 ppm (Mayer, 1982).

In conclusion, extract with code H2N was the potential to isolate a single compound and then develop as the lead compound for an anticancer drug.

BSLT method was a preliminary test for screening cytotoxic activity. It was a simple, cost-effective, no need any material for applied and can be routinely performed to estimate the toxic effect of the extract. When we know the ability to extract toward *A. salina*, the extracts probably active against of cell tumor line in human.

The major advance in cytotoxic activity was MTT assay. These methods are sensitive, highly reliable, and use some human cell line. In this study, we use some cell line, i.e., Hela (cervical cancer cell), WiDr (colon adenocarcinoma), T47D (breast adenocarcinoma), and Vero (normal cell). The MTT assay was performed by a colorimetric reaction that measure viability, proliferation, and activation of the cell. In this assay was based on the converting yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which insoluble in water and mediated by dehydrogenase enzyme in endoplasmic reticulum and mitochondria. The absorbance of the formazan purple crystals can be detected by using ELISA reader. The formation production was equal to the number of viable cells and inversely to the degree of cytotoxicity (Fotakis *et al*., 2006). In other words, the decrease of the %viability cell was proportionate with increasing the cytotoxic effect.

Cytotoxic activity of the compounds can be determined by IC_{50} value. The determination of IC_{50} can be calculated by determinating of the viability percentage of a cancer cell in the

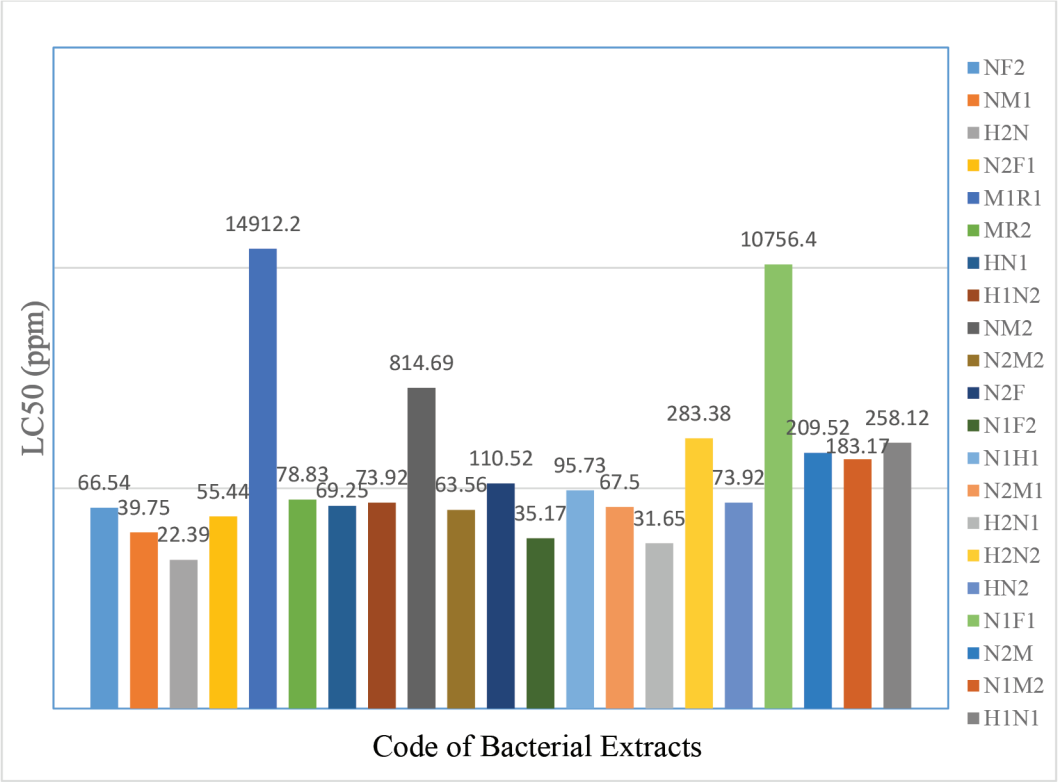

Figure 1. LC_{50} value of symbiotic bacteria extract from derived marine sponge *Haliclona fascigera.*

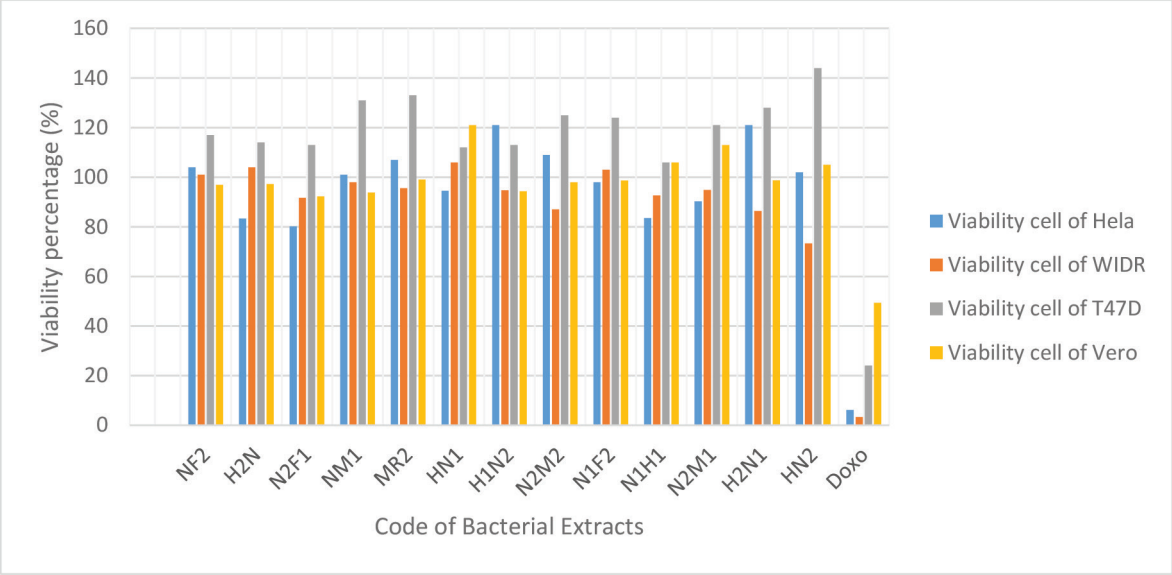

Figure 2. Viability percentage of ethyl acetate extract on the various human cancer cell line.

culture medium that contained ethyl acetate extract of symbiotic bacteria with a concentration of 100 µg/ml previously (Handayani *et al*., 2018). The percentage of viability extract is highly variable against each cancer cell; however, all of bacteria extracts did not show a good percentage of viability (>50%) (Fig. 2). In MTT assay result, all of bacteria extract have no good cytotoxic activity so that determination of IC_{50} was carried out yet.

Bacteria extract (code H2N) were selected to further characterization and identification. From the macroscopic and microscopic examination, also biochemical assay based on Cowan and Steel's Manual of Identification of Medical Bacterial Third Edition, it is known that the bacteria extract H2N is *Bacillus* sp.3.

Some previous research also stated that genus *Bacillus* has good bioactivity as a cytotoxic agent. Four bacteria cultures which code HA-68 (*Bacillus substilis*), HA MS-105 (*Bacillus safensis*), HA-MS-119 (*Lactobacillus murinus*), and HA-21 (*Bacillus sp*) that isolated from marine sponge *Amphimedon ochracea* have a cytotoxic activity to three human cancer line: HepG2 (hepatocellular carcinoma), HCT (colon carcinoma), and MCF-7 (breast carcinoma) with LC_{50}, respectively, HA-68: 10.42, 4.3, 5.5; HA-MS-105: 46.9, 28.6, 21.3; HA-MS-119: 10.42, 6.3, and 22.1; and HA-21: 13.2, 9.3, 12.2 ppm (Aboul-Ela, 2012). *Bacillus* sp. was also a potential source for isolating cytotoxic compound. Zhang *et al*. (2014) were succeeded to isolate three new cyclic acylpeptide, i.e., Mixirin A, B, and C from the fermentation of ethyl acetate extract of *Bacillus* sp. This compound has a cytotoxic effect against human intestinal tumor cell (HCT-116) with LC_{50}value: 0.68, 1.6, and 1.3 ppm, respectively (Zhang *et al*.–2004).

In this research, there is no correlation between BSLT and MTT's result. It may be due to the cytotoxic activity of bacteria extract are selective to other human cancer cell line. Therefore, this bacteria extracts can be tested to another cell line to obtain cytotoxic compound. Phytochemical screening of bacteria extract (code H2N) suggested the presence of terpene group.

CONCLUSION

Twenty-six bacteria extracts isolated derived from marine sponge *H. fascigera* that report on Handayani *et al*. (2015) was tested for their cytotoxic activity. *Bacillus* sp.3 (H2N) isolate was showed the most LC_{50} value in BSLT test, unfortunately do not show the good cytotoxic activity in Hela, WiDr, T47D, and Vero Cell Line.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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