Cytotoxicity of marine-derived fungi collected from Kepulauan Seribu Marine National Park

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Abstract. Marine-derived fungi are of great interest as new promising sources of biologically active products due to its diversity in chemical structures and biological activities. The objective of this study was aimed to determine the cytotoxicity of marine sponge-derived fungi that collected from Kepulauan Seribu Marine National Park. The fungi were fermented in a static liquid culture of malt extract broth medium (containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and seawater) for 3 weeks at 27-29°C. Bioactive secondary metabolites of fungi were extracted using ethyl acetate. Cytotoxicity of the extract was performed by using MTT assay. A total of 17 isolates were tested against T47D cell at a concentration of 30 µg/mL, among them, isolate of MFP270 exhibited the strongest cytotoxicity. Further analysis showed that mycelium extract of MFP270 had stronger cytotoxicity (IC50 = 28.3 µg/mL) than broth extract (IC50 = 645 µg/mL). The MFP270 marine fungal was identified as Aspergillus sp. base on its morphology features.

Keywords: Aspergillus sp., cytotoxicity, Kepulauan Seribu marine national park, marine fungi

1. Introduction

The number of fungi in the world is estimated to be around 1.5 million species, but only 5-10% have been studied. The biodiversity of fungi in the tropic regions is estimated to be higher than in sub-tropic regions. Indonesia has diverse marine waters that are about 2/3 of the area (Hawksworth 2000). Indonesian marine biodiversity is one of the highest in the world including microbial diversity. According to Gandjar et al (2006), the high diversity of fungi in Indonesia due to its humid environment and temperatures that support fungi growth. Approximately 200,000 fungi species are estimated to exist in Indonesia (Rifai 1995).

Studies have shown that secondary metabolites obtained from marine fungi have diverse chemical structures, where 70-80% of them have interesting biological activity (Schulz et al 2008). The increasing number of new compounds isolated from marine fungi shows the growing interest of the researcher to explore these microbes as a source of new bioactive compounds (Kjer et al 2010). Most
bioactive secondary metabolites from marine fungi have anticancer and antibiotic activity (Saleem et al 2005).

So far, there are still many secondary metabolites from marine microbes that have not been explored even though it is known to be very interesting compared to terrestrial ecosystems (Silber et al 2016). Research on marine fungi bioprospecting in Indonesia is still limited both its biodiversity and chemodiversity. Based on this reason, the research on fungi bioprospecting from the Indonesian marine environment is important. Some research in this field, for example, was done by Chasanah et al (2009), Nursid et al (2011, 2015, 2019).

The discovery of the natural product as for anticancer drugs generally is done by cytotoxicity screening using cancer cell lines. The use of the cancer line in biodiscovery of anticancer drugs has several advantages such as easy to handle, rapid, repeatable, and also it has unlimited replication. One of the most widely used cells in cytotoxicity screening are T47D cells (human breast ductal carcinoma) (Felth et al 2011). The objective of this study was to determine cytotoxicity marine-derived fungi collected from the Kepulauan Seribu National Park, Indonesia.

2. Materials and methods

2.1. Sampling
The invertebrates were collected from Kepulauan Seribu Marine National Park, Indonesia on June 2014 by scuba diving. Sponge samples were rinsed with sterile seawater then put in a sterile plastic bag and immediately preserved at cold temperatures. All samples were stored at -20°C.

2.2. Fungal isolation
Isolation of fungal from the host was done using malt extract agar (MEA) medium containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.5% agar. The isolation method was performed according to Kjer et al (2010). To minimized contamination, the host surface was sprayed with 70% ethanol, then aseptically, sponges were cut to 2-4 mm³ in size. The pieces were placed into a petri dish containing the MEA medium. Petri dish was incubated at 27-29°C for 3-7 days. Each fungus grown was moved to a new petri dish based on the shape and color of hyphae. The isolation process was carried out repeatedly until a pure strain was obtained.

2.3. Fungal cultivation
Each of the fungal strains was then cultivated in malt extract broth (MEB) containing 0.3% malt extract, 0.3% yeast extract, and 0.5% peptone. For this purpose, each fungus that covers the surface of the inoculated petri dish was aseptically cut into small pieces of 1x1 cm². These pieces were then aseptically transferred into a flask containing 10 mL of the sterilized liquid medium and incubated at room temperature for 2-3 days. After that, each fungal strain growing on the 10 mL of liquid medium was aseptically transferred into a flask containing 100 mL of sterilized liquid medium and cultivated at room temperature for 3 weeks in static conditions (Nursid et al 2011).

2.4. Cultivation of selected fungal
The selected fungus was maintained on malt extract agar. To produce sufficient extract, the fungal was cultured in static liquid culture of malt extract broth medium containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and seawater. The fungus was cultured (1 L flasks) for 3 weeks at 27°C-29°C (Nursid et al 2015).

2.5. Extraction
The extraction of the secondary metabolites from the fungal cultures was conducted using Kjer et al (2010) with some modifications. The growth of fungal was stopped by adding 100 mL of ethyl acetate
to the culture flask and macerated around 12 hours. The fungal cells were then disrupted using sonicator (Sonics Vibra cell model CV-33) for 90 minutes. The mixture of ethyl acetate, mycelium and broth then were filtered using filter paper. The culture filtrate then was transferred into a separation funnel to separate ethyl acetate and aqueous phases. The aqueous phase was reextracted twice using 100 mL of ethyl acetate. All of the ethyl acetate phases were collected and concentrated by using a vacuum rotary evaporator (Buchi). The remaining ethyl acetate in the extract was then dried with nitrogen gas to get the crude extract. For 1 L culture, mycelium and broth were extracted separately, mycelium was extracted by using the mixture of dichloromethane: methanol 1:1 (v/v) whereas broth was extracted by using ethyl acetate.

2.6. Cytotoxicity assay

The assay was conducted according to Ebada et al (2008). Human breast ductal carcinoma (T47D cell line) were routinely grown and maintained in RPMI medium with 10% FBS and 1% penicillin-streptomycin. The cell was incubated in a moisture-saturated atmosphere containing 5% CO₂. To each well of the 96-wells microplate containing 100 µL of cell suspension (1.5x10⁴ cells) was added 100 µL of fungal extract in various concentration and the plate was then incubated in a CO₂ incubator at 37°C for 24 h. After the addition of 100 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide saline solution (0.5 mg/mL) to each well of the microplate, the plate was then incubated for 4 h under the same conditions in the CO₂ incubator. After incubation, the optical density was measured at 570 nm in a microplate reader (Thermo Scientific). The inhibition concentration 50 (IC₅₀) values defined as the concentrations of a compound which inhibited 50% of the cell growth. The IC₅₀ value was determined by using Minitab probit analysis version 14.0. Doxorubicin was used as a positive control.

3. Result and discussion

The number of marine fungi isolated was 17 strains. These fungi were isolated from the surface of marine invertebrates i.e sponges, ascidians and soft coral (table 1). These invertebrates were taken from Kelapa and Panggang Islands, Kepulauan Seribu National Park (KSNP). All marine fungi were cultivated on 100 mL MEB media for 3 weeks (figure 1). The extraction of metabolites (broth and mycelium) was carried out using ethyl acetate. The extract obtained was then tested for its cytotoxicity against T47D cells at a dose of 30 µg/mL.

Figure 1. Cultivation of marine fungal on the 100 mL and selected fungal that cultivated on 1 L culture, 1st day (a), 7th day (b), 14th day (c) and 21st day (d).

The cytotoxicity test showed that there are 8 strains that can inhibit the growth of T47D cells (% inhibition > 50% (figure 2A). The fungal strain that showed the best cytotoxicity was the MFP-270 that isolated from the ascidian surface.
The MFP-270 marine fungal then were cultivated in a 3 L flask culture (containing 1 L MEB medium). After 3 weeks, the culture was harvested, mycelium and broth were extracted separately. Cytotoxicity test showed that mycelium extract has strong cytotoxicity with the IC\textsubscript{50} value of 28.3 \(\mu g/mL\) compared with broth extract (IC\textsubscript{50} value of 645.5 \(\mu g/mL\)). Morphology features of T47d cells before and after treated with MTT as described in figure 3.

**Table 1.** The fungal strain that isolated from KSMNP.

| No | Fungal Strain | Host     | Location         |
|----|---------------|----------|------------------|
| 1  | MFP-260       | Sponge   |                  |
| 2  | MFP-261       | Sponge   |                  |
| 3  | MFP-262       | Sponge   |                  |
| 4  | MFP-263       | Sponge   |                  |
| 5  | MFP-265       | Sponge   |                  |
| 6  | MFP-266       | Sponge   |                  |
| 7  | MFP-268       | Sponge   |                  |
| 8  | MFP-270       | Sponge   |                  |
| 9  | MFP-271       | Ascidian | Kelapa Island    |
| 10 | MFP-271       | Ascidian |                  |
| 11 | MFP-273       | Ascidian |                  |
| 12 | MFP-274       | Soft coral |                |
| 13 | MFP-280       | Ascidian | Panggang Island  |
| 14 | MFP-281       | Ascidian |                  |
| 15 | MFP-282       | Ascidian |                  |
| 16 | MFP-284       | Ascidian |                  |
| 17 | MFP-287       | Ascidian |                  |

**Figure 2.** MTT assay of marine fungi extract against T47D cancer cell line at a dose of 30 \(\mu g/mL\) (A) and dose-response curve of T47D cell after treated with mycelium and broth extract of MFP 270 marine fungal (B) \(=\) mycelium, \(=\) broth.

The results of this study show that marine fungal MFP-270 had promising cytotoxicity (IC\textsubscript{50} value of 28.3 \(\mu g/mL\)). In cytotoxicity testing, a crude extract of a natural product is considered active if it has...
an IC₅₀ value < 30 µg/mL (Munro et al. 1987, Chicca et al. 2008). Besides MFP-270 strain, other strains that also interesting to the next study were MFP-268, MFP-271, MFP-272, and MFP-282. These marine fungi can inhibit the growth of T47D cells with a percentage of cell death more than 50%.

All of these potential fungal strains were isolated from the surface of the marine ascidian. Ascidian belongs to the Tunicate sub-phylum, more than 3000 species have been described. They have been found in diverse ecological niches, from shallow water to deep sea. Microbes associated with ascidians represent potential sources of bioactive secondary metabolites. Many compounds purified from ascidian-associated microbes had potent bioactivity such as Ecteinascidin 743 (the trade name Yondelis) (Chen et al. 2018). In addition to Ascidian, other marine invertebrates so far are still a promising source for isolation of marine fungi that produce potential secondary bioactive metabolites, including sponges, corals, echinoderm, etc. Many marine fungi can be easily cultivated to achieve a high yield of secondary metabolites so that they can be a sustainable alternative to chemical synthesis (Bovio et al. 2018).

Microscopic observations (figure 4) of MFP 270 as follow: conidia and vesicles typically shaped like mace, long shaped conidiophores from green to brown. Conidiophores are hyaline and smooth-walled. The vesicles are quiet round and 10-20 µm in diameter. Fialids form in the metula. Conidia are round to elliptical, hyaline to light yellow and smooth-walled. This morphological feature of MFP270 marine fungal is close Aspergillus genus.

**Figure 3.** Inverted microscope photograph of T47D cells after treated with MFP-270 marine fungal after and before exposure to MTT.
Aspergillus is included in the Ascomycota phylum. Generally, it has morphological characteristics as follows: filamentous, hyphae are insulated or have septa, and are abundant in nature (Gandjar et al 2006). Aspergillus grows optimum at a temperature of 35-37°C, a minimum temperature of 6-8°C, and a maximum temperature of 45-47°C. Besides, in the process of growing these fungi, require sufficient oxygen (Madigan et al 2009).

In recent years, marine fungi associated with sponges have become a source of new bioactive molecules. Many secondary metabolites with a broad spectrum of bioactivity that have been isolated from fungi associated with sponges, including alkaloids, terpenoids, polyketides, and peptides (Ding et al 2019). In addition to ascidian and sponges, soft corals also are potential hosts as sources of fungal biodiversity. Soft coral is included in the Cnidaria phylum. According to Raimundo et al (2018), the coral group is the second most prolific source of natural products obtained from marine invertebrate after sponge.

Figure 4. A colony of MFP 270 marine fungal in MEA media (A) and its microscopic features (B) (magnification 200 x).

Most of the bioactive secondary metabolites of marine fungi isolated from the Aspergillus and Penicillium groups. Both genera have a strong tolerance to salinity, grow quickly and are relatively easy to obtain from many substrates. Aspergillus and Penicillium are known to produce metabolites with a wide variety of bioactivity (Ding et al 2019). Marine fungi can develop specific metabolite that is different from terrestrial fungi because they can live in extreme environmental conditions such as high salinity, pressure, temperature variations, competition with bacteria, viruses and other fungi (Mabrouk et al 2008). Overall, it can be concluded that mycelium marine fungi MFP 270 has strong cytotoxicity activity. Scale 10-20 L culture is needed to obtain sufficient crude extract so that isolation and identification of compounds responsible for bioactivity can be carried out.

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