SPONGE-ASSOCIATED FUNGI ISOLATES FROM ANCORINA SP. SHOWED ANTI-CANCER ACTIVITY AGAINST HELa CELL LINES

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ABSTRACT
Nowadays, natural products from marine life, especially sponge-associated fungi (SAF), have been getting extensive attention as candidates for anti-cancer agents. Although chemotherapy has been widely used as one of the cancer treatments, recently, multdrug resistance as one of its drawbacks has appeared. Our preliminary research successfully isolated 16 fungi isolates from Ancorina sp. habituating Kukup (KU) Beach, Yogyakarta, Indonesia. The ethyl acetate extracts of SAF KU4 fungi grown in enrichment broth media in seawater base either as media-free mycelium or whole media were analyzed for the cytotoxicity level and the apoptotic effect on the apoptotic effect cervical cancer cell line (HeLa). SAF KU4 was cultured in Wickerham sea-based media and then extracted using ethyl acetate. The IC₅₀ values were measured by MTT assay. IC₅₀ of the media-free extract and whole extract were 158.13 and 283.95 µg/mL, respectively. Ethyl acetate extracts of media-free mycelium and whole extract were moderately cytotoxic but could induce apoptosis in HeLa cell lines.

Keywords: Ancorina sp., Apoptosis, Cytotoxicity, HeLa cell, SAF KU4

INTRODUCTION
Cancer is still one of the leading health problems and is getting more attention in biomedical research, especially in obtaining new compounds with anti-cancer activity. Furthermore, the cause of cancer and its manifestation are still unclear and need to be elucidated (Bee soo et al., 2014; Dey et al., 2015). Chemotherapy is an optional treatment for cancer patients since it is well-documented that chemotherapeutic drugs induce multiple-drug resistance in cancer cells. Chemotherapy drugs produced by synthetic chemistry have become a concern since these drugs are lethal to both cancer and healthy cells; thus, interest in natural products as anticancer has increased recently (Haudhur, 2003; Aung et al., 2017). Natural products isolated from fungi are promising since the first isolation of cyclosporin A from Tolypocladium inflatum was approved for clinical treatments as an immunosuppressant in 1983 (Bugni & Ireland, 2004).

Meanwhile, the high rediscovery of isolated compounds has interested researchers in exploring unique habitats such as the marine environment. Microbes, i.e., fungi, alive in this environment have constantly been exposed to extreme conditions, like temperature, salinity, pressure, etc., that can produce various compounds with unique structure and biological activity (Bugni & Ireland, 2004; de Carvalho and Fernandes, 2010; Deshmukh et al., 2018). Sponge-associated fungi produce natural products with active substances showing antibiotic, anti-helminthic, anti-nematicide, and anti-cancer activities. For instance, Neochelinil A and Physiocin, two active compounds isolated from Microsorum sp., and algae-associated fungi, Lomentaria catenata, are known to have anti-cancer activity and are being developed as new potential candidates for chemotherapy (Wijesekara et al., 2013, 2014).

Up to date, the exploration of novel compounds from sponge-associated fungi is fascinating and has the potency to identify new drugs for cancer. This study isolated 16 sponge-associated fungi from Ancorina sp. inhabiting Kukup Beach, Yogyakarta, Indonesia, and screened them for anticancer properties. Here we report the anti-cancer activity of an Aspergil lus named SAF KU4, one of the 16 isolates that showed anti-cancer activity with the lowest IC₅₀ tested against HeLa cells. Further, we also standardized the conditions that enhance the production of these anti-cancer active compounds from SAF KU4.

MATERIAL AND METHODS

Materials
HeLa cell lines were from LPPT (Laboratorium Penelitian dan Pengujian Tepada/The Integrated Research and Testing Laboratory, Universitas Gadjah Mada. SAF KU4 stock isolate was stored at 4°C in the slant of malt extract agar. Dimethylsulfoxide (DMSO) (NacalaTeasuque), 3-(4,5-dimethyl-2-yl)-2,5-diphenyloxetrazolium bromide (MTT) (Sigma-Aldrich), RPMI 1640 (Sigma-Aldrich), Fetal Bovine Serum (Sigma-Aldrich), Penicillin-Streptomycin (Sigma-Aldrich), Fungizone (Gibco), Phosphate Buffer Saline, Stop solution (10% SDS in 0.01N HCl), Acridine Orange (Sigma-Aldrich)/Ethidium Bromide (Sigma-Aldrich), ethyl acetate (EtOAc), malt extract agar (Sigma-Aldrich), monohydrate glucose (Sigma-Aldrich), peptone (Oxoid), and yeast extract (Oxoid).

Subculture and identification of SAF KU4
SAF KU4 was isolated from the sponge, Ancorina sp., inhabiting sea rocks at Kukup Beach, Yogyakarta, Indonesia in 2014. For preculture, SAF KU4 was grown in Malt Extract Agar (15 g of agar, 15 g of malt extract, 1000 mL of seawater). Identification was proceeded by the lactophenol staining. A 10-day old culture of SAF KU4 fungi with hyphae was fixed to object glass and dropped by lactophenol (Sigma-Aldrich). Furthermore, the structure hyphae, spore shape, mycelia turbidity, and mycelial branching were observed under the light microscope (Olympus) with a maximal magnification of 1,000x. The pictures were taken by a camera (Sony Camera).

Cultivation of SAF KU4 and secondary metabolite extraction
Two flasks, each containing 300 mL Wickerham Medium (5 g of yeast extract, 3 g of malt extract, 5 g of peptone, 20 g of monohydrate glucose, 1000 mL of seawater) were used to inoculate 3% (v/v) of SAF KU4 fungus and cultivated for 21 days at room temperature with agitation at 200 rpm. After 21 days, one flask was extracted after removing mycelia (media-free mycelia) with ethyl acetate (EtOAc) (1:1/v/v), and these were macerated for seven days (Noirmart et al., 2017). The ethyl acetate phase was separated from broth and evaporated until obtaining the media extract. Another flask containing the mycelia in the medium was disrupted by ultrasonication, and the lysate was filtered by Whatman Paper No. 1 in Vacuum Buchner funnel. The filtrate devoid of cell debris was extracted separately with
EfOAc (1:1/v/v) and macerated for 7 days (Noinart et al., 2017). The ethyl acetate phase was separated from the medium and evaporated to get the whole extract.

**Cytotoxicity assay**

The cytotoxicity assay referred to the previously described method (Ramadhan et al., 2017). HeLa cells were cultured with RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.5% fungizone and maintained at 37°C with 5% CO₂. The cytotoxicity level was measured by MTT assay in triplicate (n=3). About 100 µL of cell lines were seeded in 96-well plates at a 2x10³ cell/well density. After 24-hour incubation, cells were treated with different concentrations of two extracts using 1:2 serial dilution (600-1.7 µg/mL) while using stock concentration at 1,000 µg/mL of extract in DMSO. Then, cell cultures were incubated for another 24 hours at 37°C with 5% CO₂. After 24-hour incubation, the medium was removed, and 100 µL of MTT solution (5 mg/mL) was added to each well. After 4-hour incubation, 100 µL of stop solution (10% SDS in 0.1 N HCl) was added to solubilize the formazan salt. The optical density (λ=550 nm) was measured and used to calculate the IC₅₀.

**Apoptosis Assay**

About 5x10³ HeLa cells/well were seeded on the coverslip in 24-well plates. After 24h, cells were treated with extracts at two concentrations of about 85 and 170 µg/mL for the media extract and 100 and 200 µg/mL for the whole extract. These extract concentrations were determined based on the IC₅₀ value from the cytotoxicity assay. After 24-hour incubation, cells were washed with phosphate buffer saline and stained with acridine orange/ethidium bromide. The cell was envisioned and photographed under a fluorescence microscope and determined for apoptotic and healthy cells (Ribble et al., 2005). Cell percentage was determined by taking 200 cells in a field (n=1) to visualize the image and count the number of both cells in apoptotic and healthy status. Then, it was compared to make the percentage.

**Statistical analysis**

Probit analysis was conducted to determine the IC₅₀ of both two extracts.

**RESULTS AND DISCUSSION**

**Morphology of SAF KU4**

Fungi can be determined by observing the hypha and conidiophore structures under the microscope. We assumed SAF KU4 was classified into the Ascomycota class in the genus Aspergillus based on the morphological observation. This genus has a foot cell produced by extending the aseptate conidiophore and modified into vesicles (Bennet, 2010; Samson, 1994). SAF KU4 was first isolated from the sponge Ancorina sp. (Figure 1A) inhabiting Kukup beach, Yogyakarta, Indonesia. The sponge is found at the intertidal zone attaching to the shoreline rocks. The microscopic structure of the fungi under the microscope was separated from the whole extract (Figures 1B & 1C). SAF KU4 has modified hyphae as a vesicle and asexual spore structure as conidiophore with transparent unbranched hyphae. Under the microscopic structure analysis, SAF KU4 has an aspergillium-like spore-bearing structure which is a key character to identifying Aspergillus genus accurately.

**Extraction and Cytotoxicity Assay of SAF KU4 Secondary Metabolites**

We successfully obtained 0.18 g extract of both whole and media extracts, which showed an oily appearance with a brownish color. Then, assays of two extracts were continued against HeLa cancer cell lines. The cytotoxicity assay was determined by MTT method. Our preliminary research revealed that SAF KU4 extract, cultured in malt extract with 14 days incubation, showed IC₅₀ around 300 µg/mL. This value was then referred to assay SAF KU4 extract cultured in enrichment Wickerham media with prolonged incubation until 21 days. We then used extract concentrations for analysis from 600-1.7 µg/mL. Based on the assays, we obtained the cytotoxicity curves in Figures 2(A) and 2(B). Using the probit analysis, MTT assay on HeLa cell lines showed the IC₅₀ of media extract and whole extract were 158.13 and 283.95 µg/mL, respectively.

**Figure 2** Effect of media extract (A) and combination extract (B) on HeLa cancer cell lines.

**Apoptosis Staining in HeLa cell lines**

The Acridine Orange/Ethidium Bromide method was used to distinguish between healthy and apoptotic cells. Here, HeLa cell lines were treated using two times lower and higher IC50 concentration values. The effect of two extract treatments in HeLa cell lines is shown in Figure 3. Several variations in apoptotic and healthy cells were observed between media and combination extract treatments. In addition, lower concentration from the IC₅₀ value showed a high number of healthy cells compared to two times concentration higher from the IC₅₀ value.

**Figure 3** HeLa cell lines condition in acridine orange/ethidium bromide staining after treatments. A) control; B) media extract at concentration 85 µg/mL; C) media extract at concentration 170 µg/mL; D) whole extract at concentration 100 µg/mL; E) whole extract at concentration 200 µg/mL. Observation took under a fluorescent microscope (40x). The white arrow shows the healthy cell, and the yellow one is the apoptotic cell.

In Figure 4, the number of apoptotic and healthy cells in the different extracts were shown. Media extract with a concentration 170 µg/mL showed about 61% HeLa cell line in apoptotic condition, and then, it was decreased to 56% at 85 µg/mL. The whole extract showed about 61% of HeLa cell line was apoptotic at 200 µg/mL, and it showed a decrease in the number of apoptotic cells by about 51% at 100 µg/mL.
remaining nutrients from the environment (Calvo et al., 2002). Those secondary metabolites can either be accumulated inside or excreted outside of the cells, which depends on the function of the cell. If toxic compounds are produced inside the cell, they will be passed outside the media and protect the cell from death, making cells alive and getting nutrient. However, the favorable compound was still maintained inside the cells and used as a growth substance (Vining, 1990).

The presence of active compounds from SAF KU4 was investigated by comparing the extract of media and whole extract by assaying the bioactivity of the secondary metabolites from extracellular and the combination of extracellular and intracellular compounds. The results showed that ethyl acetate extract from media gave the lowest IC₅₀ compared to the whole extract, and it still had a potency as the anti-cancer agent. Meanwhile, the IC₅₀ from mycelium extract was reported previously at 164 µg/mL (Ramadhan et al., 2017). This IC₅₀ showed comparable cytotoxicity compared to media and the highest cytotoxicity compared to the whole extract. Here, we suspect that the active compounds from SAF KU4 are excreted outside the cells. However, we also predict some active compounds are also still located inside the cells, proven by the comparable IC₅₀ between media and mycelium ethyl acetate extracts. The whole extract showed the highest IC₅₀ compared to media and mycelia extracts. Yet, it still showed the lowest IC₅₀ value compared to the results from the preliminary research with an IC₅₀ value of 383.88 µg/mL. We assume that compound-compound interaction may be the main reason the IC₅₀ value from the whole extract is highest compared to medium and mycelium extracts. One study identified this mechanism and reported that compounds could interact with each other to make antagonistic effects on cells that will change the ability of active compounds to inhibit the growth of the targeted cells (Yin et al., 2014).

Meanwhile, in our research, we found that modification on culture medium, prolonged incubation time, and agitation process during the culture process changed the IC₅₀ value of the SAF KU4 extract.

The modifications on media by enriching the nutrients, giving a prolonged time incubation, and adding agitation increased the production of active compounds (Miao et al., 2006). Aspergilloide, the fungus known as the source of secondary metabolites mostly at the preliminary stage (Calvino-Morilla et al., 2012). Production of secondary metabolites mostly occurs at the stationary phase due to a lack of nutrients; thus, the microorganisms compete to get any available

### Figure 4

The percentage of HeLa cells in the healthy condition (black) and apoptotic condition (gray) after treatment of media extract (A) and whole extract (B). It could be observed that apoptotic cells increased in a high concentration of extracts.

Sponge extracts of Ancorina sp. were previously reported to have anti-cancer activity (Nuriliani et al., 2013; Tunjung & Sayekti, 2019), but the activity of sponge-associated fungi extract is not reported well. Here, we first reported isolated sponge-associated fungi from Ancorina sp. that showed anti-cancer activity (Ramadhan et al., 2017). Previously, we successfully isolated 16 isolates of fungi from the sponge living at Kukup beach. Antibacterial activity against Staphylococcus aureus and Salmonella typhi was used as preliminary method to observe the antibiotic activity. Two candidate isolates, SAF KU3A and SAF KU4, showed the highest antibiotic activity in bacterial growth inhibition from sixteen isolates. We predicted that we would have promising activity as the anti-cancer agent. Further analysis, by using cervical cancer cell line HeLa, ethyl acetate extract from SAF KU4 showed the highest cytotoxicity value compared to SAF KU3A (Ramadhan et al., 2017), which then, was chosen as the candidate to investigate the activity against HeLa cancer cell lines with modification in cultivation medium, prolonged the cultivation time, and added agitation process. filamentous fungi are known as the source of bioactive compounds (Chavez et al., 2015). As the antibiotic story, Penicillin, the first well-known antibiotic from a microorganism, is isolated from fungi known as Penicillium notatum. Meanwhile, recently the number of resistance problems, even for the bacteria (antibiotic) and cancer cell (anti-cancer), has increased periodically, demanding new drugs needed to be approved by the authorities. The attempt to harness fungal secondary metabolites for drug discovery gives an approach to isolating new compounds from new habitats of isolation, such as fungal associated with a sponge. Sponge-associated fungi have been known to produce novel and unique secondary metabolites (Tian et al., 2018; Zhou et al., 2011). The interaction of fungi and sponge is mediated by a specific site (1→3)-β-D-glucan binding protein. This protein is in the sponge surface and makes a compatible site for fungi to attach to the sponge body and being associated (Suryanarayanan, 2012). This binding protein is also hypothetically synthesized and located on the surface of Ancorina sp., which makes a possible interaction with SAF KU4.

In the first preliminary research, we obtained the IC₅₀ higher than 300 µg/mL against HeLa cell lines (Ramadhan et al., 2017). It was known that modifying activities and additions in growth media would affect active metabolite production (Miao et al., 2006). Increasing activity and production of active compounds from a microorganism, especially from fungi, can be engineered by modification of incubation time (Frissva et al., 2007), stress given during culture, i.e., salt concentration (Wang et al., 2011), or modification on a culture medium (Marmann et al., 2014). In our studies, malt extract was used for cultivation media at the preliminary stage. It was also reported that enrichment of carbon, hydrogen, oxygen and nitrogen sources in cultivation medium raised the secondary metabolite production and mass of fungi (Yang et al., 2007). Carbon, hydrogen, and oxygen are the primary backbone of secondary metabolite scaffold, and nitrogen can be found occasionally on the compound and makes unique structures to be produced (Nursid et al., 2010). Further, we tried to modify the medium for the culture by using Wickerham media as the enrichment media, and we added agitation. Aeration on liquid culture is proven to actively increase the fungi biomass and increase the production of secondary metabolites. Aspergilloide, the reported secondary metabolite produced by Aspergillus glaucus HB 1-19, showed an increase in production after being given the agitation at 90 rpm (Cai et al., 2012). Therefore, maintaining SAF KU4 with agitation can further increase the production of active secondary metabolites.

### CONCLUSIONS

The modifications on media by enriching the nutrients, giving a prolonged day incubation, and adding agitation increased secondary metabolites production by SAF KU4 fungi isolated from the marine sponge. The ethyl acetate extracts of SAF KU4 induced apoptosis in HeLa cancer cell lines. The IC₅₀ of media and whole extracts were 158.13 and 283.95 µg/mL, respectively.

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### REFERENCES


