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POTENTIAL CHEMOPREVENTIVE AGENT: STUDY OF APOPTOSIS IN THE EXTRACTS OF SPONGE-ASSOCIATED FUNGI FROM YOGYAKARTA AGAINST CERVICAL CANCER *HeLa* CELL LINE

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ABSTRACT

Background: *Cervical cancer is one of the leading-cancers affecting women. Cancer drugs that do not originate from natural ingredient, chemotherapy drugs, have side and resistant effects. Thus study about the natural products treating cancer cells is needed. Secondary metabolites isolated from sponge-associated fungi are expected to have a potency to fight cancer cells. In addition, the production of anticancer compounds from microorganisms has several advantages, including rapid growth and can be manipulated to increase productivity. The isolation and testing cytotoxicity against 3 fungal isolates from Yogyakarta have been done on the previous research. All three isolates have a potential candidate as anticancer drug.*
Aims: *The purpose of this advanced study was studying bioactive compounds induced apoptosis pathway of sponge-associated fungi against cervical cancer HeLa cells.*
Methods: *This study has been carried out for approximately 5 months. The method conducted in this research including the sponge cultivation (covers growth and isolation of secondary metabolites), the mycelium extraction of fungi, the cytotoxicity assay against HeLa cells using MTT Assay and Apoptosis Staining was to see the induction of apoptosis pathway.*
Results: *Based on the research showed that ethyl acetate extract from mycelium is 0.22 grams. The cytotoxicity assay from mycelium extract showed IC50 value of 164 µg/mL against HeLa cell line.*
Conclusion: *The findings is carrying to a possibility to develop the extracts of sponge-associated fungi as candidate of anti-cancer compound. By apoptosis staining, showed the cells coloured green are still alive, and cells undergoing apoptosis have nucleus that appears orange to red. We assuming that the apoptosis was caused by the possibility of peptide compounds that induce apoptosis through the mitochondrial pathway, by increasing the activity of the protein expression of apoptosis, which are Bcl-2 and Bcl-xl.*

INTRODUCTION

Cancer problems is a health problem with case rate and death increasing each year. Cancer cells can continue to divide, not sensitive to the anti-proliferation signals and avoid programmed cell death (apoptosis) due to mutations in growth factor receptor gene [1]. Kind of cancer in Indonesia with the largest case rate is cervix. Cervix (cervical) cancer is a cancer that often affects both women after breast cancer. This cancer usually affects women who are married and aged 30 years and over. Besides the age factor risk factor for cervical cancer include sexual intercourse at a very young age, often with multiple partners, and Human papillomavirus (HPV), and carcinogenic compounds.

Women who smoke will be more easily infected with HPV because of a decrease in immune system to fight the virus. HPV 16 causes most cervical cancer is over 50%, though followed by 9% HPV 18 and HPV 31, 6% [2].

In a variety of biomedical research on cervical cancer HeLa cell line used a continuous cell line that has been used for thousands of studies on cervical cancer.Generally, cancer drugs that does not originatefrom natural ingredient have side effects and cause resistant effect [3].

Sponge is a primitive multicellular animal known produces secondary metabolites to self defence against predators [4]. Alkaloids, terpenes, acetogenin, nitrogen compounds, halides cyclic and cyclic peptides are several kinds of bioactive compounds has been isolated from sponges [5]. The bioactive compounds potentially as antimicrobial, antiviral and anticancer [6]. [7] has observed that the bioactive compounds extracted from the sponge was taken in the intertidal zone Wediombo Beach, Gunung Kidul, Daerah Istimewa Yogyakarta potentially to inhibit the growth of HeLa cells. [8] do cytotoxicity test against isolates of fungi associated with sponge and has potential as anticancer.

Sponge life forming symbiosis with microorganisms, archaea, bacteria, cyanobacteria, fungi and microalgae. Microorganisms that growing in surface or in the body of the sponge live in environmental with high competition to acquire place and nutrients. Microorganisms are mainly bacteria and fungi produce more secondary metabolites than free life microorganisms. The identified bioactive compounds from sponges have similar secondary metabolites produced by microorganisms associated with the sponge [4,9].

Sponge-associated fungi produce secondary metabolites that have antibacterial and anticancer activity that is not produced by fungi of the same species that live in terrestrial environments. Filamenteous Ascomycetes are fungi that are generally found associated with the sponge, in particular the order Eurotiales, Capnodiales, Pleosporales and Hypocorales. Trichoharzin, compounds isolated from *Trichoderma harzianum* associated with sponge *Mycale cecilia* was first discovered metabolites, while gymnastatin A, B and C are metabolites that are cytotoxic first isolated from fungi associated with sponge [10]. Microorganism's potential as bioactive compounds was concern now. This anticancer compound hopefully has potency and high specificity to against cancer cells. The anticancer compounds are expected to spur induction pathway of apoptosis that can be used as a chemopreventive agent on cancer cells treatment.

METHODS

Extraction Fungi

A total of 300 mL Erlenmeyer flask cultures of fungi were filtered using *Vacuum Buchner* in order to obtain the mycelium and the supernatant. Then mycelium macerated with ethyl acetate and destroyed by ultraturax. Ethyl acetate phase evaporated to obtain ethyl acetate extract of condensed phase.

MTT Assay

Cytotoxicity test from various extracts concentrations tested using 3-(4,5-dimetiltiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) using incubation time for 72 hours. Plate assay was read using a spectrophotometer at λ = 520nm. The data obtained is used for the calculation of the dose indicated by calculating the concentration of extract that kills 50% of the cells population (IC_{50}).

Double Staining Apoptosis Test by Acridine Orange/Ethidium Bromide (AO/EB)

Apoptosis test performed after MTT Assay. Steps being taken were as much $5x10^4$ cells / 200 mL planted into a 24 wells plate that has previously planted with a *cover slip*. Then do the incubation at 37° C for 24 hours in a 5% CO_2 incubator. Furthermore, medium in wells containing the cells is replaced with RPMI 1640 medium and added a complete new test solution with reference to the levels of IC_{50} obtained and incubation again for 24 hours. The culture medium was taken and the cells were washed with PBS. After washing, the cover slip is lifted from the wells and placed upside down on a glass object that has been poured with acridine orange/ethidium bromide before. Morphological observation of cells that have been stained with a fluorescence microscope with 100x magnification using a wavelength of 515-565 nm to see and detect the presence of HeLa cells that undergo apoptosis or HeLa cells are still alive.

Table 1. IC_{50} values based on Pertiwi et al. (2014)

Solvent	Extract	IC_{50} (µg/Ml)	
Etil Acetate	SAF KU4	383.88	958.00
	SAF KR4	414.54	100.19
	SAF KU3A	163.68	125.34
H2O	SAF KU4	3353.01	8109
	SAF KR4	261.06	539.03
	SAF KU3A	0	1054.69

RESULTS

Based on the test results of anti-bacterial activity, the research carried out at this stage in vitro cytotoxicity assay using HeLa cell line to see the anti-cancer activity, and vero cells were used as controls.In Table 1. it can be seen from the crude extract IC_{50} Ethyl Acetate (EtOAc) mycelia and media of the three Sponge-Associated Fungi isolates KU4, KR4, and KU3A. Based on IC_{50} values obtained, it can be seen that the crude extract KU4 EtOAc isolates can be developed to serve as an anti-cancer compound. In the next stage, the stages do further research on KU4 isolates of fermentation in Wickerham medium with long fermentation time for 21 days based on study of (10).

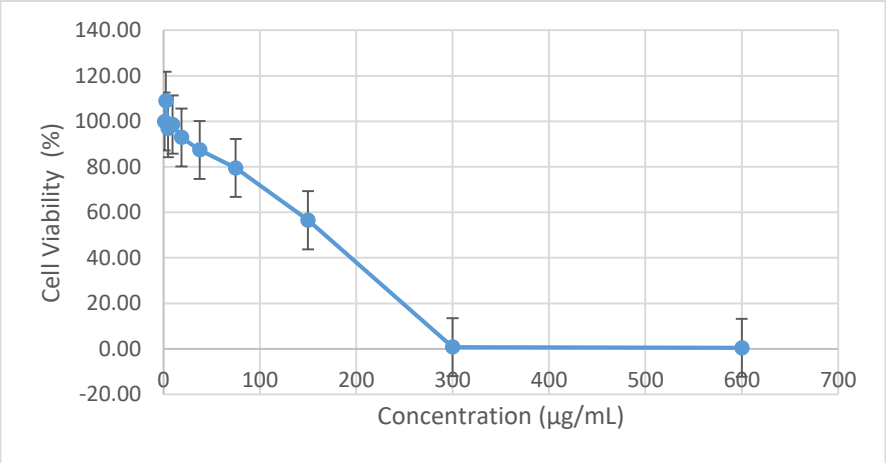

Figure 1. The relationship of mycelium extract concentration against the viability of Hela cell line.

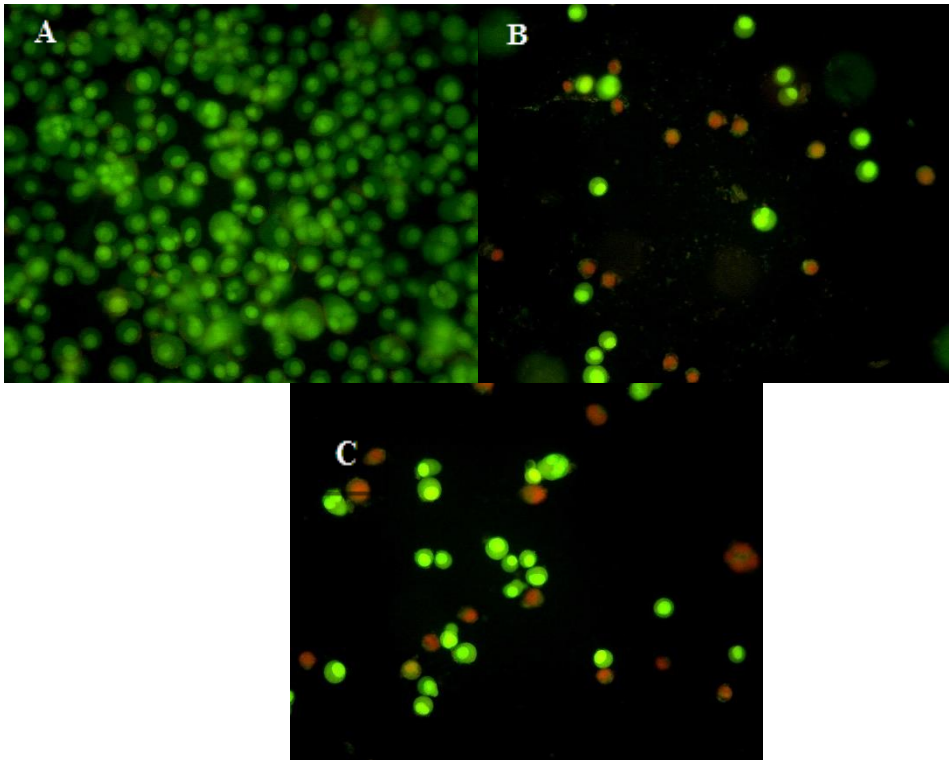

Figure 2. Results of apoptosis staining, A) control cells, B) concentration of 164 mg/mL, and C) the concentration of 82 mg/mL. The cells which are still alive appears green, and cells undergoing apoptosis will have the nucleus of the cell that appears orange to red.

Cytotoxicity assay of KU4 isolates mycelium crude extract were calculated using MTT assay.Based on the assay that was done, the results obtained in the form of graphs cytotoxicity assay concentration and cell viability are presented in Figure 1. After obtaining the IC_{50} values then continued in the stages of apoptosis staining using acridine orange/ethidium bromide (EtBr) as Figure 2.

DISCUSSION

This study is a follow-up research conducted by [8] who successfully isolated 16 strains Sponge-Associated Fungi obtained from *Ancorina* sp. This genus has been studied by [11], its ethanolic extracts has good ability as anti-virus and anti-cancer. In the study of [8], from 16 isolates of strains obtained, conducted testing of the anti-bacterial by using test bacteria from a strain of *Streptococcus aureus* and *Salmonella thyphii*. At the stage of testing an anti-bacterial, as many as three isolates with isolates code of KU4, KU3A, and KR4, has anti-bacterial activity with inhibition zone values generated sequentially, is 5.54 cm; 2.5 cm; and 1.58 cm. Based on the test results of anti-bacterial activity, the research carried out at this stage in vitro cytotoxicity assay using HeLa cell line to see the anti-cancer activity, and vero cells were used as controls. Cytotoxicity assay were performed using MTT Assay method. At this stage, as many as three isolates strain which has been obtained then, do the stages of fermentation using a medium Malt Extract (saline) for 10 days, to obtain secondary metabolites, and then extraction at medium and mycelia (mixture) of the three

fungal isolates using solvent Ethyl Acetate (EtOAc). After the crude extract is obtained, then the stages of MTT Assay.

In further research, will be tested against crude extract of mycelia isolates KU4. The stages that have been made as:

Subcultures of KU4 isolate

At this stage, isolates were grown back on medium KU4 Malt Extract Agar (MEA) using sea water obtained from coastal areas in the southern region of Yogyakarta. Isolates of KU4 incubated at room temperature with a temperature range of ± 27ºC. During the incubation period, can be seen that isolates of KU4 growth well, and had a white hypha. After subculture stage is done, isolates of KU4 then entered the stage of cultivation in Wickerham medium using sea water solution.

Cultivation Isolates KU4

Stages of cultivation is a stage of fermentation in KU4 isolates. At this stage, KU4 isolates were grown in saline water Wickerham media. Then continuedby incubation at room temperature, with conduct a shaker on media. This is done so that the aeration process is going well, and all parts of fungal mycelia may well hit by the medium. The incubation process is carried out for 21 days based on research of [10]. The incubation process is determined on the type of fungus is cultivated fungus growth. In general, mushroom cultivation to obtain a secondary metabolite can be carried out in the period 7-28 days. At this stage, 1 ose of subculture KU4 isolates were taken and inoculated on Wickerham saline water media. Furthermore, the observation of KU4 isolates growth in this medium. During the observation process, can be seen, that the KU4 isolates culture can grow well, with the formation of globular mycelium on the media.

Secondary metabolites extraction of KU4 isolate

After the cultivation process conducted over 21 days, KU4 culture contained in Wickerham saline water media enters the extraction stage of secondary metabolites. This stage using maceration method that draws on research of [10] and [12] with modifications according to Figure 3.

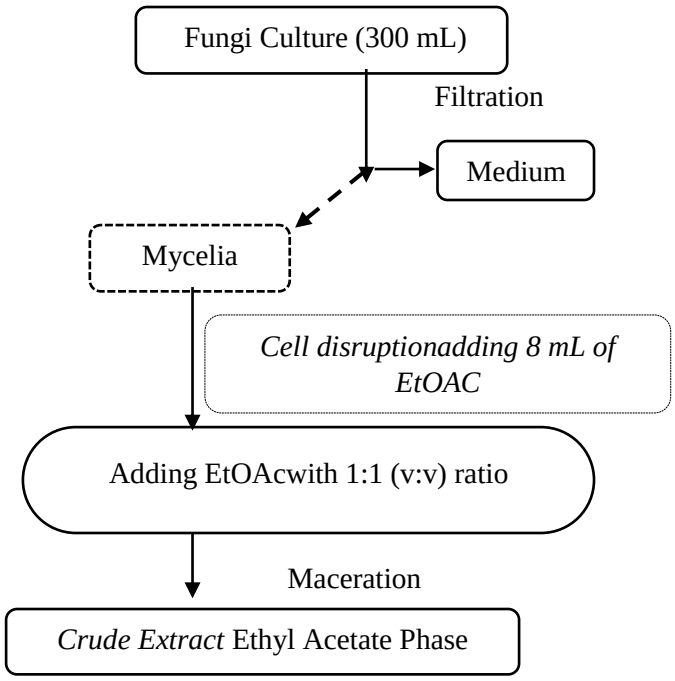

Figure 3. The secondary metabolite extraction method of KU4 isolates from EtOAc phase of the mycelium based on research of (10) and (12) with modifications.

At this stage, KU4 culture that has been aged 21 days, then filtered to separate the medium and KU4 mycelia. Then, mycelia which has been separated from the media added by 8 mL ethyl acetate then carried out using an Ultra Turax cell destruction. After that, macerated mycelia which have been destroyed by adding about 300 mL EtOAc and performed an extraction process using maceration method. Maceration process is used because it is still unknown properties of secondary metabolites produced. [11] explains that the nature of the crude ethanolic extract compounds from *Ancorina* sp. sponge have thermolabile nature, so it is also possible to isolate the compounds produced by KU4, which are fungi associated with this sponge, also possess thermolabile. After this stage, crude extract obtained from mycelium of KU4 isolate is crude extract with character is green and not smelling of 0.22 grams.

The cytotoxicity assay of crude extract from KU4 isolates mycelia

At this stage, mycelium crude extract obtained is dissolved using DMSO. A total of 6 mg of crude extract was weighed and dissolved in 20 µL DMSO. Then as many as 2 µL dissolved in 998 µL RPMI Complete Medium thus obtained will be tested as concentration of 600 µg/mL. Then proceed to the stage of dilution doses up to 1.17 µg / mL. Determines the concentration of the tested extract was based on IC_{50} values in previous studies that 383.88 µg/mL, resulting in advanced research of this concentration given above IC_{50} values in the previous study continued in serial dilution to a concentration of 1.17 µg/mL. A total of 20.000 cells were grown in 96 well plates, then in each well given tested extract concentration.

Cytotoxicity assay of KU4 isolates mycelium crude extract were calculated using MTT assay. In this method, living cells will reduce the compound MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium) with has colour of yellow to formazan crystalline compound with the colour of

purple after a given of stop solution. The amount of formazan crystals formed equivalent to the number of living cells.

In Figure 1. it can be seen that the IC_{50} values obtained from the extract crude of KU4 isolates mycelium against cervical cancer HeLa cell line amounted to 164 µg/mL. Based on IC_{50} values obtained, will be followed at this stage of Apoptosis Staining. [13] explains that the extracts that have IC_{50} values of less than 125 µg/mL are potential candidates for cancer therapies agents, while extracts which have IC_{50} values in the range of 125 µg/mL to 5000 µg/mL had a category of moderate to be developed into a drug for cancer therapy, and extracts that have IC_{50} values of more than 5000 µg/mL were less likely to be developed as a cancer therapeutic agent. In this case, crude extract of KU4 isolates mycelium can be developed further to become a candidate anti-cancer compounds, but required further processing such as crude extract fractionation and purification of compounds.

Apoptosis staining

Having obtained the IC_{50} values then continued in the stages of apoptosis staining using acridine orange/ethidium bromide. This will cause the staining of the cells are still alive appears green, and cells undergoing apoptosis will have the nucleus of the cell that appears orange to red. Concentrations given at this stage is the IC_{50} concentration and a half of IC_{50} concentration. At this stage the result as in Figure 2.

The surviving cells will be permeable to acridine orange so it will be green, whereas in cells undergoing apoptosis, cell membrane damage caused by a disruption of metabolism cause ethidium bromide can sign that will color the cell nucleus and thus appears orange to red. Based on the staining that has been done, it can be seen that the mycelium crude extract with EtOAc phase of SAF KU4 Isolates can induce apoptosis in HeLa cell line. In some literatures were described the possibility of peptide compounds that induce apoptosis through the mitochondrial pathway, by increasing the activity of the protein expression of apoptosis, which are Bcl-2 and Bcl-xl.

CONCLUSION

Based on the research that has been done shows IC_{50} value of mycelium crude extract at 164 µg/mL and were able to induce apoptosis in HeLa cell line.

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REFERENCES

[1] Bast RC, Kufe DW, and Pollck RE. Holland-Frei cancer medicine. 6th ed. BC Decker; 2000.
[2] Robbory SJ, Anderson MC, and Russel P.Pathology of the female reproduction tract.Philadelpia:Elsevier Science Limited; 2002.
[3] Williams L.A and Anderson A. New symptom measure in chronic myeloid leukemia. european hematology association. 16th Congress, Haematologica. 2006;96(2): S313-S314.
[4] Devi P, Solimabi W, Cheryl R, and Lisette DS. The *sponge*-associated bacterium *Bacillus licheniformis* SAB 1: a source of antimicrobial compounds.*Mar.Drugs*. 2010; 8,1203-1212.
[5] Muniarsih T dan Rachmaniar R. Isolasi substansi bioaktif antimikroba dari spons asal Pulau Pari Kepulauan Seribu. Prosidings Seminar Bioteknologi Kelautan Indonesia I '98. Jakarta: Lembaga Ilmu Pengetahuan Indonesia; 1999.
[6] Muniarsih, T. Metabolit sekunder dari spons sebagai bahan obat-obatan. Oseana; 2003.
[7] Arianto, IA. Sitotoksisitas ekstrak etanolik, metanolik, dan kloroform spons *Geodia* sp. terhadap sel HeLa (cervical cancer cell line). Skripsi. Yogyakarta: Fakultas Biologi. Universitas Gadjah Mada; 2012.
[8] Pertiwi, GA, Pramana AAC, Setiawibawa RAA, Zachary AS, dan Putra HE. Saatnya fungi melawan kanker: kajian senyawa bioaktif fungi yang berasosiasi dengan sponge terhadap proliferasi sel kanker HeLa teruji in vitro. Yogyakarta: Laporan Akhir PKMP. Universitas Gadjah Mada; 2014.

[9] Bewley CA. and Faulkner DJ. Lithistid *sponges*: star performers or hosts to the stars. *Angew Chem (Int Ed Eng)*.1998 [cited 2015 May 15] ;6(37): 2162–2178.
[10] Rusman, Y. Isolation of new secondary metabolites from sponge-associated and plant-derived endophytic fungi. Disertation. 2006.
[11] Nirwantono, R. Aktivitas antiretrovirus ekstrak etanolik sponge *Ancorina* sp. terhadap virus HIV-1. Yogyakarta: Skripsi. Fakultas Biologi. Universitas Gadjah Mada; 2014.
[12] Tarman, K. Biological dan chemical investigations of indonesian marine-derived fungi and their secondary metabolites. Greifswald: Innagural Dissertation: Ernst-Moritz-Arndt-Universitat; 2011.
[13] Manosroi J, Dhumtanom P. and Manosroi A. Anti-proliferative activity of essential Oil Extracted from Thai Medicinal Plants on KB an P388 Cell Lines. Elsivier; 2006.