

## CYTOTOXICITY OF AROMATIC COMPOUND FROM AN ENDOPHYTIC FUNGUS, *CLADOSPORIUM* SP. EN-S01

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Received: 10 Jul 2018, Revised and Accepted: 07 Sep 2018

### ABSTRACT

**Objective:** Endophytes have the potential to synthesize various bioactive secondary metabolites. The aims of the study were to isolate the major compound from endophytic *Cladosporium* sp. EN-S01., identify its function group, then evaluate its cytotoxicity.

**Methods:** The endophytic fungus was grown in potato dextrose broth and extracted using ethyl acetate. Secondary metabolites were isolated by chromatographic separation and re-crystallization, and function group was determined by infrared spectroscopy. *In vitro* cytotoxicity was evaluated by the prestobblue assay.

**Result:** The isolated aromatic compound evaluated by prestobblue assay against breast cancer cell line MCF-7 showed cytotoxicity with IC<sub>50</sub> value of 746,03 ppm.

**Conclusion:** Our findings indicate this aromatic compound might be useful lead compounds to develop cytotoxic drugs.

**Keywords:** Cytotoxicity, *Cladosporium*, Endophytic, Fungus, Prestobblue, Aromatic

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DOI: <http://dx.doi.org/10.22159/ijcpr.2018v10i6.30964>

### INTRODUCTION

Cancer is a genomic disease characterized by uncontrolled cell proliferation and can destroy body tissues. This disease ranks second cause of death after heart disease. The most common type of cancer in women is breast cancer [1]. Until now, cancer can be treated surgically, radiation therapy, hormones, target proteins or chemotherapy [2]. The treatment is not yet fully effective because it still affects normal cells. The search for more effective cancer treatment is done using natural chemical compounds that have cytotoxic activity.

This study will use endophytic fungus isolates from *Cladosporium* sp. EN-S01 which is isolated from macroalgae *Sargassum cinereum*. Preliminary tests have been conducted to evaluate the potency of this crude extract of fungus as an anticancer candidate. Breast cancer cell cytotoxicity testing was carried out on MCF-7 cell lines because these cell lines are very stable and can represent most invasive breast cancer cells. Wulandari *et al.* (2018) proved that the crude extract with fermented ethyl acetate solvent from *Cladosporium* sp. EN-S01 shows the potential for cytotoxic activity against MCF-7 breast cancer cell lines with IC<sub>50</sub> values of 8.46 µg/ml in very active category [3].

Important stages in the process of searching for new drugs (new drug discovery) in the fields of medical biotechnology and molecular biology are the discovery of drug molecules derived from plants, animals, and microorganisms by isolating and identifying the structure of pure compounds. Knowledge of the structure of pure compounds facilitates the study of lead compounds in molecular modeling. Anticancer validation of pure compounds is needed to be able to strengthen the potential of compounds to be developed into candidates for anticancer drugs [4].

Based on the things that have been described, it is necessary to identify pure compounds from secondary metabolites from fermented *Cladosporium* sp. EN-S01. The sample suspected to contain anticancer activity need to be re-evaluated for their cytotoxicity against MCF-7 cancer cell lines.

### MATERIALS AND METHODS

#### Fungal material

*Cladosporium* sp. EN-S01 was isolated from the marine brown algae *Sargassum cinereum*, collected from Pramuka Island, Kepulauan Seribu Marine National Park, Indonesia. This fungus was identified by morphological features, including the characteristic of ascospores and colonies. The pure cultures were deposited in the Laboratory of Microbiology, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

#### Cultivation, extraction, and isolation of secondary metabolite

*Cladosporium* sp. EN-S01 was cultivated in 1 l Erlenmeyer flask containing saline water, Potato Dextrose Broth, CaCO<sub>3</sub>, yeast extract, and chloramphenicol. The culture was incubated at 25 °C for 5 d. The filtered broth was extracted with ethyl acetate then evaporated under vacuum in a rotary evaporator, to give 1 g of the dark brown viscous mass of the crude ethyl acetate extract. Crude ethyl acetate extract was subjected to column chromatography for further fractionation using n-hexane and ethyl acetate. After crystallization gave fine needles crystal.

#### Infrared spectroscopy

The sample for IR analysis was using 2 mg of solid form sample, then crushed in small mortal together with 100 mg of dried potassium bromide become pellet. The pellet was placed in the IR spectrophotometer sample area for further analysis.

#### Cell culture

MCF-7 provided by Biological Activity Test Laboratory, Central Laboratory, Universitas Padjadjaran. Human cancer cell line was maintained as monolayer cultures in RPMI, supplemented with 10% Fetal Bovine Serum and 1% of the antibiotic solution under an atmosphere of 5% CO<sub>2</sub> at 37 °C. Cell was trypsinized confluent. A stock solution of the sample was prepared in 0, 1, 10, 100, and 1000 µg/ml.

#### Resazurin reduction assay

The cytotoxic from a secondary metabolite of fermented *Cladosporium* sp. EN-S01 was evaluated through prestobblue technique. The assay

was done with plated the cells in 96-multiwell culture plates at a density of  $0.8 \times 10^4 - 1 \times 10^4$  cells/well. Twenty-four hours after plating, the medium was discarded, and fresh medium containing the sample at different concentrations (1, 10, 100, and 1000  $\mu\text{g/ml}$ ) and control (0  $\mu\text{g/ml}$ ) was added. After 24 h incubation with the sample, the reagent of prestoblu was added at a final concentration of 0, 5 mg/ml and incubated for 2 h. The results are expressed as the percentage of cell proliferation relative to control (untreated cells) carried out in duplicate. Percentage of cell viability (CV) was calculated manually using the formula:

$$\% \text{ Cell Inhibition} = 100 - \text{Abs (sample)} / \text{Abs (control)} \times 100$$

Positive values (between 0 and 100%) can be interpreted as inhibition of cell proliferation. A dose-response curve was plotted to enable the calculation of  $\text{IC}_{50}$ . The  $\text{IC}_{50}$  values were determined using Probit analysis.  $\text{IC}_{50}$  corresponds to the concentration of the extract that decreases the number of viable cells by 50%. In this case, the absorbance in control corresponds to 100 % viability.

## RESULTS AND DISCUSSION

IR spectrum analysis is needed to determine the presence of functional groups in a sample. The results of IR spectrum analysis of fermented *Cladosporium* sp. EN-S01 compounds is shown in wavenumbers 3350 and 3264  $\text{cm}^{-1}$  indicate the O-H stretch, so this compound has an O-H functional group. In addition, there was a double bond which was marked by the stretch of C-H  $\text{sp}^2$  at a wave number of 3080  $\text{cm}^{-1}$ . The carbonyl group in this compound is characterized by a stretch of C = O conjugated at wave number 1688  $\text{cm}^{-1}$ . The absorption at wavenumber 1521  $\text{cm}^{-1}$  is a typical area of C = C aril, absorption at wavenumber 817  $\text{cm}^{-1}$  indicates the presence of C-H outside the field. Uptake in the wave numbers 1521 and 817  $\text{cm}^{-1}$  are characteristics of aromatic groups.

Test results of pure compounds on MCF-7 breast cancer cell lines with Presto Blue kits are shown in fig. 1. Pure compounds fermented by *Cladosporium* sp. EN-S01 has the effect of inhibiting proliferation of MCF-7 cell lines starting from a concentration of 1  $\mu\text{g/ml}$  as much as 17.51%. The percentage of inhibition of MCF-7 cell lines tended to increase to 23.38-26.20% when the concentration was increased to 10 or 100  $\mu\text{g/ml}$ , then continued to increase by 53.66% at a

concentration of 1000  $\mu\text{g/ml}$ . The percentage of inhibition of proliferation of MCF-7 cell lines is in line with the increase in sample concentration.

The  $\text{IC}_{50}$  value is obtained from the calculation process with probit analysis from the concentration-response range and inhibition of pure compounds from fermented *Cladosporium* sp. EN-S01 for cell death in inhibiting proliferation of MCF-7 cancer cell lines. Confirmation of accurate numbers that can be read on the percentage curve can be seen that the  $\text{IC}_{50}$  value is in the concentration range of 100-1000  $\mu\text{g/ml}$ . The results of calculations and probit analysis obtained  $\text{IC}_{50}$  values of 746.03  $\mu\text{g/ml}$ .

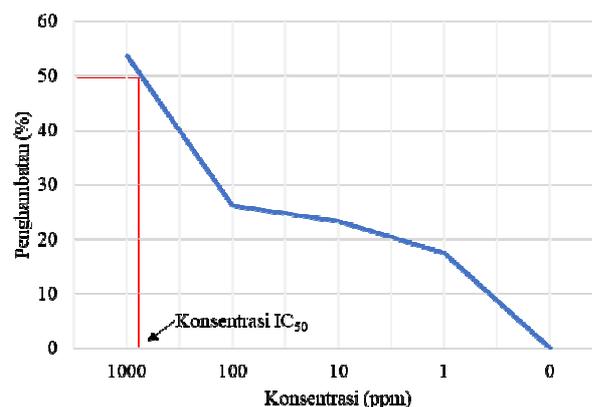


Fig. 1: Effect on antiproliferative of the aromatic compound from *Cladosporium* sp. EN-S01 on MCF-7 cell line

Research on cytotoxicity test of *Cladosporium* spp. Fermented compounds. MCF-7 breast cancer cell lines have not been widely reported. Table 1 shows information on the cytotoxicity test of several compounds, supplemented by the results of this study as a comparison.

Tabel 1: Cytotoxicity of the compounds from endophytic *Cladosporium* spp.

Species	Source	Compound	Assay	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ ) alley (1988)	Refrence
<i>C. cladosporioides</i> EN-399	Makroalga <i>Laurencia okamurai</i>	Cladosporol H	MTT	3,36 (active)	[5]
<i>C. cladosporioides</i> EN-399	Makroalga <i>Laurencia okamurai</i>	Cladosporol J	MTT	4,06 (active)	[5]
<i>Cladosporium</i> sp. KcFLg'	Bakau <i>Kandeliacandel</i>	Cladosporone A	Trypan Blue	7,50 (inactive)	[6]
<i>Cladosporium</i> sp. EN-S01	Makroalga <i>Sargassum cinereum</i>	Crude extract	MTT	8,46 (very active)	[3]
<i>Cladosporium</i> sp. EN-S01	Makroalga <i>Sargassum cinereum</i>	Aromatic compound	PrestoBlue	746,03 (tidakaktif)	Primary data

Li et al. (2017) isolate cladosporol H and cladosporol J from *C. cladosporioides* EN-399 from macroalgae *Laurencia okamurai*. Cytotoxic results using MTT assay showed that these compounds had  $\text{IC}_{50}$  values of 3.36 and 4.06  $\mu\text{g/ml}$ , respectively [5]. This value belongs to the active category according to Alley (1988). Ai et al. (2015) isolate cladosporone A from *Cladosporium* sp. KcFLg' origin of mangrove *Kandeliacandel*. The cytotoxic test of these compounds uses trypan blue assay which basically coloring dead cells. The  $\text{IC}_{50}$  value obtained is 7.50  $\mu\text{g/ml}$  and belongs to the inactive category. From the table, it can be shown that with the use of the prestoblu for sample of this study have  $\text{IC}_{50}$  values of 746.03  $\mu\text{g/ml}$  in the very inactive category.

Cell viability and cytotoxicity of compounds or extracts were assessed through measurement of mitochondrial activity. The most common mitochondrial activity was detected using tetrazolium and resazurin reduction assay. Tetrazolium reduction

assay, for example, MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), is an assay technique in the form of exposure to tetrazolium salts into cells after the incubation period after exposure of the test compound is complete. The exposure results are detected in colorimetry. Tetrazolium salt reacts with succinic dehydrogenase in the living cell mitochondria and produces purple formazan crystals. Because the crystals are insoluble, but colorimetric readings require dissolved formazan, then DMSO is described. This procedure is at risk of cytotoxic because if the DMSO concentration is overused. Resazurin (7-hydroxy-10-oxidophenoxazin-10um-3-one) reduction assay, for example, prestoblu, is a water-soluble dye. Resazurin reacts with the enzyme NADPH/NADH to live cell dehydrogenase to become resofurin. Resofurn dissolves in water so that it can be detected directly in colorimetry or fluorometry. The technique of using shorter and non-cytotoxic resazurin against test cells is the reason this technique is superior to tetralozium [7].

Boncler *et al.* (2014) compared the results of a plant compound test on human umbilical venous endothelial cell lines (HUVECs) between MTT assay and prestobblue. The results of colorimetric measurements using prestobblue produce a higher response value. However, the colorimetric and prestobblue MTT response values were similar in flourometry. These results conclude that viability testing using prestobblue in colorimetry should be avoided. But the viability test using MTT and prestobblue fluorometry is recommended [7].

The crude extract from *Cladosporium* sp. EN-S01 has an IC50 value of 8.46 µg/ml against MCF-7 (Wulandari *et al.*, 2018). This value includes a crude extract of strong anticancer potency but based on IC<sub>50</sub> pure compound from fermented *Cladosporium* sp. EN-S01 is not included in the active compound category, even though the compound is a major compound from its crude extract.

Evaluating the bioactivity of a compound indicates whether the activity is caused by a single or mixed compound. During the evaluation process, if the activity of a compound is lost or reduced from the crude extract, the possibility of pure compound activity is related to the synergy between a number of compounds contained in the crude extract, which, when separated, is not individually activated [4]. Thus, the inactivity of this aromatic compound is because this compound is synergistic with a mixture of other compounds in crude extracts.

#### CONCLUSION

In summary, a pure compound of secondary metabolites from fungus *Cladosporium* sp. EN-S01 from marine macroalgae *Sargassum cineurem* has a chemical structure of polyhydroxy aromatic. The IC50 value of pure compound fermented by *Cladosporium* sp. EN-S01 on MCF-7 breast cancer cell lines was 746.03 ppm, included in the inactive category. Further, investigations will be interesting to test the cytotoxic activity of compound against other cell lines and re-test with MTT assay.

#### ACKNOWLEDGMENT

The authors would like to acknowledge Academic Leadership Grant-Universitas Padjadjaran-2017 for providing the necessary funds to conduct the study.

#### AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: APW, DH, PA, Performed the experiments: RRINE, Analyzed the data: APW, RRINE, DH, Wrote the paper: RRINE

#### CONFLICT OF INTERESTS

Declared none

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