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by MonicaEva

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Discovering anticancer compound of ethyl acetate extract from RL1 code endophytic fungi culture derived by *Phyllanthus niruri* Linn leaves through cell cycle modulation in T47d cells

Yuyun Yuniati[1,*], Leny Yuliati[1], Eva Monica[2], Rollando Rollando[2]

[1] Program of Chemistry, Faculty of Science and Technology, Universitas Ma Chung, Malang, Indonesia

[2] Program of Pharmacy, Faculty of Science and Technology, Universitas Ma Chung, Malang, Indonesia

* Corresponding author: yuyun.yuniati@machung.ac.id

Abstract. Breast cancer is one of the most commonly diagnosed cancers and the leading causes of cancer death in women worldwide. Endophytic fungi becomes anticancer metabolite sources which alternatively in plants. The ethyl acetate extract from RL1 code of endophytic fungi from *Phyllanthus niruri* Linn has cytotoxic activity to T47D cells in vitro. This research objective is to discover active constituent of anticancer which selective in the ethyl acetate extract through cell cycle modulation of T47D cell. Identification of RL1 code endophytic fungi morphology was determined by using microscope. Secondary metabolite production was carried out through fermentation using PDB as medium during two weeks and the medium was extracted with ethyl acetate. Separate and purify method was done using TLC, TLC preparative, and liquid chromatography. Active isolate was characterized by IR, LC-MS, ^{1}H NMR, ^{13}C-NMR, and DEPT. In cytotoxic assay, T47D and Vero cells were cultured in the presence of pure isolate and was evaluated by MTT assay, whereas IC_{50} and Selectivity Index were used as the parameters to evaluate effectivity of anticancer. Cell cycle distribution was determined by flow cytometry and then analysed by using ModFit LT 3.0 program. Based on microscopic analysis, RL1 code of endophytic fungi is *Aspergillus* sp. The infrared, mass spectra, ^{1}H-NMR, ^{13}C-NMR, and DEPT signals confirm that isolate 4 is *N*-(3'-chloro-5'-oxobutyl)-1-methyl-5-phenyl-1*H*-pyrrole3-carboxamide. Isolate 4 have dose-dependent cytotoxic activity to T47D and Vero cells with their IC_{50} values of 8,3 and 124 μg/mL, respectively and Selectivity Index is 12,2. Isolate 4 at concentration of 0,9 μg/mL inhibit T47D cell cycle in S-phase. Discovering biosynthetic pathways and molecular mechanisms of cell cycle arrest in isolate 4 still needs to be further investigated.

Keywords: Cancer, Endophytic fungi, Fermentation

1. Introduction

Cancer is the leading cause of death in the world, which is 7.6 million death (around 13% of all deaths) in 2008, and estimated increasing to reach 13.1 million deaths in 2030 [1]. Breast cancer ranks first in cancer cases in women worldwide, with an incidence of 1,676,633. This cancer is the most common cause of cancer deaths in women [2]. One problem that often arises in cancer treatment is chemotherapy drug resistance (drug resistance) [3]. One agent Cancer chemotherapy that has caused resistance is

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doxorubicin [4]. Doxorubicin was originally developed as an agent antibiotics that have antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Candida albicans* [5]. Over time, doxorubicin is currently is a chemotherapy agent that is widely used in the treatment of epithelial cancer [6].

One of the plants used by the community traditionally as a multipurpose medicinal plant is at *Phyllanthus niruri* Linn. Empirically, *Phyllanthus niruri* Linn used by the people of Indonesian as a cure for infection and cancer [7]. Use of plant as a source of internal medicine large quantities can endanger the sustainability of the plant so that need to find other sources of bioactive compounds that are easier and more efficient. One of source of bioactive compounds is endophytic fungi [8].

In previous studies it was known that ethyl acetate extract results endophytic fungi fermentation RL1 code from *Phyllanthus niruri* Linn has activity antibacterial against Bacillus subtilis, Escherichia coli, and Staphylococcus aureus. This reinforces the potential for antibiotics. Beside from screening anticancer activity against several cancer cells, it is known that the RL1 endophytic fungi ethyl acetate extract is able to inhibit growth in vitro with the greatest activity against T47D cells. In this study, an active isolate search will be carried out on ethyl acetate extract of the endophytic fungi culture RL1 code from *Phyllanthus niruri* Linn that kills T47D breast cancer cells and structure determination the active compound, and its effect on the T47D cell cycle.

2. Material and Methods

2.1. Identification, Fermentation and Extraction

Endophytic fungi RL1 code which was isolated and purified from the *Phyllanthus niruri* Linn was identified based on the mycelium morphology. Observation of morphology of endophytic fungal mycelium with RL1 code a microscope was carried out at the Faculty of Biology, Brawijaya University. Secondary metabolite production was carried out by fermentation in the PDB media for 14 days and fermented filtrate partitioned liquid-liquid with ethyl acetate with a media comparison: ethyl acetate (3:1) three times. Compounds soluble ethyl acetate was allowed to evaporate to dryness.

2.2. Fractionation and Purity Test

Ethyl acetate extract was fractionated using Preparative Thin Layer Chromatography (PTLC) with silica gel 60 PF_{254} stationary phase and chloroform: methanol (3:7 v/v) mobile phase. Three stages of purity testing, namely first, identification by PTLC using three different types of mobile phases with different polarity, namely chloroform: methanol (6:4 v/v), ethyl acetate: hexane (6:9 v/v), and ethyl acetate: methanol (9:1 v/v). Second, identification of two-dimensional TLC with elution phase I i.e., ethyl acetate: methanol (1:9 v/v) and elution II mobile phase namely ethyl acetate: hexane (9:1 v/v). Third, using liquid chromatography (LC-MS) with the mobile phase of methanol: water ratio 95:5. The most active fraction identified by spray reagent, spectrophotometer FTIR, LC-MS, ^{1}H-NMR, and ^{13}C-NMR.

2.3. Cytotoxic Test and Cell Cycle Modulation

T47D breast cancer cells were grown with RPMI culture media while Vero normal cells in M199 culture media, each containing 10% FBS (Gibco), 1% penicillin-streptomycin (Gibco), and 0.5% fungizon (Gibco). For cytotoxicity test using the MTT method. Cell viability was determined by absorbance at λ 595 nm using a plate reader. Absorption absorbance data were converted into percent viability and used to calculate IC_{50}. The Selectivity Index (SI) is a quotient between Vero cell IC_{50} and T47D IC_{50} cells. Modulation of the T47D cell cycle was carried out using a 6-well plate with a number of cells of $5x10^5$ cells/wells which were incubated for 24 hours in a CO_2 incubator. The isolate 4 with a concentration of 0.4 µg/mL was added and incubated again overnight. At the end of the incubation time, all cells were harvested and given a PI solution to be immediately run on the flow cytometer.

3. Results and Discussion

The identification results show endophytic fungi RL1 code is the *Aspergillus sp*. Research is needed more about the species of the BS1 fungi. Fungi cultivation of Aspergillus sp. done in Potato Dextrose Broth liquid media use 500 mL erlenmeyer. The fermentation process was done in closed system (batch) and this process was carried out until the fungi in the stationary phase which is marked by changes in media consistency becomes thicker and the amount and volume of mycelium in the fermentor did not increase. Secondary metabolite compounds produced by Aspergillus fungi sp. extracted on culture media which has been separated from the mycelium. The extraction yield is 0.42% b/v.

Fractionation of ethyl acetate extract using PTLC method. Chromatogram which was obtained was seen under UV $_{254}$ and UV$_{366}$ (Fig. 1), then separated into five fractions, namely fractions 1, 2, 3, 4 and 5. Inside criteria choosing anticancer compounds against breast cancer cells is based on potential, selectivity, isolation and adequacy of compounds for tested and further developed. Fraction 4 is more selective in killing cancer cells T47D breast compared to Fraction 3 (table 1). This can be seen from selectivity index was Fraction 4 which is 1.5 times higher than SI Fraction 3. Fraction 4 is relatively purer with 1 spot on identification by TLC when compared to fraction 3 which produces three spots. Even though the fraction yields 3 (30.9% b/b) higher than fraction 4 (4% b/b) but needs to be considered purity level 3 which still has at least three spots.

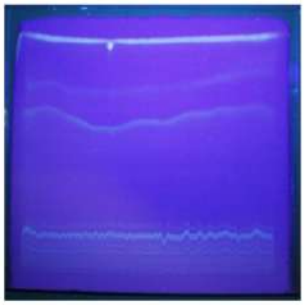
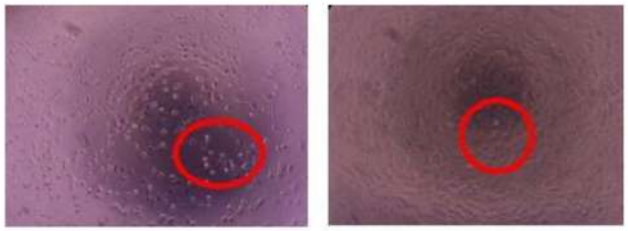


Figure 1. Chromatogram of TLC preparative from ethyl acetate extract in UV$_{366}$.



(a) (b)

Figure 2. Effect of fraction 4 treatment on T47D cells. (a) Normal cells, (b) 5 μg/mL.

Table 1. Cytotoxic activity of fraction test results.

Fraction	IC_{50} T47D Cell (μg/mL)	IC_{50} Vero Cell (μg/mL)	*Selectivity index* (SI)
1	123.02±2.45	293.34±0.23	2.38
2	83.93±0.83	112.93±3.91	1.34
3	45.84±2.94	153.82±0.83	3.35
4	8.3 ±0.01	124±0.05	12.2
5	19.83±0.11	360.54±0.65	18.18

IC_{50} = *Inhibit concentration*

From the results of the purity test, fraction 4 is said to be purely in TLC and relative purely by liquid chromatography so that isolates are called 4. Identification of isolates 4 showed positive results with blue fluorescent spots under the light UV_{366} after spraying with cerium sulphate. According to Guti'rrez [9], results the positive indicates the presence of alkaloids or compounds that contain nitrogen. The results of IR, MS, ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra confirmed the presence of pyrrole alkaloid compounds with chemical structure N-(3'chloro-5'-oxobutyl)-1-methyl-5-phenyl-1H-pyrrole-3-carboxamide. Isolate 4 had cytotoxic activity against T47D cells and Vero cells with IC_{50} values of 8.3 µg/mL and 124 µg/mL, respectively. Isolate 4 inhibits the S phase at a concentration of 0.9 µg/mL.

4. Conclusion

Isolate 4 is *N*-(3'-chloro-5'-oxobutyl)-1-methyl-5-phenyl-1*H*-pyrrole3-carboxamide. Isolate 4 have dose-dependent cytotoxic activity to T47D and Vero cells with their IC_{50} values of 8,3 and 124 μg/mL, respectively and Selectivity Index is 12,2. Isolate 4 at concentration of 0,9 μg/mL inhibit T47D cell cycle in S-phase.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6