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Combinational effects of ethylacetate extract of *Zanthoxylum acanthopodium* DC. with doxorubicin on T47D breast cancer cells

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Abstract: Andaliman fruit (*Zanthoxylum acanthopodium* DC.) is an ethnic plant from North Sumatera, Indonesia. This plant include of Zanthoxylum genus, Rutaceae family. This research having purpose to evaluate the effects of ethylacetate extract (EAE) of *Zanthoxylum acanthopodium* DC. fruits and combination with doxorubicin on cytotoxicity and cell cyle inhibition on T47D cell lines. The in vitro cytotoxicity effects were determined using MTT assay. Analysis of cell cycle distribution was performed using flowcytometer and the data was analyzed using ModFit LT 3.0 program. Cytotoxicity activity and combination of EAE with doxorubicin were evaluated using the MTT assay. The combination represents higher inhibitory effect on cell growth than the single treatment of doxorubicin on T47D cell lines. The combination changes the accumulation of cell cycle phase on G_0-G_1. Based on the results, EAE is potential to be developed as co-chemotherapeutic for breast cancer by cell cycle arrest. However, the apoptosis and molecular mechanism needs to be explored further.
Keywords: Andaliman fruit, *Zanthoxylum acanthopodium* DC, cell cycle, ethylacetate, flowcytometry.

1. Introduction

The diversity of medicinal plants in Indonesia is one of chances in development potential of Indonesia in the globalization era[1]. The use of medicinal plants in the community is increasing in several decades[2,3]. Indonesia has thousands of islands with various plants in it and the manners of community using plants as treatment for every disease traditionally[1].

Andaliman fruits (*Zanthoxylum acanthopodium* DC.) is an ethnic plant include Zanthoxylum genus, Rutaceae family[4]. Zanthoxylum showed no cytotoxic effect on normal cells (Vero cells) so that potential as an anticancer drug[5]. Andaliman fruit contains many compounds that are antioxidants[6]. Breast cancer is a type of cancer that most often affects women and leading cause of death in women, and based on the US data in 2010 breast cancer is the most common cancer with 209.060 new cases[7].

In previous study, the n-hexane, ethylacetate and ethanol extract showed a cytotoxic effect on breast cancer MCF-7 cell lines with IC_{50} value of 159.747 µg/mL; 136.490 µg/mL and 957.499 µg/mL[8]. Extract that having IC_{50} values <100 µg/mL is categorized as potent extract[9]. Based on the IC_{50} value of ethylacetate

extract >100 µg/mL but having the lowest IC_{50} value if compare with other extracts, the study was attempted to combine the ethylacetate extract with doxorubicin and evaluate the mechanism on cell cycle inhibition on T47D cell lines.

2. Material and methods

2.1 Plant material

Fresh fruits of *Zanthoxylum acanthopodium* DC. was collected from Onan Rungu village, Samosir regency, Sumatera Utara province, Indonesia. *Zanthoxylum acanthopodium* DC. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium. Doxorubicin (Ebewe). DMSO (Sigma), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), propidium iodide kit (Becton Dickinson).

2.2 Preparation of ethylacetate extract (EAE)

The air-dried and powdered leaves of *Zanthoxylum acanthopodium* DC. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder were dried in the air and extracted with ethylacetate (3x3 d, 7.5 L) at room temperature on a shake. The filtrate was collected, and then evaporated under reduced pressure to give a viscous extract and then freeze dried to give a dried extract.

2.3 Cytotoxicity assay

The combination of ethylacetate extract and doxorubicin were submitted to cytotoxicity test. In that way, T47D cell line was grown in RPMI 1640 medium, while Vero cell line was grown in M199 medium containing 10% FetaL Bovine Serum (Gibco), 1% penicillin-streptomycine (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37^{o}C. The inoculums seeded at 10^4 cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by EAE in combination with doxorubicin. After incubation 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h in 37^{o}C. Viable cells react with MTT to produce purple formazan crystals. After 4 h, SDS 10% as stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using ELISA reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells [10]. The selectivity index was calculated using equation where IC_{50} on Vero cells were divided with IC_{50} on T47D cells [11].

2.4 Flowcytometry assay

2.4.1 Cell cycle inhibition assay

T47D cells ($5x10^5$ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with EAE, doxorubicin and their combination, and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using tripsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20^{o}C for 2 h. The cells were washed thrice with cold PBS and resuspended then centrifuged 3000 rpm for 3 min and PI kit (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37^{o}C for 30 min. The samples were analysed using FACScanflowcytometer. Based on DNA content, percentage of cells in each of stage in cell cycle (G1, S and G2/M) were calculated using ModFit Lt. 3.0.s[12].

2.5 Statistical analysis

All data were analyzed using regression using SPSS 20.

3. Results

This research was aimed to investigate the efficacy of EAE as a co-chemotherapy on doxorubicin treatment. EAE, doxorubicin and their combination were investigated for their cytotoxicity effect on T47D cell lines, and selectivity was measured on Vero cells. MTT method was using to determined cell viability after incubation for 24 h. In every treatment (EAE, doxorubicin and their combination) was showed the inhibition of cells growth. The IC_{50} value of EAE 24.476 µg/ml and doxorubicin 348.72 nM, and the combination was

showed higher inhibitory effect if compare with single treatment. The optimum combination index (synergistic effect) was showed in 2/8 IC_{50} value of EAE and 1/16 IC_{50} value of doxorubicin (7.5 µg/mL- 25 nM) categorized with strong synergistic effect (CI <0.1). These effects supposed to be related to cell cycle modulation.

To measure the selectivity of EAE, we were executed cell viability assay on Vero cells. Single treatment of EAE showed cytotoxicity effect on Vero cells with IC_{50} 148.203 µg/mL. We were compared IC_{50} of EAE on Vero cells to T47D cells to find selectivity index (SI) [13]. SI of EAE is 6.05, SI> 3 is supposed to be selective to T47D cell lines. The result showed that EAE is selective to T47D cells instead of Vero cells.

To evaluate the effect of EAE in combination with doxorubicin to increased cell death by modulating cell cycle, we concentrated on it for further studies using flowcytometry method. The effect of combination is given in Figure 1. Whereas, their combination were exhibited higher at G_0/G_1 (80.83%) on 2/8: 1/16 IC_{50}. These fact was indicated that EAE can increase doxorubicin cytotoxic effect at G_0/G_1 phase.

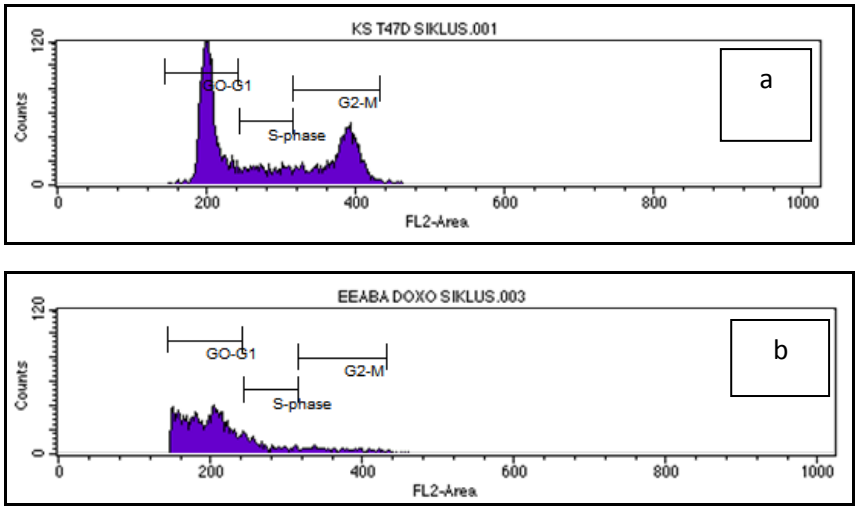

Figure 1. Cell cycle analysis using flowcytometry. T47D cells were treated by their combination (EAE and doxorubicin) for 24h and stained using propidium iodide. (a) control cells; (b) combination of doxorubicin 25 nM and EAE 7.5 µg/mL. Combination of EAE and doxorubicin exhibited G_0/G_1 phase and decreased T47D cell population.

4. Discussion

The fruits of *Zanthoxylum acanthopodium* DC. are used in North Sumatera to seasoning[14]. Although some compound have been identified as possesing medicinal properties, none of these compounds has ever reached clinical trials.

The cytotoxicity estimate of natural product is related to content of active compound in these plants including *Zanthoxylum acanthopodium* DC.Flavonoids and triterpenoids/steroids estimated as active compound[15]. We were evaluated the activity of ethylacetate extract on cytotoxicity and cell cycle of T47D cells line with single treatment and in combination with doxorubicin. We were also investigated selectivity of EAE on Vero cells. EAE showed selectivity on T47D cells line if compared to Vero cells using SI value[13].

Doxorubicin is one of chemotherapeutic agent showing strong activity on T47D cell lines with IC_{50} value of 348.72 nM. T47D cells line undergo resistant to doxorubicin pass through to p53 mutation [16,17]. To decrease the toxic effect and prevent resistance from doxorubicin to T47D cells, combination of small concentration of doxorubicin with EAE is required. In this study, combination of EAE and doxorubicin were showed very strong synergism activity on T47D cell lines. EAE was enhanced the cytotoxicity activity of doxorubicin on T47D cell lines if compared to single treatment of either EAE or doxorubicin. The strongest synergistic effect was suggested to be related to a cell cycle modulation.

In the cell cycle analysis, combination of EAE with doxorubicin were exhibited higher G_0-G_1 phase accumulation if compared to single treatments of EAE or doxorubicin. This analysis was also showed cells undergo apoptosis, showed by occurenced apoptosis during inhibition of cell cycle on G_0-G_1 phase[18].

However, the molecular mechanism of cell cycle modulation by this combination need to be explored more detail. Based on the results, we were concluded that combination of ethylacetate extract of *Zanthoxylum acanthopodium* DC. fruits and doxorubicin very stong synergically increases the cytotoxicity activity of doxorubicin through cell cycle arrest. The extract is potential to developed as co-chemotherapeutic agent for doxorubicin in breast cancer therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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