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Original Article

COMBINATIONAL EFFECTS OF ETHYLACETATE EXTRACT OF *PICRIA FEL-TERRAE* LOUR AND DOXORUBICIN ON T47D BREAST CANCER CELLS

DENNY SATRIA[1]*, MAINAL FURQAN[2], SUMADIO HADISAHPUTRA[2], ROSIDAH[2]

[1]Department of Pharmaceutical Biology, [2]Department of Pharmacology, [1,2]University of Sumatera Utara, Medan, 20155, Indonesia
Email: denny.satria.dennysatria@gmail.com

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ABSTRACT

Objective: To evaluate the effects of ethyl acetate extract (EAE) of *Picria fel-terrae* Lour. Leaves and combination with doxorubicin on cytotoxicity, cell cycle, apoptotis and suppression of cyclin D1 and Bcl-2 expression on T47D cell lines.

Methods: The *in vitro* cytotoxicity effects were determined using MTT assay. Analysis of cell cycle distribution was performed using flow cytometer and the data was analyzed using ModFit LT 3.0 program. Apoptotis assay was analyzed using flow cytometer using Annexin V. The suppression of cyclin D1 and Bcl-2 expression on T47D cell lines was identified using immune cytochemistry.

Results: 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 from G_0-G_1. The combination also increases apoptosis. The combination also showed suppression of cyclin D1 and Bcl-2 expression in T47D cell lines.

Conclusion: Based on the results, EAE is potential to be developed as co-chemotherapeutic for breast cancer by inducing apoptosis, cell cycle arrest and suppressing cyclin D1 and Bcl-2 expression. However, the molecular mechanism needs to be explored further.

Keywords: Combination, *Picria fel-terrae* Lour., T47D, doxorubicin.

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 the community using plants as treatment for every disease traditionally [1]. Poguntano (*Picria fel-terrae* Lour) in east and southeast Asia has been used traditionally as a stimulant, diuretic, anti malaria, anti hyperglycemia, fever, herpes infection, cancer and inflammation for over 200 years [4]. Breast cancer is a type of cancer that most often affects women and the 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 [5].

In previous study, the n-hexane, ethyl acetate and ethanol extract showed a cytotoxic effect on breast cancer T47D cell lines with IC_{50} value of 509.744 µg/ml; 97.92 µg/ml and 306.435 µg/ml [6]. Extract that having IC_{50} values<100 µg/ml is categorized as potent extract [7]. Based on the IC_{50} value of ethyl acetate extract<100 µg/ml. The study was attempted to combine the ethyl acetate extract with doxorubicin and evaluate the mechanism on T47D cell lines.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Picria fel-terrae* Lour were collected from Tiga Lingga village, Dairi regency, Sumatera Utara province, Indonesia. *Picria fel-terrae* Lour. 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), H_2O_2 (Lab Vision Plus), chromogen 3,3-diaminobenzidin (DAB) (Novo Castra).

Preparation of ethyl acetate extract (EAE)

The air-dried and powdered leaves of *Picria fel-terrae* Lour. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and extracted with ethyl acetate (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.

Cytotoxicity assay

The combination of ethyl acetate extract and doxorubicin was 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°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°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 [8]. The selectivity index was calculated using an equation where IC_{50} on Vero cells were divided with IC_{50} on T47D cells [9].

Flowcytometry assay

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 °C for 2 h. The cells were washed thrice with cold PBS and re suspended then centrifuged 3000 rpm for 3 min and PI kit (containing PI 40 µg/ml and RNAse 100 µg/ml) added to sediment and re suspended and incubated at 37 °C for 30 min. The samples were analysed using FACS can flowcytometer. Based on DNA

content, percentage of cells in each of stage in cell cycle (G1, S and G2/M) was calculated using ModFit Lt. 3.0. s.

Apoptosis 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 centrifuged in 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 suspended then centrifuged 3000 rpm for 3 min and Annexin V kit added to sediment and suspended and incubated at 37 ^{o}C for 30 min. The samples were analyzed using FAC Scan flow cytometer [10].

Immuno cytochemistry

T47D cells (5x10^4 cells/well) were seeded on cover slips in 24-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. After incubation, the cells were washed with PBS and then fixed with cold methanol at 4^{o}C for 10 min. After that, the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature, incubated using primary antibody Bcl-2 and cyclin D1 for 1 h, then washed thrice with PBS, then incubated with secondary antibody for 10 min. The cells were washed with PBS, then incubated in 3,3-diaminobenzidin (DAB) solution for 10 min, and washed with aquadest. Afterward, the cells were counterstained with Mayer-Haematoxylin for 5 min, and the coverslips were taken and washed with aquadest, and then immersed with xylol and ethanol 70%.

Statistical analysis

All data were analyzed using regression using SPSS 20.

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 99.404 µg/ml and doxorubicin 1.8 µg/ml, and the combination was showed higher inhibitory effect if compare with single treatment. The optimum combination index (synergistic effect) was showed in 1/8 IC_{50} value of EAE and 1/8 IC_{50} value of doxorubicin (12.5 µg/ml-0.25 µg/ml) categorized with strong synergistic effect (CI<0.1). These effects supposed to be related to apoptotic induction and 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} 451.084 µg/ml. We were compared IC_{50} of EAE on Vero cells to T47D cells to find selectivity index (SI) (12). SI of EAE is 4.537, 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 flow cytometry method. The effect of combination is given in fig. 1. Whereas, single treatment of doxorubicin on ½ IC_{50} caused cell accumulation at G_0/G_1 (58.77%), and single treatment of EAE on IC_{50} caused cell accumulation at G_0/G_1 (62.33%). Furthermore, their combination was exhibited higher at G_0/G_1 (71.24%) on 1/8: 1/8 IC_{50}. This fact was indicated that EAE can increase doxorubicin cytotoxic effect at G_0/G_1 phase.

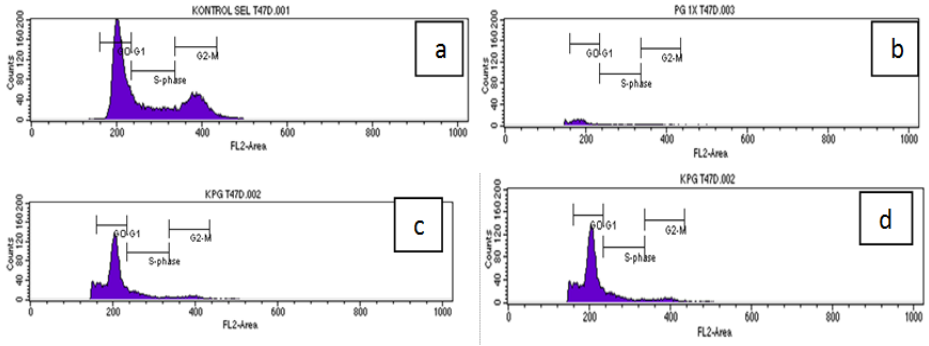

Fig. 1: Cell cycle analysis using flowcytometry. T47D cells were treated by doxorubicin, EAE, and theircombination for 24h and stained using propidium iodide. (a) control cells; (b) EAE 100 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of doxorubicin 0.125 µg/ml and EAE 12.5 µg/ml. Combination of EAE and doxorubicin exhibited G_0/G_1 phase and decreased T47D cell population

Cyclin D1 and Bcl-2 (anti apoptotic protein) are proteins that play a role in cell cycles and apoptotic process. In the study, effect of EAE, doxorubicin and their combination on cyclin D1 and Bcl-2 expressions was evaluated using immunocytochemistry. Expression of cyclin D1 and Bcl-2 protein is positive characterized by brown stained nuclei in the cells (fig. 3 and fig. 4).

In untreated cells (negative control) high intensity for cyclin D1 and Bcl-2 was found. A single treatment of EAE decreased on cyclin D1 and Bcl-2 expression, single treatment of doxorubicin did not decrease cyclin D1 and Bcl-2 expression. Combination of EAE and doxorubicin was decreased cyclin D1 and Bcl-2 expression. It was showed that combinational treatment decreases cyclin D1 and Bcl-2 expression.

DISCUSSION

The leaves of *Picria fel-terrae* Lour. are used in North Sumatera to treat hyperglycemia patient. Although some compound has been identified as possessing medicinal properties, none of these compounds has ever reached clinical trials. Moreover, the anticancer effect of *Picria fel-terrae* Lour. have not been validated *in vitro* to date based on their use in Indonesia or other system of medicine.

The cytotoxicity estimate of the natural product is related to content of active compound in these plants including *Picria fel-terrae* Lour. Flavonoids and triterpenoids/steroids estimated as active compound [12]. We were evaluated the activity of ethylacetate extract on cytotoxicity, cell cycle, apoptotic induction, and inhibition of cyclin D1 and Bcl-2 expression of T47D cells line with the 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 agents showing strong activity on T47D cell lines with IC_{50} value of 1.8 µg/ml. T47D cells line undergoes resistant to doxorubicin pass through to p53 mutation [14, 15]. 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 was 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 an apoptotic induction and cell cycle modulation. In apoptotic study, combination of EAE with doxorubicin increased the cells undergo apoptosis in late apoptosis phase/early necrosis if compared to single treatment on T47D cell lines. Apoptosis is a process of programmed cell death with changes on morphology, membrane blebbing and chromatine [16]. Inhibition of anti apoptosis protein expression as Bcl-2 strengthens the mechanism of apoptosis from the combination of EAE with doxorubicin [17].

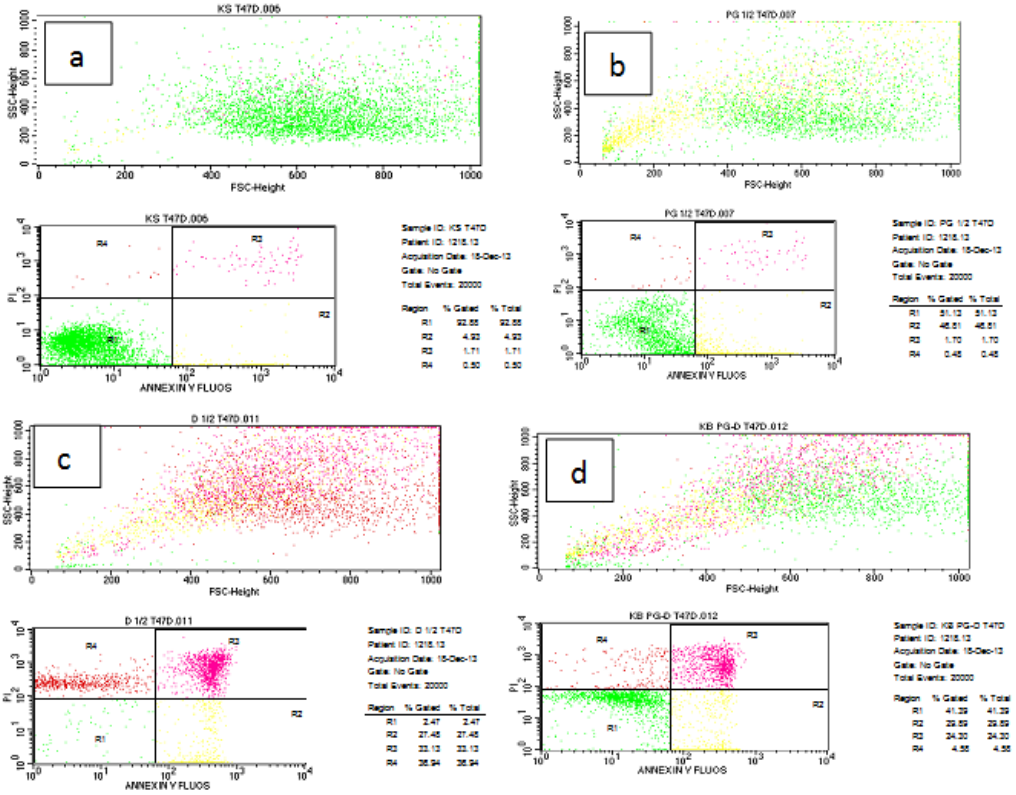

Fig. 2: Apoptotic analysis of EAE, doxorubicin and their combination on T47D cell lines (a) control cells; (b) EAE 100 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of doxorubicin 0.125 µg/ml and EAE 12.5 µg/ml. Evaluation of apoptosis induction was performed using flowcytometry method with Annexin V. as shown in fig. 2. The cells in the upper and lower right quadrants represent late apoptotic/necrotic and early apoptotic cells, respectively. The percentage of EAE, doxorubicin and their combination in early apoptotic 46.81%; 27.48% and 29.89%, in late apoptotic/early necrotic 1.70%; 33.13% and 24.30%, and in late necrotic 0.48%; 36.94% and 4.58%.

In the cell cycle analysis, combination of EAE with doxorubicin was 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 occurrence apoptosis during inhibition of cell cycle on G_0-G_1 phase [18].

Inhibitions of cyclin D1 protein expression strengthen the mechanism of modulating cell cycle especially in inhibition of cell cycle on G_0-G_1 phase. Cyclin D1 is a cyclin that role in G_0-G_1 phase with established complex with CDK-4 or CDK-6 to controlled G_1 to S phase transition [19].

Inhibition of cell cycle with combination EAE with doxorubicin were decreased level of cyclin D1 which resulted in inhibition of pRb phosphorylation so that E2F can not apart from pRb, and cells can not transcribes genes that needed in cell cycle process or cell proliferation [20,21].

However, the molecular mechanism of apoptosis induction and cell cycle modulation by this combination need to be explored more detail. Based on the results, we were concluded that the combination of ethyl acetate extract of *Picria fel-terrae* Lour. leaves and doxorubicin very stong synergically increases the cytotoxicity activity of doxorubicin through apoptosis, cell cycle arrest, decrease cyclin D1 and Bcl-2 protein expression.

The extract is potential to develop as co-chemotherapeutic agent for doxorubicin in breast cancer therapy.

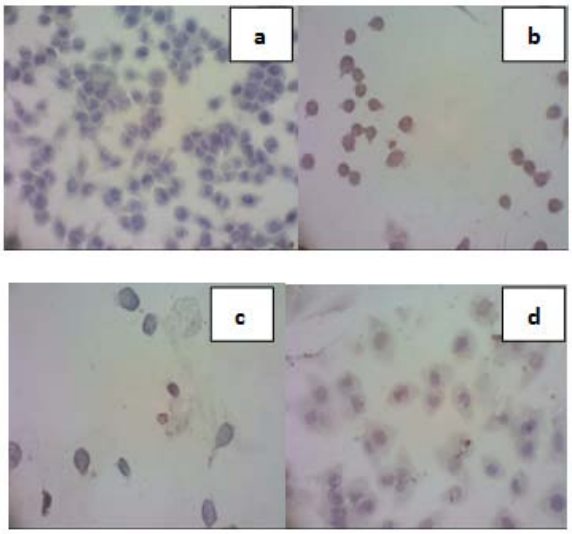

Fig. 3: Expression of cyclin D1 on T47D cells using immunocytochemistry. (a) control cells; (b) EAE 100 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of doxorubicin 0.125 µg/ml and EAE 12.5 µg/ml

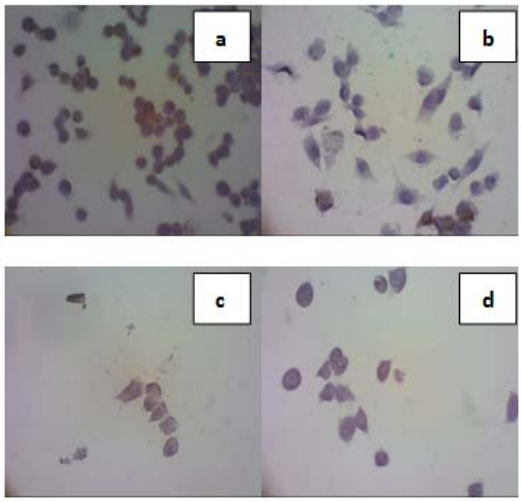

Fig. 4: Expression of Bcl-2 on T47D cells using immunocytochemistry. (a) control cells; (b) EAE 100 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of doxorubicin 0.125 µg/ml and EAE 12.5 µg/ml

CONCLUSION

According to the result obtained EAE is potential to be developed as co-chemotherapeutic for breast cancer by inducing apoptosis, cell cycle arrest and suppressing cyclin D1 and Bcl-2 expression.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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