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Research article

Combination effect of ethylacetate extract leaves of *Moringa oleifera* L. and Doxorubicin against MCF-7 cell lines

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Key words: Combination effect, *Moringa oleifera* L., MCF-7, Doxorubicin, Ethylacetate.

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Abstract

Objective: This study aim is to determine the effect of ethylacetate extract (EAE) of *Moringa oleifera* L. leaf and its combination with doxorubicin on cytotoxic effect, combination effect, cell cycle, apoptosis on MCF-7 cell lines. **Methods**: In vitro cytotoxic assays was determined by MTT (Microculture Tetrazolium Tehnique) assay, combined test was analysis with Compusyn software version 1, cell cycle inhibition and apoptosis were determined with Flowcytometry. **Result**: Cytotoxic activity of EAE with IC_{50} value 149.29 µg/mL, combination test of very strong synergistic at 1/8 IC_{50} EAE-1/4 IC_{50} doxorubicin concentration. The combination were caused cell accumulation in the G_0/G_1 phase, increased early and late apoptosis on MCF-7 cell lines. **Conclusions**: Based on the results combination giving a very strong synergistic effect. Therefore more research is needed on the mechanism.

Introduction

The therapy strategies available to cure breast cancer including cytotoxic agents (chemotherapy) have been considerable, but these cytotoxic agents have adverse effects on healthy tissue. One of the most widely used chemotherapy is doxorubicin [1], but its use provides side effects against normal tissue [1-3]. Dose reduction is can reduce the side effects of doxorubicin [4]. Therefore it becomes a challenge to improve clinical application of chemotherapeutic agents to be more effective. One of the strategies now gaining attention is the use of co-chemotherapy which has a synergistic effect with chemotherapeutic agents to improve their efficacy and reduce their toxicity to healthy tissue. Therefore, research on chemoprevention agents has synergistic effects when combined with anticancer drugs.

One of the chemoprevention agents that can be used is the leaves of *Moringa oleifera* L., or better known as daun kelor in Indonesia. This plant is known to be potent anticancer by increasing apoptosis and inhibiting the proliferation of cancer cells [5-6]. Moringa leaves contain flavonoids (kaempferol, quercetin, myricetin), alkaloid (moringine), benzyl isothiocyanate (BITC), phenetyl isothiocyanate (PEITC) and glucosinolate which is particularly useful as cancer cell chemoprevention [6-9]. Previous research on the effect of ethanol extract of Moringa leaves gave a synergistic effect of increasing the cytotoxicity of doxorubicin in Hela cells, where this extract increased apoptotic induction compared to doxorubicin alone [10], while breast cancer cells had never been done. The aim of this study want to examine whether *Moringa oleifera* L. leaves has synergistic efficacy with chemotherapy agent doxorubicin so it could be decrease its effective dose which also reduces the toxicity of chemotherapy agent.

Materials and methods

Plant and chemicals materials

Fresh leaves of *Moringa oleifera* L. were collected from Bukit Batrem Village, East Dumai District, Riau Province, Indonesia. *Moringa oleifera* L. was identified in Herbarium Medanense (MEDA) University of Sumatera Utara no. 1785/MEDA/2017. Chemicals used were Doxorubicin (Ebewe), DMSO (Sigma), [3- (4,5-dimethylthiazole-2-yl) -2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), Propidium Iodide kit (Biolegend), Annexin V (Biolegend).

Preparation of Ethylacetate Extract (EAE)

The technique was used in graded maceration, 600 grams of leaf powder *Moringa oleifera* L. was macerated with n-hexane (3x 6L), powder dried in air and re-extracted with ethylacetate (3x 6L), filtrate collected and filtered using filter paper Whatman no 42 (pore size 2.5 µm) and concentrated with rotary evaporator then inserted in freeze-dried to produced dry extract [11]. This study use ethylacetate as a solvent due to its characteristic as a semi

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polar solvent which can pull out polar and non polar compound

Phytochemical screening of ethylacetate extract

Phytochemical screening carried out on ethylacetate extract leaves of *Moringa oleifera* L includes examining the chemical secondary metabolites of alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids.

Dosage of extract and docorubicin

The treatment of extract used several concentration series of 1000 µg / mL; 500 µg / mL; 250 µg / mL; 125 µg / mL; 62.5 µg / mL; 31.25 µg / mL and 15.625 µg / mL. The treatment of doxorubicin used several concentration series of 24.00 µg / mL, 12.00 µg / mL; 6.00 µg / mL; 3.00 µg / mL; 1.50 µg / mL; 0.75 µg / mL and 0.375 µg / mL.

Cytotoxicity, selectivity index and combination index

MCF-7 cells and Vero cells used are from the Department of Parasitology Medical Faculty of Gadjah Mada University Yogyakarta. MCF-7 cells were grown on DMEM media and Vero cells with M199 media supplemented with 10% (Gibco) Fetal bovine, Penicillin 1% Streptomycin 1% (Gibco) and Fungizone 0.5% (Gibco) were incubated at 37°C, CO_2 5%. The inoculums seeded on a 96 well plate (Iwaki), each well 1 x 10^4 cells/0.1 mL. Cell culture were incubated at 37°C, 5% CO_2 for 24 hours. After 24 hours the media was discarded and the cell plus EAE, doxorubicin, and its combination were incubated for 24 hours then the medium was removed and 0.5 mg / mL of MTT was added and incubated for 4 hours at 37°C, 5% CO_2. after crystal formazan was formed and 10% SDS was added to dissolve the formazan crystals, then incubated for 24 hours at room temperature and shielded from light. The absorbance was measured with microplate reader at λ 595 nm [12]. The resulting absorbance was converted to a percentage of cell viability, then the selectivity index (IS) EAE was determined against MCF-7 cells. Further IC_{50} single and combination treatment results were analyzed with Compusyn software version 1 to determine the Combination Index (CI) [13-14].

The equation to determine the viability of cells

$$\%\text{Viability} = \frac{\text{Absorbance of treatment} - \text{absorbance of medium}}{\text{absorbance of control cells} - \text{absorbance of medium}} \text{x} 100\%$$

The equation to determine selectivity index (SI)

$$\text{SI} = \frac{\text{IC50 on Vero cells}}{\text{IC50 on MCF7 cells}}$$

Cell cycle inhibition assay

MCF-7 cells ($5x10^5$ cells/mL) were seeded into 6-well plate and incubated for 24 hours then treated with EAE, doxorubicin, and its combination and then incubated for 24 hours. After 24 hours the media was moved into the conical tube and then into well plus trypsin 0,025%, then washed with 2x PBS, collected into the conical and centrifuged at 2500 rpm for 5 minutes. The supernatant was thrown away, in the pellet added cold ethanol 70% for 2 hours for cell fixation.Then added with PBS, centrifuged at 3000 rpm for 3 min, the supernatant was removed, in pellets was added PI kit (containing 40 µg /g/µmL PI and RNAse 100 mL) and resuspended. Then mixture was incubated at 37°C for 30 minutes. The Sample was analyzed by FAC Scan Flowcytometer. Based on its DNA content the percentage of cell accumulation in the cell cycle (G_1, S, and G_2/M) were calculated using ModFit Lt.3.0 [15].

Apoptosis assay

MCF-7 cells ($5x10^5$ cells/mL) were seeded into 6-well plate and incubated for 24 hours then treated with EAE, doxorubicin, and its combination and then incubating for 24 hours. After 24 hours the media is fed into the conical tube and then into well plus trypsin 0,025%.Then washed with PBS, collected into the conical and centrifuged at 2500 rpm for 5 minutes. The supernatant was thrown away, in pellets added PBS, the suspension was centrifuged at 3000 rpm for 3 min, the supernatant was removed and Annexin V kit added to the pellet and resuspended then incubated at 37°C for 30 minutes. The Sample was analyzed by FAC Scan Flowcytometer [12, 15].

Statistical analysis

Analyzing of the cytotoxic test data using SPSS 24 software by probit analysis and the combination test was analyzed with Compusyn Software version 1.

Result and discussion

Extraction of *Moringa oleifera* L leaves

The extraction of 600 grams of Moringa dried leaf obtained 20.025 grams of thick extract and stored at 2-8°C.

Phytochemical screening result of that ethylacetate extract leaves of *Moringa oleifera* L

Phytochemical screening result showed that ethylacetate extract leaves of *Moringa oleifera* L positively contains of flavonoids, saponins, tanins, glycosides and steroids/triterpenoid.

Inhibitory concentration 50% (IC_{50})

The cytotoxic effects of EAE, doxorubicin, and its combination against MCF-7 cells and their selectivity with Vero cells were determined by the MTT assay. In each EAE treatment, doxorubicin, and its combination were showed cell growth inhibition as indicated by IC_{50} values. The IC_{50} value of EAE 149.29 µg/mL and doxorubicin 5.80 µg/ml, and the combination was showed a higher inhibitory effect if it compare with the single treatment. The optimum combination index (very strong synergistic effect) showed in 1/8 IC_{50} value of EAE and 1/4 IC_{50} (18.66 µg/mL – 1.45 µg/mL) was categorized with very strong synergistic effect (CI < 0.1). These effects is related to cell cycle modulation and apoptotic induction.

The selectivity of EAE was determined with an executed cell viability assay on Vero cells. The single treatment of EAE was showed cytotoxicity effect on Vero cells with IC_{50} 983.97 µg/ml. We were compared IC_{50} of EAE on Vero cells to MCF-7 cells to determine selectivity index (SI). Selectivity index of EAE was showed 6.59, SI>3 is supposed to be selective to MCF-7 cell lines. The result was showed that EAE is selective to MCF-7 cells instead of Vero cells [16].

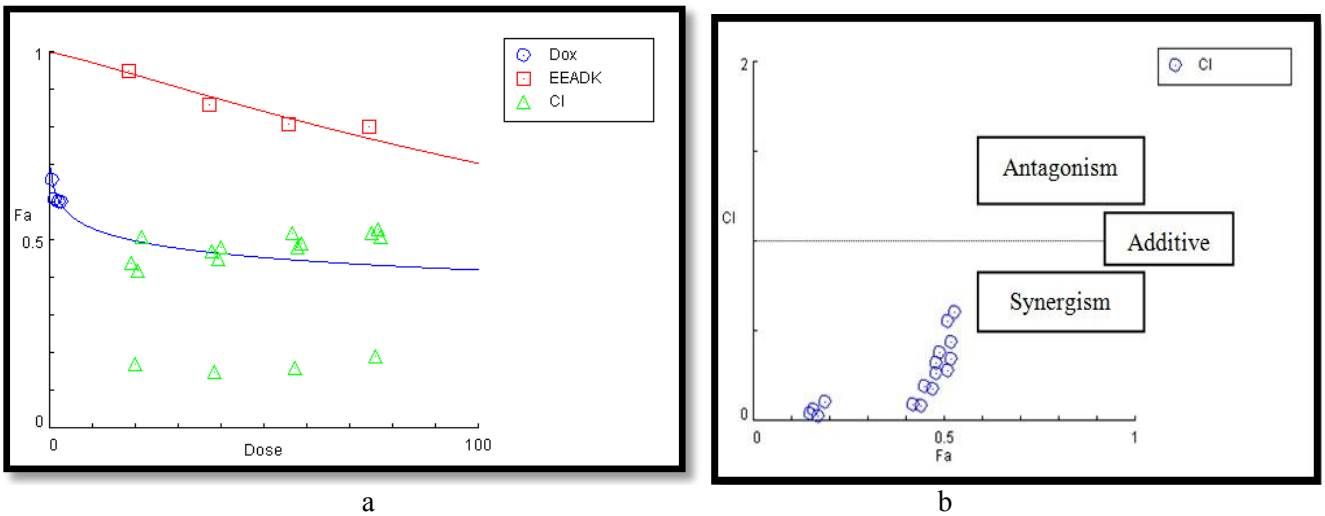
Combination assay

The results of EAE cell viability values, doxorubicin, and their combinations were analyzed with Compusyn software version 1 which gave the result of the combined index (CI) value. The results obtained can be seen in table 1 and figure 1, where the grade of CI in each treatment with the value of CI > 1 antagonism, CI = 1 additive and CI < 1 synergism [13-14].

Doxorubicin is one of the chemotherapy agents with the potent activity of IC_{50} 5.8 µg/mL to decrease toxic effect and prevent doxorubicin resistance to MCF-7 cells needed decrease dose of doxorubicin. In this study the combination of EAE and doxorubicin showed a very strong synergistic effect with CI value is 0.023 at the EAE-doxorubicin concentration (18.66 µg/mL-1.45 µg/mL). EAE increases the cytotoxic activity of doxorubicin in MCF-7 cells compared to single treatment. This synergistic effect is related to natural product contained in *Moringa oleifera* L. leaves such as flavonoid, isothiocyanate and glucosinolate is active compound to the induction of apoptosis and inhibition of cell cycle [6,9].

Table 1. Combination index values (CI) EAE-doxorubicin on MCF-7 cell lines.

EAE(µg/mL)	Doxorubicin (ug/mL)			
	2.90	2.18	1.45	0.73
74.63	0.551	0.603	0.102	0.436
55.97	0.379	0.323	0.064	0.341
37.31	0.265	0.186	0.039	0.179
18.66	0.277	0.088	0.023*	0.083



a b

Figure 1. The graphic representations obtained from the Compusyn Report for EAE and doxorubicin combinations. (a) Dose-effect curves; (b) Combination index plot.

Effect on cell cycle

Determination of EAE, doxorubicin, and its combination in inhibiting cell cycle using flowcytometric method [15]. The effect of EAE, Doxorubicin, and its combination are given in Figure 2, whereas with treatment of single doxorubicin at 2.90 µg/mL caused cell accumulation at G_0/G_1 phase (55.00%), EAE at 74.63 µg/mL caused cell accumulationat G_0/G_1 phase (63.77)%, and combination treatment G_0/G_1 cell accumulation(54.48%) at concentration 18.66 µg/mL EAE–1.45µg/mL Doxo showed higher value compared than the cell control was 47.64%. These results indicate that EAE may increase the cytotoxic effect in the G_0/G_1 phase.

Inhibition of the cell cycle by EAE is probably caused by the effect of the active content contained in the leaves of *Moringa oleifera* L. that can inhibit the activation of Nuclear factor-kappa B (NF-kB). Which is NF-kB is a transcription factor that has a important role in cell growth and death cell, and development [17-19].

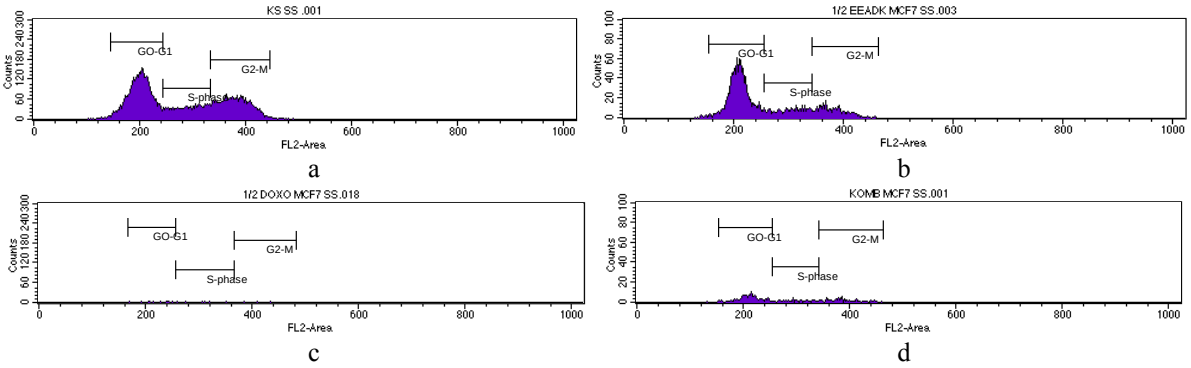


Figure 2. Cell cycle analysis of EAE, doxorubicin and their combination on MCF-7 cell lines (a) control cells; (b) EAE ½ IC_{50} (74.63 µg/mL); (c) doxorubicin ½ IC_{50} (2.90 µg/mL); (d) combination of EAE-Doxo (18.66 µg/mL–1.45 µg/mL). EAE single treatment and combination exhibited G_0/G_1 phase and decreased MCF-7 cell population.

Effect on apoptosis

Determination of apoptotic induction was performed using the flowcytometry with addition Annexin V as shown in figure 3. Percentage control, EAE ½ IC50, doxorubicin ½ IC_{50}, and its combination for early apoptotic 4.25%, 0.18%, 41.47% and 17.04%; in late apoptosis /early necrotic 2.60%, 42.83%, 13.89%, and 12.80%; and in late necrotic 2.50%, 56.99%, 40.08% dan, 5.58%. In the apoptotic study comparison of combination treatment with single showed a combination increased early apoptotic phase and late apoptotic/ early necrosis compared the single. Apoptosis is the process of cell death programmed with cell morphological changes [20]. EAE and its combination may increase apoptosis possibly due to EAE effects on inhibitory NF-kB activation which may lead to decreased expression of antiapoptotic proteins such as Bcl-2 [18-19].

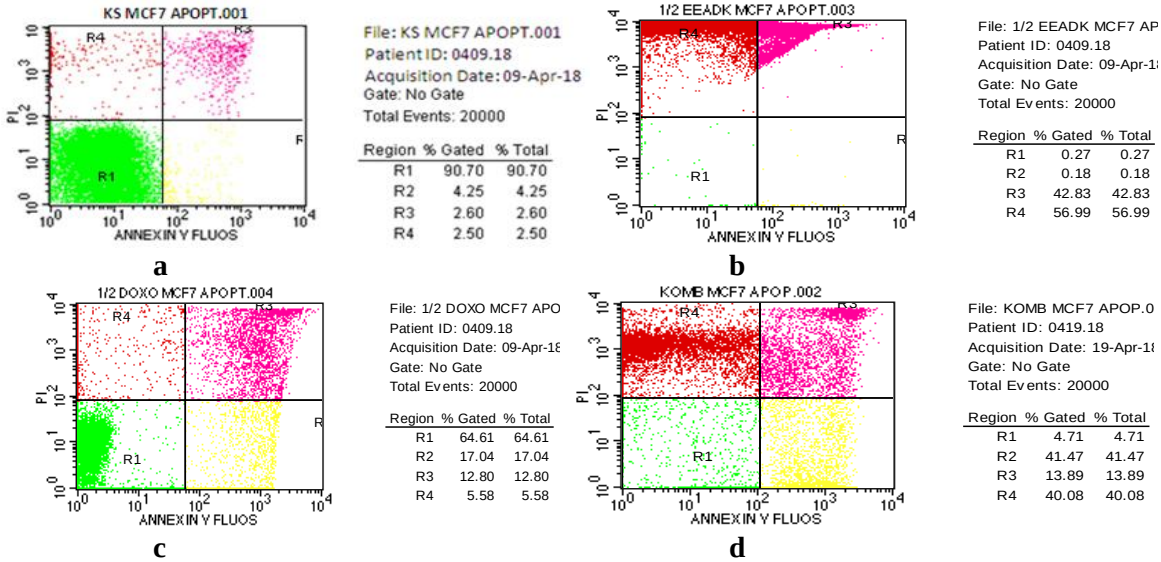


Figure 3. Apoptosis analysis of EAE, doxorubicin and their combination on MCF7 cell lines (a) control cells; (b) EAE ½ IC_{50} (74.63 µg/mL); (c) doxorubicin ½ IC_{50} (2.90 µg/mL); (d) combination of EAE-Doxo (18.66 µg/mL-1.45 µg/mL).

Conclusion

Based on the results we obtained EAE had very strong synergistic effect with doxorubicin therefore potentially used as a co-chemotherapy agent for breast cancer therapy with its activity induces apoptosis, and inhibits cell cycle.

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