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Combination Therapy of Eurycomanone and Doxorubicin As Anticancer on T47D and MCF-7 Cell Lines

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ABSTRACT

Doxorubicin (DOXO) is an anticancer that is often used clinically which induces cardiotoxicity in the patient. Therefore, finding a new compound that acts as co-chemotherapy is a current challenge. Eurycomanone (EURY) isolated from *Eurycoma longifolia* Jack have been shown to have anticancer activity in several studies. This study aimed to observe the effect of EURY, DOXO, and its combination on the cell cycle of T47D and MCF-7 breast cancer cell lines. Anticancer activities of EURY and DOXO were examined by MTT colorimetry assay. The effect of combination was analyzed by the Combination Index (CI) method using Compusyn software. The apoptotic activity was analyzed using flow cytometry. To determine CI, a fixed concentration ratio between EURY and DOXO was used from the IC_{50} value at concentrations (1/2; 1/4; 1/8; 1/16 of IC_{50}). We found that EURY had IC_{50} values at 0.377µg/mL on T47D and 4.7µg/mL on MCF-7 cells, while DOXO had IC_{50} values 1.845µg/mL on T47D and 5.074µg/mL on MCF-7cells. The optimal combination of EURY and DOXO on T47D was at 1/16 IC_{50} EURY – 1/6 IC_{50} DOXO with CI of 0.3442, whereas on MCF-7 was at 1/16 IC_{50} of EURY – ½ IC_{50} DOXO with CI 0.0221. Apoptosis was significantly higher in MCF-7 cells treated with EURY alone than DOXO alone with $p<0.001$. On T47D cells, DOXO alone resulted in more cell death than EURY alone, although not statistically significant. Cell death was higher in both cancer lines when EURY and DOXO were combined compared to DOXO alone with $p<0.05$ in both comparisons.

Keywords: Co-chemotherapy; doxorubicin; eurycomanone; MCF-7; T47D

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INTRODUCTION

Breast cancer is the leading cause of cancer mortality in women globally. In 2018, breast cancer affected 2.1 million women with 627,000 deaths worldwide with Fiji had the highest mortality rate[1]. Inherited mutation of *BRCA1* and *BRCA2* are known genetic predispositions for breast cancer. Woman with a mutated *BRCA1* or *BRCA2* has about 70% chance of getting breast malignancy by age 80. Family history, for example, having a first-degree relative with breast cancer doubles the risk, while having two first-degree relatives with the disease increases the risk about 3-fold. Other conditions, such as hormonal contraceptives, hormone replacement therapy, alcoholism, overweight, lack of physical activity, and lack of breastfeeding have been reported to slightly increase the risk of breast cancer[2].

Anthracycline is the most effective anti-tumor antibiotic that derived from *Streptomyces peucetius varietas caesius*[3]. Doxorubicin (DOXO), one of the first generation of anthracyclines, is a well-known anticancer drug, widely used for various types of tumours, such as leukemia and breast cancer, bone cancer, and ovarian cancer therapy, due to its broad-spectrum anti-tumor activity[4]. DOXO interferes with Topoisomerase II enzyme function, which results in an irreversible double-strand break of the DNA. Other mechanisms, including free radical formation and reactive oxygen species (ROS) generation, contribute to its cytotoxic effect in inducing DNA damage[5].

Although DOXO is classified as one of the most effective drugs against solid tumors, its clinical use as monotherapy is limited due to its side effects and the emergence of resistance. Various side effects that may occur, for instance, cardiotoxicity, nausea, vomiting, immunosuppression, alopecia, and hepatotoxicity[6]. The most notable side effect, cardiotoxicity, is presented as arrhythmias, hypotension, a decrease of myocardial contractility, myocarditis, and pericardial effusion. These side effects might develop as an acute reversible condition, which appears shortly after one or two doses of the drug. However, DOXO may also cause congestive heart failure and dilated cardiomyopathy when the cumulative dose of DOXO exceeds ~450 mg/m^2 [7]. Therefore, finding a combination compound that acts as co-chemotherapy with DOXO is a current scientific challenge. Co-chemotherapy is a beneficial method for reducing the anticancer drug dose and subsequently lowering its side effects[8].

Eurycoma longifolia Jack, which belongs to Simaroubaceae family, is a popular herbal medicine in Southeast Asia. *E. longifolia* has several pharmacologic activities, such as anticancer[9-11], antimalaria[12], antidiabetic[13], antibiotics[14,15] and anti-inflammatory[16,17]. This plant is rich in quassinoids, triterpenes, squalene derivatives, biphenylneolignans, canthin-6-ones, and β-carboline alkaloids[10,18]. *E. longifolia* showed potential anticancer activity against solid tumors, including breast cancer [19], cervical cancer[20], prostate cancer [11] and lung cancer[21]. The most studied compound of this plant is eurycomanone (EURY), a quassinoid that has anticancer effects against various types of human cancers including HepG2, HM3KO, Hela, CaOV-3, A2780, MCF-7 and A549 cells[22,23]. The anticancer activity of EURY is due to its ability to induce apoptosis by upregulating p53 and Bax, downregulating Bcl-2, and via a caspase-9-independent pathway[19,23]. Other study has also identified the inhibitory activity of EURY on various cancer cell lines, such as T47D cells (IC_{50} 1.17 ± 0.09µg/mL), MCF-7 (IC_{50} 3.96 ± 0.02µg/mL),

Hela (IC_{50} 2.95 ± 0.08µg/mL) and WIDR (IC_{50} 1.45 ± 0.01µg/mL). This study also reported that EURY is safe on the normal African green monkey kidney epithelial cell line (Vero cells (IC_{50} 609.89 ± 29.77 µg/mL).[24] These results showed the potential of EURY as an anticancer, which can be further developed into a combination product. Despite numerous reports regarding its cytotoxic effects, the effects of EURY in combination with DOXO on breast cancer cell line have not been reported. Therefore, in this study, we investigated the combination of EURY and DOXO and its anticancer activity on T47D and MCF-7 breast cancer cell lines.

MATERIALS AND METHODS

Preparation of cancer cell lines

MCF-7, T47D and Vero cells (ATCC, Virginia, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), Rosewell Park Memorial Institute (RPMI) 1640 and M_{199} medium, respectively (Gibco, Thermo Fisher Scientific, USA). All media were supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin-streptomycin, then incubated at 37°C for 24 hours in a humidified 5% CO2 incubator. Growth media were changed daily. After the number of cells were sufficient (70–80% confluent), cells were harvested and counted to a concentration of 5 x 10^4 cells/100 µL, which indicated the cells are ready to be used. Then, 100 µL of 10^4 / mL cells were then seeded to 96-wells microplates for testing.

Preparation of EURY and DOXO concentrations

EURY (ChromaDex, California, US) and DOXO (EBEWE Pharma, Unterach, Austria) were prepared aseptically starting with the preparation of a stock solution by dissolving every 10 mg of the drug tested in 100 mL culture media containing 0.5% v/v DMSO. Serial concentration of the each drug was prepared by mixing the stock solution with growth medium in which EURY separated in 500; 250; 125; 62.5; 31.25; 15.63; 7.80 µg/mL and DOXO in 100; 50; 25; 12.5; 6.25; 3.13; and 1.56 µg/mL.

Cytotoxic analysis

The anticancer activity of the tested drugs was carried out using the MTT Colorimetry Assay Method. In this assay, MCF-7 and T47D cells acted as a treated sample, Vero cells acted as a control sample and well seeded with growth medium only acted as a medium (blank) sample. Four microplates (seeded with MCF-7, T47D, Vero cells and growth medium) were filled with 100µL of the tested drugs (EURY and DOXO) with different concentrations, then incubated for 24 hours at 37°C, 5% CO_2 and 90% humidity. At the end of the incubation, the media was carefully removed, 100µL of fresh media and 10µL MTT (5 mg/mL) were added to each well. Viable cells will react with MTT to form purple formazan crystals. After 4 hours incubation at 37°C, this reaction was stopped by adding 100µL SDS 10% stopping solution in 0.01N Hidrochoride acid. Microplates are wrapped in aluminium foil and stored in a dark place at room temperature for 24 hours. Absorbance was read at λ 595 nm by a microplate ELISA reader. The experiment was repeated in three independent experiments in triplicate format for each treatment. Cell absorbance in MTT assay was used to calculate the percentage of cell viability. Cell viability was calculated by the following formula:

$$\% \text{ Viable cells} = \frac{\text{Treated cell absorbance} - \text{medium absorbance}}{\text{control cell absorbance} - \text{medium absorbance}}$$

The lower percentage of cell viability proves that this combination is stronger in driving cancer cell death in vitro.

The percentage of cell viability were used to determine the IC_{50} value (the concentration of the drug needed to inhibit the growth of 50% cells) by using linear regression analysis. The IC_{50} values were then used to determine the selectivity index (SI). The selectivity index was calculated by the following formula:

$$\text{Selectivity index (SI)} = \frac{IC_{50} \text{ of Vero cells}}{IC_{50} \text{ of cancel cells}}$$

Combination anticancer activity test

The combination anticancer test were carried out to determine the effect of various tested drug concentrations on the viability of cancer cells (T47D and MCF-7cell lines). Serial concentrations of each EURY and DOXO were determined based on IC_{50} of the drugs on each cancer cell. Then, the respective values were made in the concentration ratio 1/2; 1/4; 1/8; 1/16 of IC_{50}. The concentrations of DOXO and EURY obtained were then used in sequence as a drug combination.

The cytotoxic analysis was carried out as described above by incubating each cancer cell with a combination of 50 µL EURY and 50 µL DOXO in various concentrations. This experiment was performed in triplicate with three times independent repetitions. The cell viability was calculated and then used in determining the Combination Index by using CompuSyn software version 1[25]. The combination index of CI <1, CI = 1, and CI > 1 indicated the synergism, additives, and antagonism effects of a combination of drugs, respectively.

Apoptosis assay

The samples were divided into eight groups: T47D only (T47D control group, G_1), T47D treated with IC_{50} EURY(G_2), T47D treated with IC_{50} DOXO(G_3), T47D treated with IC_{50} DOXO – EURY (G_4), MCF-7 only (MCF-7 control group, G_5), MCF-7 treated with IC_{50} EURY(G_6), MCF-7 treated with IC_{50} DOXO(G_7), and MCF-7 treated with IC_{50} DOXO – EURY (G_8).

T47D and MCF-7 cells with a density of $5x10^5$ cells/mL were seeded into a 6-well plate and incubated for 24 hours. Then, the cells were treated with IC_{50} EURY, IC_{50} DOXO, and IC_{50} DOXO - EURY, followed by overnight incubation. Afterward, the cells were harvested, washed twice with PBS, and stained with the Annexin-V-FLUOS labeling solution (Roche, Mannheim, Germany) as per the manufacturer's recommendation. The samples were assessed for apoptosis by using the BD FACSCalibur Flow cytometer (Becton Dickinson, California, US) and analyzed by FlowJo software (FlowJo, LLC., Oregon, USA).

Statistic analysis

All experiments were performed in triplicate and repeated in three independent times. Data were presented as mean ± SEM. The percentage of apoptosis were further analyzed by one-way ANOVA and Tukey's test at 5% significance level using SPSS ver. 24 software with $p<0.05$ were considered significant.

RESULTS

The IC_{50} and SI values of EURY and DOXO on T47D and MCF-7 cell lines

The IC_{50} values of EURY on T47D and MCF-7 cells were 0.377 µg/mL and 4.7 µg/mL, respectively. The single treatment of EURY showed a selective cytotoxic effect on Vero cells with IC_{50} = 235.19 µg/mL. The IC_{50} values of

DOXO on T47D, MCF-7, and Vero cells were 1.845µg/mL; 5.074 µg/mL and 57.62 µg/mL, respectively. The EURY selectivity index (SI) against T47D and MCF-7 cells are 623.85 and 50.04, respectively.

Combination effect of EURY - DOXO on T47D and MCF-7 cell lines

Table 1 showed that the combination index of EURY and DOXO had a greater ability to suppress T47D and MCF-7 breast cancer cells' viability compared to individual drug therapy. The combination index of EURY with a concentration of 1/16 IC_{50} and DOXO 1/16 IC_{50} (0.023 µg/mL - 0.115µg/mL) can be categorized as a strong synergistic effect against T47D cells (CI = 0.3442 < 1).

Table 1 : The combination index values (CI) of EURY-DOXO on T47D cells

EURY (µg/mL)	DOXO (µg/mL)			
	1/2 IC_{50}	1/4 IC_{50}	1/8 IC_{50}	1/16 IC_{50}*
1/2 IC_{50}	0.5018	0.8183	0.8183	0.7964
1/4 IC_{50}	0.5993	0.6945	0.6945	0.7241
1/8 IC_{50}	0.4880	0.4971	0.4971	0.5179
1/16 IC_{50}*	0.5068	0.7049	0.7049	0.3442

*Indicates that this combination showed the strongest synergistic effect against T47D cells as the CI value was the least among other concentration (CI = 0.3442 < 1).

The combination of 1/16 IC_{50} EURY and ½ IC_{50} DOXO (0.293 µg/mL-2.54 µg/mL) also showed a strong synergistic against MCF-7 cells with a combination index value of 0.0221 (CI = 0.0221 <1) (Table 2).

Table 2 : The combination index values (CI) of EURY-DOXO on MCF-7 cells

EURY (µg/mL	DOXO (µg/mL)			
	1/2 IC_{50}	1/4 IC_{50}	1/8 IC_{50}	1/16 IC_{50}*
1/2 IC_{50}	0.2104	1.1102	1.4289	10.7756
1/4 IC_{50}	0.2990	0.9791	0.3887	10.2727
1/8 IC_{50}	0.3291	6.8578	1.1908	2.3426
1/16 IC_{50}*	0.0221	0.2806	1.0799	0.0918

*Indicates that this combination showed the strongest synergistic effect against MCF-7 cells as the CI value was the least among other concentration (CI = 0.221 < 1).

Apoptosis assay

The representative of T47D cells undergoing apoptotic stages based on treatment were illustrated on Figure 1.

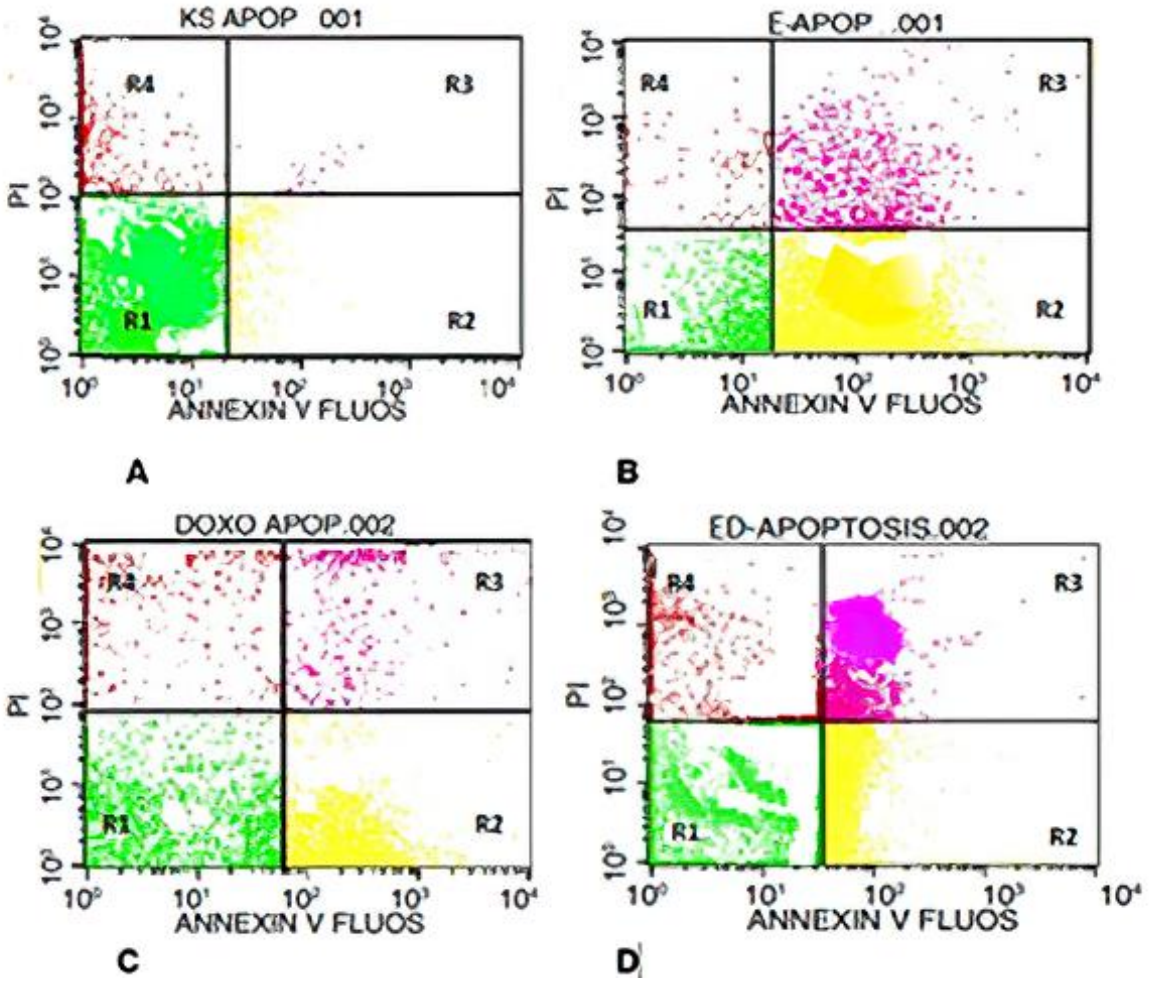

Figure 1. Results of flowcytometry analysis, where A = T47D cell, control cell; B = Eurycomnone; C= DOXO; D = EURY – DOXO; R1 (normal cells), R2 (early apoptosis cells), R3 (late apoptotic cells), and R4 (necrosis cells).

Figure 2 showed the apoptotic stage of T47D cancer cells after treatment. The viable cells were declined in all groups. A significant decrease of viable cells was observed in G_2 ($p<0.001$). The cells within G_2 mostly still underwent the early apoptosis stage, compared to G_1 where most cells were still viable ($p<0.05$). Most of the cells within G_3 was still viable cells compared to the other treatment groups, although not statistically significant. G_4 has a significantly higher percentage of late apoptotic cells and death cells among the other groups ($p<0.001$ and $p<0.05$, respectively).

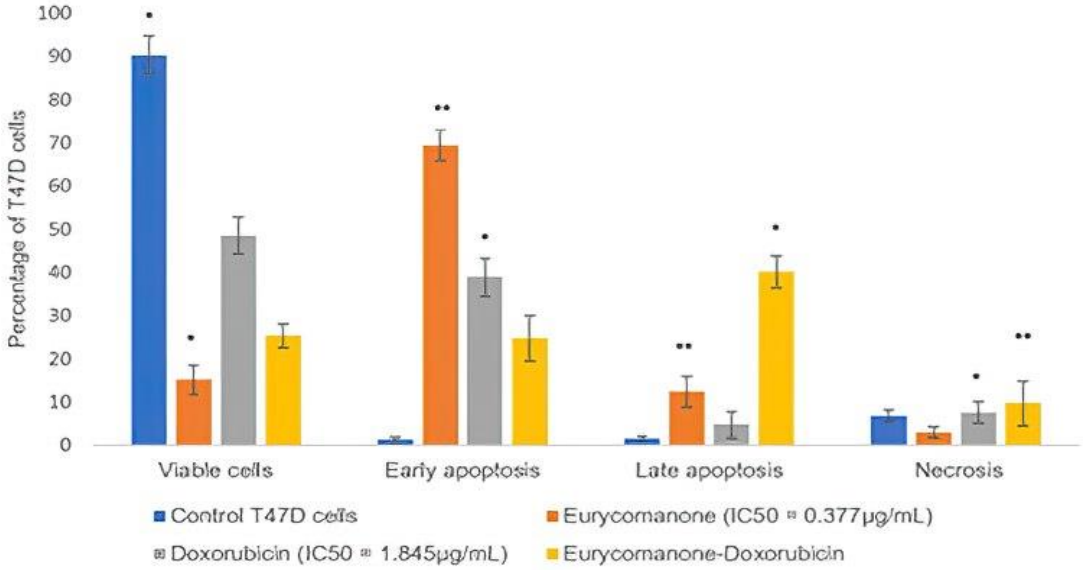

Figure 2. The percentage of T47D undergoing apoptosis cycle based on treatment.
* Statistically significant at $p<0.001$, ** Statistically significant at $p<0.05$

The illustration of MCF-7 cells undergoing apoptosis or necrosis by treatment can be seen in Figure 3.

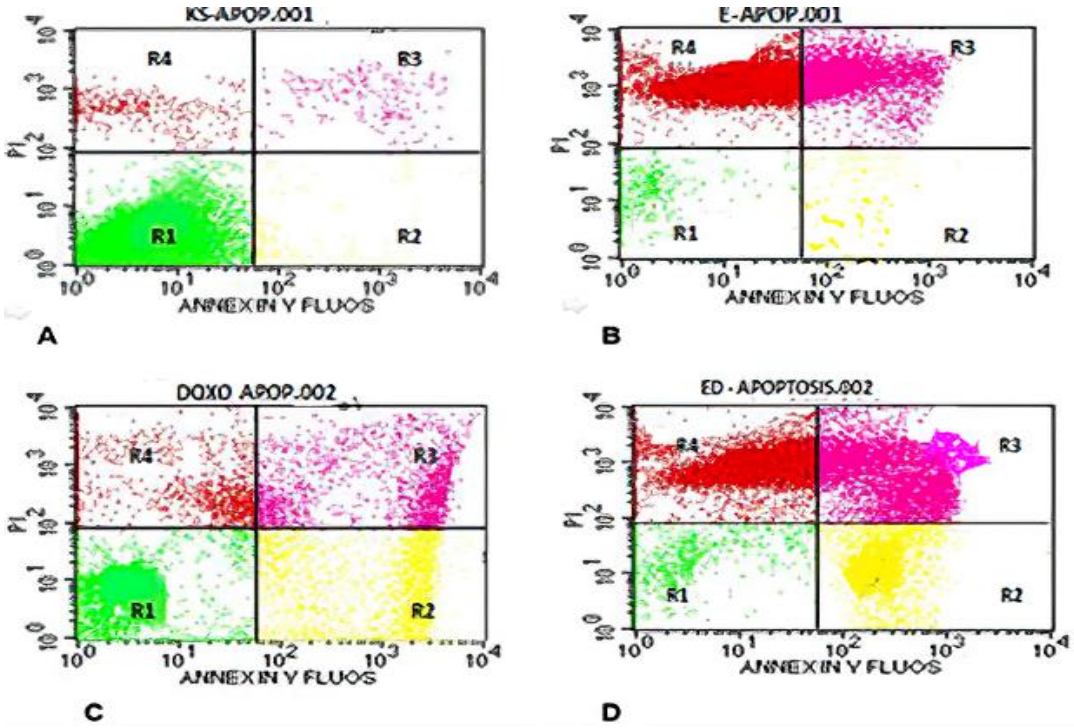

Figure 3. Results of flowcytometry analysis, where A = MCF-7 cell without treatment, control cell; B = MCF-7 treated with eurycomnone; C= MCF-7 treated with DOXO; D = MCF-7 treated with EURY – DOXO; R1 (normal cells), R2 (early apoptosis cells), R3 (late apoptotic cells), and R4 (necrosis cells).

Figure 4 showed the apoptotic stage of MCF-7 cancer cells after treatment. A significant number of cells within G_6 underwent the late apoptosis stage compared to the control where most cells were still viable ($p<0.05$). The cell death in G_6 was significantly higher than G_5 and G_7 ($p<0.001$ on both comparisons). Significant intact cells were observed within G_7 compared to G_6 and G_8 ($p<0.05$ and $p<0.001$, respectively). Cell death among G_7 was lower than the other treatment groups, although not statistically significant. G_8 showed a significant number of cells undergone late apoptotic stage and cell death ($p<0.001$ and $p<0.05$, respectively).

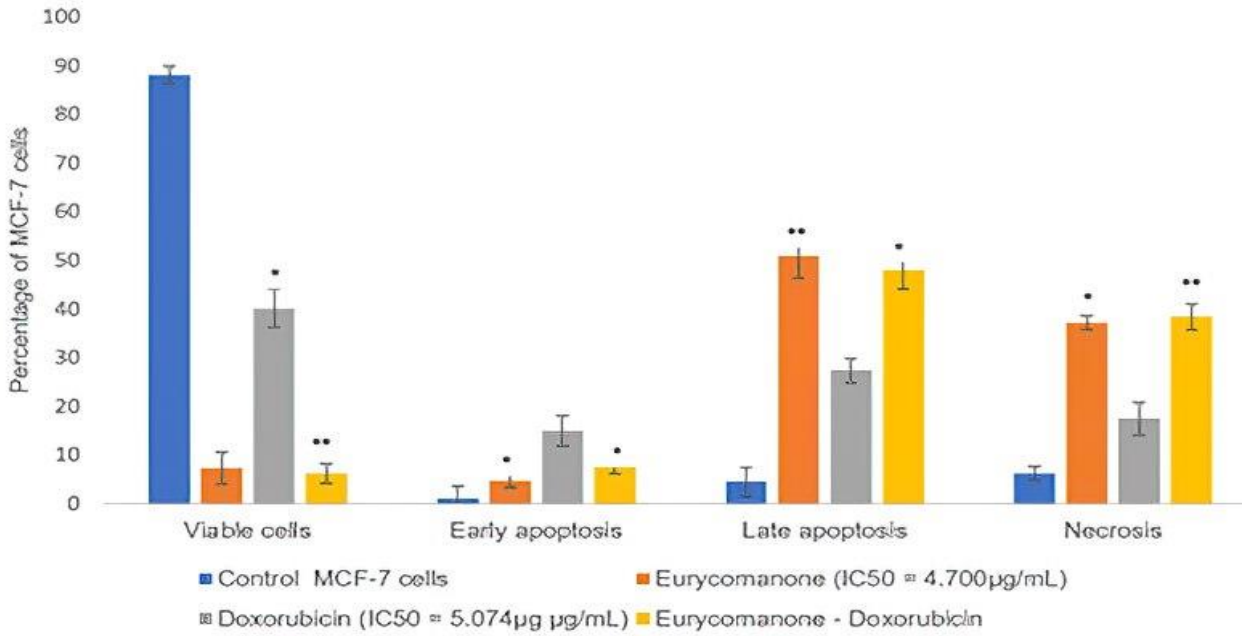

Figure 4. The percentage of MCF-7 undergoing apoptosis cycle based on treatment.
* Statistically significant at $p<0.001$; ** Statistically significant at $p<0.05$

DISCUSSION

Chemotherapy is one of the main cancer treatments. However, most anticancer agents used have some side effects, for instance, DOXO, which induce cardiotoxicity. Hence, recently, natural-based chemotherapy has been the focus of anticancer research. Therefore, in this study, we investigated the anticancer activity of a combination of DOXO and EURY on breast cancer cells MCF-7 and T47D.

Our study showed that eurycomanone was successfully inhibiting 50% of breast cancer cells with IC_{50} on T47D and MCF-7 cells were 0.377µg/mL and 4.7µg/mL, respectively. Furthermore, the cytoselectivity of eurycomanone was proven against Vero cells with IC_{50} of 235.19µg/mL. The selectivity index of EURY against T47D and MCF-7 > 3, which proved that EURY is selective for T47D and MCF-7 cells. Another study reported that EURY has low cytotoxicity on normal liver cells than tamoxifen, which showed better selectivity of EURY than tamoxifen[23]. The previous EURY study showed that EURY has cytoselectivity which inhibits the growth of cancerous cells, MCF- 7 with an EC50 value 2.2 µg/ml but not non-cancerous cells MCF-10A[26]. The side effect of current chemotherapy might be reduced by this kind of cytoselectivity, which may lead research in developing a new co-chemotherapy drug against cancer.

The combination index of EURY and DOXO on T47D and MCF-7 were 0.3442 and 0.0221, respectively. The combination index below 1 showed that EURY-DOXO has a synergistic effect and an enhanced anticancer effect on both breast cancer cells. Another study has reported that the ethanol extract of *E. longifolia* Jack root enhances DOXO effect on cancer cells by increasing p53 expression and decreasing Bcl-2 expression in the breast tissue of rats[27].

This study also showed that EURY alone significantly induced more cell death than DOXO alone on MCF-7 cells with $p<0.001$. Interestingly, the opposite occurred in T47D cells, where DOXO alone resulted in cell death than EURY alone, although not statistically significant. However, cell death was higher in both cancer lines when EURY and DOXO were combined compared to treatment with DOXO alone with $p<0.05$ in both comparisons.

The antiproliferative activity of EURY on breast cancer cells was reflected by its apoptotic effect. The apoptosis mechanism of MCF-7 and T47D cells by EURY includes down-regulating BCL-2, activating caspases-6, 7, 8, and 9, which leads to activation of tBID and FADD followed by cleavage of PARP and Lamin[18,26,28]. The antiproliferative activity of EURY has also been studied in other cancer cells[10,20,21,23]. Therefore, future research should be explored EURY as a co-chemotherapy drug, including the effective formula for optimal delivery and efficacy.

CONCLUSION

This research shows that a combination of eurycomanone and doxorubicin synergically increases the cytotoxic effect through cell death induction on both MCF-7 and T47D cancer cells. Based on this result, eurycomanone is potential to be developed as a co-chemotherapeutic agent for doxorubicin.

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AUTHOR'S CONTRIBUTIONS

HY conceptualized and designed the study, conducted the experiments, and wrote and reviewed the manuscript. DS designed and conducted the experiments and analyzed the data. SS conceived the study, designed the experiments, analyzed the data, and wrote the manuscript. MF analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have declared that they have no conflict of interests.

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