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POTENTIAL KETAPANG (*Terminalia catappa*) LEAF EXTRACT AS A DOXORUBICIN CO-CHEMOTHERAPY AGENT ON BREAST (T47D) AND CERVIX (HeLa) CANCER CELL LINES

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ABSTRACT
Doxorubicin (DOX) is chemotherapy for breast and cervical cancer with serious side effects. Ketapang (*Terminalia catappa*) is a potential plant as a co-chemotherapy agent. The purpose of this research was to examine the sensitivity of DOX as a cytotoxicity drug in combination with ethanolic extracts of ketapang leaves (EKL) against T47D and HeLa cancer cells. Cytotoxicity was determined using the MTT assay, with DOX concentration series (0.625-40 nM for T47D and 0.5-6 M for HeLa) and EKL (50-1000 µg/mL) used in combination with the study. DOX and EKL combination assays utilizing their respective IC_{50} values were performed in T47D cells and HeLa cells, and the results were used to calculate the Combination Index (CI). Furthermore, the doubling time method was used to investigate the combination of DOX and EKL proliferation inhibition on both cell lines. DOX and EKL had IC_{50} values of 158 nM and 30 µg/mL for T47D, respectively, and 3.4 M and 640 µg/mL for HeLa cell growth. While DOX and EKL have a synergistic effect on T47D cells, their combined effect on HeLa cells is cytotoxic and dose-dependent. EKL increases the inhibitory effect of DOX on the proliferation of T47D and HeLa cancer cells. In T47D cells, the combination of DOX and EKL has a higher potential for cytotoxic and antiproliferative activity than in HeLa cells.

Keywords: *Terminalia catappa*; Cytotoxicity; Doxorubicin; T47D; HeLa

1. INTRODUCTION

Breast and cervical cancers are the most common types of cancer among women worldwide. Breast cancer is the second most common type of cancer in terms of new cases and the fifth leading cause of death worldwide (Siegel et al., 2019). In comparison, cervical cancer is a kind of female reproductive system cancer that is frequently fatal (Siegel et al., 2019). Indonesia has the eighth highest cancer incidence in Southeast Asia, with breast cancer being the most prevalent in women, followed by cervical cancer.

Chemotherapy is a frequently utilized medication because it can be used to treat malignancies that have progressed to the metastatic stage by suppressing the cell cycle and apoptosis tracking processes (Hanahan, 2022). The use of doxorubicin as chemotherapy has been documented to result in cancer cell resistance and harmful effects on healthy tissues, failing cancer therapy (Braciuliene et al., 2022; Thorn et al., 2011). Resistance to chemotherapeutic treatments can develop as a result of cells developing defense mechanisms in response to the suppression of the cell regulation system during particular phases, resulting in insensitive cells (Bukowski et al.,

2020; Shah & Schwartz, 2001; Zhou et al., 2021). Apart from resistance, chemotherapeutic medicines are not selective since, in addition to cancer cells, they can damage rapidly dividing healthy cells such as hair cells, nails, skin, and blood cells, resulting in severe side effects (Altun & Sonkaya, 2018; Mayer & Burstein, 2007). One strategy for resolving these issues is to produce co-chemotherapy drugs based on natural chemotherapeutic agents, most notably chemicals derived from herbs, that may be coupled with doxorubicin chemotherapy treatments to mitigate their negative effects.

In Ketapang leaves, one of the plants may be developed as a chemopreventive agent (*Terminalia catappa*). Ketapang leaves are members of the Combretaceae family. They include important components such as phenols, flavonoids, and carotenoids that have been shown to have antibacterial, antifungal, antidiabetic, antioxidant, hepatoprotective, and anticancer properties (Morioka et al., 2005; Venkatalakshmi et al., 2016). Additionally, Ketapang extract acts as a chemopreventive agent against cancer by inhibiting colon cancer cell multiplication (Morioka et al., 2005). In vivo testing of ketapang leaf water extract (10-100 μg/mL) demonstrated a 68 percent reduction in metastasis in a mouse model of lewis lung carcinoma (LLC) via lowering TIMP-2 and PAI-1 enzyme levels (Chu et al., 2007). Ketapang leaf methanol extract is cytotoxic to EAC (*Ehrlich Ascites Carcinoma*) cells (Saroja et al., 2012). Thus, ketapang leaves are ingredients that may contain active chemicals that have the potential to be developed as anticancer agents.

However, its action in T47D breast cancer cells, HeLa cervical cancer cells, and doxorubicin has not been reported. Hence in the present study, we investigated the cytotoxic and antiproliferative effects of EKL as co-chemotherapy on T47D breast cancer and HeLa cervical cancer cell line.

2. METHODS

2.1. Material

Ketapang leaves were collected in Banyumas, Indonesia. PT. Sanbe Farma provided the doxorubicin (DOX). T47D and HeLa cancer cells were received from the Faculty of Medicine, Universitas Gadjah Mada, Laboratory of Tropical Medicine, Section of Parasitology. T47D and HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) and HeLa cells in Roswell Park Memorial Institute (RPMI) medium with 10% (v/v) Fetal Bovine Serum (FBS) and 2% (v/v) antibiotic penicillin-streptomycin. Dimethyl sulfoxide, trypsin-EDTA 0.25 percent (Gibco), MTT reagents (3- (4,5-dimethyltiazol-2-il)-2,5-diphenyltrazolium bromide), and sodium duodecyl sulfate (Merck) 10% in 0.1 N HCl are employed as cytotoxic and proliferative reagents (Merck).

2.2. Methods

2.2.1. Preparation of the Extract

To avoid destroying the chemicals contained in the leaves, 500 grams of wet ketapang leaves are dried in indirect sunlight to prevent them from turning brown. After drying, pollination of Simplicia ketapang leaves is carried out with the help of a pollinator. The maceration of ketapang leaves necessitates the use of around 70% ethanol. The filtrate and residue obtained from the maceration process are separated. A Rotary Vacuum Evaporator is then used to thicken the filtrate, which is then evaporated over a water bath at 60 degrees Celsius.

2.2.2. Cytotoxic and Proliferation Assay

The MTT (3-(4,5-dimethylthiazol-2-il)-2,5-difeniltetrazolium bromide) technique was used to culture cells, observe single and combined cytotoxic activities, and evaluate ethanolic extracts of ketapang leaves (EKL) proliferation in the presence of DOX (ISO, 2009). Cells are extracted and spread evenly throughout 96 wells at a concentration of 1 x 10^4 cells/well. By contrast, the proliferation kinetics test employs a cell density of 5 x 10^3 cells/well. For 24 hours,

cells were stimulated to adapt and adhere to wells. Following that, 100 ml of culture fluid containing only DMSO (control) or the test chemical was cultured for an additional 24 hours. The proliferation assay was conducted for 0;24;48;72 hours. Living cells react with MTT reagents to create purple formazan crystals. Following 4-6 hours, 10% SDS (Sodium Dodecyl Sulphate) is added to dissolve the formazan crystals. Cells are cultured at room temperature and sheltered from light for an overnight period. After incubation, the plate was agitated horizontally (with a shaker) for 3 minutes and then measured at λ 595 nm using an ELISA reader.

2.2.3. IC_{50} Analysis (Inhibition Concentration 50 Percent)

The absorbance data is translated to a percentage of living cells and statistically assessed using the correlation test followed by the percentage of living cells. The formula for calculating the percentage of alive cells is as follow Eq. (1) (Artanti et al., 2020).

$$\% \text{ Viability of cells} = \frac{Sample\ Absorbance - Medium\ Control\ Absorbance}{Cell\ Control\ Absorbance - Medium\ Control\ Absorbance}\ x\ 100\% \quad (1)$$

IC_{50} is determined by the logarithmic equation between the absorbance value and the extract concentration. IC_{50} is a concentration that causes the death of 50% of the cell population to be known for potential cytotoxicity. The calculation data for T47D and HeLa cells are entered into the probit program to calculate IC_{50}.

2.2.4. Combination Index (CI)

The method commonly used to evaluate drug combinations is the CI using Eq (2) (Reynolds & Maurer, 2005).

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (2)$$

Where:

DX, the concentration of the single component, is required to provide the same impact as the combined concentrations, D1 and D2, of the same chemical.

The concentration of the two chemicals (1 and 2) is used in conjunction with one another as a combination treatment (**Table 1**).

Table 1. Interpretation of CI value (Reynolds & Maurer, 2005)

CI Values	Interpretation
<0.1	Very strong synergism
0.1 – 0.3	Strong synergism
0.3 – 0.7	Synergism
0.7 – 0.9	Mild to moderate synergism
0.9 – 1.1	Additive
1.1 – 1.45	Mild to moderate antagonism
1.45 – 3.3	Antagonism
>3.3	Strong to very strong antagonism

2.2.5. Cell Proliferation Analysis

The data acquired following treatment were analyzed using Ms. Excel 2016 to determine the equation of the regression line between incubation time and absorbance.

3. RESULTS AND DISCUSSION

The cytotoxic assay was used to establish the IC_{50} value of the ethanolic extracts of Ketapang leaves (EKL). The IC_{50} values for T47D breast cancer cells from EKL and DOX were 30 μg/mL and 158 nM, respectively, but the IC_{50} values for HeLa cervical cancer cells from EKL

and DOX were 640 µg/mL and 3.4 M, respectively. Microscopy investigations of T47D and HeLa cells revealed the phenomena of cell death as a result of EKL therapy, as shown by changes in cell shape. Living cells appear to be linked to the plate's bottom and other cells; they are brightly colored and have elongated spherical forms. In comparison, cells that die away from the dish's bottom are dark-colored and have a more spherical shape. This occurs when cells lose cytoplasm as a result of injury to the cell membrane, rendering them incapable of transmitting light from the microscope.

The combined effect of EKL and DOX on cell viability is evaluated using the CI calculation method (**Figure 1**). The combination of EKL 3.75 µg/ml with DOX at 56.25 and 75 nM concentrations provided a cytotoxic effect by obtaining % cell viability values of 23.98% and 29% in T47D cells (**Figure 1a**). The combination of the two also showed a better reduction in cell viability in the proliferation test compared to a single treatment. Meanwhile, HeLa cells showed a high decrease in cell viability values (cell viability values <30%) at low to high EKL concentrations combined with DOX (**Figure 1b**). According to these figures, the results of a combination of EKL and DOX study on T47D demonstrated synergy, with the best CI value of 0.62 obtained at a concentration of EKL 3.75 µg/ml (1/8 IC_{50}) and DOX 56.25 nM (3/8 IC_{50}). Due to the synergy between EKL and doxorubicin, the dose of DOX required to achieve the same potential can be decreased to 3/8 IC_{50}. As a result, the dosage required is reduced, and adverse effects can be minimized. In HeLa cells, on the other hand, the CI value cannot be quantified potential synergism (**Table 2**).

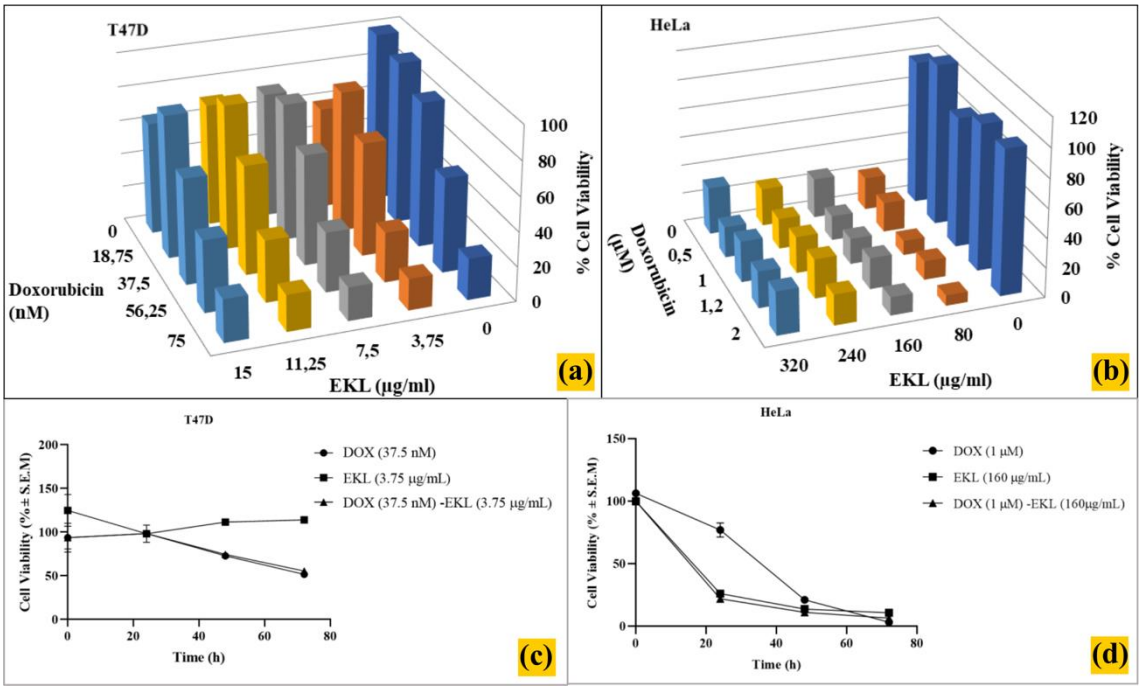

Figure 1. Graphic viability of T47D (A) and HeLa (B) cells in combination treatment of EKL and doxorubicin after incubation for 24 hours; Effects of EKL and doxorubicin treatment on T47D (C) and HeLa (D) cell proliferation

Proliferation tests can be used to measure the rate of cell growth affected by the activity of EKL and doxorubicin on the proliferation kinetics at various incubation durations. The parameter utilized in this test is the rate at which cells divide into two at the selected level. At 24 and 48 hours, a combination of EKL 3.75 µg/ml with DOX 37.5 nM inhibited cell growth more than single DOX (**Figure 1c**). However, after 72 hours, the absorbance was restored to normal levels when only DOX was used. Even when DOX is utilized as a chemotherapeutic agent, EKL's proliferation effect is more prominent, indicating that EKL has a strong proliferative effect since

it may still cause cell proliferation when combined with a cytotoxic agent. Proliferation of HeLa cells with DOX one m treatment increased from 0 to 24 hours and then reduced from 24 to 72 hours (**Figure 1d**). The combination of EKL and DOX inhibits HeLa cells more effectively than single DOX, but not considerably more effectively than single EKL, demonstrating the ability of EKL and DOX to inhibit HeLa cells.

Table 2. Combination Index Value by EKL and Dox on T47D and HeLa

T47D				
EKL (µg/mL)	**DOX (nM)**			
	18.75	**37.5**	**56.25**	**75**
3.75	1.36	0.93	0.62	0.7
7.5	1.52	1.03	0.74	0.76
11.25	nd	1.14	0.82	0.83
15	nd	1.20	0.98	0.93
HeLa				
EKL (µg/mL)	**DOX (M)**			
	0.5	**1**	**1.2**	**2**
80	1.13	nd	nd	nd
160	nd	nd	1.96	nd
240	2.86	2.07	1.43	3.24
320	3.58	1.7	2.20	1.67

*nc = no determined

The cytotoxic effect of ethanolic extracts of Ketapang leaves (EKL) alone and in combination with Doxorubicin (DOX) against T47D and HeLa cells was determined using MTT, which forms a dark blue formazan when reacting with the enzyme reductase found in living cell mitochondria (Ghasemi et al., 2021; Mahdavi et al., 2019). While MTT reagents do not affect dead cells since the mitochondria do not breathe, because the tetrazolium ring is not broken, formazan does not form, giving the color a purple hue. However, the color stays yellow (Ghasemi et al., 2021; Kusuma et al., 2010). If the purple color becomes high intense, the number of live cells increases, this absorbance data from the MTT method demonstrates the link between treatment with various dose levels and the number of live cells.

EKL cytotoxic test results on T47D cells indicate cytotoxic at low concentrations but increase cell viability at high ones. T47D cells are estrogen-producing cells that express estrogen receptors (ER) (Bouris et al., 2015; Ho et al., 2016). Because estrogenic qualities can promote cell viability, the prospect of increased viability exists due to EKL's estrogenic capabilities. Estrogenic substances have been shown to promote cell proliferation, tissue development in reproductive organs, and the transcription of specific genes (Pamplona-Silva et al., 2018). Increases cell viability as EKL levels rise, as protein accumulation for proliferation is one of the estrogenic effects generated when an estrogenic chemical is combined with estrogen receptors (Yunas et al., 2013). An additional, more focused study is required to demonstrate this. While EKL activity on HeLa cells demonstrates a lower potential effect, this is due to HeLa cells' unique properties compared to T47D cells. So, their activity on HeLa cells requires additional research.

Flavonoid and tannin molecules may have an active role in anticancer activity. Ketapang leaf extract includes tannins, and punicalagin can prevent bleomycin-induced CHO-K1 cell HGPRT cells from mutation (Chen et al., 2000). Ketapang leaves containing tannins have anticancer properties through their ability to prevent liver cancer and function as antioxidants. On EAC cells (Ehrlich Ascites Carcinoma), the flavonoid component of ketapang leaf extract has anticancer activity) (Saroja et al., 2012). Additionally, the tannin component punicalagin found in ketapang leaf extract possesses significant antioxidant activity (Sahala & Soegihardjo, 2012). The flavonoids found in ketapang are antioxidants and have been shown to suppress the proliferation of cancer cells (Lour & Meiyanto, 2007).

The IC_{50} values for EKL and DOX were utilized to adjust the cytotoxic and proliferation assays. Co-chemotherapeutic is a therapeutic method that combines EKL with a DOX chemotherapy drug. The combination of chemotherapeutic agents attempts to maximize treatment efficacy while minimizing DOX-related adverse effects. In an ideal world, the combination of drugs would have a synergistic impact on cancer cells, yet its toxicity could be tolerated in order to be clinically more effective than a single treatment. EKL has the ability to act as a co-chemotherapy agent with doxorubicin DOX in T47D cancer cells, as demonstrated by the research findings. However, this is not observed in HeLa cells, implying that additional molecular elucidation is required to develop EKL as a co-chemotherapy drug. The proliferation kinetics test provided additional proof of this combination's efficacy. According to the results obtained, cells treated with a combination of EKL and DOX appear to have the inhibitory activity of T47D and HeLa cell growth when compared to control cells, as demonstrated by the doubling time of T47D and HeLa cells treated with a combination of EKL and DOX remaining constant.

4. CONCLUSION

According to the findings of the study, a combination of Doxorubicin (DOX) and ethanolic extracts of the ketapang leaves (EKL) has more potent cytotoxic activity in T47D breast cancer cells than in HeLa cervical cancer cells, presumably due to their proliferation inhibitory activity in T47D breast cancer cells. Further investigation into the molecular mechanisms underlying these activities, on the other hand, is required.

5. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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