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Combinational Effect of *Eleutherine palmifolia* (L.) Merr Extract and Doxorubicin Chemotherapy on HeLa Cervical Cancer Cells

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Abstract. *Eleutherine palmifolia* (L.) Merr (*E. palmifolia*) is a typical Central Borneo plant that empirically has many benefits in the health sector. *E. palmifolia* is known to have flavonoids which have anticancer activities. Cancer is the leading cause of death throughout the world with the most cancer cases, namely breast and cervical cancer. One of the reasons for cancer failure is due to resistance to the use of chemotherapy drugs. One way to overcome to combine chemotherapy agents with chemoprevention agents from natural products. This study aims to evaluate the potential combination of doxorubicin and extract *E. palmifolia* on anticancer activity and HeLa cell apoptosis induction. Single anticancer activities and combinations were determined by using the MTT method. The effect of the combination on apoptosis induction known by the flow cytometry method. The results showed that the anticancer activity of the best combination obtained by a combination of 50 ppm doxorubicin and extract *E. palmifolia* 100 ppm. The apoptosis test results showed that the best combination of doxorubicin and extract *E. palmifolia* could not increase the apoptosis of HeLa cervical cancer cells.

INTRODUCTION

One of the herbs that are useful as medicine is *Eleutherine palmifolia* (L.) Merr. (*E. palmifolia*). *E. palmifolia* has potential as a chemoprevention agent and can be used as an anticancer. It has reported that *E. palmifolia* ethyl acetate extract can inhibit the T47D cell cycle in the G0-G1 phase, extract from *ethanolic E. palmifolia* can selectively inhibit the growth of cervical cancer cells. IC₅₀ (Inhibitory Concentration 50) from *E. palmifolia* was 40.36 µg / ml with an SI value of 4.06 [1]. The presence of flavonoids in these plants is thought to be a class of compounds responsible for the apoptotic activity. Flavonoids may inhibit the expression of the enzyme topoisomerase I and II, which play a role in screening catalysis and relaxation of DNA. Thus the topoisomerase enzyme inhibitor complex will stabilize and cause the topoisomerase enzyme to become stuck. Pinching the topoisomerase enzyme will cause damage and continue with the apoptosis process. Regarding the working mechanism, *E. palmifolia* has the same mechanism of action with several cancer drugs, including the doxorubicin [2].

Doxorubicin has several working mechanisms, including inhibition of topoisomerase II and DNA intercalation resulting in inhibition of DNA and RNA synthesis [3]. Compared to other antibiotics in the anthracycline group, doxorubicin is an antibiotic that has the broadest spectrum of clinical activities. However, the side effects of doxorubicin are also quite, and the most fatal is cardiomyopathy. Besides, doxorubicin has hepatotoxic side effects [4]. The long-term use of doxorubicin can cause resistance due to overexpression of P-glycoprotein (Pgp), which is a

protein that plays a role in removing the drug from the cell, so the cytotoxic potential of doxorubicin in cancer cells will decrease [5-7]. Thus giving a dose of doxorubicin is limited to 550 mg/m² [8]. Doxorubicin is used to treat cancer, including cervical cancer.

EXPERIMENTAL DETAILS

The material used in this study is *E. palmifolia* (*Eleutherine palmifolia*) taken from the city of Batu, East Java. Plants determined in the LIPI UPT of the Purwodadi Botanical Gardens Conservation Center with a Certificate of Determination number 0064 / IPH.6 / HM / I / 2017. Cancer used in this study is cell *line* HeLa cervical cancer. The cells were obtained from the *Cancer Chemoprevention Research Center* (CCRC), Faculty of Pharmacy, Gadjah Mada University. The materials used for cytotoxic tests are Dimethyl sulfoxide (DMSO), 0.025% trypsin, Phosphate buffer saline (PBS), Reagent MTT and Doxorubicin. The material used for apoptosis is BD Pharmingen *propidium iodide* (PI) *staining kit*, BD Annexin V: FITC *Apoptosis Detection* and filter.

This study has been declared ethical approved by the Health Research Ethics Commission of the Faculty of Medicine, Brawijaya University, East Java, Indonesia number: 49 / EC / KEPK-S1 / 02/2017. Extraction powder of *E. palmifolia* was carried out using 96% ethanol as a solvent. The solvent volume is 2500 mL. The ratio between ingredients and solutions is 1:20. The extraction method used ultrasonic extraction for 20 min [9]. The filtrate was evaporated using a *rotary evaporator*. The concentrated extract dried in the oven.

Anticancer Combination Test with MTT Method

Cells were placed and added 5x10⁵ RPMI media into *96-well plate*; six lower right wells emptied for media control. The sample solution was made by dissolving DMSO. Each solution made in concentrations of ½ IC₅₀, ¼ IC₅₀, 3/8 IC₅₀, and 1/8 IC₅₀. IC₅₀ known from extract *E. palmifolia* is 40.36µg/ml [1], whereas IC₅₀ doxorubicin is 6.33 µg / ml [10]. The cells were taken from the incubator, and the cell media is removed using a *96-well plate* reversed above the dump and pressed slowly on the tissue. Then the sample solution is inserted into a *96-well plate*. Cells incubated for 24 hours. Cell media were discarded and washed with PBS. MTT solution added as much as 100 µL to each well. Then incubated again for 3-4 hours until formazan formed. After formazan established, the condition of the cells was observed in an inverted microscope, and *stopper* SDS 10% in 0.1 N HCl was added. The plates are wrapped in aluminium foil and incubated overnight in a dark place. Then analyzed by *ELISA reader*.

Cell Apoptosis Analysis by Flow Cytometry

The 5x10⁵ cells/wells were planted in a 6-well plate and incubated until normal condition. The cells were treated with DMSO solvent (0,25%) and reincubated for 24 hours. At the end of the incubation time, the medium was taken and transferred into a tube and centrifuged (371g; 3 min). The supernatant was removed. The PBS was added to the well and PBS was transferred to the microtube, then centrifuged and the supernatant layer was removed. This stage was repeated once more and the cells were harvested with trypsin. The cells were transferred into the same microtube and then centrifuged (371 g; 3 min). The cells were rinsed with PBS and centrifuged again, and the PBS was discarded to get the harvest cells. The sediment was added carefully with PI-Annexin V reagent and immediately homogenized. The microtube containing the cell suspension was wrapped in aluminium foil and incubated in a 37 °C of water bath for 5 min. The cell suspension was homogenized again and transferred into a flow cytometer tube using a nylon filter, and then ready to be analyzed with a flow cytometer [11].

Analysis of MTT Test Result

The data obtained are in the form of absorbance of each well converted into living cell percent:

$$\text{Percentage (\%)} \text{ of live cells} = \frac{(\text{abs.Treatment} - \text{abs.Media control})}{(\text{abs.Abs-control cell.Media control})} \times 100\% \quad (1)$$

Description: Abs: absorbance

Percentage live cells are calculated to obtain the value of the equation used to calculate the IC₅₀. Combined cytotoxicity determined by calculating the interaction index between chemotherapeutic agents with *E. palmifolia*, using equation 2:

$$CI = (D) 1 / (Dx) 1 + (D) 2 / (Dx) 2 \quad (2)$$

Where D1 and D2 are concentrations of samples used in the treatment combination. (Dx) 1 and (Dx) 2 are single concentrations that can produce effects as large as those given a combination treatment [12]. The CI or Combination Index numbers obtained are interpreted as follows: <0.1 very strong synergistic effect 0.1 - 0.3 strong synergistic effect 0.3 - 0.7 synergistic effect 0.7 - 0.9 mild-moderate synergistic effect 0.9 - 1,1 approaching additive effects 1,1 - 1,45 mild antagonistic effects - moderate 1,45 - 3,3 antagonistic effects > 3,3 strong antagonistic effects - very strong

Data Analysis of Cell Apoptosis with Flow Cytometry

Data analysis was performed with cell quest programs to see the percentage of living cells, initial apoptosis, final apoptosis, and necrosis. The results of the experiment compared between HeLa cells induced by a combination of *E. palmifolia* and doxorubicin with a single treatment of doxorubicin, a single treatment of *E. palmifolia* and cell control. Per comparison is done by using ANOVA *one way* followed by test *post hoc* Tukey's HSD [13].

RESULTS

Anticancer Activity Combination of Doxorubicin and *E. palmifolia* Extract

Parameters of anticancer activity in this study seen from cell viability. Cell viability is the number of cells that live in each combination. In this research, 16 combination doses were used with treatments below IC₅₀. The smallest cell viability is a combination of *E. palmifolia* with a concentration of 20 ppm and doxorubicin with a concentration of 50 nM. While the largest is a combination of *E. palmifolia* with a concentration of 15 ppm and doxorubicin with a concentration of 200 nM. The viability of combination cells of doxorubicin and extract is *E. palmifolia* presented in Fig. 1.

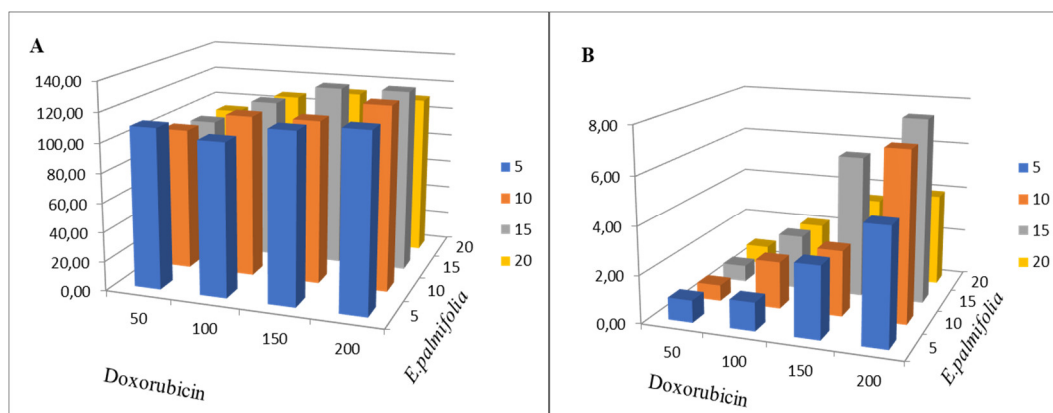


FIGURE 1. Effect of combination treatment of doxorubicin and *E. palmifolia* on HeLa cell growth: A) Cell viability due to a combination treatment of doxorubicin and *E. palmifolia* therapy B) Combination Index Treatment of combination doxorubicin therapy and *E. palmifolia* cells as much as 10⁴/wells were planted in the well plate, incubated for 24 h in RPMI media without or by treatment with a combination of doxorubicin and *E. palmifolia* with a predetermined concentration. Cell viability determined by the MTT method. The results of this analysis are representations of 2 different experiments with three replications.

After cell viability is known, a combination index is calculated. Combination index is a standard method used to evaluate drug combinations [14]. The smaller the value of a combination index, the more synergistic the combination will be - the results of the combination index calculation presented in Table 1.

The results of the combination shown that the synergistic effect obtained in two combinations 1) combination of *E. palmifolia* 10 ppm with 50 nM doxorubicin, and 2) a combination of *E. palmifolia* 15 ppm with 50 nM doxorubicin. The synergistic effect combination was obtained from the combination of *E. palmifolia* 20 ppm with doxorubicin 50 nM while the additive combination obtained from a combination of *E. palmifolia* 5 ppm and doxorubicin 50 nM. Other combinations are antagonistic because of the cell resistance to doxorubicin [16]. Other flavonoid compounds which,

when combined with doxorubicin are additive include K4 synthetic flavonoids [17]. Extract *E. palmifolia* also has an antagonistic effect when combined with methotrexate in T47D cells [18].

TABLE 1. Results of calculation of combination index

No	Combination		Cell viability	CI	Effect category [15]
	<i>E. palmifolia</i>	Dox			
1	5	50	109.42 ± 6.96	0.92	Additives
2	5	100	103.89 ± 8.8	1.18	Mild-moderate Antag
3	5	150	114.86 ± 0.45	2.98	Antagonists
4	5	200	118.83 ± 4.85	4.79	Strong antagonists
5	10	50	97.76 ± 13.64	0.67	Synergistic
6	10	100	110.76 ± 2.37	1.98	Antagonists
7	10	150	111.21 ± 8, 18	2.75	Antagonists
8	10	200	124.66 ± 2.37	7.07	Strong antagonists
9	15	50	94.02 ± 18.7	0.70	Synergistic
10	15	100	111.21 ± 5.62	2.32	Antagonists
11	15	150	124.36 ± 2.7	5.99	Antagonists
12	15	200	125.11 ± 7.65	7.78	Antagonists
13	20	50	93.12 ± 9.79	0.80	Mild synergistic
14	20	100	106.73 ± 2, 94	2.09	Antagonists
15	20	150	112.11 ± 1.55	3.49	Strong antagonists
16	20	200	110.61 ± 4.26	3.93	Strong antagonists

The Apoptosis of Combination Treatment of doxorubicin and *E. palmifolia* Extract on HeLa cell

The appearance of HeLa cells after being treated can be seen in Fig. 1. The HeLa cells are then transferred into the cones to tested for apoptosis using a tool of the flow cytometer. The results of the apoptosis study separated from the method *cell quest*. The effects of the separation presented in Fig. 2.

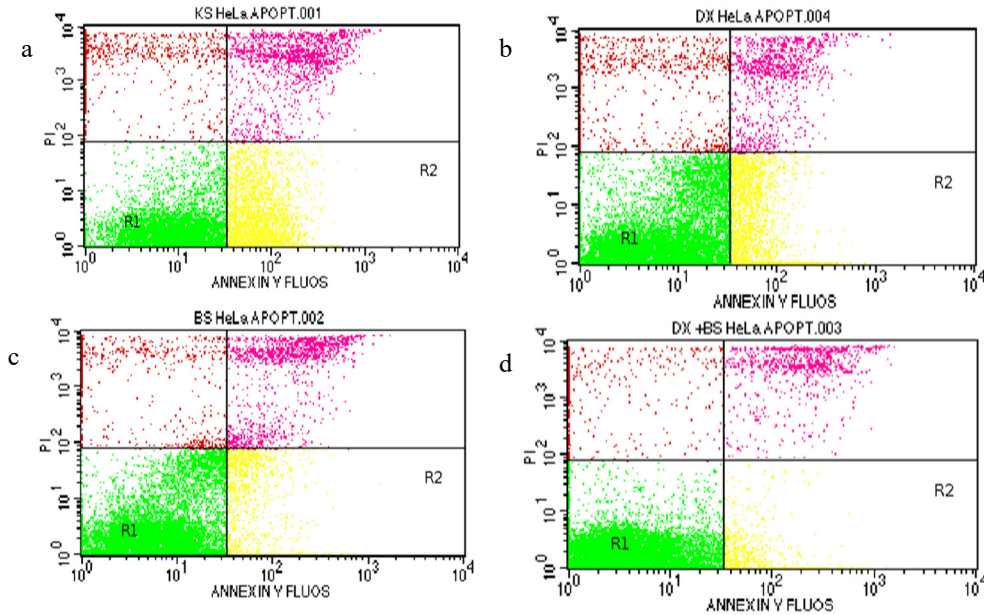


FIGURE 2. The results of the colour separation by the method cell quest on the flow cytometry test results: a) cell control; b) after a single doxorubicin induction concentration of 5 nM; c) after inducing *E. palmifolia* single concentration of 10 ppm; d) after being combined with *E. palmifolia* concentration of 10 ppm and doxorubicin concentration of 5 Nm.

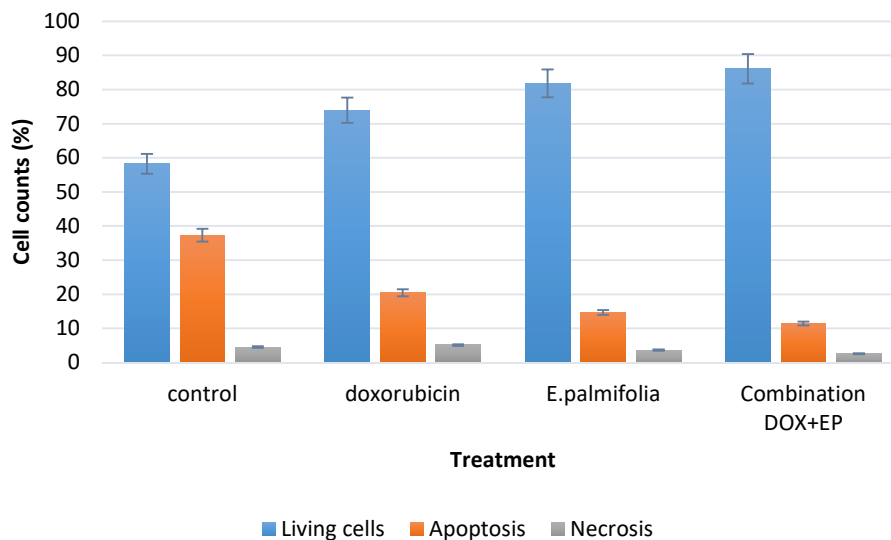


FIGURE 3. Graph of cell distribution results of a flow cytometry test

Data from apoptosis analysis were tested using SPSS 16 for windows. Data were analyzed using ANOVA test *one way*. The results of the significance of the ANOVA test *one way* are 0.00, which indicates that there are significant differences in each sample. The results of the observation showed that the percentage of living cells from combination-induced cells was higher than the cells that were induced by a single treatment. While the percentage of cells that experience apoptosis and necrosis in combination is less than a single treatment, this shows that the combination of extract *E. palmifolia* and doxorubicin cannot increase the apoptosis of HeLa cervical cancer cells. These events are expected to occur due to the presence of Fe in cell hemoproteins [19]. Doxorubicin will react with Fe to form doxorubicin -Fe complex. The structure of quinones in doxorubicin oxidized to radical semiquinone. Semiquinone radicals will react with oxygen to form superoxide and hydrogen peroxide. Superoxide and hydrogen peroxide can turn into hydroxyl radicals, which are very reactive with the presence of the doxorubicin -Fe complex [20]. The highly reactive hydroxyl radical compounds will bind to flavonoids, which are natural antioxidants [21]. This bond causes no mechanism for apoptosis.

SUMMARY

The combination of doxorubicin and extract *E. palmifolia* can provide a synergistic effect in inhibiting Hela cells in combination with *E. palmifolia* 10 ppm: doxorubicin 50 nM and the combination of *E. palmifolia* 15 ppm: doxorubicin 50 nM. The percentage of cells undergoing apoptosis in the combination treatment was 11.42%, whereas in a single doxorubicin treatment it was 20.48% and in the therapy *E.palmifolia* single was 14.66%. This shows that the combination of extract *E. palmifolia* and doxorubicin cannot increase the apoptosis of HeLa cervical cancer cells.

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