Combination Effect of Ethylacetate Extract of *Plectranthus amboinicus* (Lour.) Spreng. with Doxorubicin Against HeLa Cell Lines

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ABSTRACT

The aims of the study were to investigate the growth-inhibiting mediating effect of *Plectranthus amboinicus* (Lour.) Spreng. ethylacetate extract (PAE) in combination therapy with doxorubicin against HeLa cell lines, to analyzed the apoptotic induction and expression of cyclin D1, Bel2 and COX-2 (cyclooxygenase-2) proteins of HeLa cell lines after treatment of PAE. The cytotoxicity effects were determined by using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The effect of apoptosis on HeLa cell lines were observed by flowcytometry assay in single dose of PAE. The expression of cyclin D1, Bel2 and COX-2 proteins of HeLa cell lines after treatment of PAE were identified by using immunocytochemistry. The result showed that PAE had strong synergistic effect with doxorubicin against HeLa cells based on Combination Index analysis. PAE 28.66 µg/mL increases apoptotic induction. The PAE showed suppression of cyclin D1, Bcl2 and COX-2 expressions on HeLa cell lines. The results concluded that PAE could be a potential co-chemotherapeutic agent with doxorubicin on cervix cancer cells.

Keywords: *Plectranthus amboinicus*, doxorubicin, HeLa, combination

INTRODUCTION

The medical need for better cancer treatment is clear. In the developed world, roughly one in three people contracts cancer and around one in four of those die from the disease. The worldwide incidence of cancer is set to double from 10 to 20 million over the next two decades and the death rate will increase from 6 to 10 million\(^1\). New and more effective drug therapies are being developed due to the progress in the field of tumor biology and molecular genetics\(^2\). Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, as single modalities have a limited but important role in the overall treatment of most solid tumors\(^3\). Chemotherapy drugs are still have a lot of constrained problems, including not selective in killing cells because it affects the synthesis of nucleic acids and protein, so that normal body cells also die, side effect and costs of treatment were quite large. The use of doxorubicin as chemoterapeutic agent causes serious problems such as drug resistance and toxic effect on normal tissue which main pressing the immune system and heart toxicity\(^4\). Thus the strategies of cancer treatment using combined therapies or combined agents are considered more promising for higher efficacy, resulting in a better survival\(^3\).

Indonesia is an area that has the potential diversity of plant species as medicinal plants. One of these medicinal plants is *Plectranthus amboinicus* (Lour.) Spreng. The previous
studies had showed that the n-hexane, ethylacetate and ethanol extracts of *Plectranthus amboinicus*, (Lour.) Spreng. had antioxidant activities. The n-hexane and ethylacetate extracts exhibited strong cytotoxic effect on T47D breast cancer cells with IC₅₀ value of 44.716 µg/mL and 37.61 µg/mL, respectively. Antioxidant activity is usually correlated with cancer prevention. Thus, the extract has potential effect as a chemoprevention. The cytotoxic effect of n-hexane, ethylacetate and ethanol extracts were also examined on the HeLa cell lines. The study showed that the three extracts had cytotoxic effect on HeLa cells with IC₅₀ values 76.322 µg/mL, 143.291 µg/mL, and 88.997 µg/mL, respectively. The aims of this research are to investigate cytotoxic activity of PAE-doxorubicin combination, to analyze apoptotic induction and the proteins expression of HeLa cell lines after single treatment of PAE.

**MATERIALS AND METHODS**

**Plant material**

Fresh leaves of *Plectranthus amboinicus*, (Lour.) Spreng. was collected from Pematang Siantar, Simalungun regency, Sumatera Utara province, Indonesia. *Plectranthus amboinicus*, (Lour.) Spreng. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium.

**Preparation of Ethylacetate extract (PAE)**

The air-dried and powdered leaves of *Plectranthus amboinicus*, (Lour.) Spreng. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder were dried in the air and extracted with ethylacetate (3x3 d, 7.5 L) at room temperature on a shake. The filtrate was collected, and then evaporated under reduced pressure by rotary evaporator (Heidolph VV-200) to obtain a viscous extract and the concentrated extract was dried by freeze-dryer (Edwards).

**chemicals:** n-hexane and ethylacetate were purchased from Merck (Darmstadt, Germany), DMSO (Sigma Aldrich Chemie GmBH Germany), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma Chemical, St. Louis, MO), RPMI media and Phosphate Buffer Saline (FBS) 10% v/v (Gibco, Grand Island, NY, USA), Doxorubicin (Ebewe).

**Cytotoxicity assay**

Cytotoxicity was determined by the MTT assay. Briefly, HeLa cells were plated at 10⁴ cells/well in a 96-well plate. After incubation for 24 h at 37°C, cells were treated by *Plectranthus amboinicus* ethylacetate extract (PAE) with different concentration and incubated for 24 h. MTT solution was added to each well and further incubated for 4h at 37°C, optical density was read with an ELISA reader at 595 nm.

**Flowcytometry assay**

**Apoptosis assay**

HeLa cells (5x10⁵ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with PAE, 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 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°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°C for 30 min. The samples were analyzed using FACScan flow cytometer\textsuperscript{7}.

Immunocytochemistry
HeLa cells (5x10\textsuperscript{4} cells/well) were seeded on coverslips in 24-well plate and incubated for 24 h. After that, the cells were treated with PAE, and then incubated for 24 h. After incubation, the cells were washed with PBS and then fixed with cold methanol at 4°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, cyclin D1, and COX-2 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%. Protein expression observed by light microscope (Nikon YS100). Cells that express a particular protein will provide the brown colour, while the cells that does not give a specific protein will provide blue colour\textsuperscript{8}.

RESULT AND DISCUSSION
This research were aimed to investigate the efficacy of PAE as a co-chemotherapy on doxorubicin treatment, to analyze apoptotic induction and proteins expression of HeLa cell lines after treatment of the extract. PAE, doxorubicin and their combination were investigated for their cytotoxicity effect on HeLa cell lines. MTT method was using to determined cell viability after incubation for 24 h, and the effect of combination was analyzed by Combination Index analysis. The Combination Index (CI) analysis is one of the most popular method for evaluating drug interactions in combination cancer chemotherapy\textsuperscript{9}. The method is used to categorize the effect of the combination which is synergistic, additive, or antagonis. In every treatment (PAE and their combination) was showed the inhibition of cells growth. The IC\textsubscript{50} value of PAE 143.291 µg/mL\textsuperscript{6} 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 ¼, 3/8, and ½ IC\textsubscript{50} value of PAE and 1/2 IC\textsubscript{50} value of doxorubicin (0.6 µg/mL) categorized with strong synergistic effect (CI <0.1). These effects supposed to be related to apoptotic induction and expression of some proteins.

In this study, the apoptotic induction and expression of proteins were done at PAE single treatment, because we wanted to know its effect alone. The effect of its combination with doxorubicin need further study. Evaluation of apoptotic induction was performed by using flowcytometry assay with Annexin V. As shown in Figure 1, the cells in the upper and lower right quadrants represent late apoptotic/necrotic and early apoptotic cells, respectively. The percentage of cells treatment by PAE, in early apoptotic was 15.63%, in late apoptotic/ early necrotic 8.21% and in late necrotic 5.27%.
The observation of expression of apoptosis regulator protein Bcl2 and the protein that play important role in cell cycle, Cyclin D1 were conducted in HeLa cells by using PAE treatment. Expression of COX-2 was also done, because COX-2 may contribute to the development of human cancer. COX-2 derived prostaglandin E2 induces angiogenesis of tumor development by increasing of angiogenic factors, or decreased expression of anti-angiogenic factors, or a combination of both event\(^{10}\). Effect of PAE on cyclin D1, Bcl2 and COX-2 expressions were evaluated using immunocytochemistry. Expression of cyclin D1, Bcl2 and COX-2 proteins are positive characterized by brown stained nuclei in the cells (Figure 2).
The observation of apoptosis regulator protein Bcl_{2} was conducted in HeLa cells by using PAE. Immunocytochemistry assay with Bcl2 antibody showed the expression of Bcl2 was decreased by PAE, therefore it is strengthen the apoptosis mechanism of PAE. One of secondary metabolite in *Plectranthus amboinicus* is ursolic acid. A Study reported that ursolic acid inhibit EGFR/MAPK and suppress Bcl2 expression on colon cancer cell by activation caspase 3 and 9. Thus, it is possibly that apoptosis induction of PAE occur through the same pathway, but we need further study. As seen in Figure 2, the untreated cells (control) showed high intensity for Bcl2, cyclin D1 and COX-2. A single treatment of PAE was decreased on Bcl2, cyclin D1 and COX-2 expression. Inhibition 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 play important role in G_{0}-G_{1} phase with established complex with CDK_{4} or CDK-6 to controlled G_{1} to S phase transition. These results showed the same effect to T47D breast cancer cells. The PAE 8 µg/mL had the synergistic effect with doxorubicin againts T47D breast cancer cells, induced apoptosis and cells accumulation at G1 phase, beside the expression of cyclin D1 and COX2 showed cell cycle arrest and metastasis inhibition of T47D cells line. However, the molecular mechanism of apoptosis induction, cell cycle modulation, and antiangiogenic regulation of PAE need to be explored more detail. Based on the results, we concluded that combination of ethylacetate extract of *Plectranthus amboinicus* (Lour.) Spreng. leaves and doxorubicin sinergistically inhibit the HeLa cell lines. Based on the immunocytochemistry assays, the ethylacetate extract of *Plectranthus amboinicus* could perform as chemopreventive agent on cervical cancer.

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