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Effect of *Plectranthus amboinicus*, Lour. Spreng. *n*-Hexane Extract on T47D Cells Line: Proliferation, Apoptosis and It's Combination with Doxorubicine

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Abstract. The objectives of the study are to investigated the growth-inhibiting and apoptosis mediating effect of *Plectranthus amboinicus* (Lour.) Spreng *n*-hexane extract (PANE) on T47D cell lines. The assays were performed in the study were cytotoxicity assay and apoptosis induction of T47D cells. The cytotoxicity effects were determined by using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The effect of apoptosis were observed by ethidium bromide-acrydine orange method. The growth of T47D was inhibited by treatment with PANE on concentrations 20; 40; dan 80 µg/mL. The cell death (apoptosis) induced by PANE was characterized by orange fluorescent on the IC_{50} concentration. These results concluded that the *n*-hexane extract of *Plectranthus amboinicus*, (Lour.) Spreng. inhibited the growth of T47D breast cancer cells in dose and time dependent manners. PANE also could induced apoptosis on T47D breast cancer cells, but the combination of PANE-doxorubicine did not show the synergistic effect. The results suggesting that PANE may be a potent agent for the chemoprevention of breast cancer.

Keyword: acrydine orange, chemoprevention, combination index, doubling time, ethidium bromide, IC_{50} value ≤ 100 µg/mL.

Abstrak. *Tujuan dari penelitian ini adalah untuk menyelidiki penghambatan pertumbuhan dan penginduksi apoptosis dari ekstrak n-heksan daun bangun-bangun (Plectranthus amboinicus (Lour.) Spreng pada sel kultur T47D. Uji yang dilakukan adalah uji sitotoksisitas daninduksi apoptosis terhadap sel T47D. Efek sitotoksisitas ditentukan dengan metode MTT [3- (4,5-dimethylthiazole-2-yl) -2,5-difenil tetrazolium bromide]. Efek apoptosis diamati dengan metode akridin-oranye-etidium-bromida. Pertumbuhan T47D dapat dihambat oleh pemberian ekstrak n-heksan daun bangun-bangun pada konsentrasi 20; 40; dan 80 ug / mL. Kematian sel yang diinduksi oleh PANE ditandai oleh fluoresen oranye pada konsentrasi IC_{50}. Hasil ini menyimpulkan bahwa ekstrak n-heksan menghambat pertumbuhan sel kanker payudara T47D dalam dosis dan perilaku yang tergantung waktu. PANE juga dapat menginduksi apoptosis pada sel kanker payudara T47D, tetapi kombinasi PANE-doxorubisin tidak menunjukkan efek sinergis. Hasil menunjukkan bahwa PANE dapat menjadi agen untuk kemoprevensi kanker payudara.*

Kata Kunci: *acrydine orange, kemopreventif, combination index, doubling time, ethidium bromide, NIlai IC_{50} ≤ 100 µg/mL*

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1. Introduction

Cancer is one of the major causes of death in developed countries, together with cardiac and cardiovascular diseases. Radioactive rays and most anticancer drugs damage DNA or suppress DNA duplication to kill tumor cells growing rapidly. At the same time, they also affect normal cells to cause serious adverse effects, such as bone marrow function inhibition, nausea, vomiting, and alopecia [1], [10], [17].

Plectranthus amboinicus, (Lour.) Spreng is used for lactagogue in Bataknese, North Sumatera. Torbangun leaf soup is made by traditionally recipe as Batak cuisine exhibited increasing milk secretion of lactating mother. There are some informations about its effects such as antiinflammatory, anti clastogenic, nephroprotective and hepatoprotective. The previous studies had showed that the n-hexane, ethylacetate and ethanol extracts of *Plectranthus amboinicus*, (Lour.) Spreng. had antioxidant activity [2]. Antioxidant activity is usually correlated with cancer prevention [20]. Thus, the extract has potential effect as a chemoprevention. The n-hexane extract of *Plectranthus amboinicus*, (Lour.) Spreng. have cytotoxic effect on HeLa cell with IC_{50} 76.322 µg/mL. The measurement of the selectivity of the extract were executed by calculation of the viability cells of the Vero cell lines which is the normal cells. The IC_{50} of the extract on Vero cell were compared to HeLa cells to find the Selectivity Index (SI). The SI index of PANE is 5.43 while the SI index > 3 is supposed to be selective to HeLa cell Lines [3]. The combination of PANE with doxorubicin on HeLa cell line showed strong synergistic effect based on combination index analysis [4]. The in silico study have found that β-sitosterol which is a steroid from *Plectranthus amboinicus* (Lour.) Spreng. showed that it have the activity in inhibition of cancer growth toward T47D, MCF-7, HeLa and WiDr cell lines. The inhibitions occurred through PI3K, EGFR, ER-α, ER-β dan HER-2 pathways[5].This study aimed to evaluate the proliferation inhibition, and the synergistic effect combination of PANE-doxorubicine on T47D cell lines by using MTT assay. Meanwhile, the apoptosis induction was evaluated by acridyne orange – etydium bromide staining method.

2. Methods

2.1 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.

2.2 Preparation of *Plectranthus amboinicus,* (Lour.) Spreng. *n*-Hexane extract (PANE)

The air-dried and powdered leaves of *Plectranthus amboinicus,* (Lour.) Spreng. (1 kg) were extracted by cold maceration with *n*-hexane (3x3 d, 7.5 L). The powder were was dried in the air and at room temperature on a shake until the powder was dry. 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 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).

2.3 Cytotoxicity assay

Cytotoxicity, doubling time, and combination test of PANE-doxorubicine were determined by the MTT assay.

Briefly, T47D cells were plated at 10^4 cells/well in a 96-well plate. After incubation for 24 h at 37^oC, cells were treated by *Plectranthus amboinicus n*-hexane extract (PANE) with different concentration and incubated for 24 h. MTT solution was added to each well and further incubated for 4h at 37^0C, optical density was read with an ELISA reader at 595 nm. For doubling time assay, the incubation was also done at 48 and 72 hours [6], [19].

2.4 Detection of treatment-induced apoptosis

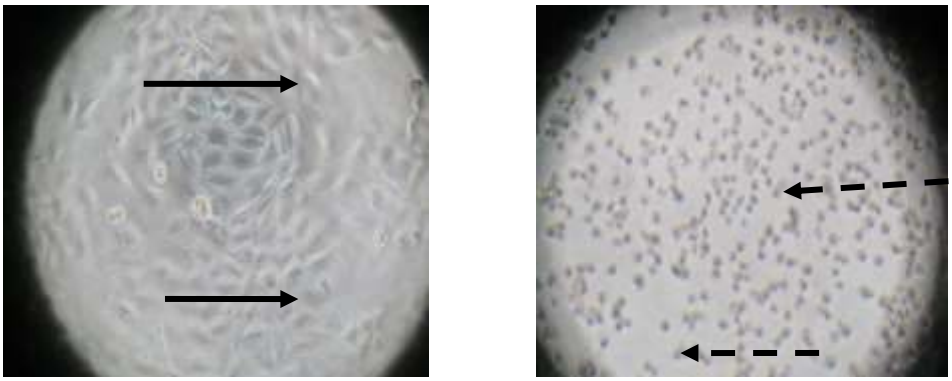
To asses the degree of treatment-induced apoptosis and or necrosis in T47D cells, the cells were exposed to *Plectranthus amboinicus,* (Lour.) Spreng. extracts and cultured. Apoptosis was determined by acrydine orange/ethidium bromide nuclear stain. Qualitative detection of apoptotic cells was performed by fluorescens microscope. briefly, 10 µL of the reaction mixture (1:1 acridine orange-ethidium bromide) was added to 250 µL of cell suspension. This was kept in the dark for 20 minutes, after which about 5 µL was dispensed onto microscope slides and examined under a fluorenscence microscope. Detection of apoptosis was based on morphological and fluorencent characteristics of the stained cells. Viable cells were indicated by bright green, apoptotic cells by orange/brown, and necrotic cells by red [7].

3. Results and Discussion

3.1 Cytotoxicity assay

T47D cell lines is a breast cancer cells which is not resistant yet on doxorubicin but recently known that it has mutated on p53 protein [8]. The controll cells have an oval glass-like appearance, they are not only sticking to each other, but also to the bottom of the well. While the dead cells because of the treatment PANE appear to be round with a black center, tend to be

scattered and float and smaller than the viable cells. While the dead cells because of the treatment of PANE look to be round with a black center, tend to be scattered, float and have smaller size than the viable cells. The morphology of T47D cells after treatment by PANE was showed on Figure 1.



Control cell T47D T47D cell treated by PANE

Figure 1. The difference of T47D cell's morphology beetween T47D control cells and treated cells by PANE at concentration 500 µg/mL (magnification 10x10)

→ : viable cell

⇢ : dead cell

The T47D cells were exposed to various concentration of PANE from 15.625 untill 500 µg/mL. The percentage of viable cells of T47D were decreased with the increasing of PANE concentration. The percentage of viable cells were showed on Table 1.

Table 1. Percentage of viable cells at various concentration

concentration (µg/mL**)**	% viable cells
250.00	04.00
125.00	49.00
62.50	63.00
31.25	57.00
15.625	53.00

Based on the viable cell, it could be calculated the inhibition concentration (IC_{50}) value of PANE by probit analysis. The IC_{50} value of PANE was 44.716 µg/mL. An extract could be potentially as a growth inhibitor of the cancer cells if it have the IC_{50} value ≤ 100 µg/mL. The lower IC_{50} value indicate the higher of its cytotoxic activity [9], [10].

3.2 Doubling time assay

To find out whether the mechanism of cell toxicity through inhibition of proliferation, the test is continued by calculating the kinetic of T47D cell proliferation using doubling time test. The kinetics of the cells proliferation were calculated by extrapolating time versus the percentage of

viable cells. Extracts that are able to extend doubling time show the ability of inhibit the proliferation of T47D through the mechanism of cell cycle arrest. Doubling time is the time required by a cell to multiply itself twice [9]. Doubling time test is done by counting the number of living cells that were treated with extracts in each unit of time. Determination of doubling time is done by extrapolating time versus percent of living cells.

Determination of PANE concentrations on doubling time assay were 2 IC_{50}, IC_{50}, dan ½ IC_{50} respectively. This determination is carried out so that not too many cells die during the 48 and 72 hour observations, the kinetics of cell proliferation can be observed until the 72 hour.

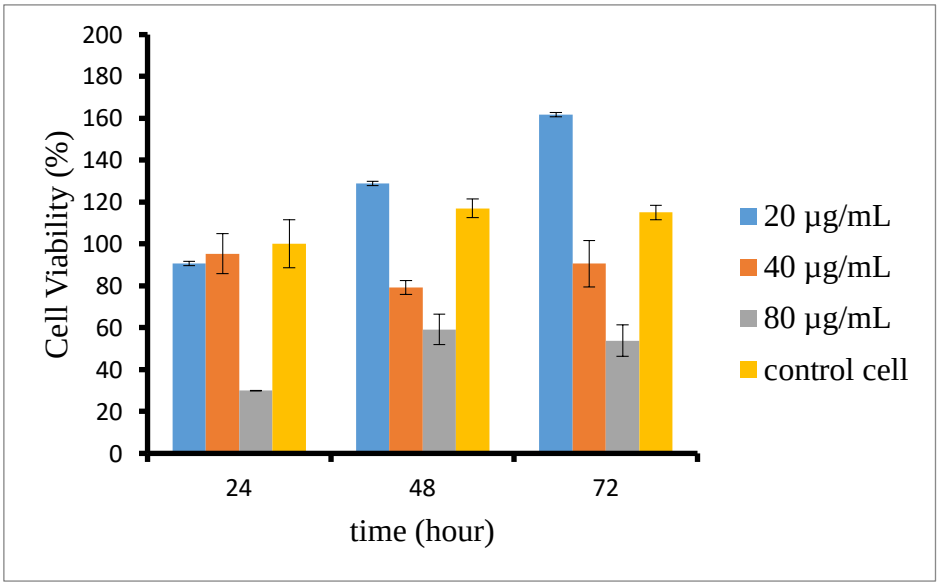


Figure 2. The effect concentrations and times of treatmen of PANE on T47D cell viability (Mean ± SEM)

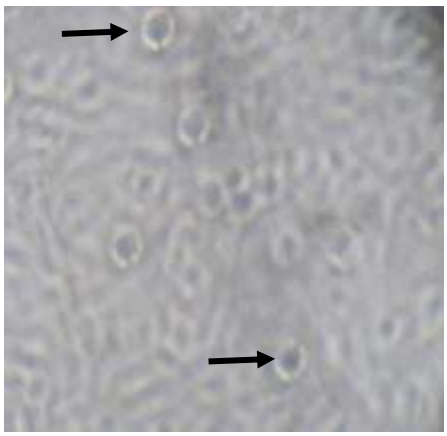
The PANE treatment showed the inhibition of T47D proliferation. The higher concentration showed the decrease of cell viability. Based on ANOVA, it can be proved that PANE on concentrations 20; 40; dan 80 µg/mL siginificantly different with control cell (0,026; 0,002; 0,000; $p<0,05$). The PANE on concentration 20 µg/mL was significantly different with concentrations of 40 µg/mL and 80 µg/mL, and between 40 µg/mL and 80 µg/mL were also significantly different (0,000; $p<0,05$). Figure 2 showed the percentage of viable cells at the 24h was significantly different with 48 and 72 hours, while the cell viability between 48 and 72 hours were not significantly different (0,134; $p>0,05$). In Figure 2, the percentage of cell viability decreases with increasing concentrations of extracts at 48 and 72 hours. The percentage of viable cells at 24 hour incubation time is significantly different from 48 and 72 hour incubation times. Increased time gives an influence in decreasing cell growth, but at the 48 and 72 hours are not significantly different. Figure 2 also showed that the PANE concentration of 20

μg/mL gives a greater percentage of living cells than the control, meaning that it is proven to be not toxic enough for cells.

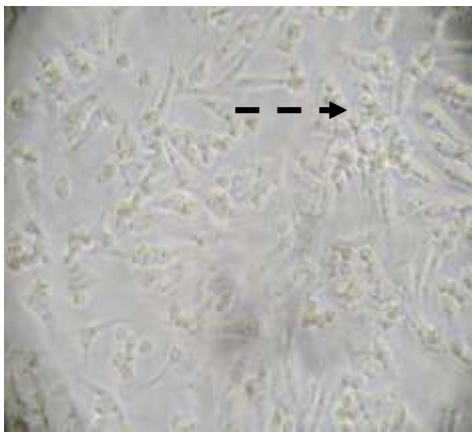
Figure 2 shows that the increase in PANE concentration can cause an inhibitory effect on the cell cycle. inhibitory effect on the cell cycle. PANE concentration 40 μg/mL did not show the addition of living T47D cells at 48 hours and 72 hours. The same thing happened at PANE concentrations of 80 μg/mL, even fewer live cells. Even though the data do not appear linear, there is a cell cycle arrest by increasing PANE concentration. It meant that the inhibition of PANE against T47D cancer cells depends on concentration.

The duration of PANE contact with cancer cells is quite influential in decreasing the percentage of T47D viable cells. At the 48th hour, the cancer cells are still dividing, so is the 72nd hour. It is probably because the genes in the cancer cells can evade the apoptotic mechanism or the gene triggering proliferation overexpresses so that the T47D cells continue to divide. T47D cells are models of breast cancer cells that have mutated p53, resulting in uncontrolled cell proliferation [21]. The cell character explains why the data look non-linear. Cancer cells multiply and can proliferate faster than the healthy cells. However, in Figure 2, it is seen that T47D cells treated by PANE experienced proliferation inhibition so that the percentage of T47D cells lived less than control cells (T47D cells without being extracted.)

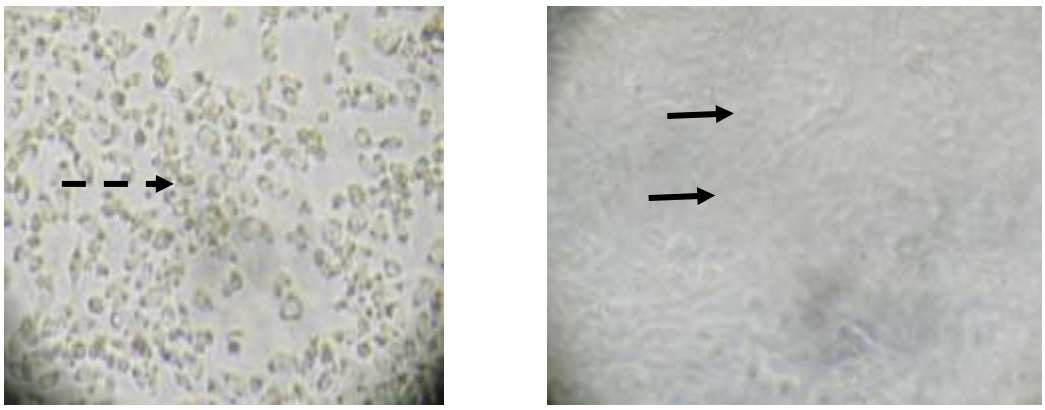
The morphology of T47D cell lines were observed after treatment of PANE on 24, 48, dan 72 hours at concentration of 40 μg/mL as seen on Figure 3.



(a)



(b)



(c) (d)

Figure 3. The morphological of T47D cell lines after treatment by PANE on (a) 24 hours, (b) 48 hours, (c) 72 hours (d) control cell of T47D.

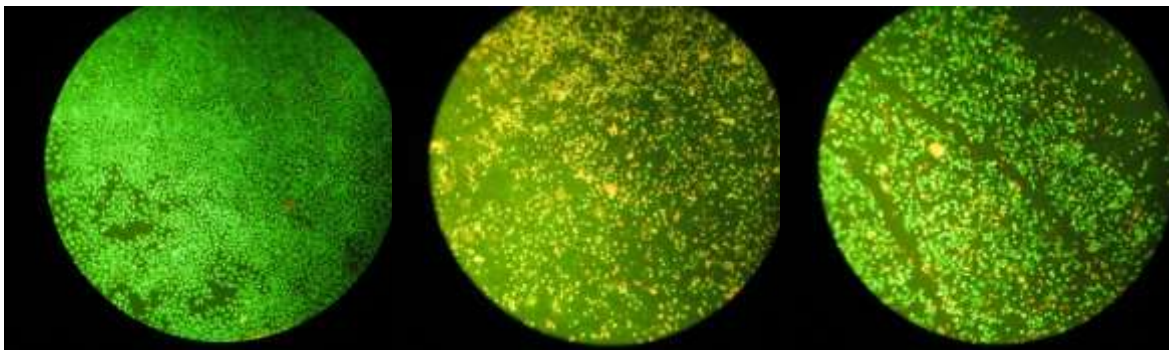
: viable cells : dead cells

It could be seen on Figure 3 that the cell death tend to be increased from 24 hours to 48 and 72 hours.

3.3 Apoptosis

A defect in the apoptosis pathway is another common event in many types of cancer including breast cancer [19]. In this study, we observed the apoptosis of T47D after treatment by PAEN. Apoptosis testing was done by using double staining method. Green fluorescent cells show living cells, while red fluorescent cells show dead cells [11].

Plectranthus amboinicus (Lour.) Spreng. contain flavonoid, terpenoid, saponin, steroid, tannin, protein, carbohydrate and volatile oils [12]. One of the active compound on this plant is *ursolic acid* (a terpenoid), known has anticancer effect. Previous research on the isolation results of ursolic acid in some traditional Chinese medicines has found that this compound could inhibit the proliferation and induced the apoptosis of colon carcinoma cell by activating caspase 3 and 9 and suppressing the phosphorylation of EGFR (*epidermal growth factor hormon*), through MAPK (*mitogen-activated protein kinase*) pathway [13], [17]. This study hypothesized that the ursolic acid containing in PANE could be the active compound that inhibit the proliferation and inducing apoptosis on t47D cell lines. It still need the further study to prove it. The previous study has proved that β-sitosetrol from *Plectranthus amboinicus* (Lour.) Spreng. was shown to inhibit cells growth toward T47D, MCF-7, HeLa and WiDr cell lines. The IC_{50} value of β-sitosterol was 0.55; 0.87; 0.76, and 0.99 mM, respectively[5]. These datas could be represent the potential effect of steroid/triterpenoid from *Plectranthus amboinicus* (Lour.) Spreng. as anticancer.



T47D control PANE on IC_{50} PANE on ½ IC_{50}

Figure 4 The microscopic view of T47D cells before and after treatment PANE and stained by etydium bromide-acrydine orange (10x10 magnification).

As shown on Figure 4, the control cell of T47D is seen the green fluorescent because they just absorb the acrydine orange, but the ethidium bromide could not enter the cells because of the cell integrity still normal. The PANE could induce apoptosis characterized by orange fluorescent. The intensity of orange fluorescent could visualize that the IC_{50} concentration showed the higher capability on inducing apoptosis than ½ IC_{50} concentration.

3.4 The cytotoxic effect of combination of PANE with Doxorubicine on T47D

PANE concentration for combination testing were 55,625; 11,250; 16,875; and 22.50 µg /mL. Variations in extract concentrations tested on T47D cells are also based on calculations from 1/8; 1/4; 3/8; and 1/2 IC_{50}. The variation in the concentration of doxorubicin used is 25; 50; 100; and 200 nM

Table 2. The combination index value of PANE-doxorubicine on T47D cell line

PANE (µg/mL)	Doxorubicine (nM)			
	25	50	100	200
5.625	1.0178	1.8077	2.045	1.7639
11.25	2.1059	2.0356	2.7138	1.8532
16.875	2.6964	1.3483	1.4692	2.1531
22.5	2.3823	1.201	1.3918	1.5998

On table 2, it can be seen that the combination of PANE-doxorubine did not toxic on T47D cell, because the combination index value was > 1, it means that the PANE on various concentrations with doxorubicine gave the antagonistic character. It could be concluded that the combination did not resulting in cell death.

Doxorubicin is an anthracycline antibiotics used as chemotherapy,which is able to interact with DNA and damage DNA function [14]. Although doxorubicin is widely used for some types of cancer, its use is limited by the side effects such as nausea, immunosuppression, and arrhythmia.

The most serious side effect from prolonged administration of doxorubicin is cardiomyopathy followed by heart failure [14]. The mechanism that mediates cardiac toxicity is caused by the

formation of reactive oxygen species, increased levels of superoxide anion and ATP depletion which causes cardiac tissue injury. While the heart does not have endogenous antioxidant [16].

4. Conclusion

The result suggest that the PANE inhibited the growth of T47D breast cancer cells in dose and time dependent manners. The *n*-hexane extract of *Plectranthus amboinicus*, (Lour.) Spreng. may play the role in tumor growth suppression by inducing apoptosis in human breast cancer cells. The combination of PANE with doxorubicine did not showed the synergistic but antagonistic effect effect on T47D breast cancer cells.

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