RESEARCH ARTICLE

**Editorial Process:** Submission:10/03/2018 Acceptance:02/10/2019

# The Anti-Proliferative and Pro-Apoptotic Properties of Ethanol *Plectranthus amboinicus* (Lour.) Spreng. Leaves Ethanolic Extract Nanoparticles on T47D Cell Lines

**Poppy Anjelisa Zaitun Hasibuan[1,2]*, Sumaiyah Sumaiyah[3]**

## Abstract

**Objectives:** *Plectranthus amboinicus* (Lour.) Spreng. is a plant which has the potential as an anti-cancer agent. To enhance the bioavailability of an extract, it is necessary to transform the extract into nanoparticles. This research aimed to create nanoparticles of the extract and investigate the anti-proliferative and pro-apoptosis effects on T47D breast cancer cell lines. **Materials and Methods:** The extraction with ethanol was performed using maceration method. The nanoparticles were prepared by using the ionic gelation method. Cytotoxic assay method evaluation of the proliferation of T47D cell line (using doubling time) was carried out using the MTT assay. Apoptosis was observed using the flow cytometry assay. **Results:** Treatment with *Plectranthus amboinicus* (Lour.) Spreng. ethanolic extract nanoparticles (PAEEN) inhibited the proliferation of T47D cell lines after 48 hours and 72 hours of incubation at concentrations of 22.3 µg/mL, 44.6 µg/mL, and 89.2 µg/mL. The viable cells were 93%, 86%, 54% (48 hours), and 98%, 71%, 57% (72 hours), respectively. The nanoparticles of extract also induced apoptosis at concentrations of ¼ $IC_{50}$ (2.16%), ½ $IC_{50}$ (1.57%), and $IC_{50}$ (2.43%). **Conclusion:** PAEEN exhibits the anti-proliferative effect on T47D breast cancer cells via apoptosis. Further study is required to confirm the mechanism of PAEEN in the cell cycle arrest and apoptosis induction on T47D cells.

**Keywords:** *Plectranthus amboinicus*- (Lour.) Spreng., antiproliferative- apoptosis- nanoparticles

*Asian Pac J Cancer Prev,* **20 (3)**, 897-901

## Introduction

Cancer is the second leading cause of death in many countries. Unfortunately, the current classical treatments are impeded by a multitude of side effects, such as the development of tumor resistance, loss of appetite, nausea, vomiting, hair loss, lowered resistant to infections, hemorrhage, and diarrhea (Lifiani et al., 2018). Therefore, finding a novel and effective therapeutic compound, enhancing its bioavailability against cancer, and at the same time being able to reduce the dosage of the medicine becomes the current scientific challenges.

The *in vitro* cytotoxic property of *Plectranthus amboinicus* (Lour.) Spreng. leaves' crude extract was tested against HeLa, MCF7, and T47D cell lines. Previous studies have shown that the n-hexane, ethyl acetate, and ethanol extracts had cytotoxic effects on HeLa cells at an inhibitory concentration of 50% ($IC_{50}$) with values of 76.322 µg/mL, 143.291 µg/mL, and 88.997 µg/mL, respectively (Bharti, et al., 2015). Furthermore, it also exhibited strong cytotoxic effects on MCF7 cell lines (Bruzea et al., 2007).

N-hexane and ethyl acetate extracts showed strong cytotoxic effects on T47D breast cancer cells with $IC_{50}$ values of 44.716 µg/mL and 37.61 µg/mL, respectively. The combination of *Plectranthus amboinicus* (Lour.) Spreng. and doxorubicin which inhibited the HeLa cell lines in 1⁄4, 3⁄(8 ), and 1⁄2 $IC_{50}$ values and 1⁄2 $IC_{50}$ value of doxorubicin (0.6 µg/mL) resulted in a strong synergistic effect with Combination Index value below 0.1 (CI < 0.1). It showed the same effect with the combination of doxorubicin which inhibited T47D cell line (Hameed et al., 2016).

Targeting a drug to its specific cells is a significant challenge in designing a drug delivery system for cancer treatment. Nanoparticles form an ideal solution for anti-cancer drugs with improved selectivity and reduced side-effects toward tumor cells. Drug-loaded nanoparticles can be created to perform more complex and cooperative targeting functions (Hasibuan et al., 2015; Hasibuan et al., 2013).

## Materials and Methods

*Plant Material*

*Plectranthus amboinicus* (Lour.) Spreng. leaves

*[1]Nanomedicine Center of Innovation, [2]Department of Pharmacology Pharmacy, [3]Department of Pharmaceutical Technology, Faculty of Pharmacy, University Sumatera Utara, Indonesia. *For Correspondence: poppyanjelisa@usu.ac.id*

were obtained from Pematang Siantar, North Sumatera, Indonesia. The leaves were identified in the Research Centre for Biology, Indonesian Institute of Science, Bogor while the voucher specimen was deposited in an herbarium. The leaves of *Plectranthus amboinicus* (Lour.) Spreng. were dried at 45°C and mashed into powder.

*Chemicals and reagents*

Ethanol purchased from Merck (Darmstadt, Germany), dimethyl sulfoxide (Sigma-Aldrich, Germany), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma Chemical, St. Louis, MO), RPMI media and phosphate buffer saline, foetal bovine serum (FBS) 10% v/v (Gibco, Grand Island, NY, USA), sodium tripolyphosphate (Sigma), and glacial acetic acid (Merck). The water used in this research was deionized water.

*The preparation of Plectranthus amboinicus (Lour.) Spreng. leaves ethanolic extract nanoparticles (PAEEN)*

Ionic gelation method was performed for the preparation of *Plectranthus amboinicus* leaves ethanolic extract nanoparticles. 0.3% PAEEN was prepared in 1.5% acetic acid. The *Plectranthus amboinicus* (Lour.) Spreng. leaves ethanolic extract nanoparticles were prepared by adding sodium tripolyphosphate (1 mg/ml) dropwise under magnetic stirring at room temperature for an hour. The mixture result was separated by centrifugation at the speed of 15,000 rpm for 20 minutes. The pellet was collected and used for characterization (Ilian et al., 2018).

1 gram of *Plectranthus amboinicus* leaves ethanolic extract was diluted into 35 mL of ethanol p.a, added with 15 mL of distilled water, chitosan in 100 mL of glacial acetic acid 1%, and 350 mL of NaTPP solution. The mixture was stirred by using magnetic stirrer for ± 2 hours. After that, the colloid of nanoparticle chitosan-NaTPP of *Plectranthus amboinicus* leaves ethanolic extract was separated by centrifugation. The result was put into a freezer (± -4°C) for ± 2 days. It was then moved into a refrigerator (± 3°C) to make it dried. The resulting nanoparticles were characterized using PSA (Particle Size Analyzer) whereas the formed solids were characterized using TEM (Transmission Electron Microscope) to determine the morphological form of its solid form (Ozcelik et al., 2004).

*Cytotoxicity and anti-proliferative activity assay*

The cells were treated with PAEEN. In this test, T47D cell lines were grown in RPMI 1640 medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycin (Gibco), and fungizone 0.5% (Gibco) in a humid atmosphere (5% $CO_2$) at a temperature of 37°C. The inoculums were seeded at $1x10^4$ cells/mL with an optimal volume of 0.1 mL per well. After 24 hours of incubation, the medium was discharged and treated with PAEEN. After 24 hours of incubation, the cells were incubated again with 0.5 mg/mL of MTT for 4 hours at 37°C. Viable cells reacted with MTT and produced purple formazan crystals. After 4 hours, 10% SDS as the stopper (Sigma) was added in 0.01N of HCl (Merck) to dissolve the formazan crystals. The cells were then incubated for 24 hours at room temperature and were kept away from light. After the incubation, the cells were shaken, and absorbance was measured using ELISA reader at λ 595 nm. The measured data from each well were converted to the percentage of viable cells (Hameed et al., 2016). The cell growth inhibition was calculated using the formula Rosidah et al., 2014; Rusdi et al., 2018; Sumaiyah et al., 2014).

Percentage of viable cell = (Absample-Abmedium)/ (Abcontrol-Abmedium) x 100%

The anti-proliferative activity was also determined using the MTT assay with the same procedure. Cells were cultured in RPMI and exposed to various concentrations of PAEEN for 4 days. The absorbance of the MTT incubated cells were measured with ELISA reader at 0, 24, 48, and 72 hours.

*Detection of Apoptosis (Flow cytometry assay)*

T47D cells were seeded into 6-well plates and incubated for 24 hours. After treated with PAEEN and incubated for 24 hours, the floating and adherent cells were collected in a conical tube using 0.025% trypsin. The cells were cleaned thrice with cold PBS and separated by centrifugation with the speed of 2,500 rpm for 5 minutes. The supernatant was separated while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 hours. The cells were cleaned thrice with cold PBS, suspended, and separated by centrifugation with the speed of 3,000 rpm for 3 minutes. Annexin V kit was added to the sediment, suspended, and incubated at 37°C for 30 min. The samples were analyzed using FAC Scan flow cytometer (Kadian et al., 2018).

*Statistical Analysis*

The results were expressed as mean ± SEM. The mean difference of the cell viability assays between experimental groups was determined using one-way ANOVA, followed by the Tukey test. All experiments were carried out in triplicate.

## Results

*The Plectranthus amboinicus (Lour.) Spreng. leaves ethanol extract nanoparticles (PAEEN)*

The study aimed to evaluate the anti-proliferative and induce apoptosis of PAEEN on T47D breast cancer cells. The preparation of *Plectranthus amboinicus* (Lour.) Spreng. leaves ethanol extract was performed using the maceration method with 96% ethanol followed by evaporation to form viscous extracts. Colloidal nanoparticles were made by mixing the viscous extracts of *Plectranthus amboinicus* (Lour.) Spreng. leaves with ethanol, distilled water, chitosan solution in glacial acetic acid, and STPP. There were nine variations of chitosan and STPP composition ratio. Colloidal nanoparticles were separated by centrifugation aimed to separate the precipitation of fingerroot nanoparticles. After that, *Plectranthus amboinicus* (Lour.) Spreng. leaves extract sediment was kept in a freezer. The characterization of *Plectranthus amboinicus* (Lour.) Spreng. leaves extract

Table 1. The Percentage of Viable Cell, Early Apoptotic and Necrotic Cell

| Condition of cells | Percentage of cells (%) | | | |
|---|---|---|---|---|
| | ¼ $IC_{50}$ | ½ $IC_{50}$ | $IC_{50}$ | Control cell |
| Viable cell (LL) | 41.46 | 4.33 | 0.77 | 92.38 |
| Apoptotic cell (LR) | 2.33 | 1.4 | 0.21 | 1.19 |
| Late apoptotic cell (UR) | 4.57 | 4.07 | 2.43 | 1.46 |
| Necrotic cell (UL) | 52.84 | 90.22 | 96.62 | 5.03 |

LL, lower left; LR, lower right; UR, upper right; UL, upper left

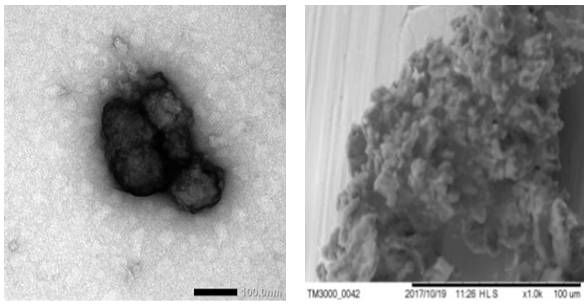


Figure 1. The Morphology of PAEEN by TEM Analysis

in nanoparticle size was performed using Particle Size Analyzer (PSA). The results of PAEEN in the nanoparticle size using PSA (Particle Size Analyzer) can be seen in Table 1. The formula using the concentration ratio of chitosan and STPP (8:1) or 0.08% chitosan and 0.01% STPP produced the smallest size of PAEEN which was 585.09 nm.

The morphology of precipitated nanoparticles was characterized using Transmission Electron Microscopy (TEM) (Martien et al., 2012). Characterization using PSA showed the nanoparticle size of 389-877 nm 98.1% with the concentration ratio of chitosan and STPP = 8:1. The average of zeta potential value was 41.87 mV. The outcome from TEM exhibits the morphology of the precipitated particle. The morphology of PAEEN can be seen in Figure 1.

The morphology of PAEEN was analyzed by using TEM. It displayed that the particles aggregated between one another. The TEM image showed that the particle size of PAEEN was in the range of 80-120 nm. The nanoparticles of *Plectranthus amboinicus* (Lour.) Spreng. leaves extract were successfully made because the size was in the range of 1 – 1,000 nm (Martien et al., 2012).

*Effect of PAEEN on T47D proliferation*

In the present study, the effect of PAEEN on T47D cell lines was examined. Cytotoxic assay of PAEEN on T47D cells was exposed to various concentrations of PAEEN (15.625, 31.25, 62.50, 125, 250, and 500 µg/mL). Cells treated with 0.1% DMSO were used as the control. The cytotoxic assay gave reproducible dose-response curves over a concentration range (Saleem et al., 2002). The cell viability decreased with the increasing concentrations of PAEEN, and the $IC_{50}$ value of PAEEN was 89.166 µg/mL.

The anti-proliferative activity of PAEEN against T47D cells at ¼ $IC_{50}$ (22.3 µg/mL), ½ $IC_{50}$ (44.6 µg/mL) and $IC_{50}$ (89.166 µg/mL) through incubation for 24, 48, and 72 hours. The selection of ¼ and ½ concentration was done so that only a few cells die at the observation time to obtain the proliferation kinetics data of T47D cells after the administration of PAEEN. Interestingly, the viability of the cells increased at 24 hours, and PAEEN suppressed the number of T47D cells. As shown in Figure 2, PAEEN inhibited the proliferation of T47D after 48 hours. The nanoparticles of the extract exhibited the most effective impact in decreasing the number of T47D cells at $IC_{50}$ concentration (89.2 µg/mL).

*Effect of PAEEN on the apoptosis of T47D breast cancer cells*

In order to determine whether the anti-proliferative effect of PAEEN was due to apoptosis, T47D cells were treated with PAEEN for 24 hours. The study aimed to evaluate apoptosis induced with the flow cytometry

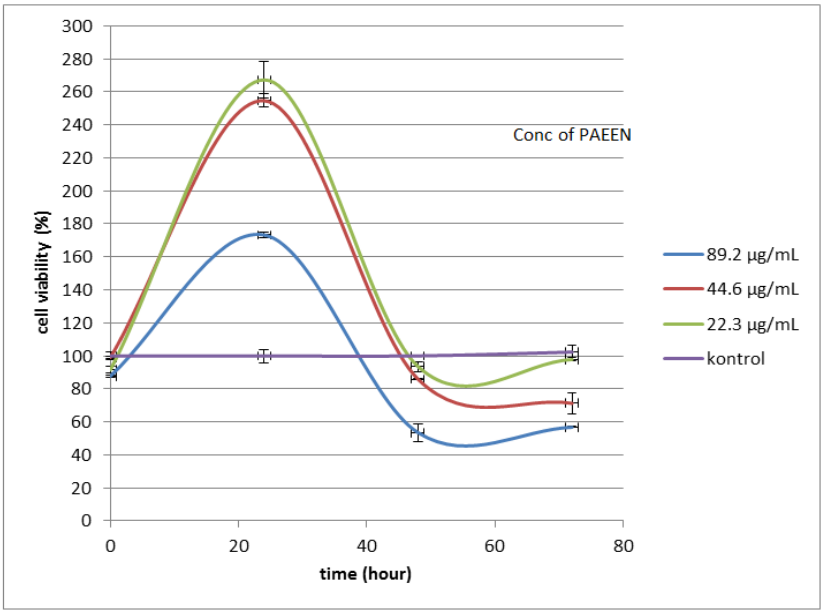
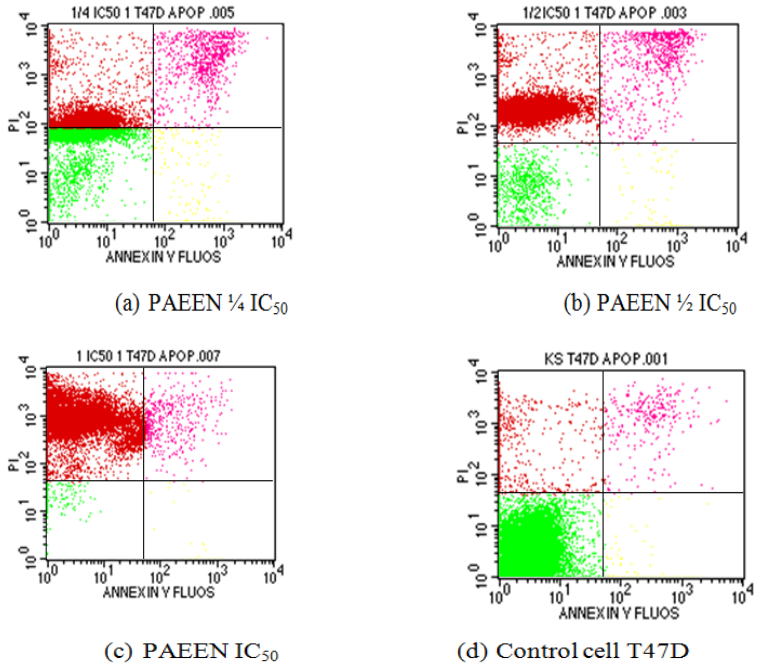



Figure 2. Effect of Different Doses of PAEEN on the Viability of T47D Cells. Data are expressed as mean ± SEM. n= 3 wells for each group.




(a) PAEEN ¼ $IC_{50}$ (b) PAEEN ½ $IC_{50}$

(c) PAEEN $IC_{50}$ (d) Control cell T47D

Figure 3. Apoptotic Analysis of T47D Treated with PAEEN for 24 hours and Stained Using Annexin-V. (a) PAEEN ¼ IC50 (22.3 µg/mL); (b) PAEEN ½ IC50 (44.6 µg/mL); (c) PAEEN IC50 (89.2 µg/mL); and (d) control cells

assay and Annexin-V. The apoptosis on T47D cells had a higher percentage than the control cells which means that the concentration of PAEEN with ¼ $IC_{50}$, ½ $IC_{50}$, and $IC_{50}$ could trigger the cell apoptosis. As shown in Figure 3, the cells in the lower right and upper right quadrants represent early apoptotic and late apoptotic or necrotic cells, respectively. The viable and late necrotic cells were represented in the lower left and upper left quadrants, respectively.

Based on the flow cytometry assay above, the percentage of viable, early apoptotic, and necrotic cells can be seen in Table 1.

PAEEN induced the early apoptosis and late apoptosis in cells with a higher percentage than the T47D control cell lines. The ¼ $IC_{50}$ PAEEN gave the highest percentage of apoptosis condition compared to the other two concentrations of PAEEN.

## Discussion

Nanoparticles form is an ideal solution for the anti-cancer drugs with improved selectivity and reduced side-effects toward the tumor cells. Drug-loaded nanoparticles can be developed to perform more complex, cooperative targeting functions (Hasibuan et al., 2016; Raj et al., 2015).

*Plectranthus amboinicus* (Lour.) Spreng. leaves extract have attracted considerable interest as alternative cancer remedies because of low toxicity and the ability to affect cells with different mechanisms, such as by preventing initiation and promotion of carcinogenesis or apoptosis induction. Cytotoxic tests on n-hexane, ethyl acetate, and ethanol extract of *Plectranthus amboinicus* (Lour.) Spreng. have been done in our previous study. The results showed that n-hexane and ethyl acetate extract had strong cytotoxic effects on MFC7 cells with $IC_{50}$ values of 63.64 µg/mL and 7.65 µg/mL, respectively. In contrast, the ethanol extract had an $IC_{50}$ value of 1,382.8 µg/mL which means that it had no cytotoxic effect (Bruzea et al., 2007). The multilevel extraction process resulted in secondary metabolites contained in *Plectranthus amboinicus* (Lour.) Spreng. leaves to be extracted in n-hexane and ethyl acetate solvents, and only a little was found in ethanol extract. This is likely to be the cause that ethanol extract has no effect. In the present study, we extracted *Plectranthus amboinicus* (Lour.) Spreng. leaves directly with ethanol solvent and made the extract into a nanoparticle size, and $IC_{50}$ value obtained was 89.166 µg/mL. The extract has the potential to be developed as an anti-cancer drug if it has an $IC_{50}$ value below 100 µg/mL (Kamuhabwa et al., 2000). Anti-proliferation of PAEEN may be achieved through inhibition of pro-oxidant agents, such as reactive oxygen species (ROS). They initiate tumor promotion and acts as the main catalyst for progression and promotion of tumor to the initiation stage (Kwon et al., 2006). Apoptosis, which is a major way to program the death of a cell, plays an important role in the regulation of tissue development and homeostasis in eukaryotes. Homeostatic between cell death and cell proliferation is required to maintain a normal state11. PAEEN may induce apoptosis through p53, down-regulation of Bcl-2, and up-regulation of Bax. The possible mechanism in which p53 regulates apoptosis involves the activation of mitochondria-regulated death pathway by elevating gene expression of pro-apoptosis in the Bcl-2 family and suppressing the expression of anti-apoptosis genes (Lotha et al., 2018).

The ability of PAEEN in triggering apoptosis might be due to its smaller size, given the greater surface area and distinct physical and chemical properties. Nanoparticle properties can occur in all biological systems, organs, or tissues (Hasibuan et al., 2013). Hence, nanoparticles

can interfere with overexpressed proteins proliferation which causes death in targeted cells. Nanoparticles also show the capacity to overcome multidrug resistance in chemotherapy (Kamuhabwa et al., 2000; Martien et al., 2013). Figure 2 shows that the cell viability was quite high at the 24th hour while the apoptosis test using the flow cytometry method shows that the number of cells experiencing death for both necrosis and apoptosis after 24 hours of incubation appeared quite high (Table 1). Although it was a contradictory phenomenon, it can be explained based on the administration time of the extract to the cells. In the anti-proliferation test, PAEEN was given after the cells were incubated for 24 hours which allowed the cells to proliferate from 0 to 24 hours. In contrast, in the apoptosis test, PAEEN was administered 24 hours before the second incubation, and the observation was performed on the following day. Thus, there had been a decrease in the viability of T47D cells which were likely to have experienced apoptosis and necrosis.

In conclusion, PAEEN could inhibit proliferation and trigger apoptosis of T47D cell lines. PAEEN exhibited anti-proliferative effects on T47D breast cancer cells via apoptosis.

*Conflicts of interest*

The authors declare that there are no conflicts of interest.

There is no requirement for ethical approval.

## Acknowledgements

We express our gratitude to the University of Sumatera Utara, Indonesia through "Hibah Talenta" Research Grant 2018 no.233/UN5.2.3.1/PPM/KP-TALENTA USU/2018 for the financial support in this study.

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