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Cyclin D1, Caspase 9 and P53 Expressions in T47D Cell Lines after Treatment of *Plectranthus amboinicus*, (Lour.) Spreng. Leaves Ethanolic Extract Nanoparticles

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Abstract

BACKGROUND: The application of nanotechnology is aimed to enhance the capability of the chemical compounds of *Plectranthus amboinicus* (Lour.) Spreng. leaves activity. The contact area of the particle surface becomes larger in nano size, which can increase the amount of the isolated active substance. The nanoparticles of this extract exhibited the most effective impact in decreasing the cell number of T47D by induction of apoptosis and arresting the cell cycle.

AIM: To evaluate the expressions of cyclin D1, caspase-9 and p53 in T47D cell lines treated by Plectranthus amboinicus, (Lour.) Spreng. Leaves Ethanolic Extract Nanoparticles (PAEEN).

METHODS: The inhibition of cyclin D1 was done by flow cytometry assay. The expression of cyclin D1, caspase-9 and p53 were observed by immunocytochemistry assay.

RESULTS: *Plectranthus amboinicus*, (Lour.) Spreng. Leaves Ethanolic Extract Nanoparticles (PAEEN) on concentration IC_{50} (89.2 µg/mL) inhibited the expression of cyclin D1 around 5.59%. Further, the immunocytochemistry assay indicated that there was an inhibition of cyclin D1, upregulation of caspase-9 and restoration of p53, indicated by their expression.

CONCLUSIONS: The *Plectranthus amboinicus*, (Lour.) Spreng. Leaves Ethanolic Extract Nanoparticles could be an effective anticancer by improving its downregulation on cyclinD1, upregulation caspase-9 and p53.

Introduction

Breast cancer is considered to be one of the common cancer-related cause of death among females. Treatment technologies such as surgery, radiation and chemotherapy are performed by administering anticancer drugs. Recommended therapy is the use of compounds from medicinal plant to reduce the rapid growth of cancer cells [1], [10], [12].

The cell cycle involves mainly four-step (G1, S, G2 and M), which lead to cell growth and cell division to produce two daughter cells. The response of growth factors in certain cell types is activated by a complex of CDK-cyclin D [2]. Genetic instability in cancer cell cause defect in the genes which are responsible for the checkpoints in the cell cycle.

It has been documented that *Plectranthus amboinicus*, (Lour.) Spreng. extract could inhibit the breast cancer cell by induction of apoptosis and arresting the cell cycle. The previous study showed that both single and combination treatment of *Plectranthus amboinicus*, (Lour.) Spreng. Ethylacetate extract 8 µg/mL with doxorubicin 1 µg/mL caused apoptosis induction and increasing in cell accumulation at the G1 phase. Expression of cyclin D1 indicated that both single application and combination of *Plectranthus amboinicus*, (Lour.) Spreng. extract with doxorubicin could arrest the cell cycle of T47D cells [3].

PAEEN inhibited the proliferation of T47D after 48 hours. The nanoparticles of this extract exhibited the most effective impact in decreasing the cell number of T47D at IC_{50} concentration (89.2 µg/mL). PAEEN displayed inhibitory activity on cell cycle of T47D cell lines at G0-G1 phase and S phase by flow cytometry assay. Cells underwent apoptosis indicated by the occurrence of inhibition of cell cycle on the G0-G1 phase and S phase.

Currently, nanoparticles based on the natural polymer such as chitosan have been widely applied as a drug delivery system due to their specific properties such as biocompatible, biodegradable, mucoadhesive and enhanced intestinal permeation [5]. The application of nanotechnology is aimed to enhance the capability of the chemical compounds of *Plectranthus amboinicus* (Lour.) Spreng. leaves activity. The contact area of the particle surface becomes larger in nano size, which can increase the amount of the isolated active substance [10].

This study aimed to evaluate the expressions of cyclin D1, caspase-9 and p53 in T47D cell lines treated by PAEEN. By doing this research, the result is expected to confirm the available data on recent studies.

Material and Methods

The extraction was conducted by maceration method. Dried leaves powder of *Plectranthus amboinicus* (Lour.) Spreng. was extracted with ethanol for 3 days at room temperature. The extract then concentrated using a rotary evaporator and was dried by freeze-dryer. The preparation of nanoparticles extract was according to the ionic gelation method.

Flowcytometry analysis of Cyclin D1 expression

FACS analysis was carried out to investigate cyclin D1 expression. Around 5 x 10^5 cells were grown in 6-well plates and cells were treated with PAEN for 24 h in incubator CO_2 5%. Trypsinized adherent cells were collected and were prepared for detection. Cells were labelled with FITC and added cyclin D1 [6].

Observation of cyclin D1, caspase-9 and p53 protein expression with immunocytochemistry

Analysis of cyclin D1, caspase-9 and p53 protein expressions using immunocytochemistry methods was performed as described previously [4]. The T47D (5 x 10^4 cells/well) were seeded on a coverslip in 24-wells plate, then incubated for 24 h at 37°C with 5% CO_2. Furthermore, the PAEN with concentrations 89.166 µg/ml was added to the cells and incubated for 24 h with 5% CO_2. The cells were washed with PBS. Then, cells were placed in the glass object for 5 min and added H_2O_2 as a blocking agent to the glass object and incubated at room temperature for 10 – 15 min. The cells washed twice with PBS and onto each glass object then added cyclin D1, caspase-9 and p53 proteins, incubated 1 h at room temperature.

The cells were washed 3 times with PBS, then added with secondary antibody (Biotinylated universal secondary antibody), and incubated at room temperature for 10 min, and washed twice with PBS. As chromogen added 3,3-diaminobenzidine, then incubated for 3–8 min. The cells were washed with distilled water and added hematoxylin solution and incubated for 5 min at room temperature. The cyclin D1, caspase-9 and p53 expressions were observed under a light microscope and documented. Cells expressing each the cyclin D1, caspase-9 and p53 in 10 fields of view in each treatment group. Cells that express a particular protein will provide the brown colour, while the cells that do not give a specific protein will provide purple colour.

Results

Analysis of Cyclin D1 Expression

Our previous study has proved that treatment of PAEEN with IC_{50} concentration (89.166 µg/mL), ½ IC_{50} (44.582 µg/mL, and ¼ IC_{50} (22.291 µg/mL) caused cell accumulation at $G_0 – G_1$ phase (data not shown). It means that there is a cell cycle arrest on the G_0-G_1 phase. In this phase, there is the activation of CDK-4 and CDK-6 with their common cyclin partner, cyclin D, that give a response to growth factor [2]. So, we investigated the expression of cyclin D1 using the flow cytometry method. The effect of PAEEN on cyclin D1 expression was showed in Figure 1.

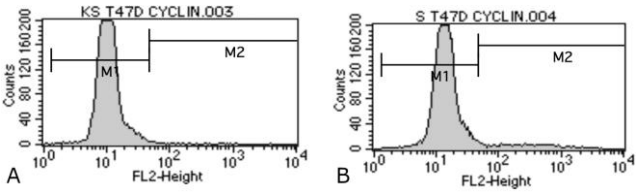

Figure 1. Analysis of cyclin D1 with flow cytometry. T47D cells were treated by PAEEN; A) Control cells; B) PAEEN 89.166 µg/mL

Treatment of PAEEN 89.166 µg/mL caused cell accumulation in M2 area (5.59%) compared with the control cell (0.45%). The data showed that there is a cell cycle arrest at the G1 phase.

Expression of Cyclin D1, caspase-9 and p53 treated by PAEEN on T47D cell lines

To confirm the mechanisms of PAEEN in cell cycle arrest and apoptosis induction on T47D cells, the molecular target protein cyclin D1, caspase-9 and p53 were investigated. The observation of expression of cell cycle regulatory protein Cyclin D1, caspase-9 and p53 were conducted in T47D cells by using PAEEN. T47D breast cancer cell line expresses caspase-3 wildtype, caspase-7 wildtype, mutant p53 and ER/PR positive [8]. In this study, the effects of PAEEN, on cyclin D1, caspase-9 and p53 were evaluated by using immunocytochemistry.

The expression of cyclin D1 proteins is positively characterised by brown stained nuclei in the cells (Figure 2). A treatment of nanoparticle of PAEEN decreased the cyclin D1 proteins expression, resulting in the higher cell growth inhibition compared to control cells.

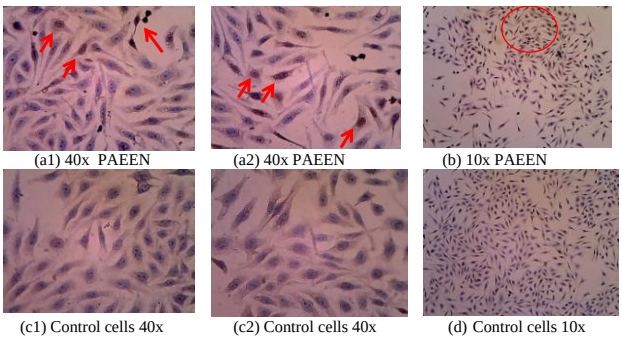

Figure 2: Expressions of cyclin D1 after treatment of PAEEN IC_{50} (89.166 µg/mL) observed by immunocytochemistry assay; A1), and A2) T47D cell lines treated with PAEEN 40 x; B) T47D treated with PAEEN 10 x; C1), and C2) control cell T47D 40 x; D) control cell T47D 10 x

Cell growth inhibition may occur via either apoptosis induction or cell cycle modulation or both occurring consecutively. Hasibuan et al., (2015) reported that the combination of *Plectranthus amboinicus* ethyl acetate extract (PAE) induced apoptosis and cell accumulation at the G1 phase. The expression of cyclin D1 indicated that both single application and combination of PAE with doxorubicin could arrest the cell cycle of T47D cell lines [3].

Our study by flow cytometry analysis showed that PAEEN led to G_0-G_1 and S phases arrest. During G_0-G_1 arrest, DNA damage is a sense, and the cell cycle paused until the DNA is repaired. If the damage is so severe, apoptosis can be induced [1], [11]. The capability of PAEEN in inducing cell death (apoptosis) can be seen by the expression of caspase-9, an initiator caspase.

The expression of caspase-9 proteins is positively characterised by brown stained nuclei in the cells (Figure 3).

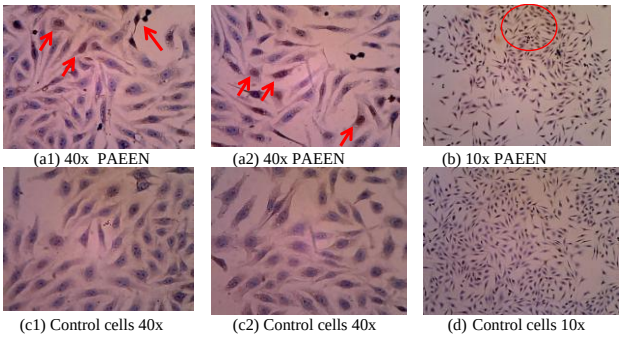

Figure 3: Expression of caspase-9 after treatment of PAEEN IC_{50} (89.166 µg/mL) observed by immunocytochemistry assay; A1), and A2) T47D cell lines treated with PAEEN 40 x; B) T47D treated with PAEEN 10 x; C1), and C2) control cell T47D 40 x; D) control cell T47D 10 x

Caspase is a class of proteases which degrade essential cellular protein and thus initiate apoptosis [2], [14]. As seen in Figure 3, High intensity of brown colour was shown on treated cells by PAEEN for caspase-9. It proves that PAEEN could induce apoptosis of T47D cell lines by upregulation of caspase 9 protein. The expression of p53 proteins was shown in Figure 4.

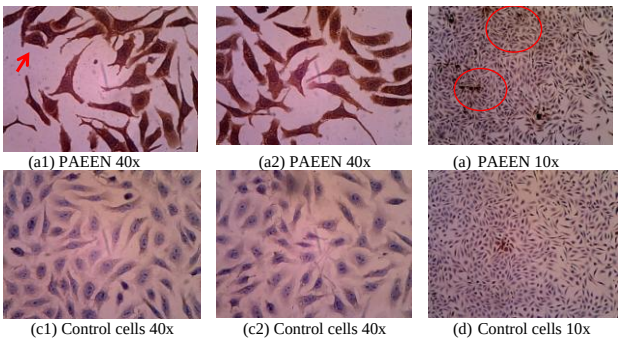

Figure 4: Expressions of p53 after treatment of PAEEN IC_{50} (89.166 µg/mL) observed by immunocytochemistry assay.

As shown in Figure 4, the treatment of PAEEN increased the p53 expression even it shows the low intensity of brown stain nuclei. P53 plays a role in cell cycle checkpoints. Its protein activation may indirectly affect cellular response through some protein regulation causing cell cycle arrest to lead DNA repair or cell death activation [6], [14].

Discussion

Notably, many anticancer agents elicit both cell cycle arrest and pro-apoptotic activity in cancer cell lines, moreover prolonged cell cycle inhibition can lead to apoptosis [9], [14]. Cell cycle arrests can be confirmed through the expression and activity of cell cycle-specific protein such as cyclin D1 [11]. PAEEN

displayed dual pro-apoptotic and cell cycle arrest activities toward T47D cell. The active compounds of PAEEN might be responsible for these effects. PAEEn contains some secondary metabolites such as *β-caryophyllene, quercetin, ursolic acid, triterpenoid acid, α-pinene, β-pinene, thymol, eugenol, carvacrol, 1,8-cineole, β-phellandrene, p-cymene, salvigenin, crisimaritin dan chrysoeriol* [17]. The secondary metabolites such as triterpenoid, quercetine, ursolic acid were shown to inhibit proliferation and induced apoptosis on many cancer cells [13], [14], [15], [16]. During DNA damage, p53 protein will be expressed and activated. It will then induce the expression of cyclin-dependent kinase inhibitors such as p21, p27, and p53 that play a role in inhibiting CDK / cyclin complex activity resulting in the disruption of the cell cycle [2]. P53 tumour suppressor gene plays an important role in apoptosis, too. The p53 gene stimulates expression of BCl_2 family, including Bax, and can bind to one or more anti-apoptosis proteins in mitochondria such as Bcl-xl [8], [15]. The mutation of p53 on T47D could inhibit the apoptosis. However, the expression of caspase-9 was increased. It proved that the mechanism of PAEEN to induce apoptosis was by causing upregulation of caspase gene.

Our result indicated that PAEEN displayed upregulation of caspase-9 protein. This might explain the apoptosis-inducing of PAEEN towards the T47D cell lines. It summarised, that PAEEN could enhance the cell cycle arrest and inducing apoptosis on T47D cell lines. It is promising to be developed as a chemopreventive agent.

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