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Cell Cycle Inhibition of Ethylacetate Fraction of *Zanthoxylum Acanthopodium DC.* Fruit against T47D Cells

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Abstract

BACKGROUND: The use of medicinal plants is increasing in several decades for relief many diseases. Indonesia consists of thousands of islands with various plants and the manners of the community using plants as a treatment for every disease traditionally.

AIM: Cytotoxic activity of ethyl acetate fraction (EAF) of *Zanthoxylum acanthopodium* fruit was tested towards T47D breast cancer cells.

METHODS: The in vitro cytotoxic activity was performed by MTT assay, and the result was expressed as the IC50 (Inhibitory Concentration), and cell cycle inhibition was investigated by flow cytometry to assess the inhibition in every phase of cell cycle, and the role of expression cyclin D1 and p53 in cell cycle inhibition were performed by immunocytochemistry.

RESULTS: EAF was showed to have high activity with a value of IC_{50} 48.94 ± 0.32 µg/mL. EAF of 25 µg/mL caused cell accumulation at G0/G1 (60.48%) and in a control cell (51.69%) and decreased expression of cyclin D1 and increased expression of p53.

CONCLUSION: The results obtained in this study provided scientific support for further investigation on compounds in *Z. acanthopodium* fruit which in the future could be used for medication.

Introduction

The world health organisation (WHO) reported that breast cancer is one of the leading causes of death and the most common cancer type amongst women worldwide in 2012 [1]. Moreover, breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths), is the most frequent cause of cancer death in women in less developed countries (324,000 deaths, 14.3% of total), and the second cause of cancer death in developed countries (198,000 deaths, 15.4%) after lung cancer [2]. The diversity of medicinal plants in Indonesia is one of the chances in the potential development of Indonesia in the globalisation era [3], [4]. The use of medicinal plant extracts for the treatment of human disease is an ancient practice and thus has greatly increased in recent years.

Traditionally, andaliman fruits (*Zanthoxylum acanthopodium* DC.) has been used as aromaticum substances, tonicum, and treat dysentery. Indian people have used andaliman to treat paralyzed and skin diseases such as abscess and leprosy. Andaliman has been used as spices at North Sumatera especially at North Tapanuli [5], [6], [7]. The plants from *Zanthoxylum* genus contain many compounds such as phenol hydroquinones, flavonoids, steroids/ triterpenoids, tannins, glycosides, volatile oils, alkaloids, coumarines, lignans, amides and terpenes [8], [9], [10], [11], [12], [13], [14], [15]. Ethyl acetate extract of andaliman fruits (EEA) was

showed to have cytotoxicity effect against MCF-7 and T47D cell lines. EEA was found to have a synergistic effect when combined with doxorubicin. EEA was showed to have anticancer activity towards mice induced with benzo(a)pyrene, having a cardioprotective effect and active on T47D resistance cells [16], [17], [18].

This study was aimed to determine cytotoxic activity and cell cycle inhibition activity of ethyl acetate fraction of *Zanthoxylum acanthopodium* DC. fruits on T47D cells.

Material and Methods

Plant and Chemicals

Fresh fruits of *Zanthoxylum acanthopodium* DC. was collected from Onan Rungu village, Samosir regency, Sumatera Utara Province, Indonesia. *Zanthoxylum acanthopodium* DC. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium with a number of 332/IPH.1.01/If.07/II/2016, DMSO (Merck), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), propidium iodide kit (Biolegend), chromogen 3,3-diaminobenzidin (DAB) (Novo Castra), monoclonal antibody cyclin D1 and p53 (Abcam).

Preparation of ethyl acetate fraction (EAF)

The air-dried and powdered fruits of *Zanthoxylum acanthopodium* DC. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3 x 3 d, 7.5 L). The powder was dried in the air and extracted with ethyl acetate (3 x 3 d, 7.5 L) at room temperature on a shake. The filtrate was collected and then evaporated under reduced pressure to give a viscous extract and then freeze-dried to give a dried extract [4], [17], [18], [19].

Cytotoxicity assay

EAF was submitted for cytotoxicity test. In that way, T47D cell line was grown in RPMI 1640 medium containing 10% FetaL Bovine Serum (Gibco), 1% penicillin-streptomycin (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37°C. The inoculums seeded at 1 x 10^4 cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by EAF. After incubation for 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as a stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using ELISA reader at λ 595 nm. The data which were absorbed from each well were converted to the percentage of viable cells [19] [20], [21]. The equation to determine the viability of cells:

$$Viability = \frac{Abs\ of\ treatment - Abs\ of\ medium}{Abs\ of\ control\ cells - Abs\ of\ medium}\ x\ 100\%$$

Cell cycle inhibition assay

T47D cells (1 x 10^6 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for 24 h. Both floating and adherent cells were collected in a conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at 4°C for 1 h. The cells were washed thrice with cold PBS and resuspended then centrifuged at 3000 rpm for 3 min, and PI kit (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37°C for 30 min. The samples were analysed using FACScan flow cytometer. Based on DNA content, the percentage of cells in each of stage in the cell cycle (G1, S and G2/M) were calculated using ModFit Lt. 3.0.s [4], [17], [22].

Immunocytochemistry

T47D cells (1 x 10^5 cells/well) were seeded on coverslips in 24-well plate and incubated for 24 h. After that, the cells were treated with EAF 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 then the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature, incubated using primary antibody cyclin D1 and p53 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-diaminobenzidine (DAB) solution for 10 min, and washed with distilled water. Afterwards, the cells were counterstained with Mayer-Haematoxylin for 5 min, and the coverslips were taken and washed with distilled water, and then immersed with xylol and ethanol 70% [4], [22], [23].

Statistical analysis

All data were analysed with regression analysis using SPSS 22.

Results

Cytotoxicity Effect of EAF

EAF was investigated for its cytotoxicity effect on T47D cell lines. The IC_{50} value of EAF was 48.94 ± 0.32 µg/mL. MTT method was used to determine cell viability after incubation for 24 h. In the treatment was showed the inhibition of cells growth.

Cell Cycle Arrest Activity

To investigate the effect of EAF on gained cell death by modulating cell cycle, we concentrated on it for further studies using flowcytometry method. The effect of EAF is given in Figure 1. Whereas, a single treatment with EAF on 25 µg/mL caused cell accumulation at G_0/G_1 (60.48%) and in control cells (51.69%). This fact was to indicate that EAF can inhibit cell grow at G_0/G_1 phase. In the cell cycle analysis, EAF was exhibited higher G_0-G_1 phase accumulation compared to control cells.

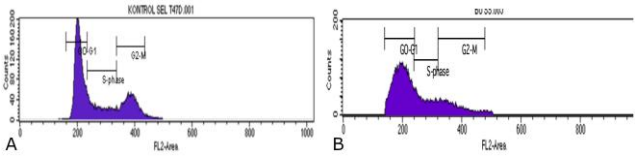

Figure 1: Cell cycle analysis using flow cytometry. T47D cells were treated by EAF for 24h and stained using propidium iodide; A) control cells; B) EAF 25 µg/mL

In the cell cycle analysis, EAF was exhibited higher G_0-G_1 phase accumulation compared to control. This analysis also showed cells underwent apoptosis, indicated by the occurrence of apoptosis during inhibition of cell cycle on the G_0-G_1 phase.

Cyclin D1 and p53 Expressions

Cyclin D1 and p53 (tumour suppressor gen) are proteins which play an important role in cell cycles process. In the study, the effect of EAF on cyclin D1 and p53 expressions were evaluated using immunocytochemistry.

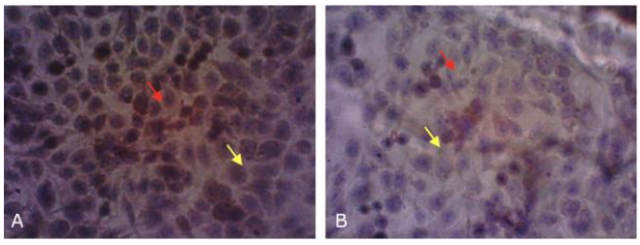

Figure 2: Expression of cyclin D1 on T47D cells using immunocytochemistry; A) control cells; B) EAF 25 µg/mL

Expression of cyclin D1 and p53 protein is positively characterised by brown stained nuclei in the cells (Figure 2 and Figure 3). In untreated cells (negative control) high intensity for cyclin D1 and low intensity for p53 was found. A single treatment of EAF was decreased on cyclin D1 and increased p53 expression.

Cells which express cyclin D1 Cells which not express cyclin D1. Inhibition of cyclin D1 protein expression strengthened the mechanism of modulating cell cycle especially in inhibition of cell cycle on the G_0-G_1 phase.

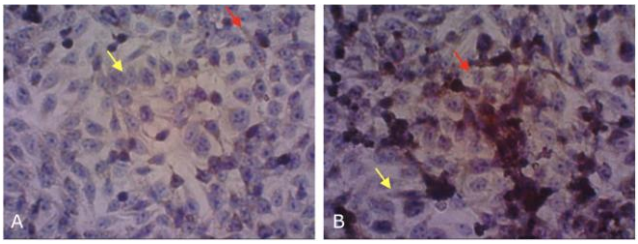

Figure 3: Expression of p53 on T47D cells using immunocytochemistry; A) control cells (Cells which express p53); B) EAF 25 µg/mL (Cells which not express p53)

Discussion

This research was aimed to investigate the efficacy of EAF in cell cycle inhibition. The cytotoxicity estimate of the natural product is related to the content of active compounds in these plants including *Zanthoxylum acanthopodium* DC. Flavonoids, alkaloids, saponins and tannins estimated as active compounds [17], [24].

This analysis also showed that cells underwent apoptosis, indicated by occurrence of apoptosis during inhibition of cell cycle on the G_0-G_1 phase [4]. Flavonoids and alkaloids could inhibit cell cycle progression [25], [26].

Cyclin D1 is a cyclin that role in G_0-G_1 phase with established complex with CDK-4 or CDK-6 to controlled G_1 to S phase transition [27]. Inhibition of cell cycle with combination EAE with doxorubicin reduced level of cyclin D1 which resulted in inhibition of pRb phosphorylation so that E2F can not apart from pRb and cells can not transcribe genes that needed in cell cycle process or cell proliferation [28], [29].

p53 plays an important function in the restriction of proliferation in abnormal cells. Functioning as a transcription factor p53 up-regulates expression of genes which contain a p53 binding element whose products contribute to cell cycle arrest [30], [31]. Activation of p53 can cause cell cycle arrest in different phases. In the majority of cases delay of G_1/S transition was previously observed [32]. The main key in p53 induced cell cycle arrest is played by $p21^{waf1}$ which binds to and inhibits a number of cyclin and Cdk (Cyclin-dependent kinase) complexes: cyclin

D1 – CDK4, cyclin E – CDK2, cyclin A – CDK2, and cyclin B – Cdc2 [30], [31], [32].

However, the molecular mechanism of cell cycle modulation by this EAF needs to be explored in more detail. Based on the results, it can be concluded that ethyl acetate fraction of *Zanthoxylum acanthopodium* DC. fruits have cytotoxicity activity through cell cycle arrest, decreases cyclin D1 and increase p53 protein expression. The extract is potential to be developed as a chemotherapeutic agent for breast cancer therapy.

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