Cell Cycle Inhibition and Apoptosis Induction Activities of N-hexane Fraction of *Cyperus Rotundus* L. Rhizome

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Abstract: Breast cancer is one of the leading cause of death and the most common cancer type amongst women in the world after cervic cancer. To evaluate the cell cycle inhibition and apoptosis induction activities on T47D cell lines of n-hexane fraction (nHF) of *Cyperus rotundus* L. rhizomes. Ethanol extract was obtained by maceration method and was fractionated with n-hexane. Cytotoxic activity was examined with MTT assay, and cell cycle inhibition, apoptosis induction and cyclin D1 expression were assessed with flow cytometry method. Cytotoxic activity of nHF was found to have IC_{50} of 71.69 ± 0.34 µg/mL, nHF at concentration 35 µg/mL caused accumulation in G_0-G_1 and S phase accumulation (56.89% and 19.36%), increased early apoptosis (26.30%) and decreased expression of cyclin D1 (26.30%). The results reveal that nHF of *Cyperus rotundus* L. rhizomes has cell cycle inhibition and apoptosis induction activities. Our further study is to isolation compounds which responsible for these activities.

1 INTRODUCTION

Breast cancer is one of the leading cause of death and the most common cancer type amongst women worldwide in 2012 (WHO, 2015). Breast cancer is the second cause of cancer death in developed countries after lung cancer. A recent study has reported that breast cancer is the first in the predicted new cancer cases, and the second most incidence death cause of women suffering from cancer in the United States (Siegel, et. al., 2015).

Cyperus rotundus L. (Cyperaceae) have been used as the drug of stomachache, disorders bowel, menstrual irregularities (Peerzada, et al., 2015). Bioassay investigations indicated which the extract of *Cyperus rotundus* L. exerts antioxidant, antibacterial, insecticidal activity and its essential oil have activity as antiradical, antimutagenic and cytotoxic. Essential oil and steroids/ triterpenoids could extracted with non polar solvent such as n-hexane and chloroform (Hemanth, et al., 2014; Hadi, et al., 2007; Vitaglione, et al., 2004; Lanciotti, et al., 2004; Kilani, et al., 2008; Tenore, et al., 2011; Nam, et al., 2016; Sonwa and Konig, 2001; Liu, et al., 2016; Kilani, et al., 2007; Jirovetz, et al., 2004; Memariani, et al., 2016). Cyclin D1 has an important role in cell cycle process in G_0-G_1 phase (Żurynń, et al., 2016). This study aimed to determine cell cycle inhibition and apoptosis induction activities of n-hexane fraction of *Cyperus rotundus* L. rhizomes.

2 MATERIALS AND METHODS

2.1 Plant and Chemicals Material

Fresh rhizomes of *Cyperus rotundus* L. was collected from Paya Tumpi, Aceh Tengah regency, Nangroe Aceh Darussalam province, Indonesia. *Cyperus rotundus* L. was identified in Herbarium Medanense, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara. Chemicals used were annexin-V (BioLegend), cyclin D1 antibody (Santa Cruz), distilled water, DMSO (Sigma), [3-(4,5-dimethylthiazole-2-yl)-2,5diphenyl tetrazolium bromide] (MTT) (Sigma), propidium iodide reagent (BioLegend).

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2.2 Preparation of Extract

Ethanol extract of *Cyperus rotundus* L. rhizomes (10g) was repeatedly fractionated with n-hexane (3x100 mL) at separating funnel. The supernatant was collected, and then evaporated under reduced pressure to give a viscous fraction and then dried on water bath to dry (Satria, et al., 2015; Anggraeni, et al., 2015; Hasibuan, et al., 2015).

2.3 Cytotoxicity Assay

The cells were treated with nHF. In this test, the T47D cell line (cancer cells which isolated from a 54 years old woman which mutation on p53) was grown in RPMI 1640 medium, 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 37ºC. The inoculums seeded at $1x10^4$ cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by EE. After incubation 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h in 37ºC. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as 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 microplate reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells (Harahap, et al., 2018; Dalimunthe, et al., 2018; Satria, et al., 2017).

2.4 Preparation of Cells for Flow Cytometry Analysis

T47D cells ($5x10^5$ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with nHF 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 (Satria, et al., 2015; Anggraeni, et al., 2015).

2.5 Cell Cycle Analysis

Cells were fixed in cold 70% ethanol in PBS at -20ºC for 2 h. The cells were washed thrice with cold PBS resuspended and incubated in ethanol 70% for 60 min then centrifuged at 3000 rpm for 3 min, and PI reagent (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37ºC for 30 min. The samples were analysed using FACScan flow cytometer (Dalimunthe, et al., 2017; Nugroho, et al., 2014).

2.6 Apoptosis Analysis

Annexin V reagent was added to sediment and suspended and incubated at 37ºC for 30 min. The samples were analyzed using FACScan flow cytometer (Satria, et al., 2017; Handayani, et al., 2017).

2.7 Cyclin D1 Expression

Sediment cells were fixed with ethanol 70% stand for 2 h in -20^{0}C and cyclin D1 antibody was added and incubated at 37ºC for 10 min. The samples were analyzed using FACScan flow cytometer (Żurynń, et al., 2016)

2.8 Statistical Analysis

Data was expressed as mean ± SD. All statistics were analyzed using the SPSS 21 software.

3 RESULTS AND DISCUSSION

3.1 Inhibitory Concentration 50% (IC_{50})

MTT method was used to determine percentage of cell viability after incubation for 24 h. In every treatment nHF was shown to inhibit cells growth. The IC_{50} value of nHF was 71.69 ± 0.34 µg/mL. The cytotoxicity estimate of natural product is related to content of active compound in these plants including *Cyperus rotundus* L. This plant contain monoterpenes, sesquiterpenes, and steroids estimated as active compounds (Nidugala, et al., 2016, Yadav, et al., 2012; Nidugala, et al., 2017).

3.2 Effect on Cell Cycle and Apoptosis

To evaluate the effect of nHF to increase cell death by modulating cell cycle, we concentrated on it for further studies using flow cytometry method. The effect of nHF is given in Figure 1. Whereas treatment of nHF in 35 µg/mL caused cell

accumulation at G_0-G_1 and S phase accumulation (56.89% and 19.36%) and for control cell (52.28% and 16.80%). This fact was to indicate that nHF can inhibit cell grow at G_0/G_1 phase. Recent study have reported which monoterpenes exert anticancer activities and as chemopreventive agents (Elson and Yu, 1994; Kellof, et al., 1996; Crowell, et al., 1999).

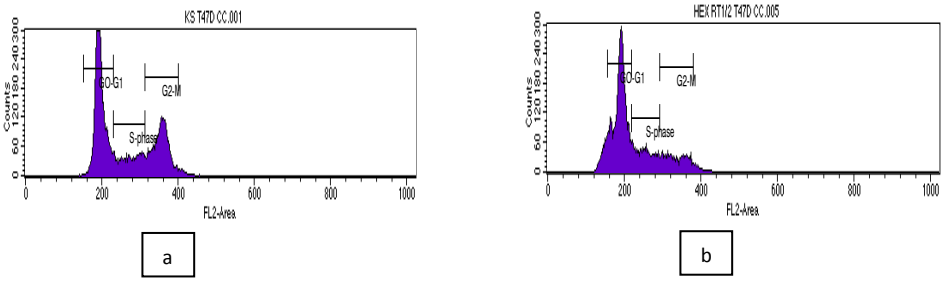

Figure 1. Cell cycle analysis using flow cytometry. T47D cells were treated by nHF for 24h and stained using propidium iodide. (a) control cells; (b) nHF 35 µg/mL.

As shown in Figure 2, the cells in the upper and lower right quadrants represent late apoptotic/ necrotic and early apoptotic cells, respectively. The percentage of nHF at 35 µg/mL and control in early apoptotic (26.11% and 3.65%), in late apoptotic/early necrotic (4.26% and 2.96%), and in late necrotic (4.88% and 2.41).

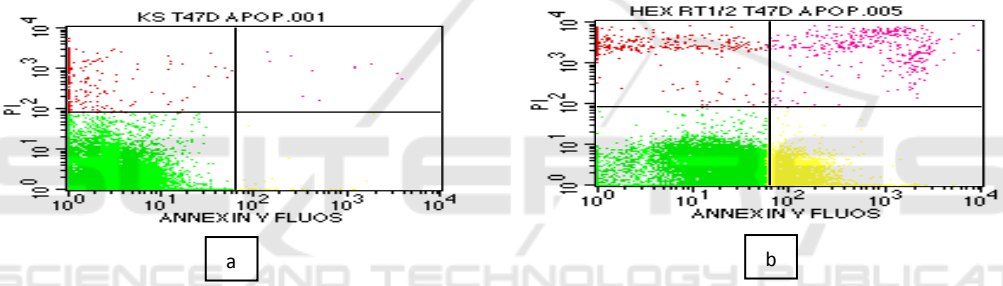

Figure 2. Apoptosis analysis using flow cytometry. T47D cells were treated by nHF for 24h and stained using Annexin-V. (a) control cells; (b) nHF 35 µg/mL.

nHF has increased the cells to apoptosis in early apoptosis if compared to control cell. Apoptosis is processed in cells which cause programmed cell death with alters on morphology, membrane blebbing and chromatine (Ruddin,et al., 1997).

3. 3 Analysis of Cyclin D1 Expression

To evaluate the effect of nHF to decrease cyclin D1 expression, we concentrated on it for further studies using the flow cytometry method. The effect of nHF is given in Figure 3. Whereas treatment of nHF in 35 µg/mL caused cell accumulation in M1 area (26.30%) and for control cell (11.19%). Evaluation of cyclin D1 expression was performed using flow cytometry method with cyclin D1 antibody as shown in Figure 3.

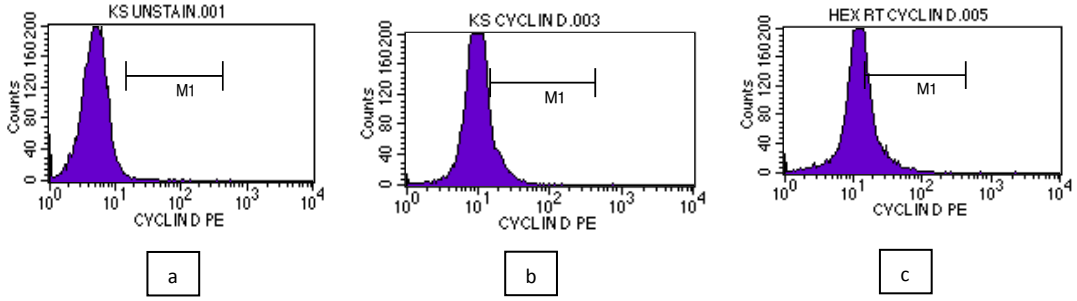

Figure 3. Cyclin D1 analysis using flow cytometry. T47D cells were treated by nHF for 24h and stained using cyclin D1 antibody. (a) control cells unstaining; (b) control cells; (c) nHF 35 µg/mL.

Geraniol is one of monoterpene which has suppressed the MCF-7 growth through induction cell cycle arrest in G_1 phase, reduce the level of cyclin D1, cyclin dependent kinase 4 (CDK4), cyclin E and cyclin A (Duncan, et al., 2004).

Based on the results above, we conclude that n-hexane fraction of Cyperus rotundus L. rhizome has cytotoxic activity towards T47D breast cancer cells which have some mechanism such as induction of apoptosis and inhibition of cell cycle especially in inhibit cyclin D1 expression.

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