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Research Article

SELAGINELLA ACTIVE FRACTIONS INDUCE APOPTOSIS ON T47D BREAST CANCER CELL

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ABSTRACT

Apoptosis is an important target on anticancer mechanism. The purpose of this research is to investigate apoptosis induction of *Selaginella plana* Hieron active fractions on T47D cells. Absolute ethanol was used to extract *Selaginella plana* powders. Ethanolic extract was dilluted by methanol:water (4:1) and then fractionated by hexane (S_Hex), methylene chloride (S_MTC), ethyl acetate (S_EA), and buthanol (S_BuOH). The proliferation of T47D cell line was detected by SRB (Sulforhodamine B) assay which was measured at a wavelength of 515nm. Flowcytometry analysis to determine apoptosis was examined by Propidium Iodide (PI) and Annexin V assay using T47D breast cancer cell line. The result showed that the IC_{50} value of S_Hex, S_MTC, S_EA, and S_BuOH on T47D cells were 107 μg/mL, 4 μg/mL, 6 μg/mL, and 17 μg/mL respectively. The active fractions (S_MTC and S_EA) at its IC_{50} concentration significantly ($P<0.05$) increased the total number of early apoptotic cells in the T47D cells 3.39% and 4.1% respectively compared to that of control (1.95%). Based on the result, methylene chloride and ethyl acetate fraction of *Selaginella plana* induced apoptosis on T47D cell.

Keywords: apoptosis, breast cancer, *Selaginella*

INTRODUCTION

Apoptosis, an active physiological process resulting in cellular self-destruction of unwanted cells, is absent in cancer cells. Apoptosis is characterized by distinct morpho-logical changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Simstein *et al*., 2003). The therapeutic application of apoptosis is currently being considered as a model for the develop-ment of anticancer drugs. It is therefore essential to identify novel apoptosis-inducing agents that are candidate for anticancer (Shafi *et al.,* 2009).

Selaginella sp including asian spikemoss (*Selaginella plana* Hieron.) has antiproliferative effect on cancer cells and antiviral activity (Silva *et al.,* 1995; Ma *et al.,* 2001; Lee *et al.,* 2008; Tan *et al.,* 2009). Flavonoid compounds on *Selaginella sp*. play an important role on cytotoxic activity against cancer cells (Lee *et al*., 2008). Flavonoid such as quercetin, luteolin, and ursolic acid are potent inhibitors of proliferation and apoptosis inducers on many cancer cells through PI3K pathway (Gulati *et al.,* 2006; Xavier *et al.,* 2009). The research hypothesizes that *Selaginella plana* Hieron active fraction is able to induce apoptosis on cancer cell.

METHODOLOGY

Preparation of the solvent fractions of *Selaginella plana* Hieron.

Preparation of the ethanolic extract and its solvent fractions followed the previous method by Harborne (1987). The dried of extract was grounded and immersed in 96 % ethanol. After 72 hours the filtrate was colle-cted. The combined filtrate was evaporated with rotary evaporator at 40°C. The ethanolic extract was diluted by methanol: water (4:1), and then partitioned with hexane. The aqueous layer was fractionaled respectively with metyhlene chloride, ethyl acetate and buthanol. The hexane (S_Hex), metyhlene chloride (S_MTC), ethyl acetate (S_EA), buthanol (S_BuOH), methanol (S_MeOH) fraction were collected and concen-trated with vacuum rotary evaporator at 40°C.

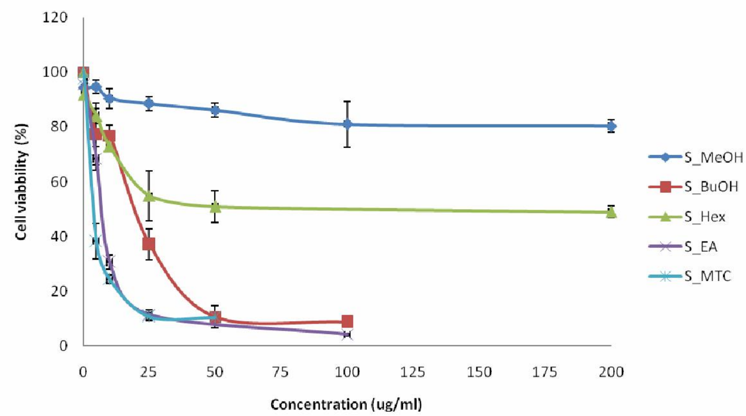

Figure 1. Percentage of viable cells of *Selaginella plana* Hieron solvent fractions in various concentrations after 24 hours. Data collection are conducted in triplicate and represented in mean $\pm$ standard deviation.

Sulphorhodamine B colorimetry for cytotoxic assay

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the cytotoxic activity to T47D human breast cancer cells (Zou *et al.*, 2008). Cells were seeded into a 96-well plate with 3000 cells per well and incubated at 37°C for 24h. The cells were treated with various concentrations of the ethanolic extract of *Selaginella plana* Hieron. and its different solvent fractions with doxorubicin as a positive control for another 24 h. The cells were then fixed with 10% trichloracetic acid for 30 minutes at 4°C, followed by drying in oven at 50°C for 1 hour and staining for 30 minutes at room temperature with 4mg/mL SRB solution. Afterwards, the cells were washed with 1% acetic acid times, followed by drying in oven 50°C for 1 hour and resuspended with 200µL 10mM buffered Tris base pH 8. Cell viability was measured by the optical density at 515 nm. The wells without ethanolic extract were used as negative controls.

Cell Cycle distribution analysis

Propidium iodide (PI) staining was used to analyze DNA content. Cells were added in 12-well plates with $1x10^5$ within 24-48 h (to yield 60–70% confluence). Cells were treated with either DMSO (0.25%) or samples (2.5 µg/mL, 5 µg/mL, and 10 µg/mL). After 24-h treatment, cells were harvested, and resuspended in PBS, fixed with 70% ethanol, labeled with PI (2 µg/mL), incubated at room temperature in the dark for 15 min, and DNA content was then analyzed using a Flowcytometry (Beckman and Coulter-EpicXL). All experiments were measure in three replications.

Apoptosis detection

Apoptotic population was determined by PI-AnnexinV assay (Annexin V-FITC Apoptosis Detection Kit Biovision). Cells were plated in 12-well plates with $2x10^5$ within 24-48 h (to yield 60–70% confluence). Cells were then treated with either DMSO (0.25%) or samples (IC_{50}). After a 24-h treatment, cells were harvested, resuspended in 1x binding buffer, labeled with PI-Annexin V, and incubated at room temperature in the dark for 5 min. Cell suspension were analyzed using a flowcytometry (Beckman and Coulter- EpicXL). All experiments were measure in two replications.

Statistical analysis

Analysis of significancy between control/untreated and treated groups were analyzed by one-way ANOVA and followed by tukey post hoc test (SPSS 11.5). The mean difference is significant at the $P< 0.05$.

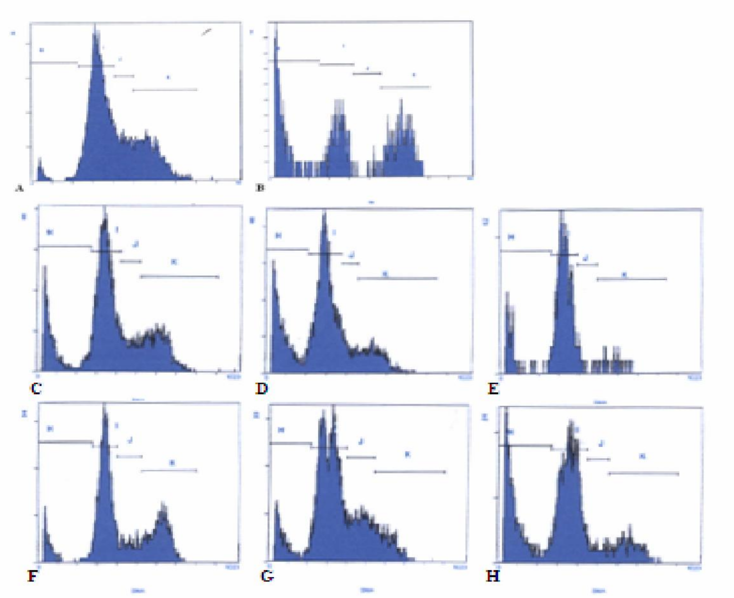

Figure 2. Cell cycle analysis of T47D cell using flowcytometry. Expose of samples increase subG1 level (%) on T47D cell cycle compared to control. Cells expose with Dox, S_MTC and S_EA for 24 hours, were resuspended in PBS, fixed with 70% ethanol, labeled with PI, and analyzed using a Flowcytometry (Beckman and Coulter- EpicXL).A. Control cells (vehicle only), B. Dox 50 nM, C. S_MTC 2.5 μg/mL, D. S_MTC 5 μg/mL, E. S_MTC 10 μg/mL, F. S_EA 2.5 μg/mL, G. S_EA 5 μg/mL, H. S_EA 10 μg/mL.

RESULTS AND DISCUSSION

The result showed that the IC_{50} value of S_Hex, S_MTC, S_EA, and S_BuOH on T47D cells were 107 μg/mL, 4 μg/mL, 6 μg/mL, and 17 μg/mL, respectively, (Fig.1; Table I). The graphic of concentration vs. cells viability (Fig.1) showed that increasing of samples concentration (except S_MeOH) significantly decreases cells viability compared to that of control ($P<0.05$). We suggest that decreasing of cells viability by solvent fraction of *Selaginella plana* Hieron was through apoptosis induction.

According to the results of cytotoxicity assay of selaginella solvent fractions, further study is to investigate the ability of active fractions (S_MTC and S_EA) to induce apoptosis on T47D cells. To verify the mechanism of cytotoxic activity of samples in breast cancer T47D cells, we observed the sub G1 level by exposing samples with propidium iodide (PI) and detected with flowcytometry (Fig. 2).The result showed that S_MTC, and S_EA at concentration of 2.5 μg/mL, 5 μg/mL, and 10 μg/mL increased significantly subG1 level compared to that of control ($P<0.05$) that related to apoptosis induction (Fig. 3). The increased level of subG1 is not proportional to the increasing samples concentration.

Furthermore, these results are supported by Annexin-based flow cytometry (Fig. 4). concentration significantly ($P<0.05$) increased the total number of early apoptotic cells in the T47D cells 4.1%, and 3.39% respectively compared to control (1.95%). According to the results, both of samples induced apoptosis against T47D cell. Samples (S_MTC and S_EA) at its IC_{50}.

Table I. The IC50 values of *Selaginella plana* Hireon solven fraction

Samples	IC50 (μg/mL)
S_Hex	107
S_MTC	4
S_EA	6
S_BuOH	17
S_MeOH	>1000

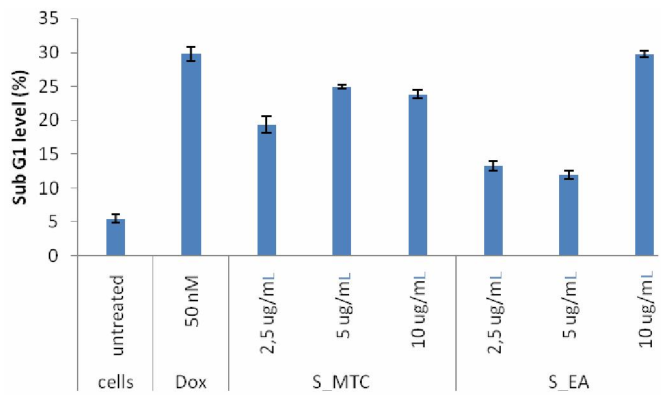

Figure 3. Increasing of subG1 level (%) on T47D cell cycle by samples. Data represent the mean values of three replicates with bars indicating standart deviation. * P<0.05 compared to control.

NFϰB transcription factor plays an important role on transcription of antiapoptotic protein (Bcl-2, IAP, and Bcl-XL) (Simstein *et al.*, 2003). Capability of agent to inhibit NFϰB would induce apoptosis. The upstream of NFϰB is Ras. Activation of Ras to Ras-GTP activates Ras effector like Ral-GEF, Rafs, PI3K, and MEKK. Activation of PI3K activates phosphoinositide-dependent kinases PDK-1 and PDK-2, followed by Akt phosphorilation and NFϰB activation (Simstein *et al.*, 2003; Markowitz *et al.*, 2007; Reuter *et al,* 2000). Phosphatidylinositol-3-kinase (PI3K) protein contributes on DNA synthesis and inhibition of apoptosis (Reuter *et al,* 2000). Exposure of flavonoid apigenin on cancer cell (invitro and invivo study) able to dephosphorylate Akt and inactivates NFϰB (Kaur *et al.,* 2008). Isoginkgetin, a biflavonoid from *Metasequoia glyptostroboides* inhibit activation of PI3K/Akt/NFkB (Yoon *et al.*, 2006).

Robustaflavone 7,4',7'-trimethyl ether (RTE) is biflavonoid from *Selaginella doederleinii* Hieron that promising as PI3K inhibitor (Handayani *et al*, 2011). All of the flavonoid induces apoptosis through this pathway. We suggest that the *Selaginella plana* Hieron active fractions also induce apoptosis through this pathway.

The other apoptosis mechanism is via Fas-L. Samples induce *DNA break,* followed by Fas-L expression. Fas-L in complex with Fas receptor activates caspase 8 leading to Bid activation to form tBid and followed by Bax localization on mitochondria outer membrane and increases cytochrome C release. Release of cytocrome C activates caspase 9, followed by caspace 3 activation and then induces apoptosis (Pope, 2002; Sun *et al.*, 2004). Flavonoid apigenin activates caspase 9 to induce apoptosis (Kaur *et al.,* 2008).

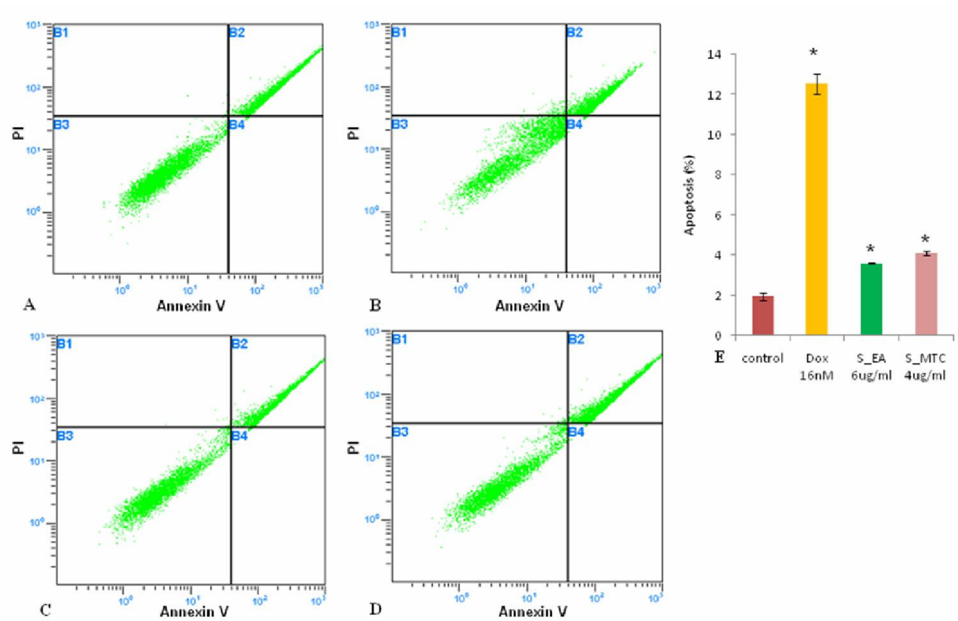

Figure 4. Sample induced apoptosis of human breast cancer T47D cells. Cells were exposed to either vehicle or samples (at its IC_{50} concentration) and incubated for 24 h. Apoptotic population was determined by Annexin-V assay. Early apoptotic cells; right bottom (B4), Late apoptotic cells; right top (B2), Viable cells; left bottom (B3).A. Control cell (vehicle only), B. Dox, C. S_EA, D. S_MTC, E. the data from early apoptotic cell (B4) represent the mean values of two replicates with bars indicating standart deviation (SD), *P<0.05 compared to control.

Neochamaejasmin A, a biflavonoid from *Stellera chamaejasme* L. induces apoptosis on prostat cancer LNCaP through Fas L pathway (Liu *et al.,* 2008). We suggest that flavonoid from *Selaginella plana* Hieron active fractions in this study possible to induce apoptosis through increasing or inhibition of protein expression that play a role in this pathway. Nevertheless, further investigation is needed to explore the mechanism of apoptosis induction of *Selaginella plana* Hieron active fractions on T47D cancer cell.

CONCLUSION

The results show that methylene chloride fraction (S_MTC) and ethyl acetat fraction (S_EA) of *Selaginella plana* Hieron induce apoptosis on T47D breast cancer cell.

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