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 planta Hieron active fractions on T47D cells. Absolute ethanol was used to extract Selaginella planta powders. Ethanolic extract was diluted by methanol:water (4:1) and then fractionated by hexane (S_Hex), methylene chloride (S_MTC), ethyl acetate (S_EA), and butanol (S_BuOH). The proliferation of T47D cell line was detected by SRB (Sulfurhodamine B) assay which was measured at a wavelength of 515nm. Flowcytometry analysis to determine apoptosis was examined Propidium Iodide (PI) and Annexin V assay using T47D breast cancer cell line. The result showed that the IC50 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 IC50 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 planta 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 morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Sinnstein et al., 2003). The therapeutic application of apoptosis is currently being considered as a model for the development 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 spiker Moss (Selaginella planta 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 planta Hieron active fraction is able to induce apoptosis on cancer cell.

METHODOLOGY

Preparation of the solvent fractions of Selaginella planta Hieron.

Preparation of the ethanolic extract and its solvent fractions followed the previous method by Harborne (1987). The dried of extract was ground and immersed in 96 % ethanol. After 72 hours the filtrate was collected. 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 fractionated respectively with methylene chloride, ethyl acetate and butanol. The hexane (S_Hex), methylene chloride (S_MTC), ethyl acetate (S_EA), butanol (S_BuOH), methanol (S_MeOH) fraction were collected and concentrated with vacuum rotary evaporator at 40°C.
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 piana Hieron, and its different solvent fractions with doxorubicin as a positive control for another 24 h. The cells were then fixed with 10% trichloroacetic acid for 30 minutes at 4°C, followed by drying in an 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.

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 measured in three replications.

Apoptosis detection

Apoptotic population was determined by PI-AnnexinV assay (Annexin V-FITC Apoptosis Detection Kit Biovisión). 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 (10 μg/mL). 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 measured in two replications.

Cell Cycle distribution analysis

Propidium iodide (PI) staining was used to analyze DNA content. Cells were added in 12-well plates with 1x10⁶ 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).

Analysis of significance 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.
RESULTS AND DISCUSSION

The result showed that the IC50 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 1). 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 platensis 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 flow cytometry (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 3.3% respectively compared to control (1.98%). According to the results, both of samples induced apoptosis against T47D cell. Samples (S_MTC and S_EA) at its IC50.
Table 1. The IC50 values of *Selaginella plana* Hieron solvent fraction

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S_Hex</td>
<td>107</td>
</tr>
<tr>
<td>S_MTC</td>
<td>4</td>
</tr>
<tr>
<td>S_EA</td>
<td>6</td>
</tr>
<tr>
<td>S_BuOH</td>
<td>17</td>
</tr>
<tr>
<td>S_MeOH</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Figure 3. Increasing of subG1 level (%) on T47D cell cycle by samples. Data represent the mean values of three replicates with bars indicating standard 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 Raf-GEF, Rafs, PI3K, and MEKK. Activation of PI3K activates phosphoinositide-dependent kinases PDK-1 and PDK-2, followed by Akt phosphorylation 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 (in vitro and in vivo study) able to dephosphorylate Akt and inactivates NFκB (Kaur et al., 2008). Isoginkgetin, a biflavonoid from *Metasagonia glyptostrooides* inhibit activation of PI3K/Akt/NFκB (Yoon et al., 2006).

Robustaflavone 7,4',7'-trimethyl ether (RTE) is biflavonoid from *Selaginella dodecandra* 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 cytochrome C activates caspase 9, followed by caspase 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<sub>50</sub> 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 standard deviation (SD), *P<0.05 compared to control.

Neochamaejasmin A, a biflavonoid from *Stellera chamaejasme* L. induces apoptosis on prostate cancer LNCaP through Fas1 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 acetate fraction (S_EA) of *Selaginella plana* Hieron induce apoptosis on T47D breast cancer cell.

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**REFERENCES**


