ANTICANCER ACTIVITY OF ETHANOLIC EXTRACT OF Selaginella plana HIERON. ON T47D CELL LINE IN VITRO

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ABSTRACT

Selaginella sp belongs to the Selaginellaceae family. It has been used in China and Indonesia as a traditional medicine. It has several medicinal properties including antibacterial, antikardiovaskular, and anticancer agent. The aim of the present study was to access the anticancer property of the ethanolic extracts of Selaginella plana Hieron. on T47D breast cancer cell line. The proliferation of T47D cell line was detected by SRB (Sulforhodamine B) assay which was measured at a wavelength of 515 nM. The result showed that the IC50 of the ethanolic extract was determined at 7.03 µg/mL. This significant activity was assumed due to its high total flavonoid content. The total flavonoid content of the ethanolic extract was 23.04%. Flow cytometry analysis indicated that the extract may undergo the cell death via apoptosis pathway. In conclusion, the ethanolic extract of Selaginella plana Hieron. has considerable activity in inhibiting T47D cell line proliferation.

Key words : Selaginella plana Hieron., Sulforhodamine B, T47D cells, anticancer, flow cytometry.

INTRODUCTION

Cancer is one of the deadly diseases in the world. Cancer cells can grow uncontrolled. These cells have ability in intrusion on and destruction of adjacent tissues (invasion), and moreover these cells may spread to other locations in the body via lymph or blood (metastasis). Some factors that can cause cancer include tobacco, diet and obesity, infections, radiation, stress, lack of physical activity, environmental pollutants, and genetics. Tobacco, diet, and obesity are the most influencing factors that cause cancer.

Many people in the developing countries still rely on herbal medicines to cure their illnesses, including cancer, instead of using synthetic medicine. One of the herbal medicines which have been used is Selaginella sp. Selaginella sp belongs to...
in the family Selaginellaceae. It has been used in China and Indonesia as a traditional medicine. It has several medicinal properties including antibacterial, antihypertensive, anticardiovascular, and anticancer.

Some of Selaginella spp. that has been known are Selaginella doederleiini Hieron, Selaginella willdenowii, and Selaginella plana Hieron. The previous study reported that the water extract of Selaginella doederleiini Hieron has moderate antimutagenic activity against benzo[a]pyrene. In addition, this plant has anticancer activity against L 929 murine cells. Another species from Selaginella sp is Selaginella willdenowii; this plant has three known biflavones, 4',7''-di-O-methylamentoflavone, isocryptomerin and 7''-O-methylrobustaflavone, that were significantly cytotoxic against a panel of human cancer cell lines. However, the study of anticancer activity of Selaginella plana Hieron has not many publications to date.

The aim of the present study was to access the anticancer property of the ethanolic extracts of Selaginella plana Hieron on T47D human breast cancer cell line. The Sulforhodamine B (SRB) assay was used to determine the anticancer activity of T47D human breast cancer cells. Cells were seeded into a 96-well plate with 10^4 cells per well and incubated at 37°C for 24h. The cells were treated with various concentrations of ESP and doxorubicin as a positive control for another 24 h. Afterwards, the cells then fixed with 10% trichloracetic acid for 30 minutes at 4°C, followed by drying in oven 50°C for 1 hours and staining for 30 minutes at room temperature with 4 mg/mL SRB solution. Afterwards, the cells were washed with 1% acetic acid for 4 times, followed by drying in oven 50°C for 1 hour and dissolved with 200μL 10mM buffered Tris base pH 8. Cell viability was measured by the optical density at 515 nM. The wells without samples were used as negative controls.

Cell Cycle Analysis by propidium iodide (PI) staining.

Cells were seeded into a 24-well plate with 10^5 cells/mL and incubated at 37°C for 24h. The DNA content of the cells were monitored by using a COULTER EPICS XL™ flow cytometer.

Assessment of Apoptosis by PI/Annexin V double staining.

Cells were seeded into a 24-well plate with 10^5 cells/mL and incubated at 37°C for 24h. The apoptotic cells were measured using Annexin V-FITC conjugate. Cells were incubated in serum-free DMEM containing 0.5 μg/mL annexin V-FITC.
and 0.5 μg/mL PI at room temperature for 5
minutes in the dark. The fluorescences of annexin
V-FITC and PI were detected by using a COULTER
EPICS XL™ flow cytometer with excitation
wavelength of 488 nm and emission wavelength of
530 nm (FL1) and 625 nm (FL2), respectively. For
each sample, 5000 cells were analyzed. The
necrotic cells lost cell membrane integrity that
permits PI entry; PI/annexin V (upper left
quadrant in the plot). Viable cells showed Pt
/annexin V (lower left quadrant in the plot); early
apoptotic cells exhibit PI/annexin V⁺ (lower right
quadrant in the plot); late apoptotic cells or necrotic
cells showed PI/annexin V⁺ (upper right quadrant
in the plot).

RESULTS AND DISCUSSION

In the present study, we investigated the
anticancer property of the ethanol extract of
Selaginella plana Hieron (S_EtOH). In this study,
the anticancer activity of the ethanol was
determined in vitro on T47D cell line. The result in
the Table 1 suggested the ethanol extract has
phenolic/flavonoid, saponin, alkaloid, and steroid
compounds, while triterpenoids were excluded.
The further study was also conducted to determine
the total of flavonoid compounds, and the result
indicated that the total flavonoid content of the
ethanolic extract was 23.04%.

<table>
<thead>
<tr>
<th>Groups of Compounds</th>
<th>Result (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic/flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>-</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
</tr>
</tbody>
</table>

The S_EtOH could inhibit the proliferation
of T47D cell lines in a dose-dependent manner as
shown in Figure 1. The IC₅₀ of the ethanol extract
and doxorubicin were 7.03 μg/mL and 16.67 nM,
respectively. This high activity may due to the
flavonoid compounds in the plants. Flavonoid in
the Selaginella sp may inhibit cancer cell lines
proliferation.

Figure 1. Percentage of viable cells treated with S_EtOH in
various concentrations after 24 hours. Samples are
conducted in triplicate and represented in mean ±
standard deviation.

For comparison, the previous study on
Selaginella doederleinii Hieron. has found that the
plant has a biflavanone, 2,2",3,3"-
tetrahydrotusafusflavone 7",7"-trimethyl ether,
and a biflavanoid, robustaflavone 7",7"-trimethyl
ether that could inhibit the proliferation of human
cancer cell lines, such as HCT, NCI-H358, and
K562(12). In addition, flavonoids isolated from
Selaginella willdenowii also could inhibit human
cancer cell lines(6).

To identify the presence of apoptosis, we
used flow cytometric analysis after PI staining of
cells to study the effect of S_EtOH on T47D cells.
The apoptotic cells were identified as sub-G1 DNA
content in the cell cycle analysis (Figure 2). The
amount of apoptotic cells measured in the sub-G1
phase were approximately 3-fold greater for the 5.0
μg/mL S_EtOH -treated T47D cells than for the
control. Meanwhile, for 2.5 μg/mL S_EtOH -
treated T47D cells, the amount of apoptotic cells
were about 2-fold greater than for the control cells.
In addition, 50 nM doxorubicin as a positive control
could induce apoptosis for T47D cells
approximately 6-fold greater than that of control
cells.

However, this cell cycle analysis method does
not distinguish between early and late apoptotic
cell. The method does not also differentiate the
amount of between apoptotic and necrotic cells.
This can be determined by annexin V and PI double
labeling method(8). We attempt to confirm the cell
death via annexin V-FITC and PI double labeling
method. T47D cells were double-labeled with
annexin V-FITC and PI, and analyzed by flow
cytometry.
Figure 2. The S_EtOH and doxorubicin - induced apoptosis in T47D cells. A flow cytometric analysis of T47D cells at the indicated concentration of ESP and doxorubicin was conducted after the cells had been incubated for 24 hours. The cell cycle distribution was determined by a flow cytometric analysis of the DNA content after staining with propidium iodide. The data are expressed as mean ± SD, n=2. *p<0.05, **p<0.01 significant vs control untreated cells.

Upon 24 hours 7 μg/mL S_EtOH treatment, the amount of early and late apoptotic cells (quadrant B4 and B2, respectively) was higher than that of untreated cells population (Figure 3). There were 9.5% early apoptotic cells, and 34.7% late apoptotic cells after 24 hours 7 μg/mL S_EtOH treatment. The upper left quadrant (B1) would contain cells that take up PI but do not bind annexin V. These cells would most likely be necrotic. There were only 1.0% necrotic cells after 24 hours 7 μg/mL S_EtOH treatment. According to the results, S_EtOH treated T47D cells may undergo the cell death via apoptosis pathway.

Apoptosis is the process of programmed cell death (PCD) that may happen in multicellular organisms. This process can change morphology cells including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis is different from necrosis, because apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage. These results clearly indicate that the anti-proliferative effect of S_EtOH on T47D cells was attributable to apoptosis process and suggest that Selaginella plana Hieron may be an important natural anticancer chemotherapeutic agent.
CONCLUSION

The ethanol extract of *Selaginella plana* Hieron. has anticancer activity on T47D human breast cancer cell line. The extract may undergo the cell death via apoptosis pathway. These findings may due to the flavonoid compounds, and study further is needed to determine the particular compounds.

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