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# 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, anticardiovascular, 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.*

## ABSTRAK

*Selaginella sp* termasuk ke dalam famili Selaginellaceae. Tanaman ini tela digunakan di Cina dan Indonesia sebagai obat tradisional. Tanaman ini memiliki beberapa khasiat di antaranya sebagai agen antibakteri, antikardiovaskular, dan antikanker. Tujuan dari penelitian ini adalah untuk mengetahui aktivitas antikanker dari ekstrak etanol *Selaginella Plana* Hieron. pada sel kanker payudara T47D. Proliferasi sel T47D dideteksi oleh metode SRB (Sulforhodamine B) yang diukur pada panjang gelombang 515nM. Hasilnya telah ditemukan bahwa nilai IC50 dari ekstrak etanol tanaman ini diketahui pada konsentrasi 7,03 µg/mL. Aktivitas yang signifikan ini diasumsikan karena kandungan total flavonoid yang tinggi. Total kandungan flavonoid pada ekstrak etanol tanaman ini adalah 23,04%. Analisis *flow cytometry* menunjukkan bahwa ekstrak ini dapat menyebabkan kematian sel melalui jalur apoptosis. Dapat disimpulkan bahwa ekstrak etanol *Selaginella plana* Hieron. memiliki aktivitas yang cukup besar dalam menghambat proliferasi sel T47D.

**Kata kunci** : *Selaginella plana* Hieron., Sulforhodamin B, sel T47D, antikanker, *flow cytometri.*

## 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[(1)].

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 the family Selaginellaceae. It has been used in China and Indonesia as a traditional medicine. It has several medicinal properties including antibacterial, antihepatitis, anticardiovascular, and anticancer[2,3].

Some of *Selaginella spp.* that has been known are Selagienlla *doederlieinii* Hieron, *Selaginella willdenowii*, and *Selaginella plana* Hieron. The previous study reported that the water extract of *Selagienlla doederlieinii* Hieron. has moderate antimutagenic activity against benzo[a]pyrene[4]. In addition, this plant has anticancer activity against L 929 murine cells[5]. 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[6]. 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 Sulforhodamin B (SRB) assay was used to evaluate the anticancer activity. Analysis by flow cytometer was also conducted in order to study apoptosis of the cells.

## EXPERIMENTAL METHODS

### Plant material.

*Selaginella plana* Hieron were obtained from one source in Bogor, West Java. The plant has been determined by Research Center for Biology, Indonesian Institute of Sciences.

### Preparation of ethanol extract of *Selaginella plana* Hieron (S_EtOH).

Preparation of the ethanol extract followed the previous method[7]. The dried of extract (3.4 kg) was grounded and immersed in 96 % ethanol. After 72 hours the filtrate was collected. The combined filtrate as evaporated with rotary evaporator at 40°C. The yield of ethanol extract was 844.4 g.

### Phytochemical Test and Determination of Total Flavonoid Componds.

Phytochemical test was conducted to analyze the present of phenolic/flavonoid, saponin, alkaloid, triterpenoid, and steroid. The method was described previously[7]. The method of determination of total flavonoid compounds followed the method from Depkes RI[8] and used luteolin as a standard.

### Sulphorhodamine B assay for cell proliferation.

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the anticancer activity of T47D human breast cancer cells[9]. 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 cells were treated with ESP and doxorubicin as a positive control for another 24 h. The cells were harvested and fixed with 70% ethanol in 4°C for 30 minutes[10]. The fixed cells were stained with 2 µg/mL of PI at room temperature in the dark for 30 minutes. The DNA content of the cells were monitored by using a COULTER EPICS XL™ flow cytometer.

### Assesment 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 cells were treated with ESP and doxorubicin as a positive control for another 24 h. After incubation, the apoptotic cells were measured using Annexin V-FITC conjugate[11]. 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 $PI^-$ /annexin $V^-$ (lower left quadrant in the plot); early apoptotic cells axhibit $PI^-$/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 1. Identification groups of compounds in ethanol extract of *Selaginella plana* Hieron (S_EtOH)

| Groups of Compounds | Result (+/-) |
|---|---|
| Phenolic/flavonoid | + |
| Saponin | + |
| Alkaloid | + |
| Triterpenoid | - |
| Steroid | + |

The S_EtOH could inhibit the proliferation of T47D cell lines in a dose-dependent manner as shown in **Figure 1.** The $IC_{50}$ 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[12].

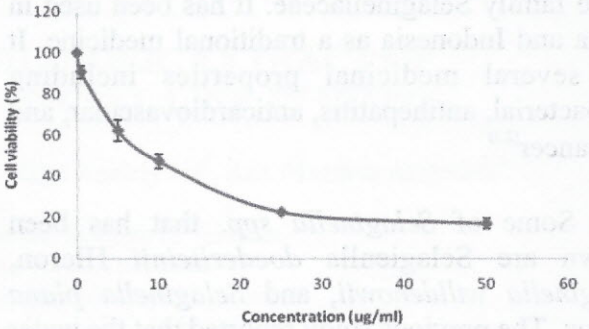


**Figure 1.** Percentage of viable cells treated with S_EtOH in various concentrations after 24 hours. Samples are conducted in triplicate and represented in mean ± standard deviatio

For comparison, the previous study on *Selagienlla doederlieinii* Hieron. has found that the plant has a biflavanone, 2,2",3,3"-tetrahydrorobustaflavone 7,',7"-trimethyl ether, and a biflavonoid, robustaflavone 7,4',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[13]. 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.

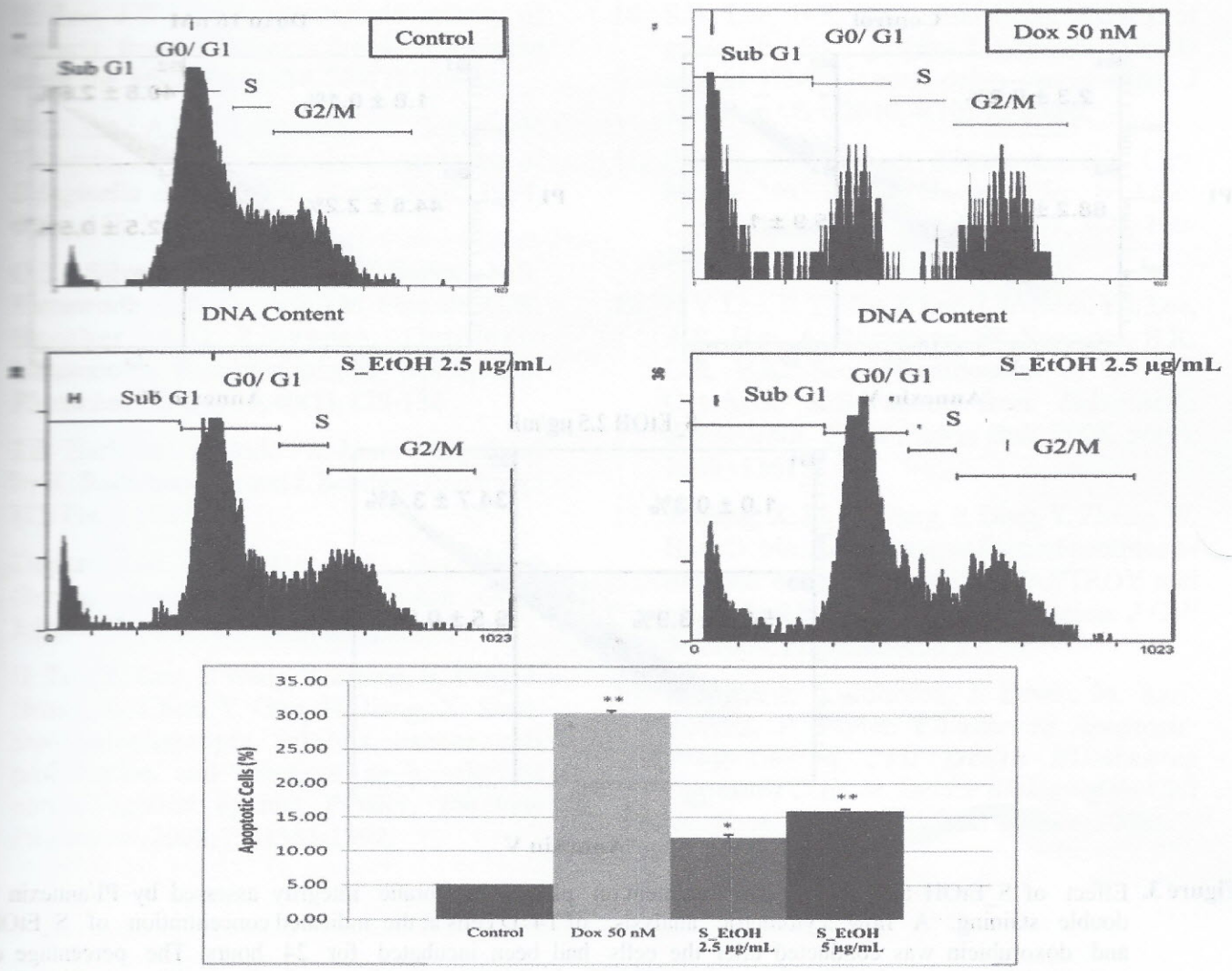


**Figure 2.** The S_EtOH and doxorubicin - induced apoptosis in T47D cells. A flow cytometric analysis of T47D cells at the indicated concentration of ESP and doxorubicin was conducted after the cells had been incubated for 24 hours. The cell cycle distribution was determined by a flow cytometric analysis of the DNA content after staining with propi dium 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[14]. 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.

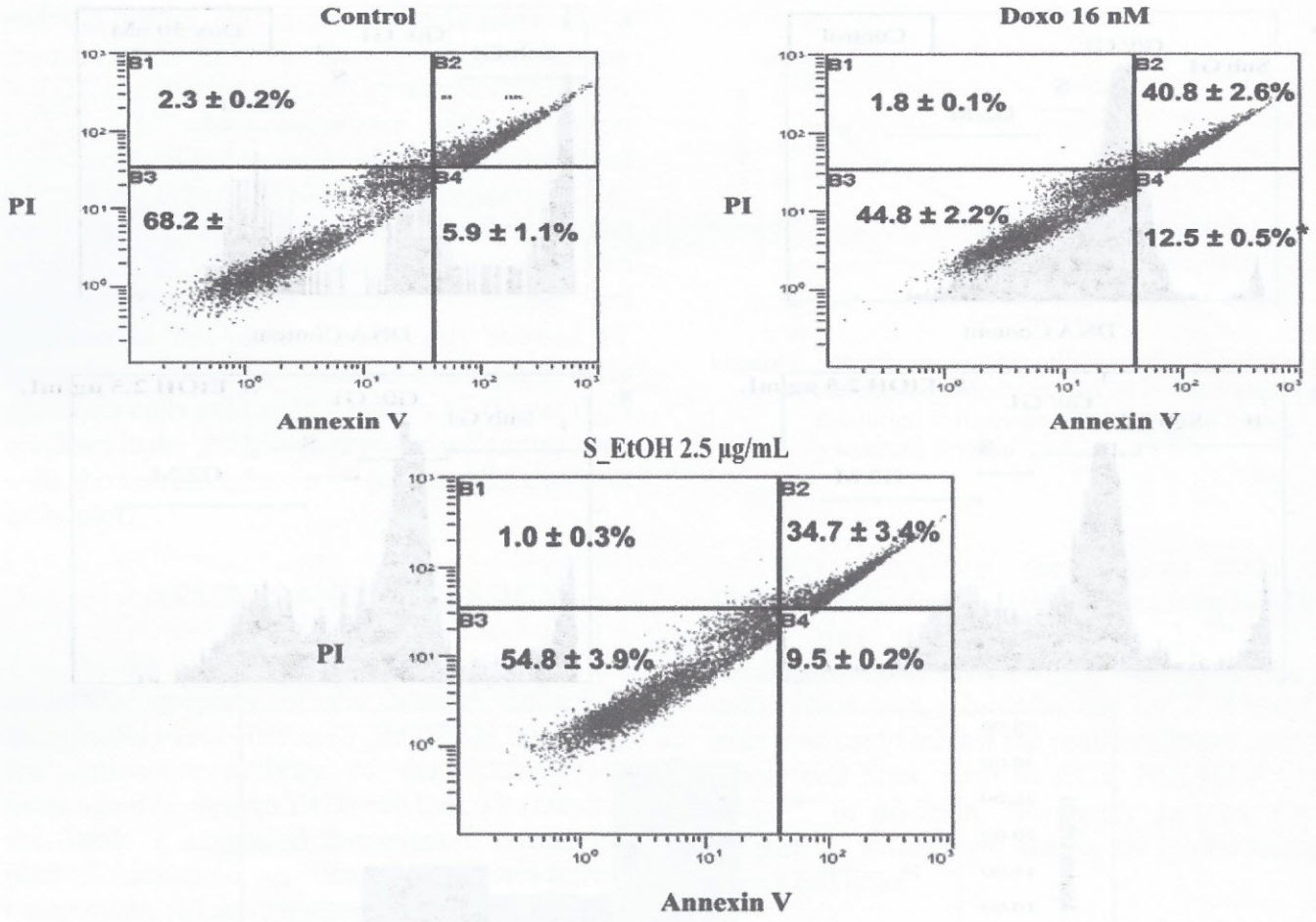


**Figure 3.** Effect of S_EtOH and doxorubicin treatment on plasma membrane integrity assessed by PI/annexin V double staining. A flow cytometric analysis of T47D cells at the indicated concentration of S_EtOH and doxorubicin was conducted after the cells had been incubated for 24 hours. The percentage of early and late apoptotic cells detected by PI/annexin V-FITC double - staining was determined by flow cytometry. Quadrant B1 indicates the amount of necrotic cells, B2 shows the amount of late apoptotic cells, B3 denotes the amount of viable cells, while quadrant B4 points to the amount of early apoptotic cells. The data are expressed as mean ± SD, n=2. *p<0.05 significant vs control untreated cells.

## 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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