Journal of Applied Pharmaceutical Science Vol. 3 (04), pp. 031-034, April, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.3405
ISSN 2231-3354

Induction of Apoptosis on MCF-7 cells by Selaginella Fractions

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ARTICLE INFO

Article history:
Received on: 27/02/2013
Revised on: 19/03/2013
Accepted on: 04/04/2013
Available online: 27/04/2013

Key words:
Selaginella plana Hieron, MCF-7, MTT, apoptosis.

ABSTRACT

The selaginella ethanolic extract shows cytotoxic activity against T47D and MCF-7 cells. The aim of this research is to evaluate the cytotoxic effect and apoptosis induction of selaginella fractions on MCF-7 cells. The *Selaginella plana* powder was extracted by absolute ethanol. 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). Cytotoxic activity was examined by MTT assay. Apoptosis examination used acrydine orange-etidium bromide staining (double staining). The result showed that the IC_{50} value of S_Hex, S_MTC, S_EA, and S_BuOH on MCF-7 cells were 30 µg/mL, 19 µg/mL, 24 µg/mL, and 2 µg/mL respectively. The active fractions (S_Hex, S_MTC, S_EA and S_BuOH) at its IC_{50} concentration increased apoptotic cells on the MCF-7 cells 35.33%, 20.33%, 24% and 45.67% respectively compared to control. Based on the result, buthanol fraction of *Selaginella plana* (S_BuOH) showed the highest apoptotic induction on MCF-7 cancer cells.

INTRODUCTION

Cell cycle arrest and apoptosis induction are targeted in the strategy of cancers therapy (Doucas *et al*., 2006). Apoptosis, or programmed cell death, is a multi-step process that is important to eliminate damaged or abnormal cells (Choi and Kim, 2009). Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but, their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention (Taraphdar *et al*., 2001).

Scientific studies indicate that the promising phytochemicals can be developed from the medicinal plants for many health problems (Dahiru *et al.,* 2006). Evidence has emerged from various studies that suggest that products derived from plants are useful in the treatment as well as in the prevention of cancer. *Selaginella plana* Hieron is the most distributed *Selaginella* in Indonesia that has not been investigated yet. The compounds of *Selaginella sp.* that have been known are flavonoid and biflavonoid (amentoflavone, robustaflavone, etc), phenolic, alkaloid, and lignan (Setyawan, 2011). The selaginella ethanolic extract shows cytotoxic activity against T47D and MCF-7 cells with the IC_{50} value are 7µg/ml and 61µg/ml respectively. *Selaginella plana* ethanolic extract induces apoptosis on both of cell lines (Risdian *et al.,* 2011; Handayani *et al.,* 2011). The aim of this research is to evaluate the cytotoxic effect and apoptosis induction of selaginella fractions on MCF-7 cells.

MATERIALS AND METHODS

Preparation of the *Selaginella plana* Hieron fractions

The dried of extract 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 to get selaginella ethanolic extract (S_EtOH).

The ethanol extract was diluted by methanol: water (4:1) and then partitioned with hexane. The aqueous layer was fractioned 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 concentrated with vacuum rotary evaporator at 40°C.

Cell culture

MCF-7 cell line was obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Gadjah Mada University and was cultured in *Dulbecco's Minimum Eagle Medium* (DMEM) medium (Gibco) with 10% Fetal Bovine Serum (Gibco) dan 1% Penicillin-Streptomycin (Gibco).

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Cytotoxic assay

MCF-7 cells were seeded in 96-well plates with 5 x 10^3 cells/well and divided into control and treatment group. Serial dilution of the samples was used at 1, 10, 50, 100, 200, 500 and 1000 µg/mL. After 24 h incubation, culture medium was removed and cells were washed in PBS (Sigma). Then, cells were incubated with 100 µL culture medium and 10 µL MTT (Sigma) 5 mg/mL in every well for 4-6 h. MTT reaction was stopped by SDS reagent (10% Sodium dodecyl sulphate (Merck) in HCl 0.1N (Merck)) and was incubate over night. The absorbance was measured by ELISA reader (Bio-Rad) at wave length of 595 nm.

Apoptosis detection

Apoptosis was detected by acrydine orange-etidium bromide staining (double staining). MCF-7 cells (5 x 10^4 cells/well) were seeded in coverslips in 24-well plates until 50-60% confluent. Then, cells were incubated with samples on IC_{50} concentration for 24 h. Culture medium was removed and cells were washed with PBS. Coverslips were moved into object-glass and added with 10 µL 1X working solution acrydine orange (Sigma)-etidium bromide (Sigma) and analized using fluoroscence microscopy (Zeiss MC 80).

Statistical analysis

Absorbance-measurement from cytotoxic assay was analyzed by Excell MS Office 2007 to get IC_{50} value. Anova single factor (Excel MS Office 2007) was used to assess differences among the treatment or the concentration ($p<0.05$). Apoptosis were observed and at least 100 cells/ field were evaluated. The result came from means of 3 fields. Apoptosis were observed and at least 100 cells/ field were evaluated. The result came from means of 3 fields.

RESULTS AND DISCUSSIONS

The cytotoxic effect of Selaginella fractions on MCF-7 cells growth were measured with the MTT assay and presented by IC_{50} value. The IC_{50} value of S_Hex, S_MTC, S_EA, and S_BuOH on MCF-7 cells were 30 µg/mL, 19 µg/mL, 24 µg/mL, and 2 µg/mL respectively. Methanol fraction (S_MeOH) did not have cytotoxic effect when the IC_{50} was higher than 100 µg/mL (Fig.1A-B; 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 control. Buthanol fraction of *Selaginella plana* Hieron presented the strongest cytotoxic activity (Fig. 1B; Table 1). The active fractions (S_Hex, S_MTC, S_EA and S_BuOH) at its IC_{50} concentration increased apoptotic cells on the MCF-7 cells 35.33%, 20.33%, 24% and 45.67% respectively compared to control (Fig.2; Table 2). Based on the result, buthanol fraction of *Selaginella plana* (S_BuOH) showed the highest apoptotic induction on MCF-7 cancer cells.

Selaginella plana ethanol extract contains phenolic/flavonoid, alkaloid, and saponin. Total flavonoid content of the ethanolic extract is 23.04% (Risdian *et al.*, 2011). Flavonoid apigenin, luteolin and quercetin have been shown to cause cell cycle arrest and apoptosis by a p53-dependent mechanism (Sandhar *et al*., 2011). Amentoflavone, a biflavonoid which also exist in *Selaginella sp.* (Setyawan, 2011), shows inhibitory effect on bcl-2 expression and upregulated p53 gene expression in B16F-10 melanoma cells (Guruvayoorappan *and* Kuttan, 2008). Different with the previous study which MTC fraction of *Selaginella plana* (S_MTC) showed the strongest cytotoxic effect and apoptosis induction against T47D cells (Handayani *et al.*, 2012), the present study showed which buthanol fraction of *Selaginella plana* Hieron (S_BuOH) that performed the strongest cytotoxic activity and apoptosis induction against MCF-7 cells. Buthanol fraction usually contains flavonoid glycoside and other polar compounds (Magaji *et al.*, 2012; Al-Taweel *et al.*, 2012; Im *et al.*, 2012). Sugar/ glycon form in flavonoid glycoside has a role on cytotoxic effect of cancer cells. Quercetin diglycoside shows a significant cytotoxic activity against the HepG2 liver carcinoma cell line (IC50 = 0.86 µg/mL), while the acetylated glycon form of quercetin diglycoside shows a lower cytotoxic activity (Al-Taweel *et al.*, 2012). Citrus extracts contains flavonoid glycosides (hesperidine, naringinin), induces apoptosis through upregulation of p53 and downregulation of bcl-2 (Meiyanto *et al.*, 2012). Since MCF-7 cell line expresses wild-type p53 (Alimirah *et al.*, 2007) and bcl-2 (Amundson *et al*., 2000), we suggest that mechanism of apoptosis from Selaginella actives fraction, especially S_BuOH that may also contain flavonoid glycoside, possibly occur by increasing of p53 tumor supressor expression and decreasing of bcl-2 expression. Both of the mechanisms perform synergistic effect to induce apoptosis.

Table. 1: IC_{50} Value of *Selaginella plana* Hieron solvent fraction against MCF-7.

Sample	IC_{50} (µg/mL)
S_EtOH*	61
S_Hex	30
S_MTC	19
S_EA	24
S_BuOH	2
S_MeOH	> 100

*Handayani *et al.*, 2011

Table 2. Apoptosis induction of *Selaginella plana* Hieron solvent fraction against MCF-7

Sample	Apoptosis cells (%)
S_EtOH*	20.67
S_Hex	35.33
S_MTC	20.33
S_EA	24
S_BuOH	45.67

*Handayani *et al.*, 2011

Apoptotic effect-mediated by cytochrome C release is dependent on the balance between antiapoptosis and proapoptosis (Ghobrial *et al.*, 2005). The antiapoptosis protein, bcl-2, is the terminal regulatory point in the process of apoptosis and the activation of cascade reaction for proteinase is terminated when the release of cytochrome from mitochondria is interfered (Han *et al*., 2008). On the other hand, expression of the p53 tumor

suppressor protein, which is a transcription factor, induces proapoptosis protein (such as Bad, Bax, and Bid) expression. High level of proapoptosis protein compared to level of antiapoptosis protein induces cytochrome C release. Consequently, activation of caspase-9 will be occur and followed by increasing of cleavage of caspase-6 and -7 as apoptosis executor (Choi *and* Kim, 2009). Nevertheless, further investigation is needed to explore the mechanism of apoptosis induction of *Selaginella plana* Hieron active fractions on MCF-7 cancer cell.

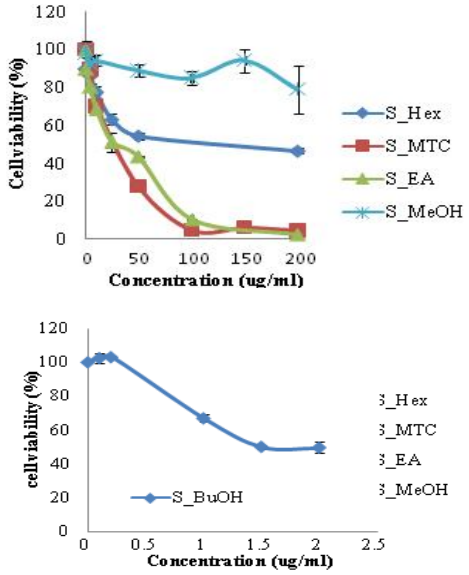

Fig. 1: Percentage of viable cells of *Selaginella plana* Hieron solvent fractions after 24 hours. A) S_Hex, S_MTC, S_EA, and S_MeOH in various concentrations (0-200 µg/ml), B) S_BuOH in various concentrations of (0-2 µg/ml). Samples are conducted in triplicate and represented in mean ± standard deviation.

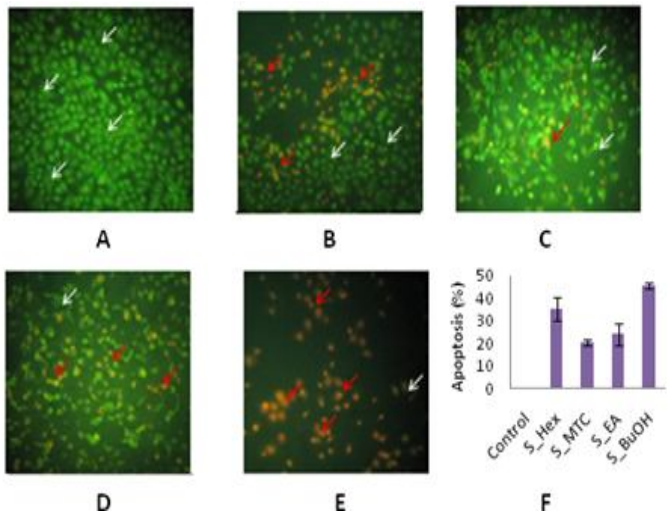

Fig. 2: Apoptosis induction of Selaginella fractions on MCF-7 cells. MCF-7 cells ($5x10^4$) incubate with each of Selaginella fraction (IC_{50}) for 24 h. (A) control cells, (B) cells with S_Hex, (C) cells with S_MTC, (D) cells with S_EA, (E) cells with S_BuOH, and (F) Graphic of apoptosis induction on MCF-7 cells due to Selaginella fractions. Cells were stained with acrydine orange-ethidium bromide and saw in fluorosence microscope. 400x magnification. viable cells, apoptosis.

CONCLUSION

Buthanol fraction of *Selaginella plana* (S_BuOH) showed the strongest cytotoxic activity (IC_{50} 2 µg/mL) and the highest apoptotic induction (45.67%) on MCF-7 cancer cells.

ACKNOWLEDGEMENT

This work was supported by The Project of Kompetitif, LIPI 2010 and all of the members of the project (Marissa Angelina M.Farm, Apt., Rina Andriyani, S.Si, Apt., and Chandra Risdian, S.Si.).

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How to cite this article:

Sri Handayani, Adam Hermawan, Edy Meiyanto, Zalinar Udin., Induction of Apoptosis on Mcf-7 cells by Selaginella Fractions. J App Pharm Sci, 2013; 3 (04): 031-034.