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Inhibitory effects of costunolide and parthenolide from Champi Sirindhorn (*Magnolia sirindhorniae*) on FLT3 protein expression in EoL-1 leukemic cells

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ABSTRACT

Background: FLT3 (Fms-like tyrosine kinase 3) belongs to the class III receptor tyrosine kinase that is involved in hematopoietic progenitor cell proliferation. It is a prognostic marker for acute myeloblastic leukemia (AML). To date, chemotherapy has been the most frequently used treatment for leukemia. It has had a very good outcome in the early stages of treatment. However, the main problem of chemotherapy is the side effects for leukemia patients, as it may also cause drug resistance after long time treatment. Magnolia (*Magnolia spp.*) is a medicinal plant and has been used as traditional medicine in China, Japan, and Thailand. It is used for treatment of gastrointestinal disorders, anxiety, allergic disease, etc.

Objectives: Effect of crude fractional extracts and purified active compounds from *Magnolia sirindhorniae* Noot. & Chalermglin (a new species of *Magnoliae spp*. which was discovered first in Thailand) were investigated for their cytotoxicity, leukemic cell proliferation, and FLT3 protein suppression in EoL-1 cells. Crude fractional extracts from leaves (fraction No. 1-3), twigs (fraction No. 4-6), and stems (fraction No. 7-9) were fractionated by hexane (fraction No. 1, 4, 7), ethyl acetate (fraction No. 2, 5, 8), methanol (fraction No. 3, 6, 9). The costunolide (1) and parthenolide (2) were purified from *n*-Hexane fraction from leaves and ethyl acetate fraction from twigs, respectively by column chromatography. Cytotoxicities against leukemic cells were determined by using MTT assay.

Results: Fraction No. 1, 2, 4, 5, 7, 8, costunolide (1), and parthenolide (2) showed strong cytotoxic effects on EoL-1 cells. Furthermore, the non-cytotoxic concentration (20% inhibitory concentration (IC_{20}) values) also decreased FLT3 protein expressions and total cell numbers of EoL-1 cells after treatments. Interestingly, fraction No. 1, 5, costunolide (1), parthenolide (2) decreased the FLT3 protein levels in a time- and dose-dependent manner.

Conclusion: In summary, costunolide and parthenolide are effective compounds from leaves and twigs of *M. sirindhorniae* to suppress FLT3 protein expression and cell proliferation.

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Introduction

Magnolia sirindhorniae Noot. & Chalermglin is a Thai medicinal plant that belongs to the family Magnoliaceae. It was found in Thailand in 1999 by Dr. Piya Chalermglin. Thai common name is "Champi Sirindhorn" (Nooteboom and Chalermglin, 2000). In Thailand it is named after HRH Princess Sirindhorn. Among Magnolia species, *M. obovata* and *M. officinalis* are very important in traditional Chinese and Japanese herbal medicines. Magnolia bark and flower have been used for treatment of gastrointestinal disorder, anxiety, and allergic disease. In addition, the bark showed anti-cancer[1], anti-inflammatory[2], and anti-oxidant activities.[3] In the central nervous system, it showed anti-stress, anti-anxiety[4], anti-depressant[5], anti-Alzheimer, and anti-stroke effects.[4] In cardiovascular system, it showed anti-esophageal obstruction, anti-gastric ulcer, anti-diarrhea, and hepatoprotective effects.[6] Moreover, magnosalin, a compound isolated from "shin-i" (Flos magnoliae), showed anti-arthritic[7], anti-angiogenetic[8], and anti-inflammatory activities.[9] The leaves of *M. sirindhorniae* collected from Khlong Luang District, Pathum Thani Province, Thailand have been reported to contain five sesquiterpene lactones including costunolide, santamarine, reynosin, parthenolide, and lipiferolide.[10]

Sesquiterpene lactone compounds had cytotoxicity on U937[1], bladder cancer cells[11], multiple myeloma[12], M DA-MB231,[13] HL-60, and L1210 cell lines.[14] Costunolide from stem bark of *M. sieboldii* has been reported to induce apoptotic cell death in a dose-dependent manner and decrease Bcl-2 protein (anti-apoptotic protein), whereas the cleavage poly-(ADP-ribose) polymerase was activated in Colon 26, 3LL Lewis, J82, T24, and HL-60 cell lines.[15] Costunolide (10 μM) from stem bark of *M. sieboldii* also demonstrated to trigger apoptosis in U937 cells by depleting intracellular reduced glutathione (GSH) and protein thiols.[1] In addition, it could inhibit growth and telomerase activity of human breast carcinoma cells (MCF-7 and MDA-MB-231) in a dose- and time-dependent manner.[16] Moreover, costunolide, isolated from roots of *Saussurea loppa* (Mu Xiang), has been reported to induce apoptosis in bladder cancer cells by mediating through ROS generation and mitochondrial dysfunction.[11] However, the activity of crude fractional extracts and purified active compounds from Thai *M. sirindhorniae* on leukemic cells have never been reported, especially its effect on molecular target protein involved in leukemic cell proliferation. Feline McDonough Sarcoma (FMS)-like tyrosine kinase 3 or FLT3 protein and its mutations in leukemic cells are also involved in leukemic cell proliferation. It primarily expressed on committed myeloid and B-lymphoid progenitors and plays an important role in their survival, proliferation, and differentiation.[17] Low levels of FLT3 protein expression have been found in normal peripheral blood mononuclear cells (PBMCs). In contrast, overexpression of FLT3 protein has been found in leukemic blood cells, especially in acute myeloid leukemia (AML) and B-cell acute lymphoblastic leukemia (B cell ALL).[18] Parthenolide, an active compound from feverfew plant (*Tanacetum parthemium*) has been investigated for the anticancer activity and reported the effect to induce apoptosis in pre-B acute lymphoblastic leukemia cell lines, including cells carrying chromosomal translocation.[19] In addition, it could induce apoptosis through mitochondrial cytochrome C release, and caspase activation in chronic lymphocytic leukemia (CLL).[20] It stimulated the modification of redox state of critical exofacial thiols in Granta mantle lymphoma cells.[21] Furthermore, it has been reported the induction of autophagy through ROS generation, GSH depletion, JNK activation, and inhibition of NF-kB activity in human breast cancer cell line, MDA-MB231.[13]

However, the effect of crude fractional extracts and purified costunolide and parthenolide from *M. sirindhorniae* on FLT3 protein expression in leukemic cells is still unknown. The present study was designed to investigate the cytotoxicity and inhibitory effects of crude fractional extracts (hexane, ethyl acetate, and methanol) and purified active compounds (costunolide and parthenolide) on FLT3 protein expression. EoL-1 cell line was used as a leukemic cell model for this study because it shows high level of endogenous FLT3 protein expression.

Materials and methods

Chemical materials

RPMI 1640 (Invitrogen™, CA, USA), fetal bovine serum (FBS), *L*-glutamine, penicillin/streptomycin were purchased from Gibco (Invitrogen™, CA, USA), MTT dye, commercial costunolide (costunolide (3)) and commercial parthenolide (parthenolide (4)) were purchased from Sigma-Aldrich (St Louis, MO, USA). Trypan blue dye solution was purchased from AMRESCO® (Solon, OH, USA). Rabbit polyclonal anti-FLT3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-GAPDH (Glyceraldehyde phosphate dehydrogenase) antibody was purchased from Santa Cruz Biotechnology (CA, USA). HRP conjugated goat anti-rabbit IgG was purchased from Invitrogen™ Life (Carlsbad, CA, USA). Enhanced chemiluminescence detection kit was purchased from Thermo Scientific (Miami, USA). Luminata™ Forte Western HRP Substrate was purchased from Millipore Corporation (Billerica, MA, USA). *n*-Hexane, ethyl acetate, and methanol were purchased from Merck (Darmstadt, Germany).

Plant materials

Leaves, twigs, and stems of *M. sirindhorniae* were collected from the Thailand Institute of Scientific and Technological Research, Khlong Luang District, Pathum Thani Province, Thailand, in April 2010. A voucher specimen No. BKF420621 was deposited at the herbarium of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Herbarium specimen has been studied and annotated by traditional methods of herbarium taxonomy.

Extraction and isolation of M. sirindhorniae

Total nine crude fractional extracts (fraction No. 1-9), fraction No. 1-3 were extracted from leaves of *M. sirindhorniae* by *n*-hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH) respectively, fraction No. 4-6 were extracted from twigs by Hex, EtOAc, and MeOH, respectively. Fractions No. 7-9 were extracted from stems by Hex, EtOAc, and MeOH, respectively. In addition, the active fractions were investigated by separation on column and preparative thin layer chromatographic (TLC) methods until purified 1 and 2 were obtained. The

two compounds (1 and 2) were identified as costunolide (1) and parthenolide (2).[10] To identify the compounds, spectroscopic analyses, including infrared spectroscopy, electrospray ionization-mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR), were performed. Two-dimensional-NMR measurements, such as correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC), also supported the identifications. Structures of costunolide (1) and parthenolide (2) are shown in Figure 1A and 1B, respectively. Costunolide (1) and parthenolide (2) were further investigated for their effects on cytotoxicity and FLT3 protein expression.

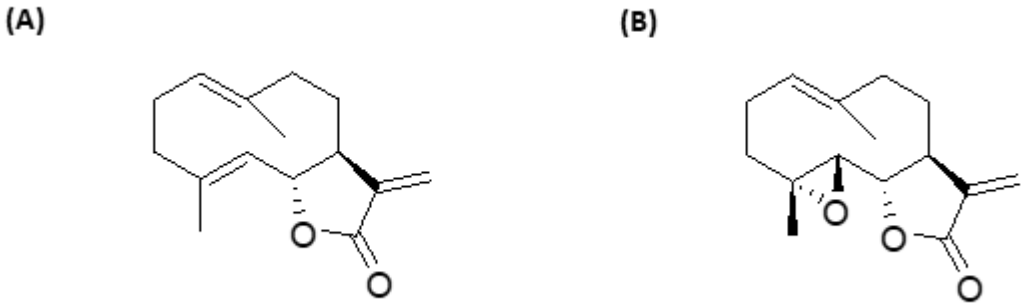

***Figure 1**. Chemical structure of (A) costunolide (1) and (B) parthenolide (2)*

Cells and cell culture conditions

EoL-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM *L*-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, and incubated under 80% relative humidity with 5% CO_2 at 37 °C.

MTT cytotoxicity assay

Cytotoxicity of crude fractional extracts (fraction No. 1-9), costunolide (1) and parthenolide (2) were evaluated using the MTT assay. Briefly, EoL-1 cells (5.0×10^4 cells/well) were cultured in 96 well plates containing 100 µL medium prior to treat for 24 hrs. After that, 100 µL of fresh medium containing various concentrations (0-100 µg/mL) of the test compounds were added to each well and incubated for 48 hrs. MTT dye solution was added (15 µL/100 µL medium) and the plates were incubated at 37 °C for 4 hrs in a humidified 5% CO_2 atmosphere. Afterward, 200 µL of DMSO were added to each well, and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader (Biotek EL 311) at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye color represent to a high number of viable cells able to metabolize MTT salts. Fractional absorbance was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in vehicle control well}} \times 100$$

Average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. The experiment was done in 3 independent experiments. The 50% inhibitory concentration (IC_{50}) of the active substances was determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture or vehicle control culture (0.2% DMSO in culture medium). IC_{50} values were mean±standard deviation (SD) and compared for their activities.

Trypan blue exclusion test

Cell viability was measured by trypan blue dye exclusion method. Cells were treated with various concentrations of crude fractional extracts (fraction No. 1-9), purified active compounds (costunolide (1) and parthenolide (2)), and commercial compounds (costunolide (3) and parthenolide (4)). Then, cells and 0.4% trypan blue dye were mixed and counted using a light microscope. All experiments were performed in triplicate.

Protein extraction and Western blotting

EoL-1 cells were treated with crude fractional extracts (fraction No. 1-9), purified active compounds (costunolide (1) and parthenolide (2)), and commercial compounds (costunolide (3) and parthenolide (4)) for 48 hrs, after cell harvesting, cells were washed twice with cold PBS, pH 7.4, and lysed with cold RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, and 0.001% protease inhibitor cocktail) for whole protein extraction. Whole protein lysate (100 µg) were loaded onto 12% SDS-PAGE and then transferred to PVDF membranes (Merck and Millipore, Burlington, MA, USA). Membranes were blocked with 5% skim milk and probed with rabbit anti-FLT3 at 1:1,000 dilution. Rabbit anti-GAPDH at a dilution of 1:1,000 was used for protein loading control. The reaction was followed by HRP-conjugated anti-rabbit IgG at 1:20,000 dilution. Proteins were visualized using an enhanced chemiluminescence detection kit (Luminata™ Forte Western HRP Substrate). Densitometry was performed using Alpha Innotech software. Band density of the loading control was used to normalize the band densities of proteins of interest to obtain the relative normalized expression level as compared to the exposed control.

Statistical analysis

All data were expressed as mean±SD from triplicate samples of three independent experiments. Statistical differences between the means determined using One-way

analysis of variance (One-way ANOVA). The differences were considered significant when the probability value obtained was found to be less than 0.05 (p<0.05) and 0.01 (p<0.01).

Results and discussion

M. sirindhorniae extracts

All *M. sirindhorniae* extracts were separately evaporated to dryness under reduced pressure at 40 °C to give 9 crude fractional extracts. Their extracts were brownish sticky solid. Crude MeOH extract of leaves showed the highest yield (6.23%), followed by crude MeOH extract of stem (5.39%), and crude EtOAC extract of leaves (4.89%), respectively (Table 1).

Active fractions were repeated column chromatography of the Hex extract of leaves resulted in the isolation of costunolide (1) and parthenolide (2), while two compounds were major constituent in these crude fractions.

Percent of costunolide contents in fraction No. 1, 2, 3, 5, 7, and costunolide (1) was identified by HPLC and compared their retention time with the internal standard costunolide (3). The results showed that percent of costunolide contents were 7.01, 1.09, 0, 0, 0, and 49.6%, respectively. In addition, parthenolide's peaks in the samples (fraction No. 5, 7, and parthenolide (2)) were also identified by HPLC and compared their retention time to the internal standard parthenolide (4). The results showed that parthenolide contents were 39.60, 20.87, and 78.36%, respectively.

Table 1 Percentage of crude fractional extracts No. 1-9.

Crude fraction No.	Plant part	Solvent for extraction	Weight (gm)		% yield
			Dry plants	Extracts	
1	Leave	*n*-Hexane	310.2	9.42	3.03
2	Leave	Ethyl acetate		15.19	4.89
3	Leave	Methanol		19.31	6.23
4	Twig	*n*-Hexane	309.5	6.53	2.11
5	Twig	Ethyl acetate		8.07	2.61
6	Twig	Methanol		12.12	3.92
7	Stem	*n*-Hexane		8.61	2.76
8	Stem	Ethyl acetate	312.3	11.76	3.76
9	Stem	Methanol		16.84	5.39

Cytotoxicity of crude fractional extracts and purified compound in EoL-1 cell line

Cytotoxic effects of crude fractional extracts (No. 1-9), costunolide (1), and parthenolide (2) from Champi Sirindhorn on EoL-1 cells have been shown in Figure 2. Crude fraction No. 1, 2, 4, 5, 7, 8, costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) showed cytotoxicity on EoL-1 leukemic cells at 48 hrs with the IC_{50} values of 35.6, 50, 32.0, 39.6, 24.0, 46.9, 5.5, 3.9, 1.1, 2.1 µg/mL, respectively. On the other hand, crude fraction No. 3, 6, and 9 had no cytotoxic effects (IC_{50}>100 µg/mL).

Effects of fractional extracts and purified compounds on FLT3 protein expression in EoL-1 cell line

IC_{20} (obtained from MTT assay) values of crude fractional extracts and purified compounds were used to examine their effects on FLT3 protein expression and compared to commercial compounds (costunolide (3) and parthenolide (4)). FLT3 protein levels were decreased after treatments with 20, 36, 100, 19, 29, 100, 10, 31, and 100 µg/mL of fraction No. 1-9 in EoL-1 cells by 43.5, 22.0, 5.7, 38.2, 62.1, 9.0, 33.8, 4.9, and 0%, respectively when compared to the vehicle control at 48 hrs (Figure 3A). These treatments also significantly decreased the total cell numbers at 48 hrs by 77.4, 62.5, 29.7, 78.4, 84.5, 28.0, 62.9, 26.1, and 15.8%, respectively (Figure 3B). Non-cytotoxic doses of costunolide (1) (3.9 µg/mL), parthenolide (2) (1.0 µg/mL), costunolide (3) (0.9 µg/mL), and parthenolide (4) (0.8 µg/mL) were examined and compared to those of fractions. Costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) decreased FLT3 protein expression by 66.0, 78.3, 91.5, and 90.4%, respectively when compared to the vehicle control (Figure 3C). Furthermore, costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) significantly decreased the total cell numbers at 48 hrs by 81.9, 84.3, 91.0, and 88.3%, respectively when compared to vehicle control (Figure 3D).

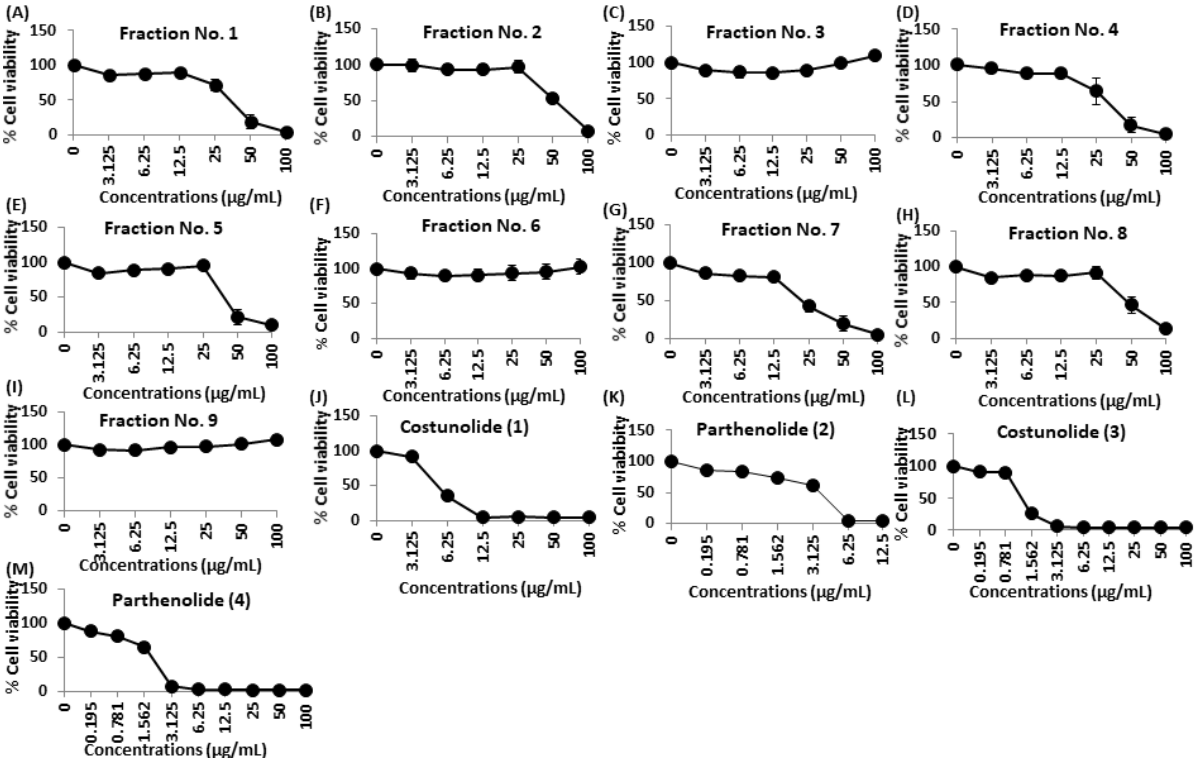

Figure 2. *cytotoxic effects of crude fractional extracts (No. 1-9) (A-I), costunolide (1), parthenolide (2) from Champi Sirindhorn (J-K), costunolide (3), and parthenolide (4) from Sigma-Aldrich (L-M) against EoL-1 cell line. Average of cell viability was obtained from three independent experiments.*

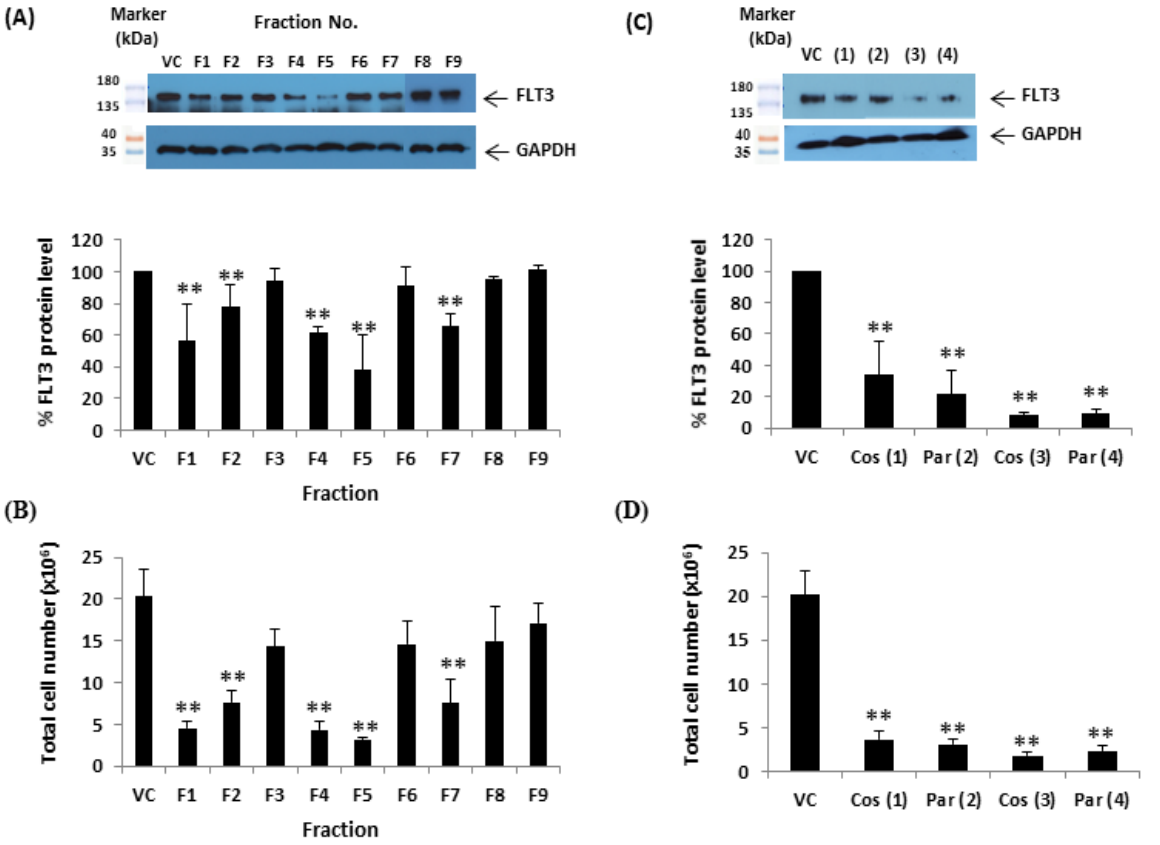

Figure 3. *Effect of fraction No.1-9 (F1-9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on FLT3 protein expression in EoL-1 cells by Western blotting. (A and B) Levels of FLT3 protein expression and total cell numbers after treatment with 20, 36, 100, 19, 29, 100, 10, 31, and 100 µg/mL of F1-9, respectively for 48 hrs. (C and D) Levels of FLT3 protein expression and total cell numbers after treatment with 3.9, 1.0, 0.9, and 0.8 µg/mL of costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4), respectively for 48 hrs. GAPDH was used as a loading control. (VC: vehicle control, F1-9: fraction No.1-9, Cos (1): purified costunolide (1), Par (2): purified parthenolide (2), Cos (3): commercial costunolide (3) and Par (4): commercial parthenolide (4)). Asterisk (**) denotes a significant difference from control group ($p<0.05$ and $p<0.01$).*

Effect of time periods and doses of fraction No. 1 (F1) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with F1 (high content of costunolide) for 6, 12, and 24 hrs decreased FLT3 protein expressions by 2.1, 0.9, and 49.2%, respectively, when compared to the vehicle control (Figure 4A). F1 significantly decreased FLT3 protein levels after treatments with various doses (10, 15, 20, and 25 µg/mL) at 24 hrs were decreased by 3.9, 22.0, 28.8, and 44.3%, respectively (Figure 4B). The total cell numbers at 6, 12, and 24 hrs were decreased by 16.9, 41.0, and 43.8%, respectively (Figure 4C). Total cell numbers in response to 10, 15, 20, and 25 µg/mL were significantly decreased at 24 hrs by 6.1, 21.4, 48.5, and 66.0%, respectively when compared to vehicle control (Figure 4D).

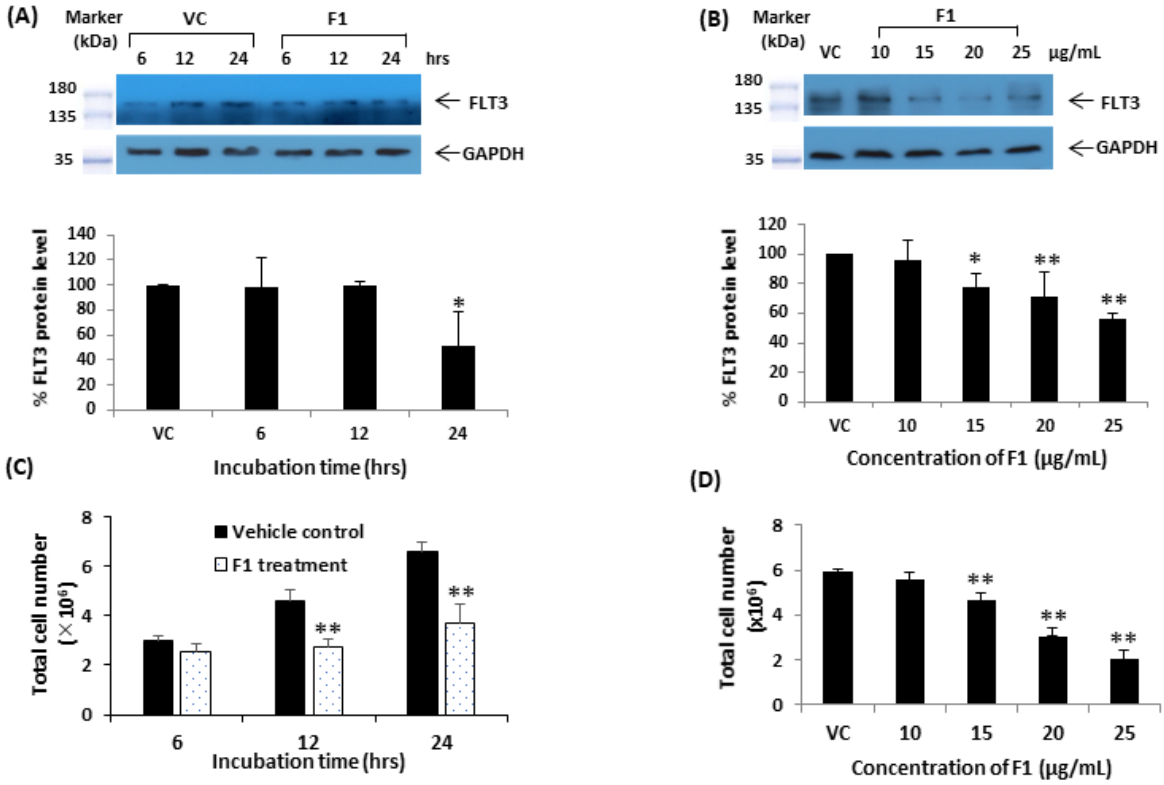

Figure 4. *Effect of various times and concentrations of fraction No.1 (F1) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with F1 at the concentration of 20 µg/mL for 6, 12, and 24 hrs. (B) EoL-1 cells were cultured with different concentrations of F1 (10, 15, 20, and 25 µg/mL) for 24 hrs. (A) FLT3 protein expression level was measured by Western blotting. GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the F1 in culture medium). C and D: Total cell numbers measured by trypan blue dye exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group ($p<0.05$ and $p<0.01$).*

Effect of time periods and doses of fraction No. 5 (F5) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with F5 (high content of parthenolide) for 3, 6, and 12 hrs decreased FLT3 protein expressions by 62.9, 69.8, and 89.8%, respectively, when compared to the vehicle control (Figure 5A). F5 significantly decreased FLT3 protein levels after treatments with various doses (20, 25, 30, and 35 µg/mL) at 12 hrs were decreased by 63.1, 72.9, 83.2, and 84.4%, respectively (Figure 5B). Total cell numbers at 3, 6, and 12 hrs were decreased by 20.0, 37.4, and 52.0%, respectively (Figure 5C). Total cell numbers in response to 20, 25, 30, and 35 µg/mL were significantly decreased at 12 hrs by 13.2, 21.0, 53.6, and 57.3%, respectively when compared to vehicle control (Figure 5D)

Effect of time periods and doses of purified costunolide (1) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with costunolide (1) for 3, 6, and 12 hrs decreased FLT3 protein levels by 70.8, 90.2, and 90.7%, respectively, when compared to the vehicle control (Figure 6A). Costunolide (1) significantly decreased FLT3 protein levels at 12 hrs by 32.4, 55.2, 73.4, and 77.7%, in response to 2, 3, 4, and 5 µg/mL, respectively (Figure 6B). The total cell numbers at 3, 6, and 12 hrs were decreased by 9.1, 28.1, and 50.7%, respectively (Figure 6C). Total cell numbers in response to 2, 3, 4, and 5 µg/mL were significantly decreased at 12 hrs by 25.2, 41.7, 58.5, and 71.5%, respectively when compared to the vehicle control (Figure 6D).

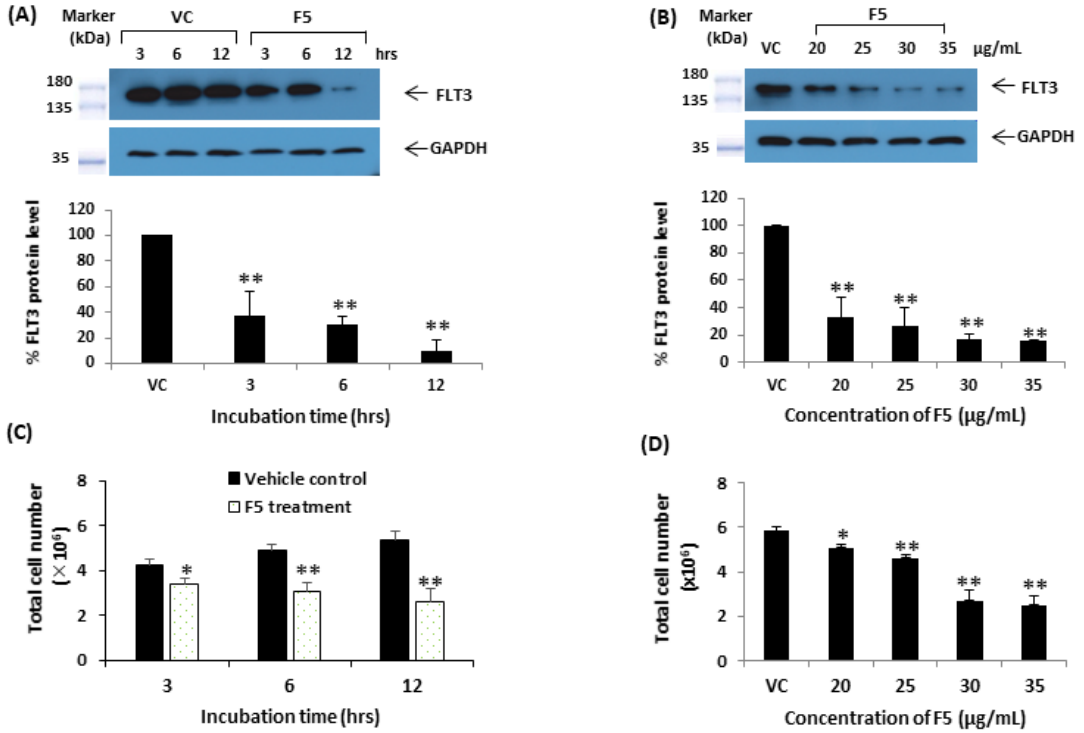

Figure 5. *Effect of various times and concentrations of fraction No.5 (F5) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with F5 at the concentration of 30 µg/mL for 3, 6, and 12 hrs. (B) EoL-1 cells were cultured with different concentrations of F5 (20, 25, 30, and 35 µg/mL) for 12 hrs. (A) FLT3 protein expression level was measured by Western blotting. GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the F5 in culture medium). C and D: Total cell numbers measured by trypan blue dye exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group (p<0.05 and p<0.01).*

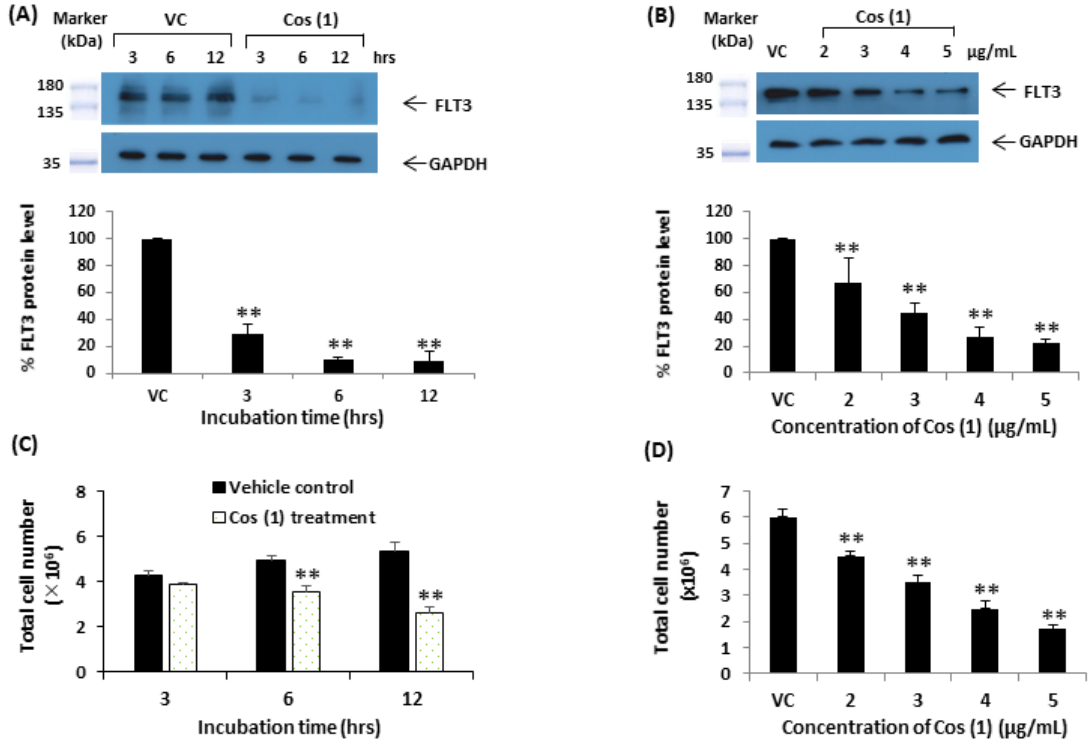

Figure 6. *Effect of various times and concentrations of costunolide (1) (Cos (1)) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with Cos (1) at the concentration of 4 µg/mL for 3, 6, and 12 hrs. (B) EoL-1 cells were cultured with different concentrations of Cos (1) (2, 3, 4, and 5 µg/mL) for 12 hrs. (A) FLT3 protein expression level was measured by Western blotting and GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels as the percentage of vehicle control (0.02% DMSO alone without the Cos (1) in culture medium). C and D: Total cell numbers measured by trypan blue exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group (p<0.05 and p<0.01).*

ffect of time periods and doses of costunolide (3) on FLT3 protein expression and total cell numbers in EoL-1 cell line

In this study, costunolide (3) was used as a model to determine its effect on time periods and doses in EoL-1 cells. Treatment of EoL-1 cells with costunolide (3) for 1, 3, and 6 hrs decreased FLT3 protein levels by 22.2, 25.4, and 47.8%, respectively, when compared to the vehicle control (Figure 7A). Cos (3) significantly decreased FLT3 protein levels at 6 hrs by 52.8, 53.8, 64.3, and 85.7% in response to 0.5, 1.0, 1.5, and 2.0 µg/mL, respectively (Figure 7B). Total cell numbers at 1, 3, and 6 hrs were decreased by 4.6, 20.0, and 39.3%, respectively (Figure 7C). Total cell numbers in response to 0.5, 1.0, 1.5, and 2.0 µg/mL were significantly decreased at 6 hrs by 18.8, 37.7, 55.0, and 76.3%, respectively when compared to vehicle control (Figure 7D).

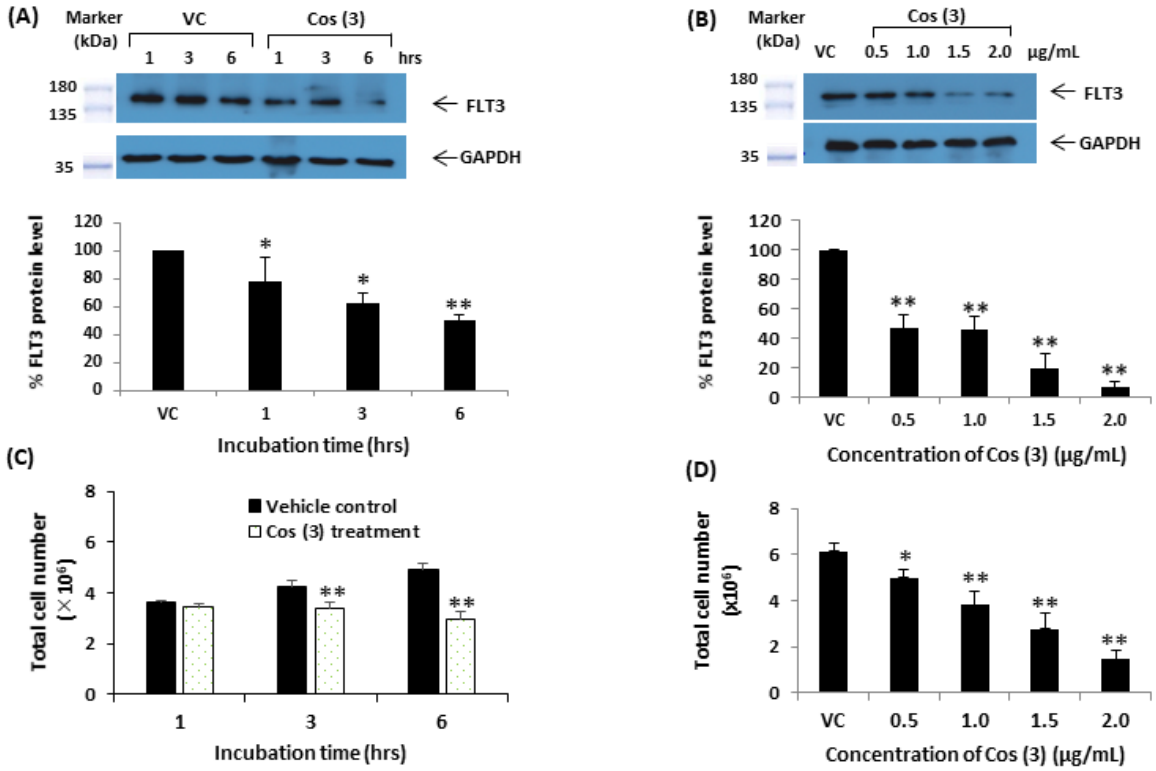

Figure 7. *Effect of various times and concentrations of standard costunolide (Cos (3)) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with Cos (3) at the concentration of 1.0 µg/mL for 1, 3, and 6 hrs. (B) EoL-1 cells were cultured with different concentrations of Cos (3) (0.5, 1.0, 1.5, and 2.0 µg/mL) for 6 hrs. FLT3 protein expression level was measured by Western blotting and GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels as the percentage of vehicle control (0.02% DMSO alone without the Cos (3) in culture medium). (C and D) The total cell numbers were measured by trypan blue exclusion method. Data are the mean ± SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group ($p<0.05$ and $p<0.01$).*

Discussion

Magnolia consists of about 240 species throughout the world[22] with 112 species distributed in tropical and subtropical parts of Asia. In Thailand, 25 species are found.[23] It has been used in a number of traditional medicine preparations in China and Japan. Magnolia is a rich source of several biological active compounds. It was reported to have at least 255 different ingredients, such as alkaloids, coumarins, flavonoids, lignans, neolignans, phenylpropanoids, and terpenoids.[24] Among these, several neolignan ingredients including magnolol, honokiol, 4-o-methylhonokiol, and obovatol have been the focus of studies examining various pharmacological effects of Magnolia. Champi Sirindhorn is a new species of *Magnolia spp*.[10] leaves of *M. sirindhorniae* consist of five sesquiterpene lactones such as costunolide, santamarine, reynosin, parthenolide, and lipiferolide.[10] This study is the first report to examine the effect of *M. sirindhorniae* extracts on FLT3 protein expression in leukemic cells. Nine crude fractional extracts (fraction No.1-9) from leaves, twigs, and stems were analyzed for their ability to inhibit leukemic cell growth under the FLT3 protein suppression which is involved in human leukemic cell proliferation and compared their activity to purified active compounds (costunolide (1) and parthenolide (2)). Furthermore, commercial costunolide (3) and parthenolide (4) were also determined to compare to those of purified compounds. EoL-1 cells were selected to furnish the analysis via the FLT3 overexpressing cell model due to high levels of FLT3 protein. Cytotoxic effects of crude fractional extracts (Hex, EtOAC, and MeOH) from leaves, twigs, and stems of *M. sirindhorniae* were assessed by MTT assay. Only crude fraction from Hex and EtOAC showed cytotoxic effects on EoL-1 cells. Furthermore, fraction No.7 (stems/ Hex) showed the strongest cytotoxicity in EoL-1 cells.

Effects of fraction No.1-9 (IC_{20} values) on FLT3 protein expression were examined by Western blotting. We found that extraction No.1 (leaves/ Hex) that has the highest

costunolide content in all fractions (7.0%) showed strong inhibitory effects on FLT3 protein expressions in EoL-1 cells. Concentration at IC_{20} value of fraction No.1 treatment was 20 µg/mL. The results showed the FLT3 suppression by a time- and dose- dependent manner after fraction No.1 treatment. In addition, extraction No.5 (twigs/ EtOAc) which has the highest parthenolide content in all fractions (39.6%) at concentration of 29 µg/mL showed the strongest inhibitory effects on FLT3 protein expressions in EoL-1 cells. The results also showed the FLT3 suppression by a time- and dose- dependent manner after fraction No.5 treatment. Moreover, 3.9 µg/mL of costunolide (1) which has the costunolide content (49.6%) decreased FLT3 protein expressions in EoL-1 by 66.0%. Furthermore, it decreased FLT3 protein by a time- and dose- dependent manner. In addition, 1.0 µg/mL of parthenolide (2) which has the parthenolide content (78.4%) decreased FLT3 protein expressions in EoL-1 by 78.3%. However, the effect of costunolide (1) to suppress FLT3 protein expression was not significantly difference when compare with parthenolide (2). Importantly, 0.9 µg/mL of the commercial costunolide (3) and 0.8 µg/mL of parthenolide (4) with the costunolide contents ≥97% and parthenolide ≥98%, respectively by HPLC decreased FLT3 protein expressions in EoL-1 cells by 91.5% and 90.4%, respectively. Taken together, it revealed that extraction No.1, 5, purified active compounds (costunolide (1), and parthenolide (2)) significantly inhibited cell proliferation without cell viability alteration via the suppression of FLT3 protein expression at non-cytotoxic doses in EoL-1 cells.

The effective compounds costunolide (1) and parthenolide (2) of *M. sirindhorniae* need further studies. The leaves extract of *M. sirindhorniae* has been reported the cytotoxicity.[10] Costunolide, parthenolide, and lipiferolide demonstrated more cytotoxicity against human breast cancer cell line (IC_{50} values of 2.17-5.24 µg/mL) than the standard drugs doxorubicin (IC_{50} value of 9.04 µg/mL) and tamoxifen (IC_{50} value of 9.61 µg/mL). In addition, the parthenolide (major compound) showed the highest activity against human epidermoid carcinoma (KB), human breast cancer (MCF7), and human small cell lung cancer cells (NCI-H187) with the IC_{50} values of 1.67, 2.17, and 0.97 µg/mL, respectively.[10] Furthermore, the cytotoxicity of Hex fraction from *M. siamensis* flower extract against EoL-1 cells has been reported with the IC_{50} value of 3.8 µg/mL[25] and showed 1.4- and 1.0- fold stronger than in costunolide (1) and parthenolide (2), respectively. However, costunolide (3) and parthenolide (4) showed 3.4- and 1.8- fold, respectively stronger than in Hex fraction from *M. siamensis* flower extract. Curcumin is the well-known FLT3 inhibitor. The cytotoxicity (IC_{50} value) of curcumin against EoL-1 cells was 6.7 µM (2.5 µg/mL)[26] which showed 2.2- and 1.6-fold stronger than costunolide (1) and parthenolide (2), respectively, whereas costunolide (3) and parthenolide (4) showed 2.3- and 1.2- fold, respectively stronger than that of curcumin. We found that, the inhibitory effect of costunolide (1) and parthenolide (2) at the concentration of 3.9 and 1.0 µg/mL, respectively (IC_{20} value) on the suppression of FLT3 protein expression in EoL-1 cells were 3- and 4-fold stronger than 1.7 µg/mL (IC_{20} value) from Hex fraction of *M. siamensis* flower extracts (which could suppress the FLT3 protein expression by 21.1%)[25] when compared at the IC_{20} values. Moreover, the IC_{20} values of costunolide (1) and parthenolide (2) could suppress FLT3 protein expression in EoL-1 cells were 5.5- and 6.5-fold stronger than 4.0 µM (1.5 µg/mL) (IC_{20} value) of curcumin.[26]

This study is the first report to show that *M. sirindhorniae* extracts demonstrate their inhibitory effects on FLT3 protein in leukemic cells. These results suggested that the active compounds costunolide (1) and parthenolide (2) of *M. sirindhorniae* inhibited FLT3 target protein related to leukemic cell proliferation, and thus can potentially be used for developing new anti-cancer drugs.

Conclusion

M. sirindhorniae is a source of costunolide (1) and parthenolide (2), the active compounds responsible for the inhibition of FLT3 protein expression in leukemic cells. The major sources of costunolide (1) and parthenolide (2) are from the leaves and twigs, respectively. This is the first report of the inhibitory effect of costunolide (1) and parthenolide (2) on FLT3 protein expressions in leukemic cells. The results were in parallel with the commercial costunolide (3) and parthenolide (4). In addition, costunolide (1) and parthenolide (2) showed the high activity for FLT3 protein suppression in EoL-1 cells. This natural product displays a potent inhibitory activity on leukemic cell proliferation and may have therapeutic potential as an anti-leukemic drug. Hence, we suggest that costunolide and parthenolide are promising compounds and can be successfully exploited in leukemic drug in future. Moreover, traditional herbal medicine industry is also the target for herbal drug product as an alternative for leukemia patients.

Conflicts of interests

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

[1]. Choi JH, Ha J, Park JH, Lee JY, Lee YS, Park HJ, et al. Costunolide triggers apoptosis in human leukemia U937 cells by depleting intracellular thiols. Cancer Sci 2002; 93(12): 1327-33.

[2]. Kang JS, Lee KH, Han MH, Lee H, Ahn JM, Han SB, et al. Antiinflammatory activity of methanol extract isolated from stem bark of Magnolia kobus. Phytother Res 2008; 22(7): 883-8.

[3]. Kong C-W, Tsai K, Chin J-H, Chan W-L, Hong C-Y. Magnolol attenuates peroxidative damage and improves survival of rats with sepsis. Shock (Augusta, Ga) 2000; 13(1): 24-8.

[4]. Weeks BS. Formulations of dietary supplements and herbal extracts for relaxation and anxiolytic action: Relarian. Med Sci Monit 2009; 15(11): RA256-RA62.

[5]. Xu Q, Yi L-T, Pan Y, Wang X, Li Y-C, Li J-M, et al. Antidepressant-like effects of the mixture of honokiol and magnolol from the barks of Magnolia officinalis in stressed rodents. Prog Neuro-Psychopharmacol Biol Psychiatry 2008; 32(3): 715-25.

[6]. Park E-J, Kim S-y, Zhao Y-Z, Sohn DH. Honokiol reduces oxidative stress, c-jun-NH2-termial kinase phosphorylation and protects against glycochenodeoxycholic acid-induced apoptosis in primary cultured rat hepatocytes. Planta Med 2006; 72(07): 661-4.

[7]. Kobayashi S, Kobayashi H, Matsuno H, Kimura I, Kimura M. Inhibitory effects of anti-rheumatic drugs containing magnosalin, a compound from 'Shin-i' (Flos magnoliae), on the proliferation of synovial cells in rheumatoid arthritis models. Immunopharmacology 1998; 39(2): 139-47.

[8]. Kobayashi S, Kimura I, Kimura M. Inhibitory effect of magnosalin derived from Flos magnoliae on tube formation of rat vascular endothelial cells during the angiogenic process. Biol Pharm Bull 1996; 19(10): 1304-6.

[9]. Kimura M, Kimura I, Guo X, Luo B, Kobayashi S. Combined effects of Japanese-Sino medicine 'Kakkon-to-ka-senkyu-shin'i'and its related combinations and component drugs on adjuvant-induced inflammation in mice. Phytother Res 1992; 6(4): 209-16.

[10]. Katekunlaphan T, Chalermglin R, Rukachaisirikul T, Chalermglin P. Sesquiterpene lactones from the leaves of Magnolia sirindhorniae. Biochem Syst Ecol 2014; 57: 152-4.

[11]. Rasul A, Bao R, Malhi M, Zhao B, Tsuji I, Li J, et al. Induction of apoptosis by costunolide in bladder cancer cells is mediated through ROS generation and mitochondrial dysfunction. Molecules 2013; 18(2): 1418-33.

[12]. Wang W, Adachi M, Kawamura R, Sakamoto H, Hayashi T, Ishida T, et al. Parthenolide-induced apoptosis in multiple myeloma cells involves reactive oxygen species generation and cell sensitivity depends on catalase activity. Apoptosis 2006; 11(12): 2225-35.

[13]. D'anneo A, Carlisi D, Lauricella M, Puleio R, Martinez R, Di Bella S, et al. Parthenolide generates reactive oxygen species and autophagy in MDA-MB231 cells. A soluble parthenolide analogue inhibits tumour growth and metastasis in a xenograft model of breast cancer. Cell Death Dis 2013; 4(10): e891.

[14]. Moon MK, Oh HM, Kwon B-M, Baek N-l, Kim S-H, Kim JS, et al. Farnesyl protein transferase and tumor cell growth inhibitory activities of lipiferolide isolated from Liriodendron tulipifera. Arch Pharmacal Res 2007; 30(3): 299-302.

[15]. Park HJ, Kwon SH, Han YN, Choi JW, Miyamoto K-i, Lee SH, et al. Apoptosis-inducing Costunolide and a novel acyclic monoterpene from the stem bark of Magnolia sieboldii. Arch Pharmacal Res 2001; 24(4): 342-8.

[16]. Choi S-H, Im E, Kang HK, Lee J-H, Kwak H-S, Bae Y-T, et al. Inhibitory effects of costunolide on the telomerase activity in human breast carcinoma cells. Cancer Lett 2005; 227(2): 153-62.

[17]. Rosnet O, Bühring H, Marchetto S, Rappold I, Lavagna C, Sainty D, et al. Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia 1996; 10(2): 238-48.

[18]. DaSilva N, Hu ZB, Ma W, Rosnet O, Birnbaum D, Drexler HG. Expression of the FLT3 gene in human leukemia-lymphoma cell lines. Leukemia 1994; 8(5): 885-8.

[19]. Zunino SJ, Ducore JM, Storms DH. Parthenolide induces significant apoptosis and production of reactive oxygen species in high-risk pre-B leukemia cells. Cancer Lett 2007; 254(1): 119-27.

[20]. Steele A, Jones D, Ganeshaguru K, Duke V, Yogashangary B, North J, et al. The sesquiterpene lactone parthenolide induces selective apoptosis of B-chronic lymphocytic leukemia cells in vitro. Leukemia 2006; 20(6): 1073-9.

[21]. Skalska J, Brookes PS, Nadtochiy SM, Hilchey SP, Jordan CT, Guzman ML, et al. Modulation of cell surface protein free thiols: A potential novel mechanism of action of the sesquiterpene lactone parthenolide. PLoS One 2009; 4(12): e8115.

[22]. Knox GW, Klingeman WE, Paret M, Fulcher A. Management of pests, plant diseases and abiotic disorders of Magnolia species in the Southeastern United States: A review. J Environ Hortic 2012; 30(4): 223-34.

[23]. Nooteboom HP, Chalermglin P. A new species of Magnolia (Magnoliaceae) from Thailand. Blumea 2000; 45(1): 245-7.

[24]. Tachikawa E, Takahashi M, Kashimoto T. Effects of extract and ingredients isolated from Magnolia obovata thunberg on catecholamine secretion from bovine adrenal chromaffin cells. Biochem Pharmacol 2000; 60(3): 433-40.

[25]. Sangkaruk R, Tima S, Rungrojsakul M, Chiampanichayakul S, Anuchapreeda S. Effects of Saraphi (Mammea siamensis) flower extracts on cell proliferation and Fms-like tyrosine kinase 3 expression in leukemic EoL-1 cell line. Bull Chiang Mai Assoc Med Sci 2016; 49(2): 286-93.

[26]. Tima S, Ichikawa H, Ampasavate C, Okonogi S, Anuchapreeda S. Inhibitory effect of turmeric curcuminoids on FLT3 expression and cell cycle arrest in the FLT3-overexpressing EoL-1 leukemic cell line. J Nat Prod 2014; 77(4): 948-54.