SUPPLEMENTARY MATERIALS

Costunolide and parthenolide from Champi Sirindhorn (*Magnolia sirindhorniae*) inhibit leukemic cell proliferation in K562 and Molt-4 cell lines

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Abstract  

The magnolia plant has been used in traditional medicine since ancient times. This study was designed to investigate the effects of active compounds from Thai Champi Sirindhorn (Magnolia sirindhorniae) on leukemic biomarker Wilms’ tumor 1 (WT1) protein expressions in K562 and Molt-4 cells. Costunolide (1) and parthenolide (2) were the major components used in this study, they were purified from ethyl acetate fractions. Costunolide (1) and parthenolide (2) had strong cytotoxic effects in K562 and Molt-4 cells measured with MTT assays. Their activities were compared to standard commercial costunolide (3) and parthenolide (4). Costunolide (1) and parthenolide (2) decreased WT1 protein levels and total cell numbers in K562 and Molt-4 cells. Both purified costunolide (1) and standard commercial costunolide (3) decreased WT1 protein levels in a time- and dose-dependent manner. Therefore, the active compounds from M. sirindhorniae were identified as promising sources for bioactive compounds for further applications in traditional medicine.

**Keywords:** Magnolia sirindhorniae; Champi Sirindhorn; costunolide; parthenolide; Wilms’ tumor 1; leukemia

**Abbreviations:** WT1, Wilms’ tumor 1; GAPDH, Glyceraldehyde phosphate dehydrogenase; Hex, n-Hexane; EtOAc, Ethyl acetate; MeOH, Methanol
1. Experimental section

1.1 Chemical materials

RPMI 1640 (Invitrogen™, CA, USA), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, 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-WT1 antibody (C-19; SC-192) and rabbit polyclonal anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (CA, USA), and 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), and 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).

1.2 Plant materials

The leaves, twigs, and stems from the *M. sirindhorniae* plant (over than 3 years old) 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 in the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The herbarium specimen has been studied and annotated with traditional methods of herbarium taxonomy.

1.3 Extraction and isolation of *M. sirindhorniae*

Nine crude fractional extracts were prepared utilizing different solvents for the various parts of the plant. Specifically, fractions No. 1 – 3 were extracted from the leaves of *M. sirindhorniae*, fractions No. 4 – 6 from twigs, and fractions No. 7 – 9 from the stems. The fractions were extracted by using *n*-hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH), respectively. In addition, the active fractions were purified with column and thin layer chromatography (TLC) until the purified compounds 1 and 2 were obtained. The two compounds (1 and 2) were identified as costunolide (1) and parthenolide (2) (Tuman et al. 2005; Katekunlaphan et al. 2014; Venditti et al. 2016). 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 of these compounds (Figures s1 – s18). The structures for costunolide (1) and parthenolide (2) are shown in Figures 1(a) and 1(b), respectively. Costunolide (1) and parthenolide (2) were further investigated for their effects on WT1 protein expressions.

1.4 Cells and cell culture conditions

The K562 and Molt-4 cell lines, a model of WT1-overexpressing leukemic cells, were cultured in a RPMI-1640 medium supplemented with a 10% fetal bovine serum, 1 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. They were then incubated under the following conditions, a 95% relative humidity with 5% CO$_2$ at 37 °C.

1.5 MTT cytotoxicity assay

The cytotoxicity of the crude fractional extracts (fractions No. 1 – 9), costunolide (1), and parthenolide (2) were evaluated using MTT assays. Briefly, the K562 and Molt-4 cells (1.0 $\times$ 10$^4$ cells/well) were cultured in 96 well plates containing 100 µL medium prior to treatment for 24 h. 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 h. The MTT dye solution was added (15 µL/100 µL medium) and the plates were incubated at 37 °C for 4 h in a humidified 5% CO$_2$ conditions. Afterwards, 200 µL of DMSO was 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, representing a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated with the following formula:

$$\% \text{ Cell survival} = \left( \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \right) \times 100$$

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. This was completed in three independent experiments. The 50% inhibitory concentrations (IC$_{50}$) of the active substances were determined to be the
lowest concentrations which reduced cell growth by 50% in those treated compared to untreated cultures or vehicle control cultures (0.2% DMSO in culture medium). The IC₅₀ values are representative of the activities and are shown as a mean ± standard deviation (SD).

1.6 Trypan blue exclusion test
Cell proliferation was measured by using the Trypan blue exclusion method. Cells were treated with various concentrations of crude fractional extracts (fractions 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 together and counted using a light microscope. All experiments were performed in triplicate.

1.7 Protein extraction and Western blotting
K562 and Molt-4 cells were treated with crude fractional extracts (fractions No. 1 – 9), the purified active compounds (costunolide (1) and parthenolide (2)), and commercial compounds (costunolide (3) and parthenolide (4)) for 48 h, after which the cells were collected, washed twice with a cold PBS, and lysed with a 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 lysates (30 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-WT1 at a dilution ratio of 1:1,000. Rabbit anti-GAPDH at a dilution ratio of 1:1,000 was used for the protein loading control. The reaction was followed by HRP-conjugated anti-rabbit IgG at 1:20,000 dilution. Proteins were shown using an enhanced chemiluminescence detection kit, and Luminata™ Forte Western HRP Substrate. The chemiluminescent signals were detected using a Typhoon TRIO Imager. Densitometry was performed using the Alpha Innotech software. The band density of the loading control was used to normalize the band densities of the proteins of interest to obtain the relative normalized expression level as compared to the exposed control.

1.8 Statistical analysis
All the data was expressed as a mean ± deviation (SD) from the triplicate samples of three independent experiments. The statistical difference between the mean was determined using Student’s t-test. 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).
2. Supporting information available: Percentage of crude fractional extracts No. 1 – 9 is shown in Table s1. IC₅₀ values after crude fractional extracts No. 1 – 9, purified costunoline (1), purified parthenolide (2), commercial costunolide (3), and commercial parthenolide (4) treatments in K562 and Molt-4 cell lines are shown in Tables s2 and s3. 1D and 2D NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. ES-MS and ES-TOFMS spectra were measured with a Finnigan LC-Q and a Bruker microTOF mass spectrometer. IR spectra were obtained using a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. Spectra of compounds 1 and 2 are shown in Figures s1 – s18. Costunolide and parthenolide contents were determined by HPLC (Figures s19 – s21, Tables s4 – s6). Supporting results are shown in Figures s22 – s26.

Figure s1. ¹H NMR spectrum of costunolide (1) in CDCl₃
Figure s2. ¹³C NMR spectrum of costunolide (1) in CDCl₃
Figure s3. COSY spectrum of costunolide (1) in CDCl₃
Figure s4. HMBC spectrum of costunolide (1) in CDCl₃
Figure s5. Expansion of HMBC spectrum of costunolide (1) in CDCl₃
Figure s6. Expansion of HMBC spectrum of costunolide (1) in CDCl₃
Figure s7. Expansion of HMBC spectrum of costunolide (1) in CDCl₃
Figure s8. HSQC spectrum of costunolide (1) in CDCl₃
Figure s9. MS spectrum of costunolide (1)
Figure s10. IR spectrum of costunolide (1)
Figure s11. ¹H NMR spectrum of parthenolide (2) in CDCl₃
Figure s12. ¹³C NMR spectrum of parthenolide (2) in CDCl₃
Figure s13. COSY NMR spectrum of parthenolide (2) in CDCl₃
Figure s14. HMBC spectrum of parthenolide (2) in CDCl₃
Figure s15. Expansion of HMBC spectrum of parthenolide (2) in CDCl₃
Figure s16. HSQC spectrum of parthenolide (2) in CDCl₃
Figure s17. MS spectrum of parthenolide (2)
Figure s18. IR spectrum of parthenolide (2)
Figure s19. Quantitative analysis of fractions No. (a) 1, (b) 2, (c) 3, and (d) standard costunolide (3) by HPLC.
Figure s20. Quantitative analysis of fractions No. (a) 5, (b) 7, (c) purified costunolide (1), and (d) standard costunolide (3) by HPLC.
**Figure s21.** Quantitative analysis of fractions No. (a) 5, (b) 7, (c) purified parthenolide (2), and (d) standard parthenolide (4) by HPLC.

**Figure s22.** The effects of fractions No.1 – 9 (F1 – 9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on WT1 protein expression in K562 cells by Western blotting.

**Figure s23.** The effects of fractions No.1 – 9 (F1 – F9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on WT1 protein expression in Molt-4 cells by Western blotting.

**Figure s24.** Effects of various times and concentrations of the fraction No.1 (F1) on WT1 protein expression in K562 and Molt-4 cells by Western blot analysis.

**Figure s25.** Effects of various times and concentrations for costunolide (1) (Cos (1)) on WT1 protein expression in K562 and Molt-4 cells by Western blot analysis.

**Figure s26.** Effects of various times and concentrations for costunolide (3) (Cos (3)) on WT1 protein expression in K562 and Molt-4 cells by Western blot analysis.

**Table s1.** Percentage of crude fractional extracts No. 1 – 9.

**Table s2.** IC$_{50}$ values after crude fractional extract No. 1 – 9 treatments in K562 and Molt-4 cell lines.

**Table s3.** IC$_{50}$ values after purified costunoline (1), purified parthenolide (2), commercial costunolide (3), and commercial parthenolide (4) treatments in K562 and Molt-4 cell lines.

**Table s4.** Costunolide content of fractions No. 1, 2, and 3 compared to standard commercial costunolide (3) that showed the costunolide ≥ 97% by HPLC.

**Table s5.** Costunolide content of fractions No. 5, 7, and purified costunolide (1) compared to standard commercial costunolide (3) that showed the costunolide ≥ 97% by HPLC.

**Table s6.** Parthenolide content of fractions No. 5, 7, and purified parthenolide (2) compared to standard commercial parthenolide (4) that showed the parthenolide ≥ 98% by HPLC.
Figure s1. $^1$H NMR spectrum of costunolide (1) in CDCl$_3$

Figure s2. $^{13}$C NMR spectrum of costunolide (1) in CDCl$_3$
**Figure s3.** COSY spectrum of costunolide (1) in CDCl₃

**Figure s4.** HMBC spectrum of costunolide (1) in CDCl₃
**Figure s5.** Expansion of HMBC spectrum of costunolide (1) in CDCl₃

**Figure s6.** Expansion of HMBC spectrum of costunolide (1) in CDCl₃
Figure s7. Expansion of HMBC spectrum of costunolide (1) in CDCl$_3$.

Figure s8. HSQC spectrum of costunolide (1) in CDCl$_3$. 
**Figure s9.** MS spectrum of costunolide (1)

**Figure s10.** IR spectrum of costunolide (1)
Figure s11. $^1$H NMR spectrum of parthenolide (2) in CDCl$_3$. 

[Image of NMR spectrum]
Figure s12. $^{13}$C NMR spectrum of parthenolide (2) in CDCl$_3$.

Figure s13. COSY NMR spectrum of parthenolide (2) in CDCl$_3$. 
Figure s14. HMBC spectrum of parthenolide (2) in CDCl$_3$

Figure s15. Expansion of HMBC spectrum of parthenolide (2) in CDCl$_3$
Figure s16. HSQC spectrum of parthenolide (2) in CDCl$_3$
Figure s17. MS spectrum of parthenolide (2)

Figure s18. IR spectrum of parthenolide (2)
| Crude fraction No. | Plant part | Solvent for extraction | Weight (g) | % yield |
|-------------------|------------|------------------------|------------|---------|
|                   |            |                        | Dry plants | Extracts |        |
| 1                 | Leave      | n-Hexane               | 310.2      | 9.42    | 3.03   |
| 2                 | Leave      | Ethyl acetate          | 309.5      | 15.19   | 4.89   |
| 3                 | Leave      | Methanol               | 16.84      | 11.76   | 3.76   |
| 4                 | Twig       | n-Hexane               | 8.61       | 6.53    | 2.11   |
| 5                 | Twig       | Ethyl acetate          | 312.3      | 16.84   | 5.39   |
Table s2. IC₅₀ values after crude fractional extract No. 1 – 9 treatments in K562 and Molt-4 cell lines.

| Crude fraction No. | Plant part | Solvent for extraction | Weight (mg) | IC₅₀ (µg/mL) |
|-------------------|------------|------------------------|-------------|--------------|
|                   |            |                        |             | K562 | Molt-4 |
| 1                  | Leave      | n-Hexane               | 100.4       | >100 | 81.5  |
| 2                  | Leave      | Ethyl acetate          | 100.7       | >100 | 54.5  |
| 3                  | Leave      | Methanol               | 102.9       | >100 | >100  |
| 4                  | Twig       | n-Hexane               | 119.8       | >100 | 76.1  |
| 5                  | Twig       | Ethyl acetate          | 104.3       | 80.4 | 73.9  |
| 6                  | Twig       | Methanol               | 110.9       | >100 | >100  |
| 7                  | Stem       | n-Hexane               | 109.0       | 83.3 | 63.0  |
| 8                  | Stem       | Ethyl acetate          | 104.1       | >100 | 93.5  |
| 9                  | Stem       | Methanol               | 108.3       | >100 | >100  |

Table s3. IC₅₀ values after purified costunoline (1), purified parthenolide (2), commercial costunolide (3), and commercial parthenolide (4) treatments in K562 and Molt-4 cell lines.

| Compounds             | IC₅₀ (µg/mL) |
|-----------------------|--------------|
|                       | K562 | Molt-4 |
| Purified costunolide (1) | 45.8 | 21.9  |
| Purified parthenolide (2) | 21.9 | 10.9  |
| Commercial costunolide (3) | 6.2  | 11.6  |
| Commercial parthenolide (4) | 5.6  | 1.6   |
Chromatographic analysis

The fractions for No. 1, 2, 3, and standard costunolide (3) were weighed and dissolved in methanol at the final concentration of 0.0808 mg/mL. Then the mixture was filtered through a 0.45 µm syringe filter and analyzed by HPLC (Agilent 1100 DAD). The HPLC system consisted of a C-18 reversed-phase column (Verticep usp 150L 4.6 mm). The costunolide was eluted with a mobile phase consisting of methanol and distilled deionized water (60:40) in 8.5 min. The standard costunolide (3) was injected five times and two times for those samples. The average for the results of each product is given in Table s4.

Table s4. Costunolide content of fractions 1, 2, and 3 compared to standard costunolide (3) that showed the costunolide ≥ 97% by HPLC.

| Fraction No. | Costunolide content (%) |
|--------------|-------------------------|
| 1            | 7.0                     |
| 2            | 1.1                     |
| 3            | 0                       |

a. Fraction No. 1

![Chromatogram](image)
b. Fraction No. 2

![Graph showing fraction No. 2](image)

Costunolide ≥ 97.0%

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c. Fraction No. 3

![Graph showing fraction No. 3](image)

Costunolide 0.00%

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d. Standard costunolide (3)

![Graph showing standard costunolide](image)

Costunolide ≥ 97.0%

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**Figure s19.** Quantitative analysis of fractions No. (a) 1, (b) 2, (c) 3, and (d) standard costunolide (3) by HPLC.
The fractions for No. 5, 7, purified costunolide (1), and standard costunolide (3) were weighed and dissolved in methanol at the final concentration of 0.0808 mg/mL. After that, the mixture was filtered through a 0.45 µm syringe filter and analyzed by HPLC (Agilent 1100 DAD). The HPLC system consisted of a C-18 reversed-phase column (Verticep usp 300L 4.0 mm). The costunolide and was eluted with a mobile phase consisting of methanol and distilled deionized water (60:40) in 22 min. The standard costunolide (3) was injected five times and two times for those samples. The average of the results for each product is given in Table s5.

Table s5. Costunolide content of fractions No. 5, 7, and purified costunolide (1) compared to standard commercial costunolide (3) that showed the costunolide ≥ 97% by HPLC.

| Fraction No. | Costunolide content (%) |
|--------------|-------------------------|
| 5            | 0                       |
| 7            | 0                       |
| Purified costunolide | 49.6                   |

a. Fraction No. 5

Costunolide 0.00%
b. Fraction No. 7

Figure s20. Quantitative analysis of fractions No. (a) 5, (b) 7, (c) purified costunolide (1), and (d) standard costunolide (3) by HPLC.
The fractions No. 5, 7, purified parthenolide (2), and standard parthenolide (4) were weighed and dissolved in methanol at the final concentration of 0.0808 mg/mL. Then the mixture was filtered through a 0.45 µm syringe filter and analyzed by HPLC (Agilent 1100 DAD). The HPLC system consisted of a C-18 reversed-phase column (Vertisep usp 250L 4.6 mm). The parthenolide was eluted with a mobile phase consisting of methanol and distilled deionized water (60:40) in 8.9 min. The standard parthenolide (4) was injected five times and two times for those samples. The average of the results for each product is given in Table s6.

**Table s6.** Parthenolide content of fractions No. 5, 7, and purified parthenolide (2) compared to standard commercial parthenolide (4) that showed the parthenolide ≥ 98% by HPLC.

| Fraction No. | Parthenolide content (%) |
|--------------|--------------------------|
| 5            | 39.6                     |
| 7            | 20.9                     |
| Purified parthenolide (2) | 78.4                     |

a. Fraction No. 5

![HPLC chromatogram](image)

**Parthenolide 39.6%**
b. Fraction No. 7

Parthenolide 20.9%

Figure s21. Quantitative analysis of fractions No. (a) 5, (b) 7, (c) purified parthenolide (2), and (d) standard parthenolide (4) by HPLC.
Figure s22. The effects of fractions No.1 – 9 (F1 – 9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on WT1 protein expression in K562 cells by Western blotting. (a, b) The levels of WT1 protein expression and total cell numbers after treatment with F1 – 9 for 48 h. (c, d) The levels of WT1 protein expression and total cell numbers after treatment with costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) for 48 h. GAPDH was used as a loading control. (VC = vehicle control, F1 – 9 = fractions 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 (*) and (**) denote a significant difference from the control groups ($P < 0.05$ and $P < 0.01$).
Figure s23. The effects of fractions No.1 – 9 (F1 – 9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on WT1 protein expression in Molt-4 cells by Western blotting. (a, b) The levels of WT1 protein expression and total cell numbers after treatment with fractions No. 1 – 9 for 48 h. (c, d) The levels of WT1 protein expression and total cell numbers after treatment with costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4), respectively for 48 h. GAPDH was used as a loading control. (VC = vehicle control, F1 – 9 = fractions 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 (*) and (**) denote a significant difference from the control groups ($P < 0.05$ and $P < 0.01$).
**Figure s24.** Effects of various times and concentrations of the fraction No.1 (F1) on WT1 protein expressions in K562 and Molt-4 cells by Western blot analysis. (a, e) K562 and Molt-4 cells were cultured with F1 at the concentrations of 50 and 54 µg/mL, respectively for 6, 12, 24, and 48 h. (b, f) K562 cells were cultured with different concentrations of F1 (40, 45, 50, and 55 µg/mL) for 12 h while Molt-4 cells were cultured with F1 (45, 50, 55, and 60 µg/mL) for 48 h. WT1 protein expression levels were measured with the use of Western blotting. GAPDH was used as loading control. (a, b, e, f) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle controls (0.02% DMSO alone without the F1 in culture medium). (c, d, g, h) The total cell numbers were measured with Trypan blue exclusion methods. Data used is the mean ± SD from three independent experiments. Asterisk (*) and (**) denote a significant difference from the control groups ($P < 0.05$ and $P < 0.01$).
Figure s25. Effects of various times and concentrations for costunolide (1) (Cos (1)) on WT1 protein expression in K562 and Molt-4 cells by using Western blot analysis. (a and e) K562 and Molt-4 cells were cultured with Cos (1) at the concentrations of 10 and 12 µg/mL, respectively for 6, 12, 24, and 48 h. (b and f) K562 cells were cultured with different concentrations of Cos (1) (6, 8, 10, and 12 µg/mL) for 48 h while Molt-4 cells were cultured with Cos (1) (8, 10, 12, and 14 µg/mL) for 48 h. WT1 protein expression levels were measured with the use of Western blotting and GAPDH was used as loading control. (a, b, e, f) Densitometry was used to quantitate the protein levels as the percentage of vehicle controls (0.02% DMSO alone without the Cos (1) in culture medium). (c, d, g, h) The total cell numbers were measured using the Trypan blue exclusion method. Data used is the mean ± SD from three independent experiments. Asterisk (*) and (**) denotes a significant difference from the control groups (P < 0.05 and P < 0.01).
**Figure s26.** Effects of various times and concentrations of costunolide (3) (Cos (3)) on WT1 protein expressions in K562 and Molt-4 cells by Western blot analysis. (a, e) K562 and Molt-4 cells were cultured with Cos (3) at the concentrations of 5 and 6 µg/mL, respectively for 6, 12, 24, and 48 h. (b, f) K562 cells were cultured with different concentrations of Cos (3) (3, 4, 5, and 6 µg/mL) for 48 h while Molt-4 cells were cultured with Cos (3) (4, 5, 6, and 7 µg/mL) for 48 h. WT1 protein expression levels were measured with the use of Western blotting and GAPDH was used as a loading control. (a, b, e, f) Densitometry was used to quantitate the protein levels as the percentage of vehicle controls (0.02% DMSO alone without the Cos (3) in culture medium). (c, d, g, h) The total cell numbers were measured using the Trypan blue exclusion method. Data used is the mean ± SD from three independent experiments. Asterisk (*) and (***) denote a significant difference from the control groups ($P < 0.05$ and $P < 0.01$).

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