

***In vitro* anti-leukemia activity of *Gynostemma pentaphyllum* (Thunb.) on HL-60 leukemia cell line**

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ABSTRACT

Objective: This study aimed to investigate the *in vitro* anti-leukemia activities of the active extract derived from the leaves of *Gynostemma pentaphyllum*, focusing on its cytotoxic properties, cell cycle regulation, and impact on key protein markers involved in leukemia cell proliferation.

Materials and methods: *G. pentaphyllum* fractional extracts were prepared through sequential extraction using hexane, ethyl acetate, and ethanol solvents through maceration. The cytotoxicity of these extracts was determined against HL-60 leukemia cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The most cytotoxic extract was further fractionated through column chromatography. The anti-leukemia activities of the active extract and its active fraction were evaluated through cell cycle analysis using flow cytometry. Western blotting was performed to assess the expression levels of key proliferation markers, Feline McDonough Sarcoma (FMS)-like tyrosine kinase 3 (FLT3) and Wilms' tumor 1 (WT1) proteins.

Results: The intermediate-polar ethyl acetate fractional extract (F-EtOAc) was found to be highly cytotoxic against HL-60 leukemia cells, with an IC₅₀ value of 43.41±2.75 µg/mL. Among the isolated fractions of F-EtOAc, fraction F10 was identified as the most active. It was shown that F-EtOAc and F10 significantly induced S-phase cell cycle arrest and reduced the expression levels of FLT3 and WT1 proteins in HL-60 cells in a dose-dependent manner (*p* < 0.001), suggesting that anti-proliferative molecular pathways contribute to their anti-leukemia potential.

Conclusion: This study's findings indicate that the ethyl acetate extract of *G. pentaphyllum* contains bioactive compounds with anti-leukemia potential, supporting further investigation into its underlying molecular mechanisms.

Introduction

Leukemias are hematopoietic malignancies characterized by uncontrolled growth and clonal expansion of immature lymphoid or myeloid precursor cells. These abnormalities disrupt normal bone marrow function and hinder cellular differentiation.¹ Acute myeloid leukemia (AML) is the most prevalent form of acute leukemia in adults, accounts for approximately 33% of all blood-related cancers.² The disease arises from impaired differentiation of myeloid, monocytic, erythroid or megakaryocytic lineage precursors.^{1,2} Acute promyelocytic leukemia (APL) was defined as one of the most aggressive form of AML.³ Severe bleeding tendencies, chloroma, fever, anemia, fatigue, and

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frequent infections are the symptoms or complications mostly found in APL patients. FLT3, frequently mutated and overexpressed in AML, plays a critical role in disease progression by driving cell proliferation and survival, making it an attractive therapeutic target.⁴ Similarly, WT1, a transcriptional regulator expressed at elevated levels in leukemic cells compared to normal hematopoietic cells, is involved in leukemia progression and serves as a marker of cell proliferation.⁵ Although cytotoxic chemotherapy induces complete remission in 50-70% of younger AML patients, disease relapse is common.⁶ Moreover, the high toxicity of standard chemotherapy is generally unsuitable for elderly patients who represents the majority of AML cases. The genetic complexity of AML plays a significant role in chemotherapy resistance^{1,4} and this drives ongoing research to explore alternative therapeutic strategies.

Medicinal plant extracts have long been recognized as sources of pharmacologically active compounds for treating wide range of diseases. Plant-derived extracts, which often contain multiple active components, can interact with various targets within tumor cells and their microenvironment, affecting critical biochemical and molecular pathways involved in disease progression.⁷ In Thailand, renowned for its rich biodiversity and geographical location, approximately 9,097 plant species have been documented.⁸ Among these, *Gynostemma pentaphyllum* (Thunb.), commonly known as Jiaogulan, has been widely used in Asian traditional medicine since the nineteenth century for its diverse therapeutic properties, including the ability to regulate inflammation, blood sugar and lipid levels.⁹⁻¹¹ A member of the *Gynostemma* genus, *G. pentaphyllum* is native to South China, Japan, Korea, India, Nepal, and other regions due to its strong environmental adaptability.¹² The plant is a perennial vine characterized by creeping rhizomes and climbing stem with leaves.⁹⁻¹³ Compared to ginseng, *G. pentaphyllum* has a shorter growth cycle, faster growth rate, and higher concentrations of bioactive flavonoids and terpenoids.¹³ These compounds primarily concentrated in the leaves,¹³ and exhibit potent antioxidant, anti-inflammatory, and anti-tumor activities against various cancers, including oral, breast, lung, and liver cancers, as demonstrated in both *in vitro* and *in vivo* studies.^{9,11} Notably, the saponin-rich extract of *G. pentaphyllum*, abundant in gypenosides compound, has shown significant anti-leukemia activities against HL-60 cells *in vitro* and *in vivo* xenograft mouse models, as reported by Lin et al.¹⁴

This study aims to evaluate the cytotoxic activity of three fractional extracts of *G. pentaphyllum* against HL-60 leukemia cell line. The most potent extract will undergo further fractionated using column chromatography, and the anti-leukemia properties will be investigated by evaluating cell cycle progression and the effect on key protein markers involved in leukemia cell proliferation.

Material and Methods

Materials

Analytical-grade solvents, including n-hexane, ethyl acetate, ethanol, and dimethyl sulfoxide (DMSO) were

purchased from Labscan (Dublin, Ireland). The biochemical assay reagents, such as MTT and Folin-Ciocalteu reagents, were procured from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI)-1640 culture medium, penicillin-streptomycin, L-glutamine, and 0.4% trypan blue solution, were purchased from Gibco, Thermo Fisher Scientific, Inc. Fetal bovine serum (FBS) was obtained from Capricorn Scientific (Hesse, Germany). Chromatographic analysis used Silica gel 60 (40-63 µm) for column chromatography and Silica gel 60 F254 plates for thin-layer chromatography (TLC), both manufactured by Merck (Darmstadt, Germany).

Preparation and fractionation of *G. pentaphyllum* active extract

Gynostemma pentaphyllum plant was taxonomically identified by a botanist at the Chiang Mai University Herbarium, Faculty of Pharmacy, Thailand, and a voucher specimen (reference no. 0023392) was deposited for reference. Fresh air-dried leaves were ground into powder and extracted sequentially with n-hexane, ethyl acetate and ethanol. Each solvent extraction was conducted over 72 hrs, divided into three cycles of 24 hrs. Then, the extracts were pooled, filtered, concentrated using a rotary evaporator, and dried in a hot air oven at 40 °C. The dried extracts were weighed and stored at -20 °C for further experiments. Cytotoxicity of three fractional extracts was evaluated against HL-60 leukemia cell model, and the most active extract was selected for further fractionation using normal silica column chromatography (NSCC) with a hexane/ethyl acetate solvent gradient. Collected fractions were monitored using TLC and tested for cytotoxicity. The most active fraction was selected for further study. The extracts and fractions were dissolved in DMSO to obtain the working concentration of 12.5 mg/mL and stored at -20 °C for further use.

Cell line and culture conditions

The acute promyelocytic leukemia cell model (HL-60) was cultured in RPMI-1640 medium containing 1 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, supplemented with 10% FBS. The cell line was maintained at 37 °C in a humidified incubator with 5% CO₂.

Cytotoxicity evaluation by MTT assay

The cytotoxicity of the extracts and isolated fractions from the active extract was assessed using the MTT assay. HL-60 cells at 1.0×10⁴ cells/well were treated with sample concentrations ranging from 0 to 100 µg/mL for 48 hrs. A vehicle control (VC) containing 0.8% DMSO was included as a control. Dose-response curves were plotted based on the mean percentage of cell survival for each concentration from triplicate experiments. The percentage (%) of cell viability was calculated using the following formula:

$$\% \text{Cell viability} = \left[\frac{OD_s}{OD_c} \right] \times 100$$

where OD_s is the mean absorbance of cells treated

with the extract and OD_c is the mean absorbance of cells treated with the VC. The 50% and 20% inhibitory concentrations (IC_{50} and IC_{20}) were determined as previously described.¹⁵

Cell cycle analysis

Cell cycle progression was assessed using propidium iodide (PI) staining to determine the nuclear DNA content after treatment. The leukemic cell model was treated with non-cytotoxic concentrations (IC_{10} , IC_{15} , IC_{20}) of the active extract and its active fraction for 48 hrs. VC was included for comparison. Cells were fixed in 70% ethanol, stained with a PI solution (0.02 mg/mL PI, 2 mM EDTA, 0.1% Triton X-100, 8 µg/mL RNase A, in PBS pH 7.4), and analyzed using a DxFLX Flow Cytometer (Beckman Coulter) and FlowJo software V10 (Ashland, OR, USA).

Western blot analysis

For protein expression analysis, leukemia cells were treated with non-cytotoxic concentrations (IC_{10} , IC_{15} , IC_{20}) of the active extract and its active fraction for 48 hrs, with VC included as control. Cells were lysed using RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were measured with the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, IL, USA). Lysates were separated by SDS-PAGE (7.5% or 12% gels) depending on target protein size, transferred to PVDF membrane, and probed with antibodies against FLT3 (Abcam; ab245116) and WT1 (Cell signaling Technology; 83535). HRP-conjugated Anti-rabbit IgG (Abcam; ab205718) was used as the secondary antibody. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Abcam; ab9485) was used as a loading control. Bands were visualized using chemiluminescence and quantified with Quantity One 1-D software (Bio-Rad, USA).

Trypan blue exclusion test

This test aimed to assess the cytotoxicity of the test samples. After leukemia cells were treated with non-cytotoxic concentrations (IC_{10} , IC_{15} , IC_{20}) of the active extract and its active fraction for 48 hrs, cells were washed with PBS, pH 7.4, and resuspended. The cell suspension

was then diluted with PBS, pH 7.4 and mixed with 0.2% trypan blue solution. Viable and non-viable cells were counted with a hemocytometer under a light microscope and the total cell number of each was calculated.

Statistical analysis

Data were analyzed using SPSS software V22 (SPSS Inc., USA). Statistical significance was determined using Student's t-tests for two-group comparisons and one-way ANOVA for comparisons of three or more groups. A $p < 0.05$ was considered statistically significant (* $p < 0.05$).

Results

Cytotoxic effects of *G. pentaphyllum* fractional extracts on leukemic cell viability

The maceration of *G. pentaphyllum* dried leaf samples yielded three distinct crude fractional extracts: F-Hex (non-polar hexane extract), F-EtOAc (intermediate polar ethyl acetate extract), and F-EtOH (polar ethanol extract). Each extract appeared as a pasty greenish semi-solid mass, with F-EtOH yielding the highest amount ($2.70 \pm 0.06\%$), followed by F-EtOAc ($1.60 \pm 0.35\%$) and F-Hex ($0.58 \pm 0.04\%$). The cytotoxic effects of extracts on HL-60 cells were examined using the MTT assay, which measure cell viability (%). The half-maximal inhibitory concentration (IC_{50}) was calculated to determine the potency of each extract in reducing cell viability. The results showed differences in cytotoxic activity among the extracts against HL-60 cells (Figure 1). Among the three extracts, F-EtOAc exhibited the highest cytotoxic activity, with an IC_{50} value of 43.41 ± 2.75 µg/mL, whereas F-Hex and F-EtOH showed IC_{50} values exceeding 100 µg/mL, indicating lower cytotoxicity. The National Cancer Institute (NCI) categorizes crude extracts based on their cytotoxicity levels; extracts with $IC_{50} > 50$ µg/mL are classified as weakly cytotoxic, those with IC_{50} between 20-50 µg/mL as moderately cytotoxic, and those with $IC_{50} < 10$ µg/mL as highly cytotoxic.¹⁶ In addition, treatment with F-EtOAc at 50 and 100 µg/mL significantly increased the cytotoxicity to the HL-60 cells when compared with F-Hex and F-EtOH ($p < 0.001$). Based on this classification, F-EtOAc demonstrated moderate cytotoxicity in HL-60 cells.

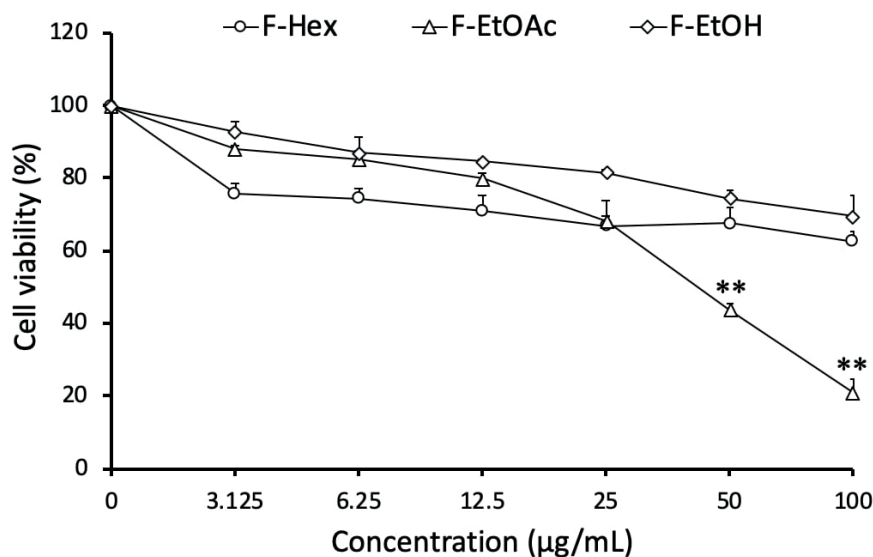


Figure 1. Cytotoxicity of *G. pentaphyllum* hexane fractional extract (F-Hex), ethyl acetate fractional extract (F-EtOAc), and ethanol fractional extract (F-EtOH) on HL-60 leukemia cell line after 48 hrs of treatment. Data are presented as mean±SD from three independent experiments, each performed in triplicate. Asterisks (**) indicate significant differences of % cell viability of HL-60 after treatment with F-EtOAc compared to those of F-Hex and F-EtOH; (** $p < 0.001$, one-way ANOVA).

Cytotoxic effects of F-EtOAc isolated fractions on HL-60 cell viability

The F-EtOAc extract was fractionated using NSCC with Silica gel 60 N (neutral) as a stationary phase and a gradient solvent system of hexane and ethyl acetate. Samples were collected and initially screened by TLC. Fractions with similar TLC profiles were pooled into ten fractions. Subsequently, the dried fractions were used to determine their cytotoxic effects against HL-60 leukemic

cells using the MTT assay. Among ten fractions, fraction F10 showed the most potent cytotoxic activity after 48 hrs, with an IC_{50} value of 27.40 ± 0.20 µg/mL. This fraction had a dry weigh of 96 mg per 4 g of F-EtOAc (w/w), yielding 2.4%. The other fractions showed minimal cytotoxic effects (Table 1). These findings indicate that the F-EtOAc extract and fraction F10 possess strong anti-proliferative potential against HL-60 cells and were examined further.

Table 1. Cytotoxicity of F-EtOAc isolated fractions on HL-60 leukemic cell line performed by MTT assay.

F-EtOAc fractions	IC_{50} value (µg/mL)
F1	>100
F2	>100
F3	>100
F4	>100
F5	>100
F6	>100
F7	85.26 ± 4.31
F8	77.32 ± 2.71
F9	47.76 ± 0.98
F10	27.40 ± 0.23

Note: IC_{50} value are presented as mean±SD from three independent experiments, each performed in triplicate.

Effect of F-EtOAc and F10 on HL-60 cell cycle progression

HL-60 cells were treated with three non-cytotoxic concentrations (IC_{10} , IC_{15} , IC_{20}) of F-EtOAc and F10 for 48 hrs, followed by PI staining to analyze DNA content. The results presented in Figure 2 showed that treatment with both F-EtOAc and F10 caused a significant dose-dependent increase in the number of cells in S-phase, suggesting

an arrest in S-phase progression. At IC_{20} concentration of F-EtOAc (12 $\mu\text{g/mL}$) and F10 (6 $\mu\text{g/mL}$), a significantly higher percentage of HL-60 cells were in the S-phase compared to the VC group ($p < 0.001$, Figure 2B). These findings demonstrate that the anti-proliferative activities of F-EtOAc and F10 on HL-60 cells are mediated, at least in part, by inducing cell cycle arrest at the S-phase.

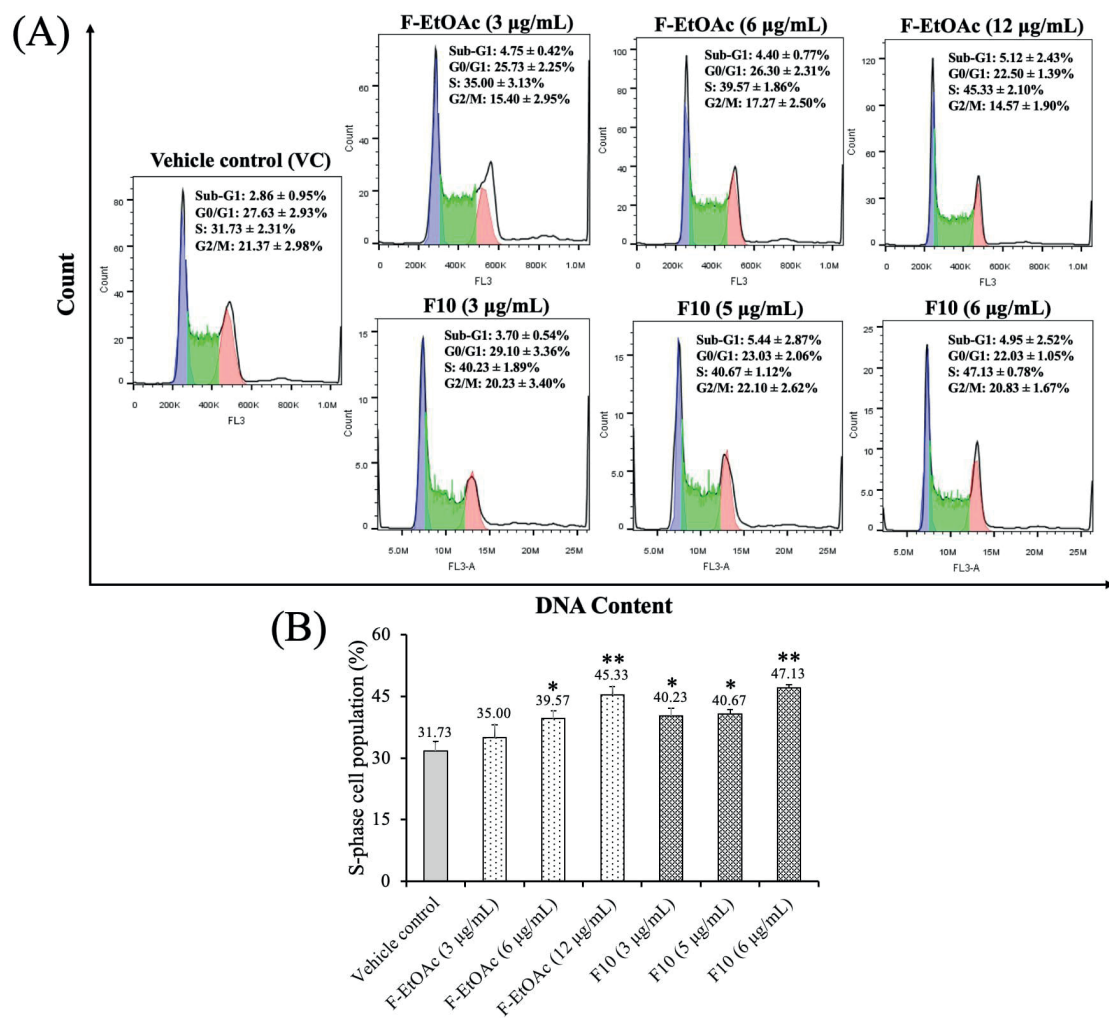


Figure 2. Cell cycle distribution of HL-60 cells. A: after treated with vehicle control (VC) and IC_{10} , IC_{15} , IC_{20} concentrations of F-EtOAc and F10 for 48 hrs, B: S-phase cell population (%) of HL-60 cells after treated with F-EtOAc, and F10 for 48 hrs in comparison with VC. The bars represent the mean $\pm$ SD from three independent experiments. Significant differences (* $p < 0.05$, ** $p < 0.001$) were determined by One-way ANOVA compared to the vehicle control (VC).

Effect of F-EtOAc and F10 on FLT3 and WT1 expression in HL-60 cells

The expression levels of FLT3 and WT1 proteins, which are important markers associated with leukemia cell proliferation, were evaluated using Western blot analysis. HL-60 cells were treated with three non-cytotoxic concentrations (IC_{10} , IC_{15} , IC_{20}) of F-EtOAc and F10 to investigate their effects on FLT3 and WT1 expression. The

results showed that both F-EtOAc and F10 significantly reduced FLT3 and WT1 protein levels in a dose-dependent manner, thereby influencing the proliferation of HL-60 cells (Figure 3). A significant decrease in FLT3 (Figures 3A) and WT1 (Figures 3B) expression was observed at the IC_{20} concentration, demonstrating pronounced inhibitory effects compared to the VC after 48 hrs of treatment.

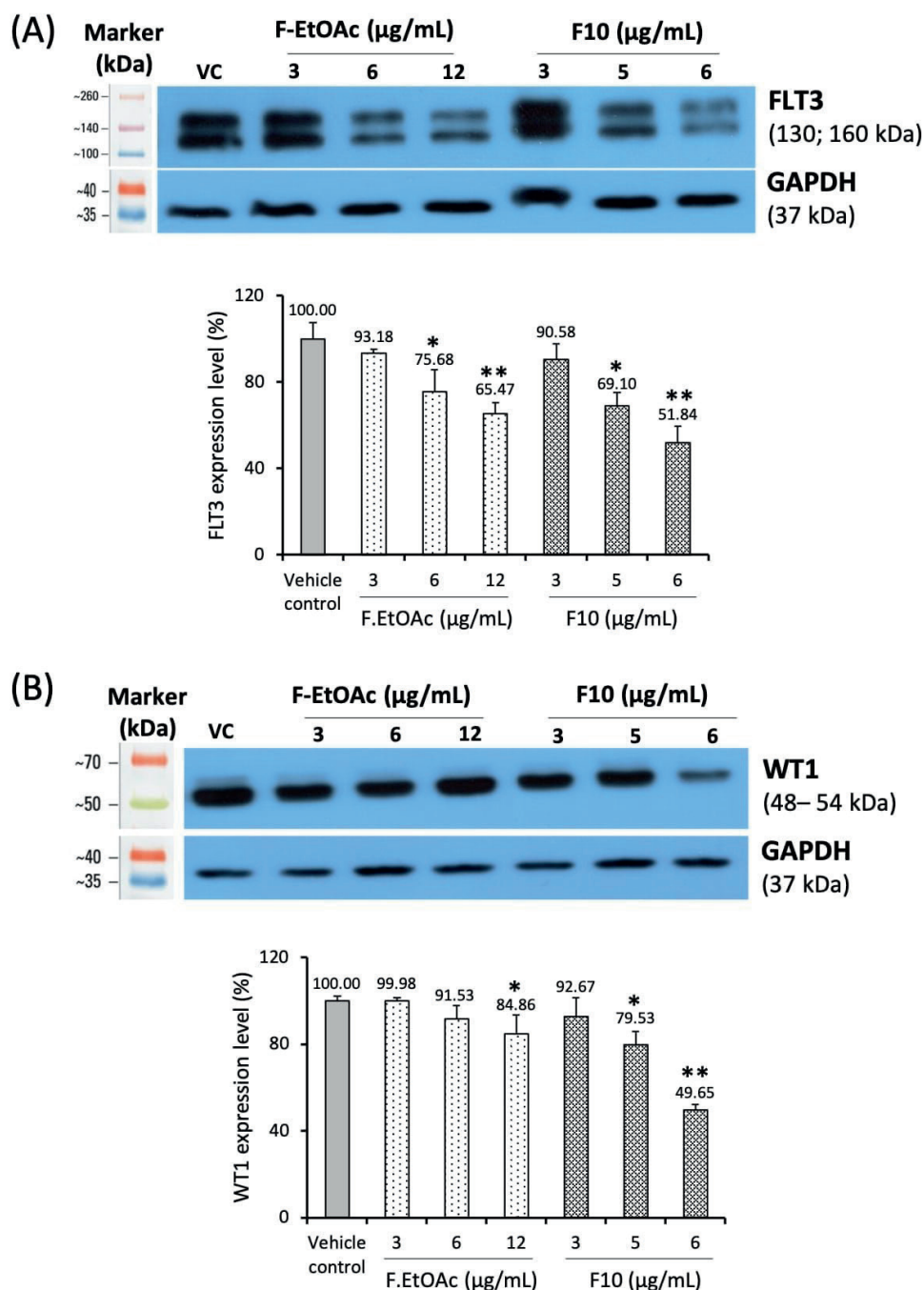


Figure 3. Expression levels of FLT3 and WT1 protein in HL-60 cells treated with the vehicle control (VC) and IC_{10} , IC_{15} , and IC_{20} concentrations of F-EtOAc and F10 for 48 hrs. A: FLT3, B: WT1 protein. GAPDH (37 kDa) was used as the loading control. Histograms represent the mean (%) protein expression $\pm$ SD from three independent experiments. Significant differences (* $p < 0.05$, ** $p < 0.001$) were determined by Student's t-test compared to the vehicle control (VC).

These findings were consistent with the reduction in total viable cell numbers, as confirmed by the trypan blue exclusion assay (Figure 4). Taken together, the results

suggest that downregulation of FLT3 and WT1 protein expression contributes to the anti-proliferative activities of F-EtOAc and F10 in HL-60 cells.

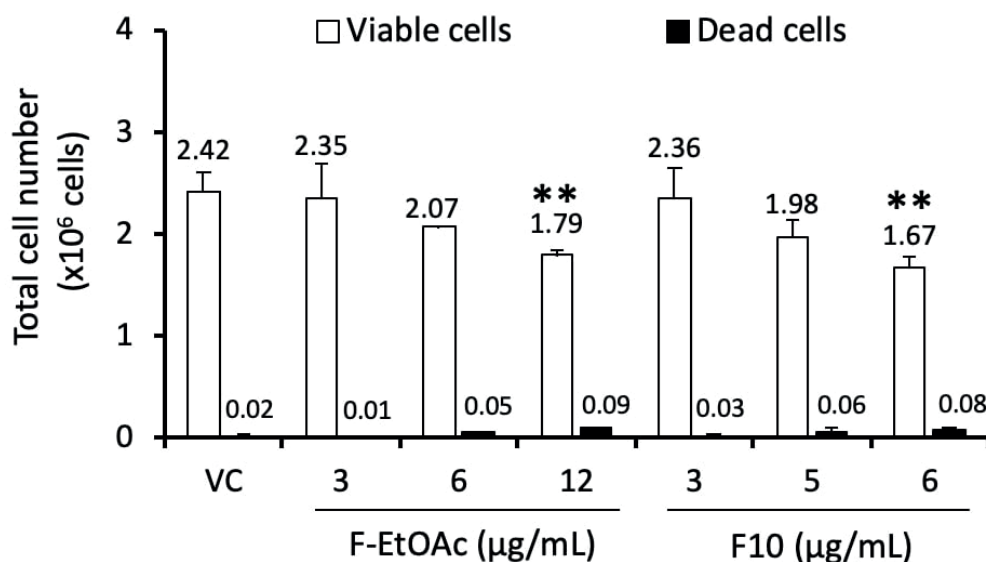


Figure 4. The effects of F-EtOAc and F10 on total cell numbers in HL-60 leukemic cells are determined using trypan blue exclusion test. Histograms show the mean±SD from three independent experiments. Significant differences (** $p < 0.001$) were determined by Student's *t*-test compared to the vehicle control (VC).

Discussion

In this study, we investigated the effects of dried leaf fractional extracts from *Gynostemma pentaphyllum*, collected from a farm in Chiang Mai Province, Thailand, on the cell viability, cell cycle progression, and expression of proliferation-associated proteins in HL-60 AML cells *in vitro*. Previous studies demonstrated that extracts from *G. pentaphyllum* herbs can inhibit the growth and induce apoptosis in various solid cancer cell lines.^{9,11,17} Among the limited studies on leukemia cells, Lin *et al.*¹⁴ and Hsu *et al.*¹⁰ showed that gypenosides-enriched *G. pentaphyllum* saponin extracts possess cytotoxic activity, modulating proliferation and apoptosis in HL-60 and WEHI-3 cells (mouse myelomonocytic leukemia cell model) both *in vitro* and *in vivo*. In our study, crude plant extracts were prepared using the maceration method, known for its high efficiency and suitability for extracting active ingredients from medicinal plants.¹⁸ Among the extracts, ethanol (F-EtOH) yielded the highest extraction percentage (2.70±0.06%), followed by ethyl acetate (F-EtOAc) and hexane (F-Hex). This result indicates that polar protic solvents are more effective in getting higher extraction yields. The superior yield with alcohol-based solvents can be attributed to their ability to dissolve both polar and non-polar compounds.¹⁹ Specifically, ethanol is particularly effective at extracting polar components, such as polysaccharides and proteins, which are likely the predominant constituents in the *G. pentaphyllum* leaf samples used in this study.

Among the three fractional extracts tested, only the ethyl acetate fractional extract (F-EtOAc) strongly inhibited cell growth in HL-60 cells. This high cytotoxic activity is likely due to the presence of specific bioactive

phytochemicals in the ethyl acetate fraction, supporting the potential of *G. pentaphyllum* as source of anti-cancer compounds. F-EtOAc showed the strongest activity against HL-60 cells, with an IC_{50} value of 43.41±2.75 µg/mL. On the other hand, F-Hex and F-EtOH fractions did not significantly affect the viability of leukemic cell lines, as the percentage of viable cells remained above 50% (Figure 1). This suggests the need for further investigation into the cellular mechanisms underlying the sensitivity of AML cells to F-EtOAc and its active components. Similar findings reported by Wang *et al.*²⁰ observed higher anti-cancer activity of the ethyl acetate fraction from *G. pentaphyllum* against A549 lung cancer cells compared to the ethanol extract, highlighting enrichment of potent compounds in the ethyl acetate fraction.

Using column chromatography, the crude F-EtOAc extract was separated into distinct fractions containing compounds based on the polarity and molecular size. Of the ten fractions derived from F-EtOAc, fraction F10 exhibited the strongest cytotoxic activity, with IC_{50} value of 27.40±0.20 µg/mL against HL-60 cells (Table 1). Fraction F10 was obtained using hexane/ethyl acetate solvent system with a ratio of 55:45, indicating the presence of semi-polar character to the compounds in the extract. This finding contrasts with one previous study that identified triterpenoid saponin compounds isolated from 75% ethanol extract as moderately cytotoxic against HL-60 cells with IC_{50} value of 17.44 µg/mL.²¹ Consistent with earlier research, nearly all cytotoxic compounds and extracts derived from *G. pentaphyllum* have shown anti-proliferative activity, with IC_{50} values ranging from 0.05 to 74.3 µg/mL.⁹ Since plant crude extracts are composed

of both active and inactive phytochemicals, their overall effectiveness is generally lower compared to purified fractions. Our findings suggest that ethyl acetate fraction of *G. pentaphyllum* may serve as interesting source of bioactive compounds that are yet to be explored for its characterization and biological activities.

In this study, cell cycle arrest was investigated as a mechanism of action for natural cytotoxic compounds, as several natural compounds inhibit cancer cell proliferation by suppressing the cell cycle progression.⁹ Molecules that can block uncontrolled proliferation or induce cell death are increasingly recognized as promising candidate for therapeutic applications in cancer treatment. The effects of the most potent extract F-EtOAc and fraction F10 on the cell-cycle progression of HL-60 cells were analyzed using flow cytometry. Recent studies suggest that the inhibitory effects of medicinal plant-derived extracts on cell proliferation may be mediated through arresting cells at the G0/G1, S, or G2/M phases or by promoting apoptosis.^{22,23} In line with this, treatment of HL-60 cells with the IC₂₀ concentration of F-EtOAc and F10 significantly arrest the cell cycle at S-phase ($p < 0.001$, Figure 2). Previous research studies reported that gypenosides from *G. pentaphyllum* inhibit cancer cell growth by inducing G0/G1-phase arrest and promoting apoptosis in various cancer cell lines, including A549,²⁴ Colo 205,²⁵ HL-60,¹⁴ and WEHI-3 cells.¹⁰ In another study, flavonoids and saponins isolated from *G. pentaphyllum* have been shown to cause cell cycle arrest at the S and G2/M phases in PC-3 prostate cancer cells by modulating the expression of cyclins.^{26,27} To further explore the molecular mechanisms underlying these effects, in our study, we examined the expression of cell proliferation-associated markers in HL-60 cells. To the best of our knowledge, no prior studies have reported the effects of *G. pentaphyllum* compounds on FLT3 and WT1 protein expression in leukemic cells. Western blot analysis showed that treatment with F-EtOAc (12 µg/mL; $p < 0.05$) and F10 (6 µg/mL; $p < 0.001$) for 48 hrs significantly reduced FLT3 and WT1 expression levels in HL-60 cells (Figure 3). This reduction in protein expression was consistent with a decreased total cell count observed using trypan blue exclusion (Figure 4), indicating a suppression of cell proliferation.²⁸ These findings suggest that F-EtOAc and F10 reduce the viability of AML cells through mechanisms involving FLT3 and WT1 inhibition. The precise molecular pathways by which *G. pentaphyllum* down-regulates FLT3 and WT1 expression, leading to S-phase cell cycle arrest, awaits further studies.

Conclusion

The present research highlights the potential anti-proliferative properties of *Gynostemma pentaphyllum* leaves against HL-60 leukemia cells, providing evidence for further investigation. The study demonstrates that ethyl acetate extract (F-EtOAc) and its active fraction F10 exhibit significant anti-leukemia activities by inducing S-phase cell cycle arrest and reducing expression of FLT3 and WT1. Overall, these findings suggest that ethyl acetate

extract of *G. pentaphyllum* contains physiologically active compounds targeting FLT3 and WT1, making it a promising source of anti-leukemic biomolecules. Further research should focus on isolation and characterization of the specific compounds responsible for the anti-proliferative effects, as well as their mechanisms of action.

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Conflict of interest

The authors declare no conflict of interest.

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