Anti-Leukemic Activity of *Smilax china* L. Root Extracts against Acute Myeloid Leukemia Cells and Inhibition of Xenograft Tumor Growth *In-vivo*

Hongyang Kang,* and Changqing Tong

Acute myeloid leukemia (AML) is a clinically challenging malignancy with limited effective therapies and significant toxicity. This study evaluated the anti-leukemic potential of ethanolic extract of *Smilax china* L. root in HL-60 cells and a xenograft mouse model. The extract markedly reduced HL-60 cell viability in a dose- and time-dependent manner (IC_{50} = 65.2 µg/mL at 24 h). Mechanistic analyses showed strong induction of apoptosis, increased Bax expression, decreased Bcl-2 levels, and an elevated Bax/Bcl-2 ratio. Cell-cycle arrest at the G2/M phase further confirmed its antiproliferative activity. The extract also significantly inhibited HL-60 cell migration and invasion. *In vivo* treatment of HL-60 xenograft-bearing mice (5 and 10 mg/kg) resulted in substantial suppression of tumor growth with minimal systemic toxicity. Tumor tissues exhibited reduced TNF-α, IL-1β, and IL-6 levels, indicating an additional anti-inflammatory effect. These findings demonstrate that *Smilax china* L. root extract exerts multi-target anti-leukemic actions by inducing apoptosis, blocking cell-cycle progression, suppressing metastasis-related behavior, and down-regulating inflammatory cytokines. *Smilax china* shows therapeutic promise as a complementary strategy for AML and warrants further investigation of its active constituents.

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Keywords: Acute myeloid leukemia (AML); HL-60 cells; Apoptosis; Bax/Bcl-2; Cell-cycle arrest; Xenograft model; Anti-inflammatory cytokines; Migration; Invasion

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy characterized by clonal expansion of poorly differentiated myeloid progenitors in the bone marrow and blood, leading to marrow failure and rapid clinical deterioration if untreated (Hwang 2020; Wachter and Pikman 2024). Globally, AML represents a substantial and growing public-health challenge: population-based estimates from GLOBOCAN and recent burden studies document increasing incidence and mortality over recent decades, with marked age- and region-specific variations (higher incidence in older adults and in higher-SDI regions) (Bray *et al*. 2024). The pathogenesis of AML is heterogeneous and driven by cooperating genetic and epigenetic lesions. Recurrent mutations in genes such as FLT3, NPM1, DNMT3A, TP53, and alterations in chromatin-remodeling and DNA-methylation pathways reshape differentiation and survival programs, while leukemic stem cells and signaling/epigenetic plasticity underpin persistence and relapse (Park 2024; Wysota *et al*. 2024). Historically, treatments aimed at curing the disease have centered on

intensive cytotoxic induction (the "7+3" cytarabine-anthracycline backbone) followed, when feasible, by consolidation with high-dose chemotherapy or allogeneic hematopoietic stem cell transplantation; these approaches yield complete remissions but are limited by age-related tolerability and treatment-related morbidity. Over the last decade, targeted and epigenetic agents (FLT3 inhibitors such as midostaurin/gilteritinib, BCL-2 inhibitor venetoclax combined with hypomethylating agents, IDH inhibitors, and other novel combinations) have substantially expanded therapeutic options and improved outcomes for select molecular subgroups (Carter *et al*. 2020; Roloff *et al*. 2022). Nevertheless, major disadvantages remain: intensive chemotherapy causes substantial toxicity and is often unsuitable for older or frail patients; targeted and HMA-venetoclax regimens, while effective initially, are frequently undermined by primary or acquired resistance driven by clonal evolution, adverse genetics (*e.g.*, TP53), drug-efflux, metabolic adaptation, and persistence of therapy-resistant leukemic stem cells — resulting in relapse and limited long-term survival for many patients (Strickland and Vey 2022; Guarnera *et al*. 2025). Together, these facts underscore an urgent need for deeper mechanistic insight and novel therapeutic strategies that are both more effective across diverse molecular subtypes and better tolerated in the predominantly elderly AML population.

Plant extracts have emerged as promising therapeutic resources in AML due to their ability to deliver a synergistic mixture of bioactive constituents with multi-targeted anticancer effects (Zhamanbayeva *et al*. 2016). Unlike isolated compounds, whole extracts often act on several leukemogenic pathways simultaneously, reducing the likelihood of resistance and offering improved biocompatibility. *Curcuma longa* extract (turmeric extract) exhibits potent anti-AML activity by suppressing NF-κB, elevating intracellular reactive oxygen species (ROS), and triggering mitochondrial-dependent apoptosis (Badagliacca *et al*. 2025). Grape seed extract, rich in polyphenols, induces S-phase arrest, activates p53 and caspase signaling, and sensitizes AML cells to chemotherapeutic agents (Lin *et al*. 2021). Additionally, green tea extract (rich in catechins) modulates cell-cycle regulators, downregulates anti-apoptotic proteins such as Bcl-2, and enhances TRAIL-mediated apoptosis (Della Via *et al*. 2023). Collectively, plant extracts offer a multitarget, low-toxicity approach with potential to complement existing AML therapies and overcome chemoresistance.

Smilax china L., a perennial climbing herb widely distributed across East and Southeast Asia, is traditionally used in Chinese and Korean medicine for detoxification, anti-inflammatory effects, and treatment of infections (Wang *et al*. 2024). Different parts of the plant exhibit diverse pharmacological activities: the leaves show antioxidant and anti-inflammatory actions by suppressing COX-2 and ROS pathways (Jiang *et al*. 2021); stems demonstrate anti-diabetic activity *via* modulation of glycation, aldose reductase, α-glucosidase, and lipase (Lee *et al*. 2017). Among all parts, the roots are the most extensively studied. Recent *in vitro* and *in vivo* studies (past 10 years) demonstrate that *Smilax china* root extracts possess potent anticancer (Wu *et al*. 2023), hepatoprotective (Mandal *et al*. 2008), and anti-inflammatory (Zhong *et al*. 2017) properties. Mechanistically, the roots induce apoptosis *via* mitochondrial pathway activation, inhibit NF-κB and MAPK signalling, suppress cell-cycle progression, and reduce metastasis by downregulating MMP-2 and MMP-9. Their strong ability to modulate oxidative stress, inflammation, and oncogenic pathways suggests meaningful therapeutic potential. Given these multifaceted biological activities—especially the pro-apoptotic and anti-inflammatory mechanisms relevant to leukemic progression—*Smilax china* L. root extract is a scientifically worthy candidate for evaluation against AML.

EXPERIMENTAL

Plant Collection

The roots of *Smilax china* L. (family: Smilacaceae) were collected from near the First Affiliated Hospital of Hebei North University, located in Zhangjiakou, Hebei Province, China. The plant material was taxonomically authenticated by a qualified botanist at the Institute. A voucher specimen (Voucher No. HNU-2025-032A) was prepared and deposited in the institutional herbarium of the Institute for future reference. Freshly collected roots were thoroughly washed with distilled water, shade-dried at room temperature for 72 h, and then ground into a coarse powder prior to extraction.

Extraction

An ethanolic extract of *Smilax china* L. roots was prepared following a standardized maceration procedure. Approximately 100 g of root powder was defatted with n-hexane and subsequently extracted with 70% ethanol (1:10 w/v) by maceration at room temperature for 72 h with intermittent stirring. The mixture was filtered through Whatman No. 1 filter paper, and the marc wash was re-extracted twice under identical conditions to ensure exhaustive extraction. All filtrates were combined and concentrated under reduced pressure using a rotary evaporator at temperatures below 45 °C to obtain a semi-solid crude extract. The concentrated extract was further dried in a vacuum desiccator to yield a stable ethanolic root extract, and the final extraction yield was calculated relative to the initial dry weight. For biological assays, the dried extract was dissolved in DMSO (0.1% v/v) to prepare stock solutions, filtered through a 0.22 µm membrane for sterility, and stored at –20 °C until further use. This ethanolic extract served as the test sample for subsequent pharmacological evaluations.

Evaluation of *In-vitro* Activity

Cell line and culture conditions

U937 (human myeloid leukemia), K562 (chronic myeloid leukemia), and HL-60 (human promyelocytic leukemia) cells were obtained from a Shanghai Institute for Biological Sciences (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay by MTT method

HL-60 cells (1×10^4 cells/well) were seeded in 96-well plates and treated with varying concentrations of *S. china* extract (5 to 200 µg/mL) for 24 h. MTT reagent (0.5 mg/mL) was added and incubated for 4 h. Formazan crystals were dissolved in DMSO (0.1% v/v), and absorbance was measured at 570 nm. The IC_{50} value was calculated, and three concentrations—0.5×, 1×, and 2× IC_{50} (30, 65, and 130 µg/mL)—were selected for mechanistic assays (Srivastava *et al*. 2017; Zhang and Li 2024).

Cell-cycle analysis by flow cytometry

HL-60 cells (2×10^5 cells/well) were treated with extract (30, 65, and 130 µg/mL) for 24 h. Cells were harvested, washed with PBS, and fixed in 70% cold ethanol overnight at −20 °C. After washing, cells were incubated with RNAse A (100 µg/mL) and stained with PI (50 µg/mL) for 30 min in the dark. Samples were analyzed using a FACS flow cytometer, and the distribution of cells in G0/G1, S, and G2/M phases was determined.

Apoptosis assay using Annexin V–FITC/PI staining)

HL-60 cells treated with 30, 65, and 130 µg/mL of extract for 24 h were collected, washed with cold PBS, and resuspended in binding buffer. Annexin V–FITC and PI were added as per kit protocol and incubated for 15 min in the dark. Flow cytometry was used to quantify early and late apoptotic cell populations.

Evaluation of *In-vivo* Activity

Animals

Age-matched male NOD/SCID-IL2Rγ^null (NSG) mice (6 to 8 weeks old) were used for tumor xenograft experiments. Animals were housed in individually ventilated cages within a pathogen-free barrier facility maintained on a 12 h light/12 h dark cycle. Sterile rodent chow and water were provided *ad libitum*. All procedures were performed in compliance with institutional guidelines and approved by the Animal Ethics Research Board of The First Affiliated Hospital of Hebei North University, China.

Xenograft transplantation and treatment protocol

HL-60 cells (2×10^6) suspended in 0.1 mL RPMI-1640 medium were injected subcutaneously into the right flank of NSG mice. Tumor growth was monitored using digital calipers, and tumor volume was calculated using the formula:

$$\text{Tumor volume } (\text{mm}^3) = \frac{1}{2} \times \text{length} \times (\text{width})^2 \quad (1)$$

In Eq. 1, the factor (1/2) is a commonly used approximation of the area of an ellipsoid enclosed within a rectangle.

When tumors reached approximately 250 mm³, mice were randomly assigned to different treatment groups (n=6). Animals received intraperitoneal injections of *Smilax china* root ethanolic extract at doses of 5 or 10 mg/kg. The injected substance was a standard anticancer drug (doxorubicin, 2 mg/kg; positive control), or vehicle control. The extract and control solutions were prepared using dimethyl sulfoxide (DMSO, 0.1% v/v) and diluted with sterile phosphate-buffered saline to obtain the required dosing concentrations. The selected extract doses were based on preliminary tolerability observations and previously reported studies evaluating plant-derived anticancer extracts in xenograft models.

Treatments were administered five times per week for two consecutive weeks. Tumor volume was measured periodically throughout the treatment period, and body weight was recorded every two days as a general indicator of systemic toxicity.

At the end of the 15-day treatment period, mice were euthanized by CO_2 inhalation followed by cervical dislocation to ensure death, in accordance with institutional animal care and ethical guidelines approved by the Animal Ethics Research Board, and tumors were excised, weighed, and processed for further biochemical analyses. Although body weight monitoring was used to assess general systemic toxicity, comprehensive toxicity evaluation including serum biochemical parameters, hematological profiling, and histopathological examination of major organs was not performed in the present study.

Tissue collection and cytokine analysis (*TNF-α, IL-1β, IL-6*)

After treatment, mice were euthanized and tumor tissues were excised, washed with cold PBS, and homogenized in RIPA buffer containing protease inhibitors. The

homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatants were collected and used to quantify TNF-α, IL-1β, and IL-6 using ELISA kits. Results were expressed as pg/mg protein.

Statistical Analysis

All experiments were performed in at least three independent biological replicates unless otherwise stated. Data are presented as mean ± standard error of the mean (SEM). IC_{50} values were calculated from dose–response curves generated using nonlinear regression analysis in GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. For *in vivo* experiments, tumor volumes and body weights were analyzed using one-way ANOVA. A p-value < 0.05 was considered statistically significant.

RESULTS

Effect of *Smilax china* L. Root Extract on the Viability of Leukemic Cells

The cytotoxic effect of *Smilax china* L. root extract on leukemic cell viability was evaluated in U937, K562, and HL-60 cells across 24, 48, and 72 h, and shown in Table 1. The extract exhibited a time- and cell-line-dependent cytotoxic response. HL-60 cells were the most sensitive, showing the lowest IC_{50} values at all time points, which progressively decreased from 65.2 ± 15.5 µg/mL at 24 h to 41.4 ± 10.3 µg/mL at 48 h and 18.1 ± 9.2 µg/mL at 72 h. K562 cells displayed moderate sensitivity, with IC_{50} values decreasing from 320.3 ± 45.3 µg/mL at 24 h to 240.5 ± 42.3 µg/mL at 48 h and 145.2 ± 20.4 µg/mL at 72 h. In contrast, U937 cells were comparatively less susceptible, exhibiting the highest IC_{50} values (430.5 ± 52.2, 322.1 ± 43.5, and 210.4 ± 22.5 µg/mL at 24, 48, and 72 h, respectively). Overall, the extract demonstrated increasing cytotoxicity with longer exposure time, particularly against HL-60 cells. Based on these findings, three concentrations of the *Smilax china* L. root extract—30, 65, and 130 µg/mL, corresponding to 0.5×, 1×, and 2× the 24-h IC_{50} against HL-60 cells—were selected for further biochemical analyses to evaluate concentration-dependent effects.

Table 1. Concentration- and Time-Dependent Cytotoxicity of *Smilax china* L. Root Extract Against Human Leukemia Cell Lines (U937, K562, and HL-60) Expressed as IC_{50} Values (µg/mL) at 24, 48, and 72 Hours

Smilax china L.	IC_{50}#		
	U937	K562	HL-60
At 24 h	430.5±52.2	320.3±45.3	65.2±15.5
At 48 h	322.1±43.5	240.5±42.3	41.4±10.3
At 72 h	210.4±22.5	145.2±20.4	18.1±9.2

Data are presented as mean ± SEM from triplicate experiments.

Effect of *Smilax china* L. Root Extract on the Apoptosis and Cell Cycle of HL-60

Treatment with *Smilax china* L. root extract induced a concentration-dependent increase in apoptosis in the tested cancer cells. Annexin V-FITC/PI flow cytometry profiles (Fig. 1A) showed a progressive rise in early and late apoptotic populations with increasing

extract concentrations (30, 65, and 130 µg/mL), compared with the untreated control. Quantitative analysis (Fig. 1A) confirmed this trend, with apoptotic rates increasing from baseline levels at 0 µg/mL to approximately 25%, 40%, and over 60% at 30, 65, and 130 µg/mL, respectively ($p < 0.004$). In addition to apoptosis induction, the extract significantly altered cell-cycle progression (Fig. 1B). A marked increase in number of cells in G2/GM phase, with concomitant reduction in the cell population of S-phase. No change was observed in G0/G1 phase. Together, these findings demonstrate that *S. china* root extract exerts potent anti-proliferative effects by simultaneously promoting apoptosis and disrupting normal cell-cycle progression.

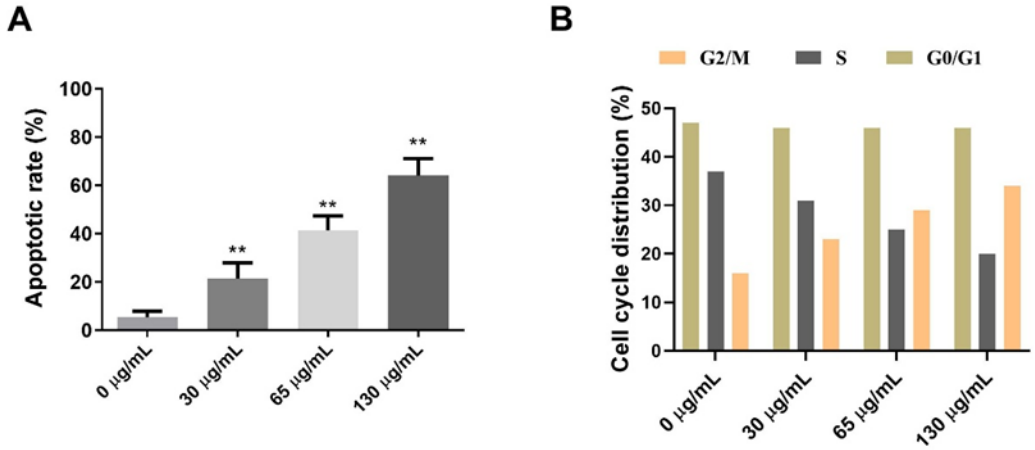

Fig. 1. *Smilax china* L. root extract induces apoptosis and G2/M cell-cycle arrest in cancer cells: (A) Quantitative analysis of apoptotic rates; and (B) Cell-cycle distribution analysis demonstrating accumulation of cells in the G2/M phase. Data are presented as mean ± SEM from three independent biological experiments. Statistical significance compared with the 0 µg/mL control group: **P = 0.004.

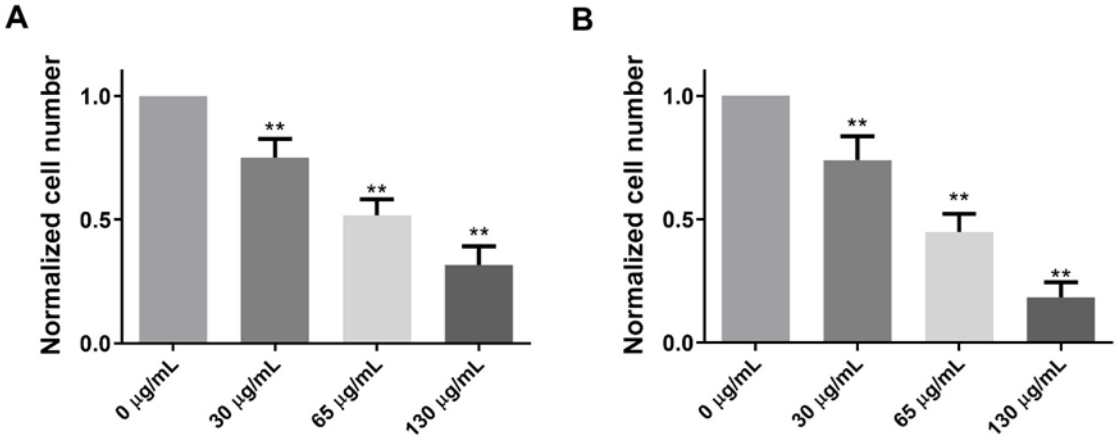

Fig. 2. Effect of *Smilax china* L. root extract on the migratory and invasive potential of HL-60 cells: (A) Transwell migration assay showing a concentration-dependent reduction in the number of migrating HL-60 cells following treatment with 30, 65, and 130 µg/mL extract; and (B) Transwell invasion assay demonstrating a similar concentration-dependent decrease in invasive cell numbers. Data are presented as normalized cell numbers relative to untreated controls and expressed as mean ± SEM from three independent experiments. Statistical significance compared with the control group: **P = 0.003.

Effect of *Smilax china* L. Root Extract on Migration and Invasion of HL-60 Cells

The ability of *Smilax china* L. root extract to modulate the metastatic behaviour of HL-60 cells was assessed using transwell migration and invasion assays. As shown in Fig. 2A, the extract significantly inhibited HL-60 cell migration in a clear dose-dependent manner. Treatment with 30 μM produced an observable reduction in the number of migrating cells, while 65 μM and 130 μM led to progressively greater suppression, reflecting a strong impairment of cell motility. Similarly, in the invasion assay (Fig. 2B), *Smilax china* extract markedly restricted the invasive capacity of HL-60 cells. A substantial decline in invasive cell numbers was evident even at the lowest dose, and the inhibition became more pronounced at 65 μM and most significant at 130 μM, indicating reduced ability of the cells to traverse the extracellular matrix barrier. Together, these results demonstrate that the extract exerts potent anti-metastatic effects by significantly blocking both migration and invasion of HL-60 cells in a concentration-dependent manner.

Effect of *Smilax china* L. Root Extract on Bax and Bcl-2 mRNA Expression

The pro-apoptotic activity of *Smilax china* L. root extract in HL-60 cells was further confirmed through RT-PCR analysis of Bax and Bcl-2 gene expression. As shown in Fig. 3A, treatment with the extract induced a strong and progressive upregulation of the pro-apoptotic gene Bax. Even the lowest concentration (30 μM) significantly increased Bax expression compared with the untreated control, while 65 μM and 130 μM produced a marked and concentration-dependent enhancement, indicating strong activation of apoptotic signalling. In contrast, expression of the anti-apoptotic gene Bcl-2 was significantly suppressed following extract treatment (Fig. 3B).

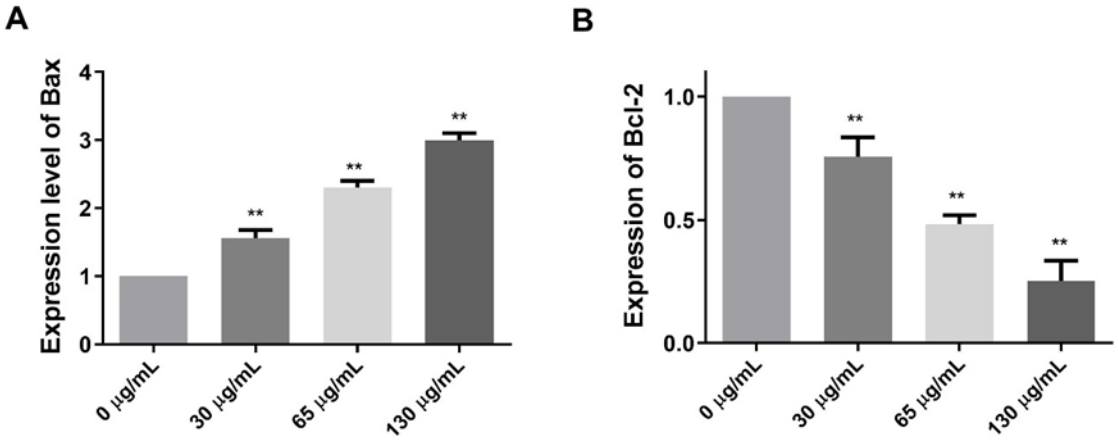

Fig. 3. Effect of *Smilax china* L. root extract on the expression of apoptosis-related genes in HL-60 cells: (A) RT-PCR analysis showing concentration-dependent upregulation of the pro-apoptotic gene Bax following treatment with 30, 65, and 130 μg/mL extract; and (B) Corresponding downregulation of the anti-apoptotic gene Bcl-2, indicating suppression of survival signaling. Gene expression levels were normalized to the internal control and expressed relative to untreated cells. Data are presented as mean ± SEM from three independent biological experiments. Statistical significance compared with control: **P= 0.005.

The Bcl-2 mRNA levels gradually decreased with increasing concentrations of the extract, with the highest dose (130 μM) showing the most pronounced downregulation.

The opposing regulation of Bax and Bcl-2 resulted in a markedly elevated Bax/Bcl-2 ratio, a key indicator of mitochondrial-mediated apoptosis. Collectively, these results demonstrate that *Smilax china* L. root extract promoted apoptosis in HL-60 cells by enhancing pro-apoptotic Bax expression while simultaneously suppressing anti-apoptotic Bcl-2, thereby shifting the balance toward programmed cell death.

Anti-tumor Activity of *Smilax china* L. Root Extract in HL-60 Xenograft Model

The anti-leukemic efficacy of the *Smilax china* L. root extract was evaluated in an HL-60 xenograft mouse model using two dose levels (5 and 10 mg/kg), with saline and a standard drug serving as controls. Throughout the 15-day treatment period, body weight was monitored to assess systemic toxicity (Fig. 4A). Mice receiving saline or the standard drug showed no significant changes in body weight, indicating normal physiological status. In contrast, animals treated with *Smilax china* extract exhibited a mild but noticeable reduction in body weight, particularly in the 5 mg/kg group, suggesting a modest treatment-related effect; however, no severe toxicity or distress was observed, indicating acceptable tolerability.

Tumor growth progression was monitored over 15 days (Fig. 4B). In saline-treated mice, tumor volume increased rapidly, reaching approximately 1200 mm^3 by day 12. Treatment with 5 mg/kg extract resulted in a moderate suppression of tumor growth, with tumor volumes consistently lower than the saline group at each time point. A more pronounced anti-tumor effect was observed at 10 mg/kg, where tumor progression was markedly delayed, and tumor volume remained substantially reduced throughout the study period. The standard drug group showed the strongest inhibition, maintaining the smallest tumor volumes, which served as an internal benchmark for therapeutic efficacy. Overall, these findings demonstrate that the *Smilax china* L. root extract exhibits significant dose-dependent anti-tumor activity *in vivo*. The 10 mg/kg dose effectively suppressed HL-60 tumor growth without inducing major toxicity, indicating that the extract possesses promising therapeutic potential as an anti-leukemic agent.

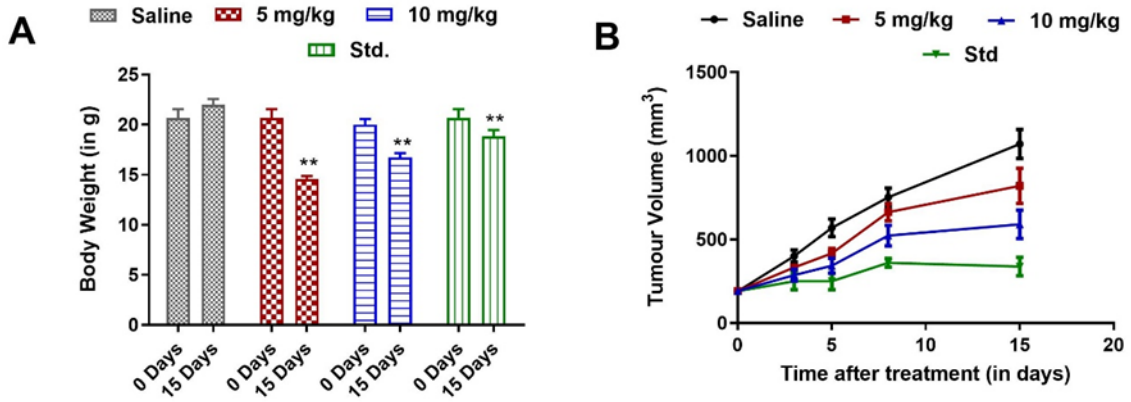

Fig. 4. *In vivo* antitumor efficacy of *Smilax china* L. root extract in HL-60 xenograft-bearing mice: (A) Changes in body weight of mice treated with vehicle control, extract (5 and 10 mg/kg), or the standard drug during the 15-day treatment period; and (B) Tumor growth curves showing dose-dependent suppression of HL-60 tumor progression by the extract, while the standard drug exhibited the greatest inhibitory effect. Data are presented as mean ± SEM (n = 6 per group). Statistical significance compared with the control group: **P = 0.006.

Effect of *Smilax china* L. Root Extract on Pro-inflammatory Cytokines in HL-60 Xenograft Tissues

Administration of *Smilax china* L. root extract produced a clear and dose-dependent suppression of inflammatory cytokines in HL-60 tumor-bearing mice. As shown in Fig. 5A, TNF-α levels were markedly elevated in the control group, whereas treatment with 5 and 10 mg/kg extract significantly decreased TNF-α concentrations ($P < 0.004$), indicating attenuation of tumor-associated inflammatory signalling. The reduction was more pronounced at 10 mg/kg, and the standard drug group displayed the lowest cytokine level, confirming effective inflammatory suppression.

A similar inhibitory effect was observed for IL-1β (Fig. 5B). Control tumors showed high IL-1β production, while extract-treated mice exhibited significantly reduced IL-1β levels in a concentration-dependent manner ($P < 0.004$). This suggests that the extract effectively limits inflammasome-associated cytokine release within the tumor microenvironment. IL-6 levels (Fig. 5C) followed the same trend, with both extract doses causing significant decreases relative to untreated controls ($P < 0.004$). The 10 mg/kg dose produced a more substantial decline, approaching the cytokine-lowering effect of the standard drug. Since IL-6 is a major mediator of tumor survival, proliferation, and systemic inflammation, its suppression further supports the anti-tumor efficacy of the extract. Collectively, these findings demonstrate that *Smilax china* L. root extract exerts potent anti-inflammatory activity in vivo by downregulating TNF-α, IL-1β, and IL-6, thereby contributing to the observed inhibition of HL-60 tumor growth.

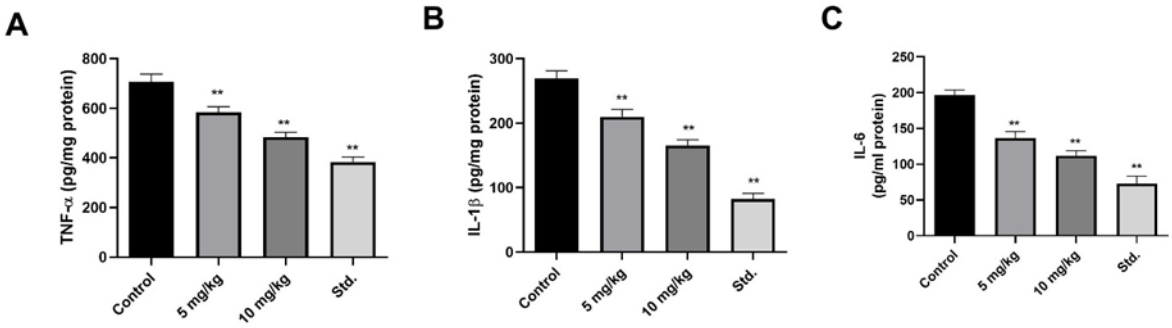

Fig. 5. Effect of *Smilax china* L. root extract on pro-inflammatory cytokines in HL-60 xenograft tissues: (A) TNF-α, (B) IL-1β, and (C) IL-6 levels in tumor tissues of mice treated with *S. china* root extract (5 and 10 mg/kg) or standard drug. The extract produced a significant, dose-dependent reduction in all three cytokines compared with the control group, indicating strong anti-inflammatory activity. Data are expressed as mean ± SEM (n = 6). **P < 0.004 vs. control.

DISCUSSION

The present study demonstrated that *Smilax china* L. root extract exerted potent anti-leukemic effects on HL-60 cells, both *in vitro* and *in vivo*. Observations included significant inhibition of cell proliferation, induction of apoptosis, and suppression of tumor growth in xenograft models. These findings are consistent with previous reports of anticancer activity for *S. china* and related *Smilax* species (Nho *et al*. 2015; Wu *et al*. 2023; Guo *et al*. 2025). For example, kaempferol-7-O-β-D-glucoside (KG) was extracted from *S. china* rhizome and showed that KG induced G_1-phase arrest and apoptosis in HL-60 cells (Xu *et al*. 2008). Similarly, extracts of *S. china* rhizome were found to block cell cycle

progression at G_2/M and trigger late apoptosis in hepatocellular carcinoma cells (Zhang *et al*. 2012). In other cancer models, *S. china* preparations have likewise reduced cell viability and tumor growth (*e.g.* in ovarian and breast cancer) by promoting cell death (Hu *et al*. 2015; Nho *et al*. 2015). Taken together, the present results align well with these reports, confirming that *S. china* contains bioactive constituents capable of suppressing malignant cell growth.

The cytotoxic effects of *S. china* extract in the present study appear to involve the intrinsic (mitochondrial) apoptotic pathway (Elmore 2007). Hallmarks of apoptosis (*e.g.*, sub-G_0 DNA content, caspase-3 activation) were observed, and these effects mirror those described for *Smilax*-derived compounds. Prior studies have shown that *S. china* extracts modulate Bcl-2 family proteins: Hu *et al*. (2015) reported that *S. china* rhizome extract activated caspase-3 and upregulated pro-apoptotic Bax while downregulating anti-apoptotic Bcl-2, Bcl-xL and IAPs in A2780 ovarian cancer cells. Likewise, the present work revealed an increased Bax/Bcl-2 ratio, suggesting that *Smilax* triggers mitochondrial permeabilization and cytochrome c release. Moreover, natural flavonoids in *S. china* may act similarly to quercetin: in HL-60 xenografts, quercetin and green tea polyphenol reduced expression of Bcl-2, Bcl-xL, and Mcl-1 while increasing Bax and caspase-3 activity (Calgarotto *et al*. 2018). These parallels imply that *S. china* flavonoids (such as astilbin or KG) might underlie the observed Bax/Bcl-2 modulation in the leukemia cells.

In addition, the present findings (and the literature) suggest that *S. china* impinges on survival signaling pathways. The rhizome extract was shown to inhibit NF-κB activity and Akt signaling in cancer cells, which would relieve suppression of apoptosis. For instance, Hu *et al*. (2015) found that *S. china* downregulated NF-κB, AKT, XIAP and cIAP-1 in ovarian cancer cells, thereby sensitizing them to apoptosis. Such NF-κB inhibition may contribute to the Bax/Bcl-2 effects observed in the present work and could also explain reduced cytokine production (see below). Consistent with this, Jiang *et al.* (2021) demonstrated that a *S. china* extract suppressed LPS-induced NF-κB/MAPK signaling in THP-1 macrophages, leading to decreased IL-1β, IL-6 and TNF-α production. Thus, *S. china* root extract likely exerts its antileukemic action by simultaneously activating intrinsic apoptosis (*via* mitochondrial pathways) and dampening survival/ inflammatory signals (*via* NF-κB and related pathways).

In cell-cycle analyses, it was found that *S. china* treatment caused accumulation of HL-60 cells in a sub-G_0/G_1 (apoptotic) or G_2/M phase, depending on dose. This is in line with prior work on *Smilax* extracts: Xu *et al*. (2008) observed G_1-phase arrest by KG in HL-60 cells, while Zhang *et al* (2012) reported G_2/M arrest in HepG2 cells treated with *Rhizoma Smilacis Chinae* extracts. Such cell-cycle perturbations likely contribute to reduced proliferation. Beyond cell-cycle control, *S. china* appears to affect cell motility and invasion in cancer. Although HL-60 leukemia cells are non-adherent, studies in solid tumors reveal anti-metastatic properties of *Smilax* extracts (Guo *et al.* 2022; Wu *et al.* 2023). Nho *et al*. (2015) showed that an ethanol extract of *S. china* markedly inhibited migration of MDA-MB-231 breast cancer cells and decreased expression of ECM-degrading enzymes (uPA, uPAR) while upregulating TIMP1/2. Similarly, water extracts of *S. glabra* (a related *Smilax*) were found to suppress migration/invasion of HepG2 and MDA-MB-231 cells by inhibiting TGF-β1 signaling (Guo *et al*. 2022). These studies suggest that *Smilax* constituents can interfere with tumor cell adhesion and motility. By analogy, the present *in vivo* results (*e.g.,* reduced leukemia infiltration of organs) may reflect impaired leukemic cell homing or survival, a hypothesis that warrants further testing. Moreover, the migration and invasion assays are commonly applied to adherent

solid tumor models; however, the present study utilized HL-60 cells, which are non-adherent suspension leukemia cells. Although transwell systems have been used to evaluate chemotactic migration of hematological malignancy cells, the interpretation of migration or invasion behavior in such suspension cell lines should be approached with caution. Therefore, the observed effects in this assay may reflect alterations in chemotactic motility rather than classical invasion characteristics typically studied in adherent cancer cells. Further validation using additional leukemia-specific functional assays or adherent tumor models would help strengthen the interpretation of these findings.

Chronic inflammation and dysregulated cytokines often support leukemia growth (Camacho *et al*. 2021). Intriguingly, *S. china* extract has well-documented anti-inflammatory effects that may complement its direct cytotoxicity. Jiang *et al.* (2021) demonstrated that *S. china* extract selectively inhibited COX-2 and significantly reduced pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in stimulated immune cells. They showed this was due to blockade of MAPK and NF-κB signaling pathways. By extension, the observed downregulation of NF-κB and IAPs in the present study could concurrently suppress autocrine/paracrine cytokine loops that support HL-60 survival (Muniyan *et al*. 2022). In other words, *S. china* treatment may reduce leukemia-promoting inflammation in the microenvironment. This immunomodulatory angle is important: besides killing cancer cells, *Smilax* extracts could re-balance the cytokine milieu (*e.g.*, lowering IL-6/TNF) that often drives leukemia cell proliferation and chemoresistance.

The present findings, together with the literature, suggest that *S. china* root extract has potential as a complementary leukemia therapy. Its ability to trigger apoptosis and cell-cycle arrest *via* modulating Bcl-2 family proteins, caspases, and NF-κB signaling parallels the action of known antileukemic phytochemicals (*e.g.*, quercetin). Moreover, *Smilax* extracts have been reported to enhance chemosensitivity: for instance, pretreatment with *S. china* extract increased the efficacy of cisplatin and adriamycin in ovarian cancer cells (Hu *et al*. 2015). This raises the prospect that combining *Smilax*-derived compounds with standard chemotherapy could improve outcomes in AML.

Nevertheless, there are important limitations. This study used a crude root extract, whose composition can vary with preparation. Identifying the specific active constituents (saponins, flavonoids, tannins, *etc*.) is necessary for reproducibility and drug development. Future studies should focus on comprehensive phytochemical profiling and standardization of the extract to identify the major active constituents and improve the reproducibility and translational relevance of the findings. Future investigations will include testing of the extract in normal hematopoietic cells to evaluate therapeutic selectivity and safety. Additionally, mechanistic insights reported in the paper are primarily based on mRNA expression of apoptosis-related genes. Protein-level validation of key apoptotic markers such as Bax, Bcl-2, cleaved caspase-3, PARP, and cytochrome c, as well as functional assays including mitochondrial membrane potential and ROS measurement, were not performed. Therefore, the proposed involvement of the mitochondrial apoptotic pathway should be considered preliminary and requires further validation in future studies. Some isolated Smilax saponins showed little activity against HL-60 *in vitro*, highlighting that synergistic or selective effects may arise only from the complex phytochemical mixture. *In vivo*, the study utilized a xenograft model with a relatively small sample size (n = 6 per group) in immunodeficient mice, which lack an intact immune system and therefore may not fully recapitulate the complexity of human leukemia. Future studies using larger cohorts and more physiologically relevant models, such as syngeneic or patient-derived xenograft systems, will be necessary to better validate the therapeutic potential of the

extract. Toxicity and pharmacokinetics of the extract are also unaddressed here. Finally, while the present work showed suppression of migration/invasion-related factors, as in other studies, there was no direct measurement of leukemic cell dissemination. Future studies should investigate the effects of *S. china* on leukemia cell homing, angiogenesis, and interactions with the bone marrow microenvironment. In addition, evaluating potential synergistic effects with clinically relevant agents such as cytarabine or venetoclax would further enhance the translational relevance of these findings.

CONCLUSIONS

1. *Smilax china* L. root extract exhibited cytotoxic activity against HL-60 leukemia cells and reduced cell viability in a concentration-dependent manner.
2. Treatment with the extract promoted apoptotic cell death and induced cell-cycle arrest, suggesting the possible involvement of apoptosis-related pathways.
3. The extract was associated with reduced levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, indicating a potential anti-inflammatory effect that may contribute to its observed biological activity.
4. In addition, the extract decreased the migratory and invasive behavior of HL-60 cells, suggesting an effect on leukemia cell motility.
5. Overall, these findings suggest that *Smilax china* L. root extract may possess potential antileukemic properties. However, further studies involving phytochemical characterization, protein-level validation, and expanded *in vivo* investigations are required to confirm the underlying mechanisms and therapeutic relevance.

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Conflict of Interest

Authors declare that they have no conflict of interest.

Use of Artificial Intelligence

During the preparation of this manuscript, the authors used the AI tool ChatGPT to assist with language editing and structuring of text. The authors reviewed, revised, and verified all generated content and accept full responsibility for the final version of the manuscript.

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