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Research Article

Pinocembrin Suppresses Leukemic Cell Growth through CDK4 Downregulation and Caspase-8 Activation

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a blood cancer that arises from immature lymphoid progenitor cells. Current treatments often cause significant toxicity and long-term side effects. Pinocembrin, a natural flavanone found in plants and honey, has shown anticancer effects in various experimental models, but its impact on leukemic cells remains incompletely understood. This study investigated the antileukemic properties of pinocembrin and its potential mechanisms of action in the Jurkat and K562 cell lines. Viability of Jurkat and K562 cells was assessed using the trypan blue exclusion and XTT assays. Cell cycle and apoptotic activity were analyzed by flow cytometry. Interleukin-2 secretion was evaluated by ELISA, and protein expression was analyzed by Western blotting. Molecular docking was performed to explore potential protein–ligand interactions. Pinocembrin decreased cell viability in both leukemic cell lines, with effects depending on time and concentration; IC_{50} values were 175 μM for Jurkat cells and 122 μM for K562 cells after 48 h. In Jurkat cells, mechanistic studies revealed G0/G1 cell-cycle arrest and reduced CDK4 levels. Additionally, pinocembrin lowered interleukin-2 secretion, increased apoptotic cell populations, and elevated cleaved caspase-8 expression, indicating activation of the extrinsic apoptotic pathway. Molecular docking suggested a possible interaction with the Fas receptor. Collectively, these findings suggest that pinocembrin prevents growth and induces pro-apoptotic effects in leukemic cells *in vitro*. Further *in vivo* validation and pharmacological optimization are required to determine its therapeutic potential in ALL.

Keywords: Pinocembrin, Leukemic T cells, Extrinsic apoptosis, Caspase-8, Cell proliferation, Antileukemic activity

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Introduction

Acute lymphoblastic leukemia (ALL) is a blood cancer that develops from immature lymphoid cells. It is marked by the rapid proliferation of abnormal lymphoblasts, which gradually replace normal blood-forming cells in the bone marrow and can invade lymphoid and other organs outside the marrow. This leads to leukocytosis, anemia, and thrombocytopenia [1, 2]. Current therapeutic approaches include chemotherapy, radiotherapy, stem cell transplants, targeted therapy, and immunotherapy. However, these often cause significant side effects [3, 4]. Consequently, alternative approaches have attracted growing interest, especially plant-derived phytochemicals [5], which are used alongside conventional therapies to reduce adverse effects and to identify new therapeutic options.

Pinocembrin (5,7-dihydroxyflavanone) is a flavanone flavonoid (Figure 1) widely found in plants, herbs, vegetables, and fruits, with high concentrations in honey and propolis [6]. It occurs at approximately 1.60–1.85 mg per 100 g in honey [7]. Previous studies have demonstrated that this compound exerts various pharmacological effects, including anti-inflammatory, antifibrotic, antioxidant, antimicrobial, neuroprotective, and anticancer activities [8-10]. Studies have shown that pinocembrin has notable anticancer effects across multiple cancer types, including colorectal cancer cells (HCT116, HT29) [11, 12], ovarian cancer cells (SKOV3) [13], human retinoblastoma cells (Y-79) [14], lung cancer cells (A549) [15], melanoma cells (B16F10, A375) [16], and breast cancer cells (MCF-7, MDA-MB-231, SKBR3) [17]. Furthermore, research indicates that pinocembrin derived from *Alpinia galanga* induces mitochondrial dysfunction in HCT116 colon cancer cells, characterized by reduced mitochondrial membrane potential, cytochrome c release, and activation of caspase-9 and caspase-3, with minimal caspase-8 processing [12].

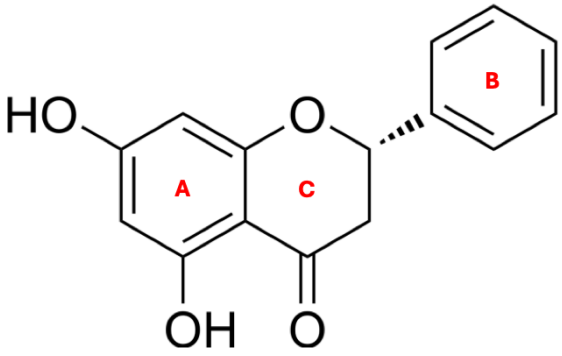

Figure 1 The chemical structure of pinocembrin. Pinocembrin (5,7-dihydroxyflavanone) is a naturally occurring flavanone ($C_{15}H_{12}O_4$) characterized by a 2-phenylchroman-4-one backbone. Its structure consists of the typical flavanone three-ring system: two aromatic rings (A and B) linked by a heterocyclic C ring. The A ring contains hydroxyl groups at the C-5 and C-7 positions, while the B ring is unsubstituted. The C ring contains a ketone group at C-4 and a saturated C2–C3 bond, generating a chiral center at C-2.

Apoptosis proceeds via two main signaling pathways: the intrinsic and extrinsic routes [18-20]. The intrinsic pathway is initiated by internal cellular signals and is controlled by Bcl-2 family proteins. It involves the mitochondria releasing proteins like cytochrome c (Cyt c) and apoptosis-inducing factor (AIF). Cytochrome c subsequently associates with apoptotic protease-activating factor 1 (APAF-1), initiating a sequence of downstream signaling activities.

In contrast, the extrinsic apoptosis pathway begins when external ligands bind to death receptors on the cell surface, such as the Fas receptor (CD95/Apo-1). Activation of the Fas receptor leads to the recruitment of the Fas ligand and the Fas-associated death domain (FADD). FADD then attracts pro-caspase-8 to form the death-inducing signaling complex (DISC), which activates caspase-8. Ultimately, both pathways result in caspase-3 activation, leading to programmed cell death [21-23].

Previous studies have shown that pinocembrin has anticancer effects in various tumor models; however, its biological impact and mechanisms in human leukemic T cells remain poorly understood. Therefore, this study aims to explore how pinocembrin affects acute leukemic T cells, particularly its influence on cell proliferation and apoptosis signaling pathways.

Materials and Methods

Cell Culture

Jurkat and K562 leukemia cells were obtained from ATCC (USA) and ANH Scientific Marketing (Thailand), respectively. Cells were maintained in RPMI-1640 medium containing L-glutamine and 10% FBS (HyClone, GE Healthcare Life Sciences, USA) at 37°C in a humidified 5% CO_2 incubator. Cultures were not maintained beyond 20 passages. Pinocembrin (Sigma-Aldrich, St. Louis, MO, USA; ≥95% purity) was prepared as a 5 mM stock solution using DMSO and kept at -20°C until further use.

Cell Viability Assay

The effect of pinocembrin on Jurkat and K562 cell viability was evaluated using the XTT assay (Abcam, Cambridge, UK). Cells were seeded at a density of 1×10^5 cells/mL and subsequently treated with different concentrations of pinocembrin (5–300 μM) for 48 h. After incubation, the XTT/PMS reagent was added following the manufacturer's instructions. The results were normalized to control cells treated with the vehicle. Additionally, for Jurkat cells, toxicity was confirmed using the trypan blue assay to count live and dead cells, validating the XTT assay results.

Cell Cycle Assay

The Propidium iodide/RNase staining kit (FxCycle™ PI/RNase Staining Solution; Invitrogen, Thermo Fisher Scientific, USA) was used for cell cycle analysis according to the manufacturer's instructions. Jurkat cells (1×10^5 cells/mL) were treated with pinocembrin at concentrations ranging from 25 to 275 μM for 24 or 48 h. Following treatment, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS; HyClone, GE Healthcare Life Sciences, USA), and fixed overnight in cold 70% ethanol at -20°C. Fixed cells were then stained with PI/RNase staining solution for 15 min at room temperature in the dark, and cell cycle distribution was subsequently analyzed using a DxFLEX flow cytometer (Beckman Coulter, USA).

Annexin V/PI Staining Assay

The extent of apoptosis was determined using an Annexin V-FITC/PI Apoptosis Detection Kit (ImmunoTools, Friesoythe, Germany). Jurkat cells (1×10^5 cells/mL) were treated with pinocembrin at concentrations ranging from 25 to 275 μM for 48 h. Cells were then harvested, washed twice with cold PBS, and resuspended in Annexin V binding buffer. Following incubation with Annexin V-FITC and propidium iodide for 15 min at room temperature in the absence of light, samples were analyzed on a BD FACSCelesta

flow cytometer (BD Biosciences, USA). Data acquisition and analysis were performed using FACSDiva software (BD Biosciences, USA).

Western Blot Analysis

Jurkat cells were seeded at 1×10^5 cells/mL and treated with pinocembrin at concentrations ranging from 25–275 μM for either 24 or 48 h. After treatment, cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS), and lysed on ice for 15 min using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a 1×protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Equal amounts of protein (10 μg) were resolved by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) prepared in TBST (Tris-buffered saline containing 0.1% Tween-20) and subsequently incubated overnight at 4°C with primary antibodies against CDK4 (#12790S, 1:3,000), p53 (#2527S, 1:1,000), phospho-p53 (#9284S, 1:1,000), cleaved caspase-8 (#9496S, 1:3,000), procaspase-9 (#9502S, 1:1,000), cleaved caspase-9 (#9502S, 1:1,000), Bax (#2774S, 1:1,000), Bcl-2 (#4223S, 1:3,000), and β-actin (#4970T, 1:10,000) (all antibodies obtained from Cell Signaling Technology, Danvers, MA, USA).

Membranes were washed three times with TBST, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Protein bands were then visualized with an enhanced chemiluminescence (ECL) detection substrate (Bio-Rad, Hercules, CA, USA) and captured with an Amersham ImageQuant 800 imaging system. Band intensities were analyzed using ImageJ software, and the target protein expression was normalized to β-actin.

Enzyme-Linked Immunosorbent Assay (ELISA)

Jurkat cells (1×10^5 cells/mL) were stimulated with PMA/PHA and then exposed to pinocembrin at concentrations between 6.25 μM and 175 μM for 24 h. After incubation, the culture media were collected, and the levels of IL-2 were quantified using an ELISA kit (BioLegend, San Diego, USA). Cytokine levels were then normalized to the total protein content of the viable cell pellets, measured using the BCA protein assay.

Molecular Docking

The 3D structure of pinocembrin (PubChem CID: 68071) was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The Fas receptor (CD95 or tumor necrosis factor receptor superfamily member 6; TNFRSF6) structure was obtained from the AlphaFold Protein Structure Database (AlphaFold prediction ID: AF-P25445-F1-v6). Molecular docking simulations were carried out using Swiss Dock (https://www.swissdock.ch/) [24, 25]. Docking results were evaluated based on binding affinity and the interaction profiles between pinocembrin and key amino acid residues within the Fas receptor. The most favorable ligand–receptor interactions were those with the lowest binding free energy (ΔG). Visualization of the docked complexes, including solid-surface models, was performed using UCSF Chimera version 1.19 (build 42556). Additionally, two-dimensional interaction diagrams were generated using BIOVIA Discovery Studio Visualizer version 25.1.0.24284 to further analyze ligand–protein interactions.

Differential gene expression in cancer and survival analysis

The GEPIA3 online tool (https://gepia3.bioinfoliu.com) was used to examine FAS expression across different tumors compared with normal tissues from the Genotype-Tissue Expression (GTEX) database. Additionally, GEPIA3 was used to assess how high versus low FAS expression affects patient survival in acute myeloid leukemia (LAML), with the high/low expression cutoff set at 50%.

Statistical Analysis

The data are shown as mean±SEM. Statistical analyses were performed using GraphPad Prism 10.6.1. Differences between groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. p values below 0.05 were deemed statistically significant.

Results

The Effect of Pinocembrin on the Viability of Leukemia cells

Treating Jurkat and K562 cells with the vehicle control (DMSO) had minimal effect on cell survival (data not shown). In the *in vitro* XTT assay, pinocembrin toxicity at 5 and 300 μM showed a dose-dependent decrease in cell survival over 48 h compared with the control (DMSO <1%). The IC_{50} values were 175.1 μM for Jurkat cells (95% CI: 170.3 to 180.0) and 121.9 μM for K562 cells (95% CI: 113.6 to 130.2), as shown in Figure 2.

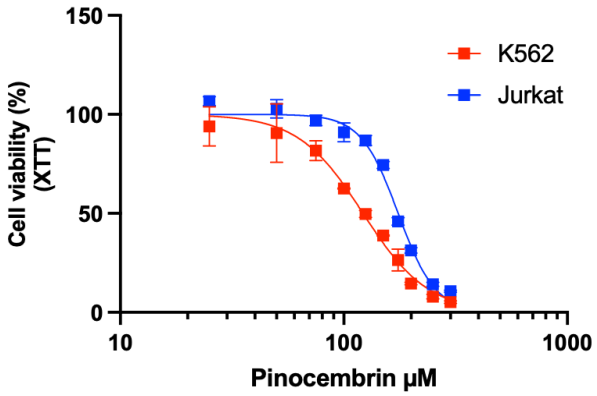

Figure 2 Toxicity of Pinocembrin in Jurkat and K562 cells. Dose-response showing cell viability after treatment with various concentrations of pinocembrin for 48 h, measured using the XTT assay. The IC_{50} for Jurkat is 175.1 μM (95% CI "170.3 to 180.0"), and for K562 is 121.9 μM (95% CI "113.6 to 130.2").

Pinocembrin toxicity in Jurkat cells was evaluated using the trypan blue exclusion assay (see Supplementary Figure 1). Treatments with 5 and 300 μM pinocembrin significantly decreased the total cell count at both 24 and 48 h. Additionally, the proportion of live cells significantly decreased following pinocembrin exposure at these time points.

After initial cytotoxicity testing in Jurkat and K562 cells, the compound showed activity in both, with a lower IC_{50} in K562 cells. Nonetheless, further mechanistic and functional studies were conducted primarily in Jurkat cells. This choice was driven not only by potency but also by biological relevance: Jurkat cells are a well-established T-cell acute lymphoblastic leukemia model featuring defined signaling pathways, apoptosis

mechanisms, and immune-related transcriptional programs aligned with the study's mechanistic goals. Conversely, K562 cells, originating from chronic myelogenous leukemia in blast crisis, have unique myeloid features and BCR-ABL–driven signaling, which could complicate pathway-specific interpretations. Consequently, Jurkat cells provided a more suitable, mechanistically interpretable system for subsequent analyses, despite their higher IC_{50} value.

The Effect of Pinocembrin on Cell Cycle in Jurkat Cells

We investigated the effect of pinocembrin on cell cycle progression using propidium iodide/RNase staining to analyze cell cycle distribution. Jurkat cells were treated with various concentrations of pinocembrin (75, 125, 175, and 225 μM) for 24 and 48 h. The findings revealed that pinocembrin significantly inhibited cell proliferation in a dose- and time-dependent manner and induced arrest at the G0/G1 phase after 24 h (Figures 3A–F) and 48 h (Figures 4A–F). Notably, at a lower concentration (75 μM), pinocembrin significantly caused G0/G1 arrest at 24 h (Figure 3F). Similarly, at 48 h, G0/G1 arrest was evident at 125 μM (Figure 4F). Overall, these results indicate that pinocembrin induces G0/G1 cell cycle arrest, thereby reducing Jurkat cell proliferation.

Furthermore, flow cytometry analysis revealed that pinocembrin markedly increased the number of apoptotic cells in a time- and concentration-dependent manner, as evidenced by more cells accumulating in the sub-G1 phase at 24 h (Figure 5A) and 48 h (Figure 5B).

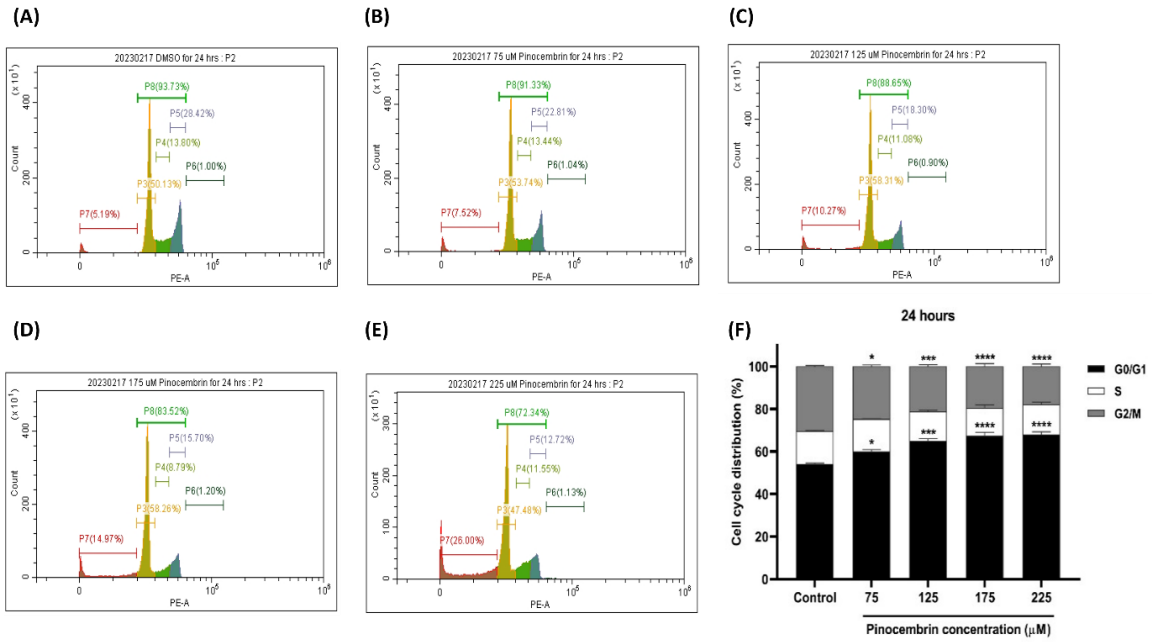

Figure 3 Cell cycle analysis with propidium iodide staining in Jurkat cells treated with pinocembrin. **(A-E)** Jurkat cells exposed to pinocembrin (75 μM to 225 μM) for 24 h were stained with propidium iodide/RNase solution and analyzed via flow cytometry. **(F)** Mean percentages of cell cycle phases (G0/G1, S, and G2/M) from three independent experiments. Statistical significance was determined using ANOVA with Tukey's *post hoc* test (* p <0.05, *** p <0.001, **** p <0.0001). Data are from at least three separate experiments.

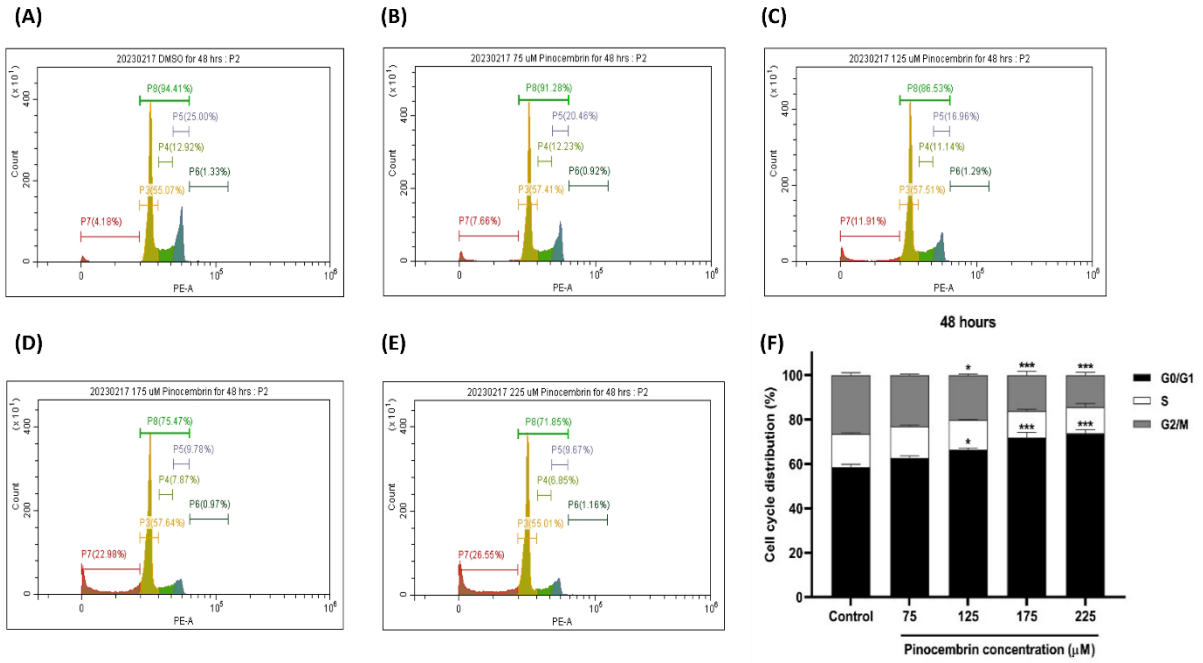

Figure 4 Pinocembrin alters cell cycle distribution in Jurkat cells. **(A–E)** Representative flow cytometric DNA histograms of Jurkat cells treated with pinocembrin (75–225 μM, 48 h) and stained with PI/RNase solution. **(F)** Quantification of the percentage of cells in G0/G1, S, and G2/M phases. Values represent the mean of three independent experiments. Statistical significance was assessed by one-way ANOVA with Tukey's post hoc test (* p <0.05, *** p <0.001 versus control).

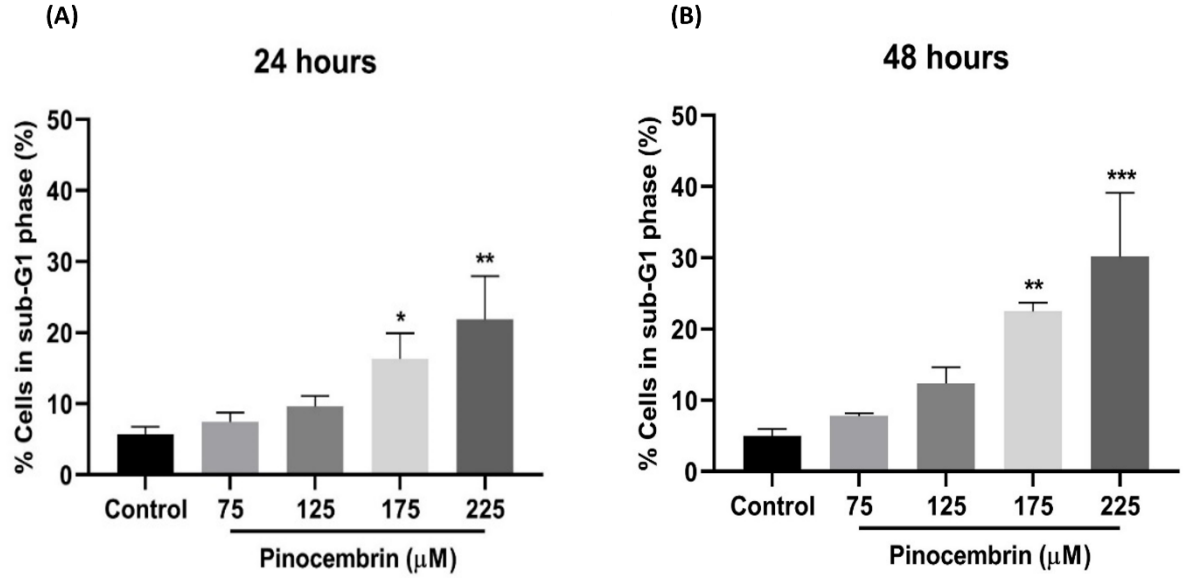

Figure 5 illustrates flow cytometry analysis of cell distribution in the sub-G1 phase in Jurkat cells treated with pinocembrin. It shows the average percentage of cells in the sub-G1 population relative to the total, based on three independent experiments, for Jurkat cells treated with pinocembrin (75 μM to 225 μM) for **(A)** 24 h and **(B)** 48 h. The statistical significance was determined using ANOVA followed by Tukey's *post hoc* test, with significance indicators: * p <0.05, ** p <0.01, *** p <0.001. Data were obtained from at least three separate experiments.

The Effect of Pinocembrin on Apoptosis Activation in Jurkat Cells

Using the previously established IC_{50} value, Jurkat cells were treated with pinocembrin at concentrations of 25, 75, 125, 175, and 225 μM over 48 h. Apoptosis was then evaluated through Annexin V/PI staining. The data revealed that pinocembrin induced apoptosis in a concentration-dependent manner (Figures 6A-F), with the percentage of apoptotic cells increasing significantly from below 10% in the control (DMSO) to nearly 80% at 225 μM (Figure 6G). These results demonstrate that pinocembrin can effectively trigger apoptosis in Jurkat cells.

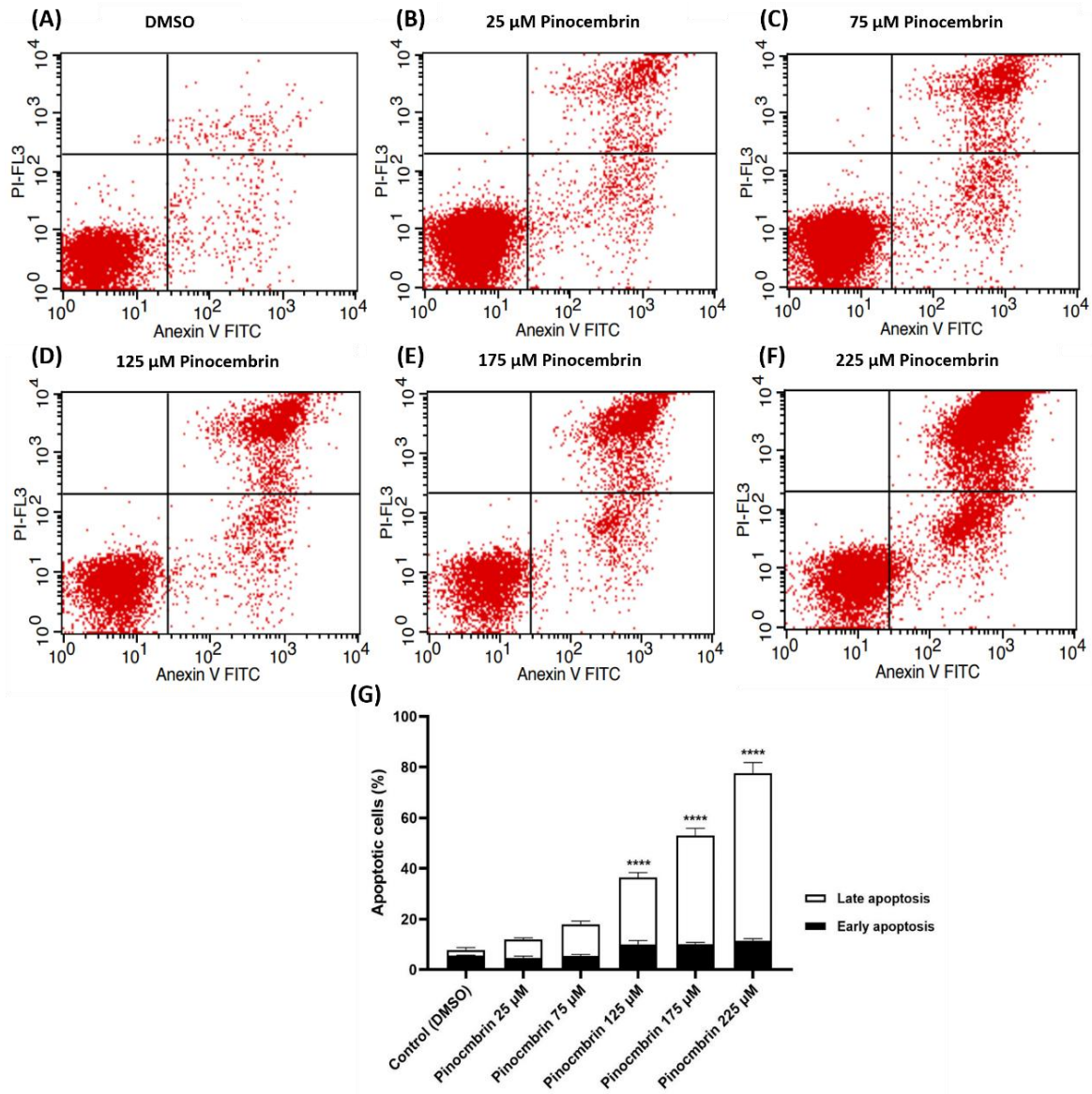

Figure 6 Assessment of pinocembrin-induced apoptosis in Jurkat cells by flow cytometry. **(A–F)** Representative Annexin V/propidium iodide (PI) staining profiles of Jurkat cells following 48 h exposure to pinocembrin (25–225 μM). **(G)** Quantification of apoptotic cells expressed as the mean±SEM from three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test, with treated groups compared to the untreated control (**** p <0.0001).

The Effect of Pinocembrin on IL-2 Secretion in Jurkat Cells

Cell proliferation in T cells is linked to cytokine production, particularly interleukin-2 (IL-2), which supports T cell growth. We therefore examined how pinocembrin affects IL-2 secretion. Jurkat cells were stimulated with PMA/PHA and then treated with pinocembrin at concentrations ranging from 6.25 μM to 175 μM for 24 h. IL-2 levels were measured by ELISA. The findings showed that pinocembrin markedly reduced IL-2 secretion in a dose-dependent manner (Figure 7).

The Effect of Pinocembrin on Protein Expression in Jurkat Cells

As previously noted, Pinocembrin inhibits cell proliferation by inducing G0/G1-phase cell-cycle arrest in Jurkat cells. Each cell cycle phase is controlled by specific cyclin-dependent kinases (CDKs), with CDK4 playing a key role in G1 by interacting with D-type cyclins. To explore this, we examined the impact of pinocembrin on cell cycle-related protein expression in Jurkat cells via Western blot analysis. Cells were treated with pinocembrin (25 μM to 275 μM) for 24 h. Results showed a dose-dependent decrease in CDK4 protein levels, especially evident in Figure 8B (Figures 8A-E). This indicates that pinocembrin may induce G1 arrest by inhibiting CDK4, thus blocking progression into the S phase and reducing cell proliferation. Notably, different Pinocembrin concentrations did not alter p53 protein expression or phosphorylation (Figures 8C-E), suggesting that pinocembrin does not activate the p53 pathway.

We also investigated how pinocembrin influences apoptosis-related protein expression in Jurkat cells (Figures 8A-G). Cells were exposed to 25 μM to 275 μM of pinocembrin for 48 h, and protein levels were analyzed using western blotting. The data demonstrated that pinocembrin notably increased cleaved caspase-8 levels in a dose-dependent manner (Figure 9B), suggesting activation of the extrinsic apoptosis pathway. Conversely, the levels of cleaved caspase-9, the pro-apoptotic protein band, and the anti-apoptotic protein Bcl-2 remained unchanged with pinocembrin treatment (Figures 8C, E, F). These results imply that pinocembrin likely does not trigger apoptosis through the intrinsic pathway. Full blots and individual repeats are available in Supplementary Figures 2-4.

Molecular Docking Analysis of Pinocembrin Binding to the Fas Receptor

As previously noted, pinocembrin markedly increased cleaved caspase-8 levels in a dose-dependent manner, signifying activation of the extrinsic apoptotic pathway. This pathway begins when extracellular ligands bind to cell-surface receptors such as the Fas receptor, leading to caspase-8 activation. To explore the molecular details further, molecular docking was conducted with the Fas receptor as the target and pinocembrin as the ligand. The binding free energy (ΔG) for this interaction was calculated at -6.3954 kcal/mol. Results showed that pinocembrin occupied the binding pocket and formed a conventional hydrogen bond (indicated by a green dashed line) with CYS129, a key contributor to binding specificity and stability.

Furthermore, CYS129 forms a π-sulfur interaction (yellow dashed line) with the ligand's aromatic ring, enhancing complex stability. A π-alkyl interaction (pink dashed line) is also seen between the ligand's aromatic ring and ARG128, offering extra hydrophobic support within the binding pocket (Figure 10A-C). Overall, these results suggest that the Fas-mediated extrinsic apoptotic pathway contributes to pinocembrin-induced apoptosis in leukemic T cells.

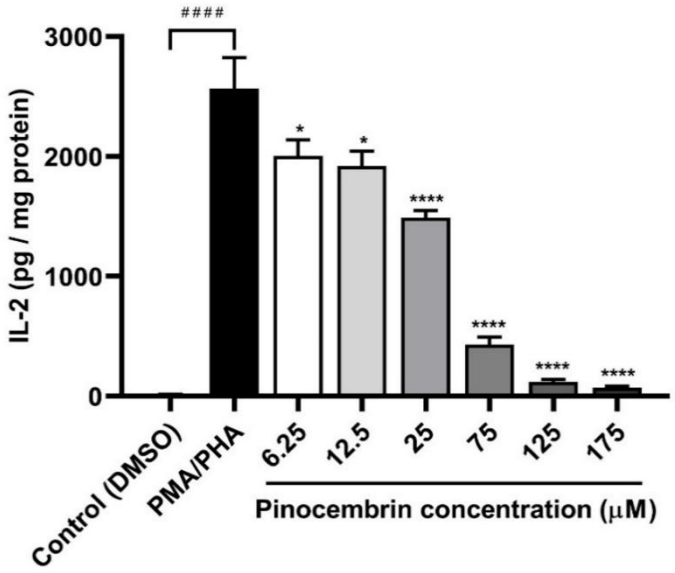

Figure 7 Effect of pinocembrin on IL-2 production in PMA/PHA-stimulated Jurkat cells. Cells were treated with pinocembrin (6.25–175 μM) for 24 h, and IL-2 secretion was quantified by ELISA. Values represent mean±SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc test* (* p <0.05, **** p <0.0001 versus PMA/PHA; #### p <0.0001 versus DMSO control).

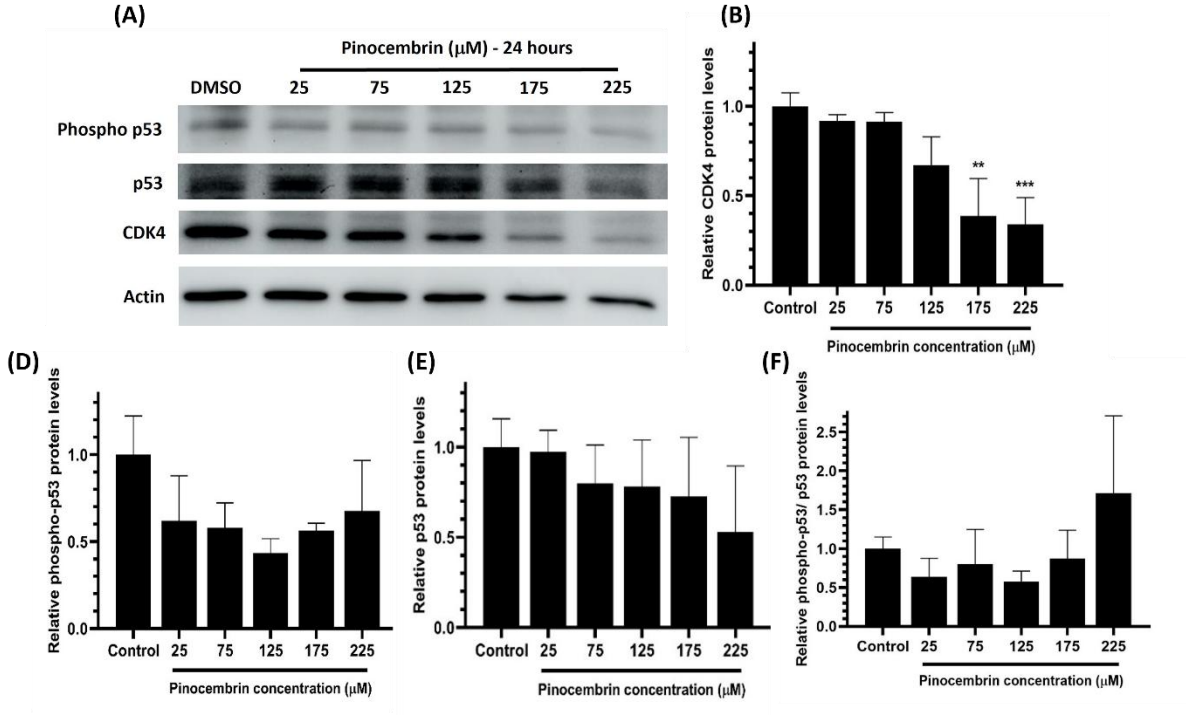

Figure 8 The effect of pinocembrin on the expression of proteins involved in cell cycle regulation in Jurkat cells. Jurkat cells were treated with pinocembrin (25 μM to 275 μM) for 24 h. The average relative protein levels from three independent experiments. Statistical analysis using ANOVA followed by Tukey's *post hoc* test for statistical significance compared with control (** p <0.01, *** p <0.001).

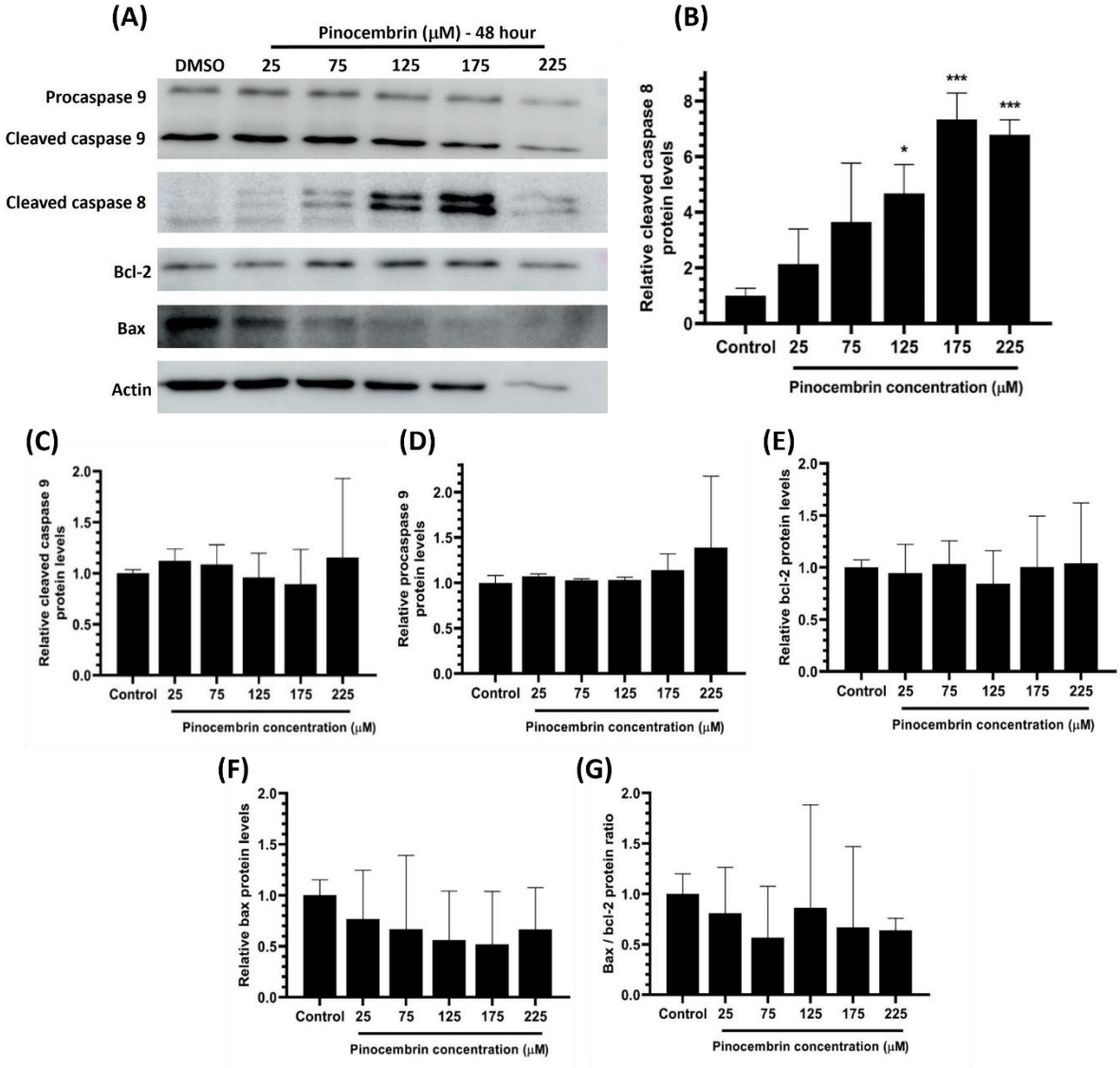

Figure 9 Effect of pinocembrin on the expression of apoptosis-associated proteins in Jurkat cells. Cells were treated with pinocembrin (25–275 μM) for 48 h, and protein expression levels were evaluated by western blotting. Quantitative densitometric analysis is presented as the mean±SEM from three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparison test, with * p <0.05 and *** p <0.001 relative to the untreated control.

Differential gene expression in cancer and survival analysis

Pan-cancer transcriptomic analysis identified distinct patterns of FAS expression among tumor types. Several cancers, including GBM, KIRC, KIRP, LAML, PAAD, and SARC, showed enrichment of FAS transcripts relative to normal tissue counterparts, whereas DLBC, KICH, LUAD, LUSC, OV, PCPG, UCEC, and UCS displayed reduced expression (Figure 11A). The strongest association was observed in acute myeloid leukemia (LAML), where FAS expression was substantially elevated compared with normal tissues (p <1.28×10^{-108}) (Figure 11B).

High levels of FAS expression do not seem to correlate with patient survival in LAML or DLBC (Figures 12A-B).

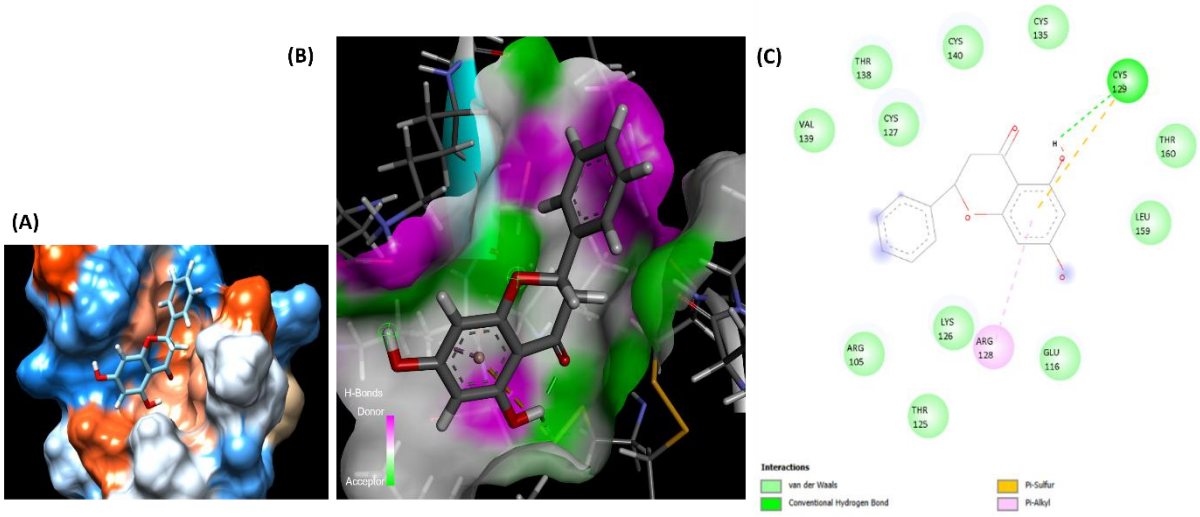

Figure 10 Docking analysis of pinocembrin binding to the Fas receptor. **(A)** Electrostatic surface model illustrating the predicted orientation of pinocembrin within the receptor binding cavity. **(B–C)** Three-dimensional and two-dimensional interaction profiles generated using BIOVIA Discovery Studio (v25.1.0.24284). The docked complex is stabilized through hydrogen-bond and π-sulfur interactions with CYS129, as well as a π-alkyl interaction with ARG128.

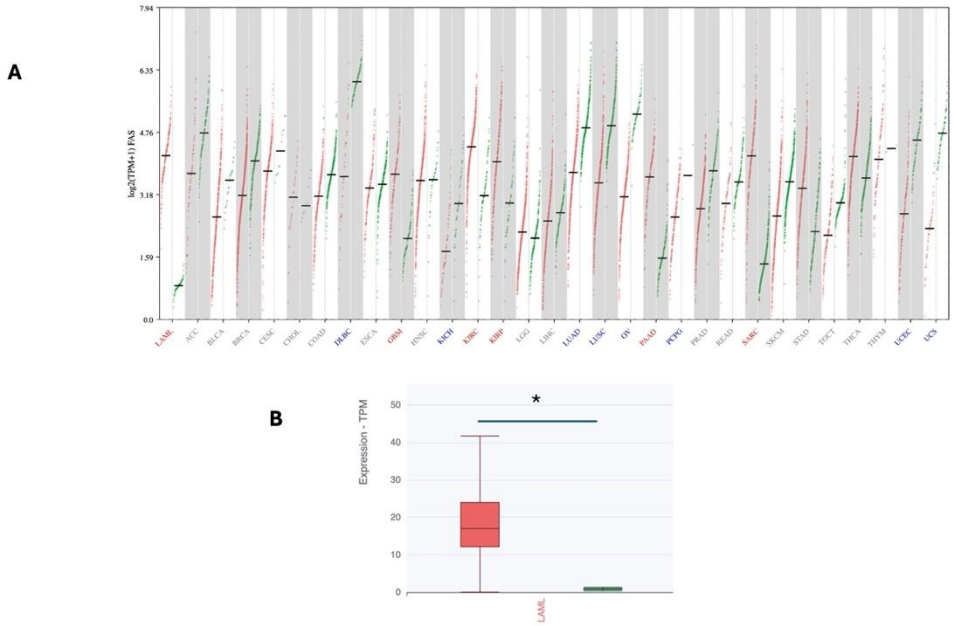

Figure 11 (A) Pan-cancer analysis of FAS expression across 31 tumor types relative to matched GTEx normal tissues. Increased expression was detected in GBM, KIRC, KIRP, LAML, PAAD, and SARC, whereas decreased expression was observed in DLBC, KICH, LUAD, LUSC, OV, PCPG, UCEC, and UCS. **(B)** Comparison of FAS transcript abundance (TPM) in LAML and matched normal tissues. Leukemia samples (n = 173) exhibited markedly higher expression (median 16.98 TPM) than normal controls (n = 70, median 0.20 TPM), consistent with significant FAS upregulation in LAML.

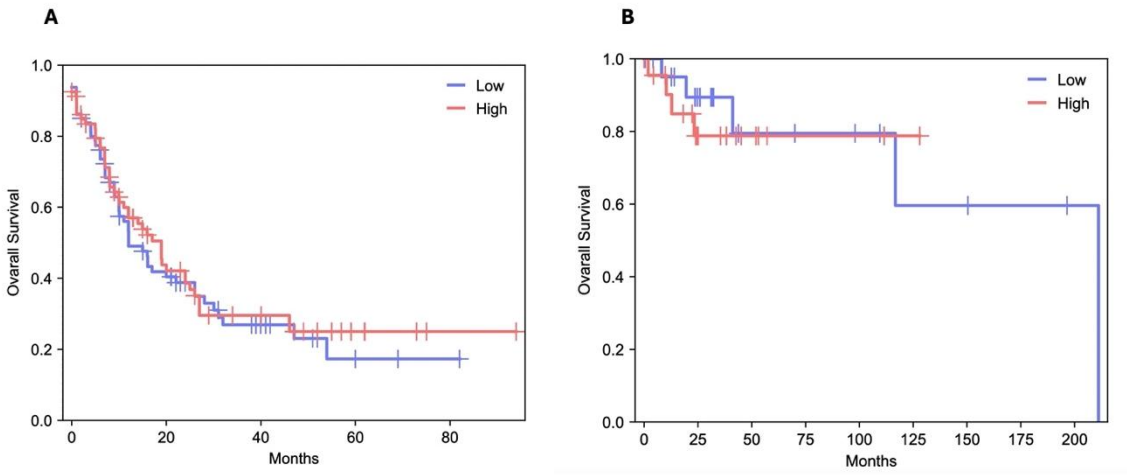

Figure 12 Kaplan–Meier survival analysis comparing overall survival in patients with high and low FAS expression. **(A)** In LAML, overall survival was similar between patients with high (red) and low (blue) FAS expression, indicating no clear association between FAS expression level and patient survival. **(B)** In Diffuse DLBC, overall survival was also comparable between high (red) and low (blue) FAS expression groups. Survival time is shown in months.

Discussion

In this study, pinocembrin showed notable cytotoxic effects on both Jurkat and K562 leukemia cells, with effects increasing over time and with concentration. A comparative analysis revealed activity in both cell lines, with K562 cells having a lower IC_{50} of 125 μM (95% CI 113.6 to 130.2) compared to Jurkat cells, which had an IC_{50} of 175 μM (95% CI 170.3 to 180.0) after 48 h. Subsequent mechanistic studies focused on Jurkat cells because they serve as a well-characterized T-cell leukemia model relevant to the signaling pathways examined. In Jurkat cells, pinocembrin significantly reduced cell numbers at 24 and 48 h, as reflected by decreased live-cell percentages and increased dead-cell percentages. The XTT assay confirmed a concentration-dependent decline in cell viability, aligning with microscopic observations of cytotoxicity.

These findings suggest that pinocembrin effectively reduces viability and induces death in leukemic cells across both lymphoid and myeloid lineages, with detailed mechanistic studies focusing on T-cell leukemia. Previous studies have also reported biological activities of pinocembrin extracted from Kaempferia pandurata, including cytotoxicity against murine leukemia P-388 cells (IC_{50} = 176.3 μM) and moderate antioxidant activity in DPPH assays (IC_{50} = 816 μM) [26]. In addition, pinocembrin did not exhibit significant toxicity in the human embryonic kidney cell line (HEK293) at concentrations below 300 μM, suggesting a degree of selectivity for cancer cells in this non-hematopoietic model [16, 27]. However, an important limitation of the present study is the absence of toxicity evaluation in normal hematopoietic cells, such as peripheral blood mononuclear cells (PBMCs), normal lymphocytes, or hematopoietic progenitor cells. Consequently, the hematological safety profile and therapeutic selectivity of pinocembrin cannot be fully assessed from the current data. Given the relatively high concentrations required to achieve cytotoxic effects in leukemic cells, future studies should include normal hematopoietic cell models to better define the therapeutic window and translational potential of pinocembrin as an anticancer agent.

Pinocembrin has been safely administered to healthy individuals in a double-blind, placebo-controlled trial at doses up to 150 mg per day. The study also investigated the pharmacokinetics of pinocembrin, with the maximum plasma concentration (C_{max}) reaching 2.46 μg/mL. Furthermore, the area under the curve (AUC) was 89.34 μg/mL/min, and the relationship was linear, indicating dose-proportional pharmacokinetics, where increases in drug exposure (as measured by C_{max} and AUC) are directly proportional to increases in dose [28]. These data suggest that plasma pinocembrin concentrations at the higher treatment doses are about 10 times lower than the observed IC_{50} in our study. While the effective concentrations of pinocembrin observed *in vitro* are unlikely to be directly achievable systemically, lower sub-IC_{50} doses may exert cumulative biological effects with prolonged exposure. Furthermore, advanced drug-delivery strategies such as nanoparticle encapsulation could enhance bioavailability, improve cellular uptake, and enable targeted delivery to leukemic cells [29, 30]. These approaches may substantially reduce the required therapeutic dose and improve translational potential.

The results showed that pinocembrin significantly inhibited Jurkat cell proliferation in both dose- and time-dependent manners and induced cell-cycle arrest at the G0/G1 phase at 24 and 48 h. Interestingly, G0/G1 arrest was observed even at a relatively low concentration (75 μM) after 24 h of treatment, suggesting that Jurkat cells are susceptible to pinocembrin's cell-cycle regulatory effects. Each phase of the cell cycle is regulated by specific cyclin-dependent kinases (CDKs), and CDK4 plays a crucial role in the G1 phase through its interaction with D-type cyclins. In this study, pinocembrin markedly reduced CDK4 protein expression in a dose-dependent manner. This downregulation likely contributes to G1 phase arrest by inhibiting CDK4 activity, thereby preventing progression into the S phase and ultimately suppressing Jurkat cell proliferation. These findings are consistent with previous observations in HepG2 hepatocellular carcinoma cells, in which pinocembrin also induced G1 phase arrest with minimal induction of cell death and downregulated cyclin D1, cyclin E, CDK4, and CDK6, key regulators of the G1-to-S phase transition [31].

The present findings demonstrate that pinocembrin effectively promotes apoptotic cell death in Jurkat leukemia cells. Both sub-G1 analysis and Annexin V/PI staining indicated a concentration-dependent increase in apoptosis, with the strongest effects observed following 48 h exposure. At concentrations between 125 and 225 μM, apoptosis increased markedly, approaching 80% of the cell population at 225 μM. These results agree with previous studies reporting pinocembrin-induced apoptosis in breast [17], ovarian [13], lung [15], prostate [32], melanoma [16], and colon cancer cell lines [12]. Taken together, the available evidence suggests that activation of apoptotic pathways is a broadly conserved response to pinocembrin treatment across multiple cancer types, including hematological malignancies.

Apoptotic signaling can be initiated through either death receptor-mediated mechanisms or mitochondrial stress pathways, which ultimately converge on the activation of executioner caspases and cellular dismantling [18, 19]. In the present study, pinocembrin treatment produced a concentration-dependent increase in cleaved caspase-8 expression, while no significant changes were observed in cleaved caspase-9, Bad, or Bcl-2 levels. This pattern suggests that apoptosis in Jurkat cells is predominantly mediated through activation of the extrinsic pathway rather than mitochondrial signaling. These findings are further supported by the molecular docking analysis, which predicted a favorable interaction between pinocembrin and the Fas receptor.

Interestingly, the apoptotic mechanism of pinocembrin appears to vary among cancer cell types. In HCT116 colon cancer cells, pinocembrin isolated from Alpinia galanga was reported to induce mitochondrial dysfunction, cytochrome c release, and subsequent activation of caspase-9 and caspase-3, with limited involvement of caspase-8 [12]. Similarly, studies in A549 lung cancer cells demonstrated increased apoptosis accompanied by elevated Bax, cleaved caspase-3, and cleaved caspase-9 expression together with reduced Bcl-2 levels [15]. Collectively, these observations suggest that while pinocembrin consistently promotes apoptotic cell death, the upstream signaling events may differ depending on cellular context. In Jurkat leukemia cells, our data indicate a greater contribution of death receptor-mediated signaling, whereas mitochondrial pathway activation appears to predominate in several solid tumor models.

IL-2 is a key cytokine involved in T-cell activation, proliferation, and survival. In the present study, pinocembrin significantly reduced IL-2 secretion in PMA/PHA-stimulated Jurkat cells, suggesting that its antiproliferative effects may be associated, at least in part, with suppression of cytokine-mediated growth signaling. This observation is consistent with the well-documented anti-inflammatory properties of pinocembrin. Previous studies have shown that pinocembrin attenuates the production of multiple inflammatory mediators, including TNF-α, IL-1β, nitric oxide, and prostaglandin E_2, in activated BV2 microglial cells through modulation of the PI3K/Akt/NF-κB signaling pathway [33]. Similar anti-inflammatory effects have also been reported in macrophage models, where pinocembrin reduced the expression of iNOS, COX-2, TNF-α, and IL-1β through inhibition of NF-κB activation and suppression of ERK and p38 MAPK signaling [34].

Pinocembrin is a major bioactive flavonoid present in honey and other natural products that are widely recognized for their antioxidant and immunomodulatory activities. Honey-derived flavonoids have been reported to reduce pro-inflammatory cytokine production, including TNF-α and IL-6, in several cancer models, potentially through regulation of NF-κB and MAPK signaling networks [35]. Given the central role of these pathways in T-cell activation and cytokine transcription, it is plausible that the reduction in IL-2 secretion observed in Jurkat cells may arise from a similar mechanism. Collectively, these findings suggest that, in addition to inducing apoptosis, pinocembrin may suppress leukemic cell growth by attenuating inflammatory and cytokine-dependent signaling pathways.

The results demonstrated that pinocembrin significantly increased cleaved caspase-8 expression in a dose-dependent manner, indicating activation of the extrinsic apoptotic pathway, initiated when extracellular ligands bind to cell-surface death receptors. To further support this mechanism, molecular docking analysis was performed using the Fas receptor as the target protein and pinocembrin as the ligand. The docking results revealed a favorable interaction, with a calculated binding free energy (ΔG) of -6.3954 kcal/mol, indicating a stable, energetically favorable interaction between pinocembrin and the Fas receptor. Pinocembrin occupied the Fas receptor binding pocket and engaged in multiple stabilizing interactions, including a conventional hydrogen bond with CYS129, which is likely critical for binding specificity. In addition, π-sulfur interactions with CYS129 and π-alkyl interactions with ARG128 further stabilized the protein-ligand complex, supported by extensive van der Waals contacts with surrounding residues.

The Fas receptor contains multiple extracellular cysteine-rich domains (CRDs) [36-38], including CRD1 (residues 56-82), CRD2 (85-127), and CRD3 (129-149) (UniProtKB: P25445). CRD2 and CRD3 are located within the extracellular region of the Fas receptor and primarily mediate ligand binding [39].

Docking analysis revealed that pinocembrin interacts predominantly with residues within or near the CRD2 domain, including ARG105, GLU116, THR125, LYS126, and CYS127, indicating that this region plays a major role in ligand binding. Additional interactions with CYS129 and CYS135 in the CRD3 domain, along with residues THR138, VAL139, CYS140, LEU159, and THR160, further stabilize the protein-ligand complex. Notably, CYS129's role as a key interacting residue highlights its potential role in Fas receptor activation and ligand recognition. The combined hydrogen-bonding and hydrophobic interactions observed in the docking analysis suggest that pinocembrin may interact with the Fas receptor, thereby promoting downstream caspase-8 cleavage and triggering extrinsic apoptosis, consistent with experimental findings.

LAML tumors exhibit significantly higher FAS receptor expression than matched normal tissue controls in the Genotype-Tissue Expression (GTEx) Project dataset. FAS is a central mediator of the extrinsic apoptotic pathway, where receptor engagement triggers caspase activation and programmed cell death. In contrast, Lymphoid DLBC shows downregulated FAS expression, suggesting a possible mechanism for apoptotic escape in this malignancy. Together, these findings highlight tumor-type-specific alterations in FAS signaling that may influence apoptotic susceptibility and immune–tumor interactions.

Notably, we show that Pinocembrin may interact with FAS and promote apoptosis in certain tumor cell types, suggesting a potential mechanism by which this compound may exert antitumor effects. Despite this, differential FAS expression does not significantly affect overall survival in LAML or DLBC, suggesting that FAS levels may not independently determine prognosis. However, the receptor could still be a therapeutic target through pharmacological activation of FAS-mediated apoptotic pathways.

Overall, the integration of biological assays and molecular docking analysis suggests that pinocembrin may promote apoptosis through the Fas-mediated extrinsic pathway. However, further experimental validation, such as receptor-binding assays or mutational studies targeting CYS129, would be necessary to confirm the Fas receptor's direct role in pinocembrin-induced apoptosis.

Conclusions

In conclusion, this study demonstrates that pinocembrin exerts anti-leukemic activity in both Jurkat and K562 models, with mechanistic interrogation conducted in Jurkat T-cell leukemia cells. Pinocembrin inhibited Jurkat cell proliferation in a time- and concentration-dependent manner, induced G0/G1 cell cycle arrest by downregulating cyclin-dependent kinase 4 (CDK4), and significantly suppressed interleukin-2 secretion, reinforcing its anti-proliferative effects. Importantly, pinocembrin robustly induced apoptosis, accompanied by increased cleaved caspase-8 expression, indicating activation of the extrinsic apoptotic pathway. Molecular docking further supported a plausible interaction between pinocembrin and the Fas receptor, providing mechanistic insight into Fas-mediated apoptotic signaling.

Collectively, these findings position pinocembrin as a multi-targeted modulator of leukemic T-cell survival, acting through coordinated cell-cycle inhibition and induction of apoptosis. Given its previously

reported selectivity for malignant over non-malignant cells, pinocembrin represents a promising scaffold for further therapeutic development in acute lymphoblastic leukemia. A limitation of this study is the lack of normal hematopoietic cells for evaluation. Therefore, although pinocembrin showed limited toxicity in HEK293 cells and demonstrated selective activity against leukemic cell lines, its effects on normal blood cells remain unknown and warrant investigation in future studies. Future investigations should prioritize *in vivo* validation, Fas activation (and trimerization), pharmacokinetic optimization, and targeted delivery strategies, such as nanoencapsulation, to compensate for the relatively high IC_{50}, enhance bioavailability, and maximize translational potential toward clinical application.

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