Effects of apigenin on the proliferation, migration, and apoptosis of human diffuse large B-cell lymphoma OCI-LY3 cells

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Keywords:	Xenograft tumor, Apoptosis, Migration, Proliferation, Diffuse large B-cell lymphoma, Apigenin
Abstract:	Objective: This study aimed to investigate the effects of apigenin on the proliferation, migration, and apoptosis of human diffuse large B-cell lymphoma (DLBCL) OCI-LY3 cells. Methods: OCI-LY3 cells were cultured in vitro and treated with varying concentrations of apigenin (20, 40, 80 μmol/L). The CCK-8 assay, Transwell assay, and flow cytometry were employed to detect the proliferation, migration, invasion, and apoptotic abilities of each cell group, respectively. Western blot was employed to measure the expression levels of apoptosis-related proteins (Bax, Bcl-2, Caspase-3). BALB/c mice were used to establish xenograft tumor models via subcutaneous injection of OCI-LY3 cells. Mice were randomly divided into a vehicle group, an apigenin group (10 mg/kg), and a cyclophosphamide group (20 mg/kg) to evaluate the effects of apigenin on tumor growth in vivo. Additionally, Ki-67 expression (a marker for tumor proliferation) was assessed by immunohistochemistry in tumor tissues. Results: Compared to the DMSO group, apigenin at 20, 40, and 80 μmol/L greatly reduced cell proliferation, migration, invasion, and Bcl-2 expression while increasing apoptosis rates and the abundance levels of Bax and cleaved Caspase-3. Apigenin also suppressed the growth of xenograft tumors and reduced Ki-67 expression in tumor tissues. Conclusion: Apigenin exerts anti-DLBCL effects both in vitro and in vivo, offering a novel therapeutic strategy for lymphoma treatment.

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Running title: Apigenin on OCI-LY3 cell functions

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Abstract

Objective: This study aimed to investigate the effects of apigenin on the proliferation, migration, and apoptosis of human diffuse large B-cell lymphoma (DLBCL) OCI-LY3 cells.

Methods: OCI-LY3 cells were cultured *in vitro* and treated with varying concentrations of apigenin (20, 40, 80 μmol/L). The CCK-8 assay, Transwell assay, and flow cytometry were employed to detect the proliferation, migration, invasion, and apoptotic abilities of each cell group, respectively. Western blot was employed to measure the expression levels of apoptosis-related proteins (Bax, Bcl-2, Caspase-3). BALB/c mice were used to establish xenograft tumor models via subcutaneous injection of OCI-LY3 cells. Mice were randomly divided into a vehicle group, an apigenin group (10 mg/kg), and a cyclophosphamide group (20 mg/kg) to evaluate the effects of apigenin on tumor growth *in vivo*. Additionally, Ki-67 expression (a marker for tumor proliferation) was assessed by immunohistochemistry in tumor tissues.

Results: Compared to the DMSO group, apigenin at 20, 40, and 80 μmol/L greatly reduced cell proliferation, migration, invasion, and Bcl-2 expression while increasing apoptosis rates and the abundance levels of Bax and cleaved Caspase-3. Apigenin also suppressed the growth of xenograft tumors and reduced Ki-67 expression in tumor tissues.

Conclusion: Apigenin exerts anti-DLBCL effects both *in vitro* and *in vivo*, offering a novel therapeutic strategy for lymphoma treatment.

Keywords: Apigenin; Diffuse large B-cell lymphoma; Proliferation; Migration; Apoptosis; Xenograft tumor

Introduction

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype of non-Hodgkin lymphoma, bearing the highest global burden of deaths related to lymphoma [1]. Patients commonly exhibit either progressive lymphadenopathy, extranodal disease, or both conditions, necessitating treatment [2]. DLBCL exhibits significant clinical and biological heterogeneity, with distinct genetic and molecular subtypes influencing its behavior and prognosis [3]. While rituximab-based chemoimmunotherapy (R-CHOP) has substantially improved survival rates [4], approximately 40% to 50% of DLBCL patients remain incurable after undergoing first-line treatment with R-CHOP [5]. These relapsed or refractory cases often have poor long-term prognoses, highlighting the urgent need for novel therapeutic strategies.

Natural compounds have gained attention in recent years for their low toxicity and broad biological activities, making them promising candidates for cancer therapy [6]. Among these, apigenin, a naturally occurring flavonoid found in various fruits, vegetables, and herbs, has demonstrated potent anti-cancer properties, including anti-proliferative, anti-migratory, and pro-apoptotic effects [7]. This compound induces cell growth arrest and apoptosis in various types of tumors through the modulation of several signaling pathways [8]. Further research has revealed that apigenin can target various signaling pathways crucial for cancer development and progression, including PI3K/Akt/mTOR, MAPK/ERK, JAK/STAT, NF-κB, and Wnt/β-catenin pathways [9]. Notably, apigenin has demonstrated significant anticancer effects in multiple cancer types, such as lung cancer [10], colorectal cancer [11], and ovarian cancer [12]. Additionally, in the treatment of breast cancer, apigenin has exhibited its unique anticancer mechanism by acting on tumor cells in a complex manner, directly inhibiting PI3K activity and simultaneously indirectly inhibiting Akt kinase activity, thereby effectively suppressing Akt function [13]. However, apigenin's specific roles and mechanisms in DLBCL remain insufficiently explored.

DLBCL poses unique therapeutic challenges due to its rapid proliferation, high invasive potential, and resistance to apoptotic signals. Targeting these features is critical for improving

therapeutic outcomes. Apigenin's low toxicity [14], natural origin, and multi-targeted actions provide a compelling case for its investigation as an alternative or adjunct therapy. It is worth noting that OCI-LY3 possesses highly aggressive, tumorigenic, and clinically drug-resistant characteristics and has been widely used in DLBCL research [15-17]. This study delved into the impacts of apigenin on the proliferation, migration, invasion, and apoptosis of DLBCL OCI-LY3 cells, while also assessing its anti-tumor efficacy *in vivo* through the utilization of a xenograft model in nude mice. By bridging the gap between *in vitro* and *in vivo* findings, this study sought to establish apigenin as a promising therapeutic candidate for DLBCL. The findings are expected to provide insights into the broader applicability of apigenin as a therapeutic agent, offering new avenues for the development of novel treatment options for DLBCL and potentially other cancers.

Materials and methods

Ethics statement

The animal experiments of this study were approved by the Laboratory Animal Ethics Committee of Second Hospital of Shanxi Medical University (approval number: 2021059) and were conducted in accordance with the National Institutes of Health Guide for the Management and Use of Laboratory Animals.

Cell culture and grouping

OCI-LY3 cells were purchased from ATCC (Manassas, VA) and cultured in DMEM (Gibco, Billings, MT, USA) added with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were kept at 37°C in a moist atmosphere with 5% CO_2. The medium was replaced daily, and cells were passaged at a 1:3 ratio when they arrived at 80% confluency. Logarithmic growth phase cells were used for subsequent experiments. Cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and treated with different concentrations of apigenin. The groups included a dimethyl sulfoxide (DMSO) group with an equal volume of DMSO (1 μL/mL), and apigenin-treated groups at concentrations of 20 μmol/L, 40

μmol/L, and 80 μmol/L (the final concentration of DMSO was the same as in the apigenin-treated groups, both at 0.1% v/v, in order to eliminate solvent interference). The incubation period for all groups was 24 hours.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was analyzed using MTT assay. OCI-LY3 cells were inoculated in 96-well plates at 5×10^3 cells/well, and were treated with apigenin [purity ≥ 99%, Sigma-Aldrich, St Louis, Missouri, USA, dissolved in DMSO (Sigma-Aldrich) at concentrations of 0, 5, 10, 20, 40, 80 μmol/L and stored at different concentrations and at -20°C] treatment, and the DMSO group was added with an equal volume of DMSO (1 μL/mL) and incubated at 37°C in a 5% CO_2 incubator for 24 hours. Subsequently, the cells were incubated with 15 μL of MTT reagent (5 mg/mL) at 37°C for 4 hours. DMSO was added to dissolve the dirty crystals. The optical density value (A) at 490 nm of each well was measured using a microplate reader (SpectraMAX Plus; Molecular Devices, Sunnyvale, CA, USA). The cell survival rate (%) = (A value of each concentration of apigenin group - A value of the blank group) / (A value of DMSO group - A value of the blank group) × 100% [15].

Transwell assay

For the invasion assay, Matrigel (BD Biosciences) was diluted and spread evenly in the upper chamber of Transwell (Corning), and then placed at 37°C for 4 hours and left to solidify. The OCI-LY3 cells of each group after different concentrations of apigenin treatment were collected, and the cell concentration was adjusted to 5×10^4 cells/mL after resuspension with serum-free medium, and 100 μL of cell suspension was added into the upper chamber of Transwell, and 800 μL of serum-containing medium was added into the lower chamber. After 24 hours of incubation, the upper chamber was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 10 minutes. Five fields of view were randomly selected under the light microscope, and the cells that crossed the membrane were counted and photographed. For the migration experiments, the steps were consistent with the invasion experiments, except that Matrigel coating was not used [18].

Flow cytometry

Cell apoptosis was detected according to the Annexin V/PI Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). After being exposed to various concentrations of apigenin or an equivalent volume of DMSO for 24 hours, the cells were collected. Approximately 5×10^5 cells were harvested from each group, washed with PBS three times and resuspended in binding buffer. To the cell suspension, 5 μL of Annexin V-FITC and 10 μL of PI were added. The mixture was incubated for 10 minutes at room temperature away from light, and then apoptosis was detected using the FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo software, with ≥ 10,000 cells analyzed [15].

Western blot

Total protein was extracted from each group of cells using RIPA lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Protein concentration was determined using the BCA protein assay kit (Invitrogen, USA). Equal amounts of total proteins were loaded onto 10% SDS-PAGE gels (Life Technology, USA), and after 1.5 hours of electrophoresis, constant pressure electrotransfer was performed to transfer the proteins onto PVDF membranes (Millipore, Billerica, MA, USA), which were then enclosed at room temperature in a buffered saline solution containing 0.1% Tween-20 and 5% skimmed milk in Tris-buffered saline (TBS) for 1 hour. Primary Rabbit polyclonal antibodies to Bax, Bcl-2, Cleaved caspase-3, and GAPDH (all in a diluted concentration of 1:1000, Cell Signaling Technology) were then added and the membrane was incubated at 4°C in the presence of the primary antibodies overnight at 4°C. The membranes underwent three washes with TBST buffer, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution: 1:5000, Cell Signaling Technology) for 1 hour at 37°C, followed by visualization of the membranes using enhanced chemiluminescence reagent (Millipore). Analysis was conducted using Image Pro Plus 6.0 software [15].

Animal experiments

A total of thirty-four-week-old male BALB/c nude mice were supplied by the Laboratory Animal Center of our University and housed in the laboratory animal centre of our hospital. All mice were allowed to eat and drink freely. OCI-LY3 cells were resuspended with 1× PBS. The cell suspension (1×10^7 cells) was injected subcutaneously at the upper inguinal margin of the mice, and when the tumour volume grew to about 50 mm^3, mice with tumors were assigned at random to the carrier group (Vehicle group), the cyclophosphamide group (CTX group), and the apigenin group (APG group), with 10 mice in each group. The CTX group was given 20 mg/kg cyclophosphamide intraperitoneal injection treatment, the APG group was given 10 mg/kg apigenin intraperitoneal injection treatment, and the Vehicle group was given an equal dose of 0.9% sodium chloride and 1% DMSO, and the drug intervention was carried out for 21 days. Tumor growth of the mice was observed, and the mice's tumor volume was measured once every 3 days. The tumor volume (mm^3) = $a^2 \times b/2$, where a is the smallest diameter, and b is the diameter perpendicular to a. The mice were executed by excess CO_2 after 21 days. The tumors were excised and weighed, and the tumor tissue was fixed in 4% paraformaldehyde for spare use.

Immunohistochemistry

Mouse tumor tissues underwent fixation with 4% paraformaldehyde, followed by embedding in paraffin. The tissues were then sliced into 4-micrometer-thick sections and mounted onto slides. Deparaffinization of the sections was carried out using xylene, followed by dehydration through a graded series of ethanol. To block endogenous peroxidase activity, the sections were incubated in 3% hydrogen peroxide for 15 minutes. Antigen retrieval was achieved by treating the sections with citrate buffer (pH 6.0) at 95°C for 15 minutes. Subsequently, the sections were incubated with normal goat serum at room temperature for 20 minutes to block nonspecific staining. Then, the primary antibody Ki-67 (diluted 1:500, purchased from Abcam) was incubated overnight at 4°C. Finally, the diaminobenzidine was used as the chromogen, with hematoxylin as the counterstain, and the sections were observed under an optical microscope [19].

Statistics

SPSS 22.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 9.4.0 (GraphPad Software, Inc., USA) were used for graphing and statistical analyses, and the results were expressed as mean ± standard deviation (mean ± SD). Statistical meaningful was determined using Tukey's post hoc test. $P < 0.05$ was considered statistically meaningful. All experiments in this study were repeated more than 3 times.

Results

Apigenin inhibits OCI-LY3 cell proliferation

Previous studies have shown the anti-tumor effects of apigenin in various cancers [20, 21]. This study investigated its effects on DLBCL. OCI-LY3 cells were exposed to apigenin at different concentrations for 24 and 48 hours, and the MTT assay was used to measure the cell viability. The results disclosed that compared with the control group, OCI-LY3 cell proliferation was inhibited by apigenin in a manner that was dependent on both concentration and time (Figure 1), with marked reductions observed at 40 μmol/L and 80 μmol/L. Based on these findings, 20, 40, and 80 μmol/L concentrations were selected for subsequent experiments.

Apigenin suppresses OCI-LY3 cell migration and invasion

It has been shown that apigenin inhibits colorectal and lung cancer cell migration and invasion [22, 23]. To investigate whether apigenin can affect OCI-LY3 cell metastasis, we performed cell migration and invasion assays using the Transwell assay. The results showed (Figure 2A-B) that apigenin at concentrations of 20, 40, and 80 μmol/L inhibited OCI-LY3 cell migration and invasion, and the number of cells migrating and invading decreased in a dose-dependent manner. To sum up, apigenin inhibited OCI-LY3 cell migration and invasion in a concentration-dependent manner.

Apigenin promotes OCI-LY3 cell apoptosis

Flow cytometry results (Figure 3A) revealed that compared with the DMSO group, the apoptosis rate of OCI-LY3 cells increased after the intervention of apigenin at concentrations of 20, 40, and 80 μmol/L, and was highest at 80 μmol/L ($P < 0.05$). Meanwhile, the results of Western blot

(Figure 3B) showed that compared with the DMSO group, the protein expression levels of Bax and Cleaved caspase-3 in OCI-LY3 cells were increased and Bcl-2 protein expression level was decreased after the intervention of apigenin at concentrations of 40 and 80 μmol/L. In conclusion, apigenin could promote apoptosis in OCI-LY3 cells, and the effect was best at 80 μmol/L.

Apigenin restrains the growth of xenografted DLBCL tumors in nude mice

Each mouse underwent the treatment procedures outlined in the methods section. Representative tumors in different groups of xenograft mice are shown in Figure 4A. The APG and CTX groups significantly reduced tumor volume and tumor mass compared to the Vehicle group (Figure 4B-C). Next, immunohistochemical staining was performed to detect Ki-67 levels in the tumors of mice in each group to assess tumor growth, and the results showed (Figure 4D) that the Ki-67 positivity of tumor tissues was reduced in both the APG and CTX groups compared to the Vehicle group. These findings indicate that apigenin can inhibit lymphoma growth *in vivo*.

Discussion

Although there is now a deeper understanding of DLBCL pathological subtypes and rituximab-based chemoimmunotherapy has proven effective, many patients experience reduced treatment efficacy due to the rapid development of drug resistance [24]. Consequently, the search for new drugs that can inhibit DLBCL continues. For instance, geniposide, an active compound derived from the traditional Chinese medicine "Zhizi," has proven its ability to restrain DLBCL cell proliferation and promote apoptosis [25]. Apigenin is gaining growing recognition as an agent for cancer chemoprevention [26]. This study systematically explored the anti-tumor effects of apigenin on DLBCL OCI-LY3 cells both *in vitro* and *in vivo*, revealing its potential as a novel curative agent.

A key finding of this study was the concentration-dependent inhibition of OCI-LY3 cell proliferation by apigenin. The MTT assay revealed noticeable reductions in cell viability at concentrations of 40 μmol/L and 80 μmol/L. These results align with previous studies on apigenin in solid tumors [10], suggesting its ability to interfere with cell cycle progression by modulating

cyclin-dependent kinases and other regulatory proteins [27]. In the context of DLBCL, where rapid proliferation is a hallmark of disease progression, apigenin's anti-proliferative effects are particularly relevant. In addition to inhibiting proliferation, apigenin markedly impaired the migration and invasion abilities of OCI-LY3 cells. Transwell assays revealed dose-dependent reductions in both migration and invasion, highlighting apigenin's potential to interfere with metastatic processes. This effect has been demonstrated in previous studies [28, 29]. These effects may be mediated through the downregulation of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9 [30], which are critical for extracellular matrix degradation and tumor cell dissemination. Given that DLBCL frequently involves extranodal metastases [31], the ability of apigenin to suppress metastatic behavior represents a promising avenue for future therapeutic development.

Apigenin's pro-apoptotic activity was another critical finding. Flow cytometry showed notable increases in apoptosis rates in OCI-LY3 cells following apigenin treatment, with the strongest effects observed at 80 μmol/L. Western blot analysis further confirmed the upregulation of pro-apoptotic proteins Bax and cleaved Caspase-3, along with the downregulation of the anti-apoptotic protein Bcl-2. These results suggest that apigenin promotes apoptosis via the intrinsic mitochondrial pathway, consistent with its reported mechanisms in other relative research [32, 33]. As resistance to apoptosis is a major obstacle in DLBCL treatment, apigenin's ability to overcome this resistance provides a compelling rationale for its inclusion in therapeutic regimens. What's more, the *in vivo* experiments further validated the anti-tumor efficacy of apigenin, which has been demonstrated in a prior study [34]. Mice treated with apigenin exhibited significantly reduced tumor volumes and weights. Immunohistochemical analysis revealed decreased Ki-67 expression, indicating reduced proliferative activity within the tumor tissues. These findings reinforce the translational potential of apigenin from *in vitro* observations to *in vivo* models, suggesting that it may serve as an effective therapeutic agent for DLBCL.

In conclusion, this study highlights the remarkable anti-tumor activity of apigenin in DLBCL,

demonstrating its ability to inhibit proliferation, migration, and invasion while inducing apoptosis of OCI-LY3 cells and suppressing tumor growth. These findings provide strong evidence supporting apigenin's capacity as a curative option for DLBCL and offer valuable insights into the mechanisms underlying apigenin's anti-lymphoma effects and its potential clinical applications. Future studies with larger sample sizes are essential to validate these findings and to establish the clinical relevance of apigenin in lymphoma therapy. By addressing these gaps, apigenin could become a valuable addition to the therapeutic arsenal against DLBCL.

Declaration

Funding

No funds, grants, or other support was received.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethics statement

The animal experiments of this study were approved by the Laboratory Animal Ethics Committee of Second Hospital of Shanxi Medical University and were conducted in accordance with the National Institutes of Health Guide for the Management and Use of Laboratory Animals.

Consent to participate

Not applicable

Code availability

Not applicable

Data Availability Statement

The experimental data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

Pengyan Wu finished the study design. Li E and Chen Wang finished the experimental studies. Jing Li finished the data analysis. Rong Wei finished the manuscript editing. All authors read and approved the final version of the manuscript.

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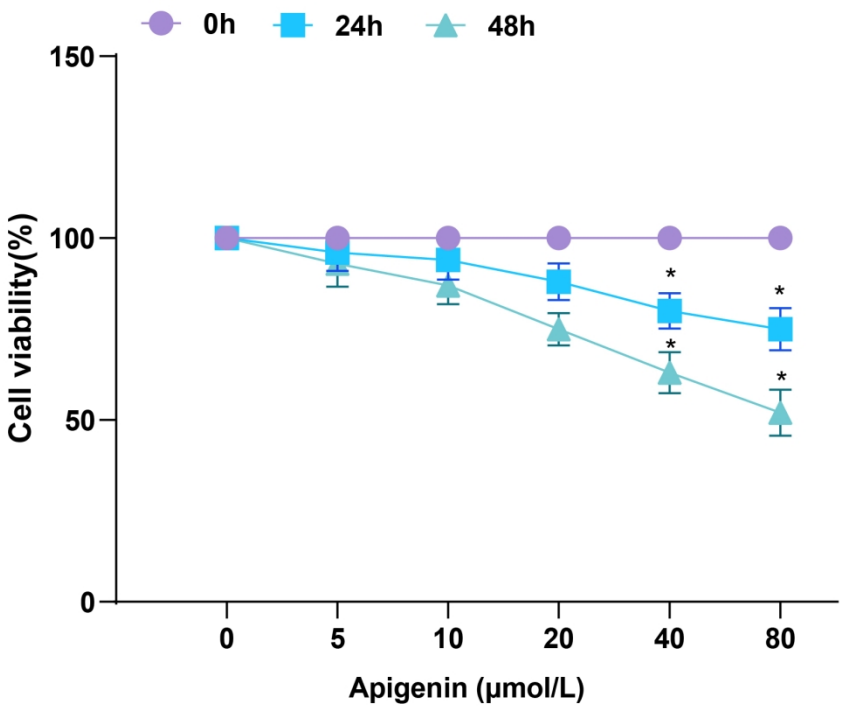
Legends

Figure 1 Apigenin inhibits OCI-LY3 cell proliferation. Cell proliferation was detected by MTT assay. *$P < 0.05$; cell experiments were all repeated 3 times.

Figure 2 Apigenin suppresses OCI-LY3 cell migration and invasion. A: Transwell assay to detect cell migration; B: Transwell assay to detect cell invasion. Comparisons among multiple groups were made by one-way ANOVA, followed by Tukey's post hoc tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; cellular experiments were all repeated 3 times.

Figure 3 Apigenin promotes OCI-LY3 cell apoptosis. A: Flow cytometry to detect OCI-LY3 cell apoptosis; B: Western blot to detect the protein expression levels of Bax, Bcl-2 and Cleaved caspase-3. Comparisons among multiple groups were made by one-way ANOVA, followed by Tukey's post hoc tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; cellular experiments were all repeated 3 times; DMSO, dimethyl sulfoxide.

Figure 4 *In vivo* effects of apigenin in OCI-LY3 cells derived mouse xenograft model. A: representative tumors of xenogenic-inhibited mice; B: tumor growth volume curves of mice in each group; C: tumor weight of mice in each group; D: immunohistochemical staining to detect the Ki-67 level of mouse tumor tissues. Red arrows indicate Ki-67 positive cells (dark brown); blue arrows indicate Ki-67 negative cells (light blue nuclei); APG, apigenin; CTX, cyclophosphamide; n = 10. Comparisons among multiple groups were made by one-way ANOVA, followed by Tukey's post hoc tests. ***$P < 0.001$.



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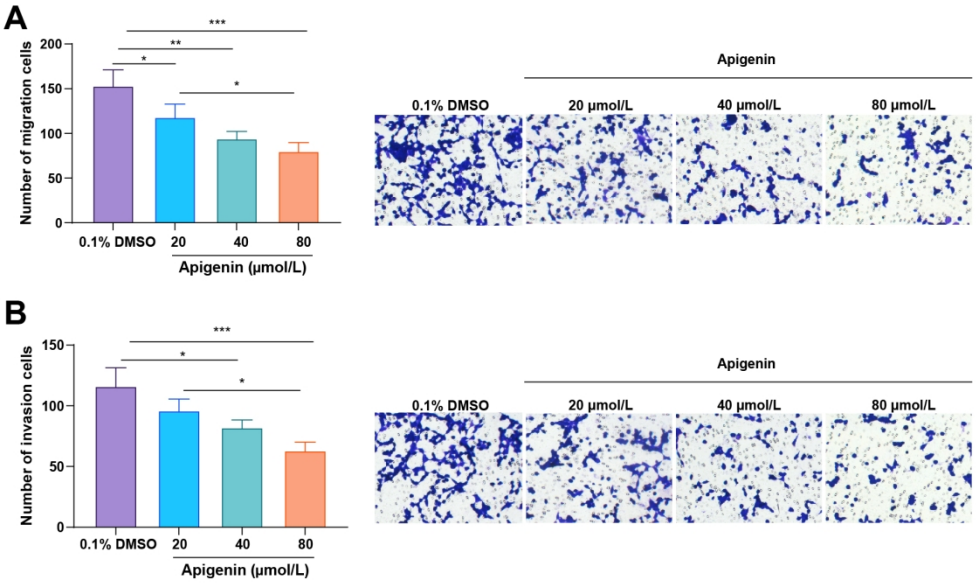

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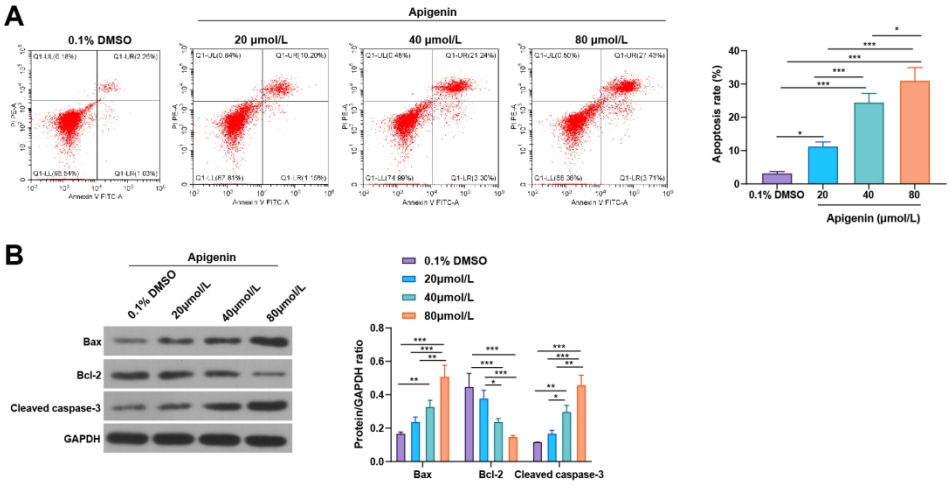

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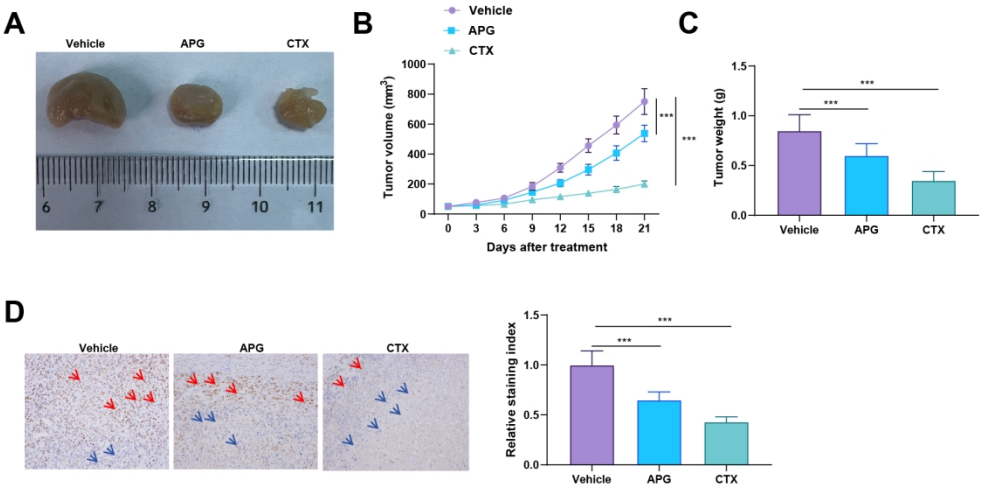

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