Research on the Anti-Tumor Mechanism of Artocarpus Lingnanensis Lectin

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Abstract: The purpose of this study was to investigate the mechanism of Artocarpus Lingnanensis Lectin (ALL) in inhibiting the growth of tumor cells EL-4. ALL was applied to mouse lymphoma cells EL-4, with PHA (100μg/mL) as a positive control drug. The toxicity of ALL (3.125, 6.25, 12.5, 25, 50, 100, 200μg/mL) on EL-4 cells was evaluated using the CCK-8 assay. Flow cytometry was used to assess the effect of ALL (25, 100 μg/ mL) on apoptosis and cell membrane potential of EL-4 cells. Western blotting was conducted to measure the protein expression levels of BAX, cleaved-Caspase-3, cleaved-Caspase-9, cleaved-PARP1. Fluorescence microscopy was employed to detect the concentration of free calcium ions. Results showed that compared to the control group, ALL had a significant inhibitory effect on EL-4 cells in a dose-dependent manner, with an IC50 value of 105.4μg/mL. Flow cytometry results indicated an increase in apoptosis rate of EL-4 cells with ALL treatment, leading to a higher proportion of depolarized mitochondrial membrane potential. Furthermore, the protein expression levels of BAX, cleaved-Caspase-3, cleaved-Caspase-9, and cleaved-PARP1 were significantly upregulated. In conclusion, ALL can promote apoptosis of EL-4 cells through the mitochondrial apoptosis pathway, inhibit their migration and invasion, and thereby exhibit anti-tumor effects.

***Keywords*:** Artocarpus Lingnanensis Lectin, EL-4 cells, proliferation, mitochondrial apoptosis

1. Introduction

Lectins are a class of non-immune proteins or glycoproteins that can specifically and reversibly bind to sugars, and have the functions of agglutinating cells and precipitating polysaccharides or glycoconjugates[1,2]. Recent studies have found that lectins have certain anti-cancer properties, as they can directly inhibit the proliferation of malignant tumor cells without affecting the growth of normal cells[3]. Lectins play important regulatory roles in the targeted recognition of specific tumor cells, as well as in signal regulation, division, immune defense, apoptosis, and migration of tumor cells[4,5]. Abnormal glycosylation is present in many tumor tissues, and based on the specific sugar-binding characteristics of plant lectins, nanoparticles carrying anti-cancer drugs can be connected to the surface of lectins, further targeting drugs to the surface of tumor cells to enhance their cytotoxicity and reduce side effects. Therefore, lectins have a broad prospect as a potential anti-cancer drug.

Artocarpus Lingnanensis Lectin (ALL) is a stable and highly agglutinating lectin purified from the seeds of Artocarpus lingnanensis, a subtropical plant in Guangxi[6]. Previous studies have shown that ALL can inhibit the proliferation of JurkatT lymphoma cells[7], indicating its anti-tumor effects. This study further reveals the anti-tumor mechanism of ALL.

2. Materials

2.1 Major Equipment

Microplate Reader (Thermo Scientific, model MULTISKAN Sky)

Flow Cytometer (BD, model LSR Fortessa)

Gel Imaging System (Bio-Rad, model Image Lab)

Fluorescence Inverted Microscope (LEICA, model DMi8)

2.2 Main Reagents

Freeze-dried Artocarpus Lingnanensis Lectin from the Department of Biochemistry and Molecular Biology, Guangxi Medical University, donated by Professor Zeng Qianyan

Mouse lymphoma cells EL-4 purchased from Wuhan Procell Biotechnology Co., Ltd.

RPMI1640 purchased from Hyclone (catalog number SH30809.01)

Fetal bovine serum purchased from Hyclone (catalog number SH30370.03)

CCK-8 reagent purchased from Biosharp (catalog number C0038)

Annexin V-FITC/PI assay kit purchased from BD (catalog number 556547)

JC-10 Mitochondrial Membrane Potential Kit(catalog number421902)

PAGE gel preparation kit purchased from Acmec (catalog number AP1200-50T)

Antibodies cleaved-Caspase-3, cleaved PARP1, β-actin purchased from Abcam (catalog numbers ab214430, ab32064, ab8226), cleaved-Caspase-9 purchased from Cell Signaling Technology (catalog number 9509T)

Horseradish Peroxidase-labeled Goat Anti-Rabbit IgG (H + L) purchased from Shanghai Biosharp Biotechnology Co., Ltd. (catalog number A0208)

3. Methods

3.1 CCK-8 Assay for Cell Toxicity

EL-4 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C with 5% CO2, and cells in the logarithmic growth phase were selected for the experiment. Cells were seeded in 96-well plates at 5×106 per well and treated with ALL at concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 μg/mL for 48 hours. CCK-8 solution (10μL) was added to each well, and the plates were incubated in a cell culture incubator for 4 hours. The absorbance (A) value was read at 450nm using a microplate reader, and the cell inhibition rate was calculated. The wells with medium and CCK-8 solution but no cells were used as blank controls, and wells with cells and CCK-8 solution but no drug were used as controls.

Cell inhibition rate (%) = (1 - experimental group OD value / control group OD value) × 100%

3.2 Flow Cytometry Assay for Apoptosis

EL-4 cells were seeded in a 6-well plate and treated with final concentrations of 25 μg/mL and 100 μg/mL of ALL for 48 hours, while the control group received no treatment. Cells were collected by centrifugation at 300g for 5 minutes, supernatant was discarded, and 1×105 cells were resuspended in 500 μL Binding Buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added to each sample and incubated in the dark at room temperature for 20 minutes. Apoptosis was then analyzed by flow cytometry within 1 hour.

3.3 Detection of Mitochondrial Transmembrane Potential Changes by JC-10

EL-4 cells were induced to undergo apoptosis with ALL at concentrations of 25μg/mL, 100 μg/mL, and PHA at 100 μg/mL, respectively. After induction, the cells were centrifuged at 300 g for 5 minutes, the supernatant was discarded, and the cells were collected. The cells were gently resuspended in PBS and counted. Cells resuspended at a concentration of 1-5 × 105. Prepare 1× JC-10 Assay Buffer and JC-10 staining working solution. The diluted 1× JC-10 Assay Buffer should be stored at 4°C. Add 500 μL of JC-10 staining working solution to resuspend the cells in the cell suspension. Incubate at 37°C in a 5% CO2 culture incubator for 15-20 minutes. Centrifuge at 300 g for 5 minutes, discard the supernatant, and wash the cells twice with 500 μL of pre-chilled 1× JC-10 Assay Buffer. Resuspend the cells in 500 μL of pre-chilled 1× JC-10 Assay Buffer. Store the samples on ice and perform dual-channel detection of FITC fluorescence and PI fluorescence using a flow cytometer within 30 minutes.

3.4 Measurement of Intracellular Calcium Ion Concentration

Cells were seeded and treated as in the “2.2” section, then collected for measurement. The calcium ion probe Fura-2AM was diluted to 1 mmol/L in DMSO, and cells in the treatment group were incubated with the calcium ion probe for 30 minutes. The intracellular free calcium ion concentration was observed under a fluorescence microscope. Results showed that treatment with ALL significantly increased the intracellular free calcium ion concentration in EL-4 cells.

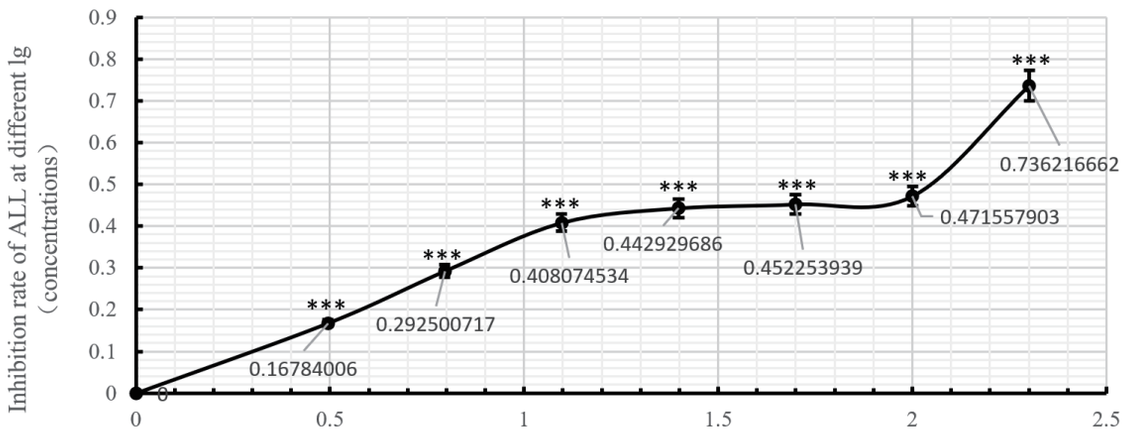
3.5 Western Blotting Experiment

Cells were seeded and treated as in the “2.2” section, then collected for protein extraction. Proteins were separated by 12.5% SDS-PAGE, transferred to PVDF membrane, and blocked with 5% skim milk at room temperature for 1.5 hours. Primary antibodies (BAX at 1:1000, cleaved-Caspase-3 at 1:500, cleaved-Caspase-9 at 1:1000, cleaved-PARP1 at 1:750, β-actin at 1:500) were added, and the membrane was incubated overnight at 4°C. After washing, the membrane was incubated with goat anti-rabbit secondary antibody (1:1000) at room temperature for 1 hour, followed by ECL development and imaging. Image J software was used for grayscale quantification, with β-actin as the internal control.

4. Results

4.1 CCK-8 Assay for Inhibition Rate of ALL on EL-4 Cells

Logarithmic values (lg) were used for concentrations of various groups: 0 (lg0), 0.495 (lg3.125), 0.796 (lg6.25), 1.097 (lg12.5), 1.398 (lg25), 1.699 (lg50), 2 (lg100), 2.301 (lg200). A scatter plot was generated with each concentration inhibition rate as the horizontal axis. As shown in Figure 1, compared to 0 μg/mL, all other concentrations exhibited a significant increase in inhibition rate (P<0.001). The IC50 value was calculated as 105.4 μg/mL using GraphPad.



***P<0.001 vs control group. N=6, biological raplicates

Figure 1. Effect of ALL on proliferation ability of EL-4 cells

4.2 Flow Cytometry Assay to Assess the Effect of Different Concentrations of ALL on EL-4 Apoptosis

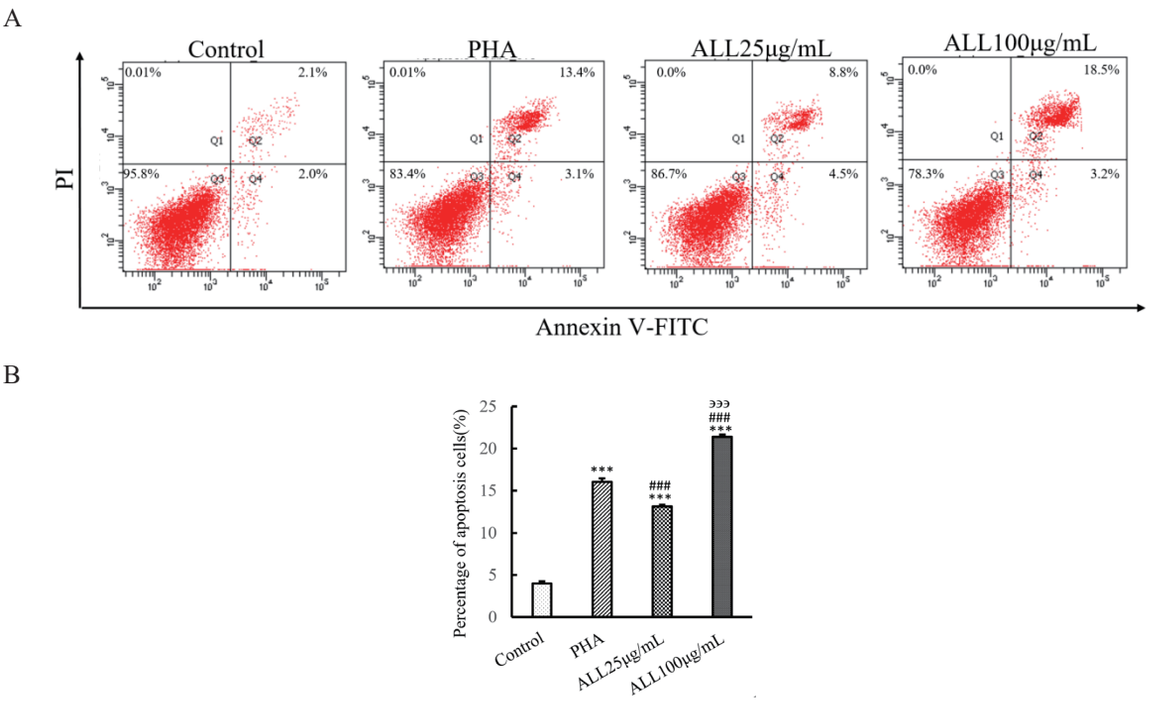
As shown in Figure 2, compared to the Control group, the early and late apoptosis totals for the PHA group, ALL 25 μg/mL group, and ALL 100 μg/mL group all significantly increased (P<0.001). The PHA group was significantly higher than the ALL 25 μg/mL group (P<0.001) but significantly lower than the ALL 100 μg/mL group (P<0.001), indicating a significant induction of EL-4 apoptosis by ALL. Furthermore, the high concentration of 100 μg/mL ALL had a superior effect on promoting EL-4 apoptosis compared to the positive drug PHA at 100 μg/mL.

4.3 Flow cytometry detected changes in mitochondrial transmembrane potential in different groups of cells using JC-10

Compared to the control group, the PHA group, ALL at 25 μg/mL group, and ALL at 100 μg/mL group showed a significant increase in the ratio of JC-10 monomer/aggregate fluorescence (P<0.01 or P<0.001). The ALL at 25 μg/mL group was significantly lower than the PHA group (P<0.05), while the ALL at 100 μg/mL group was significantly higher than the PHA group (P<0.05). The ALL at 100 μg/mL group was significantly higher than the ALL at 25 μg/mL group, suggesting that the high dose of 100 μg/mL of ALL promoted apoptosis in EL-4 cells more effectively than the low dose of ALL at 25 μg/mL, and even better than the positive drug PHA at 100 μg/mL. This indicates that ALL has a significant impact on the mitochondrial membrane potential of EL-4 cells, increasing the proportion of cells with depolarized mitochondrial membrane potential, a effect that is even stronger than that of PHA. (Figure 3)

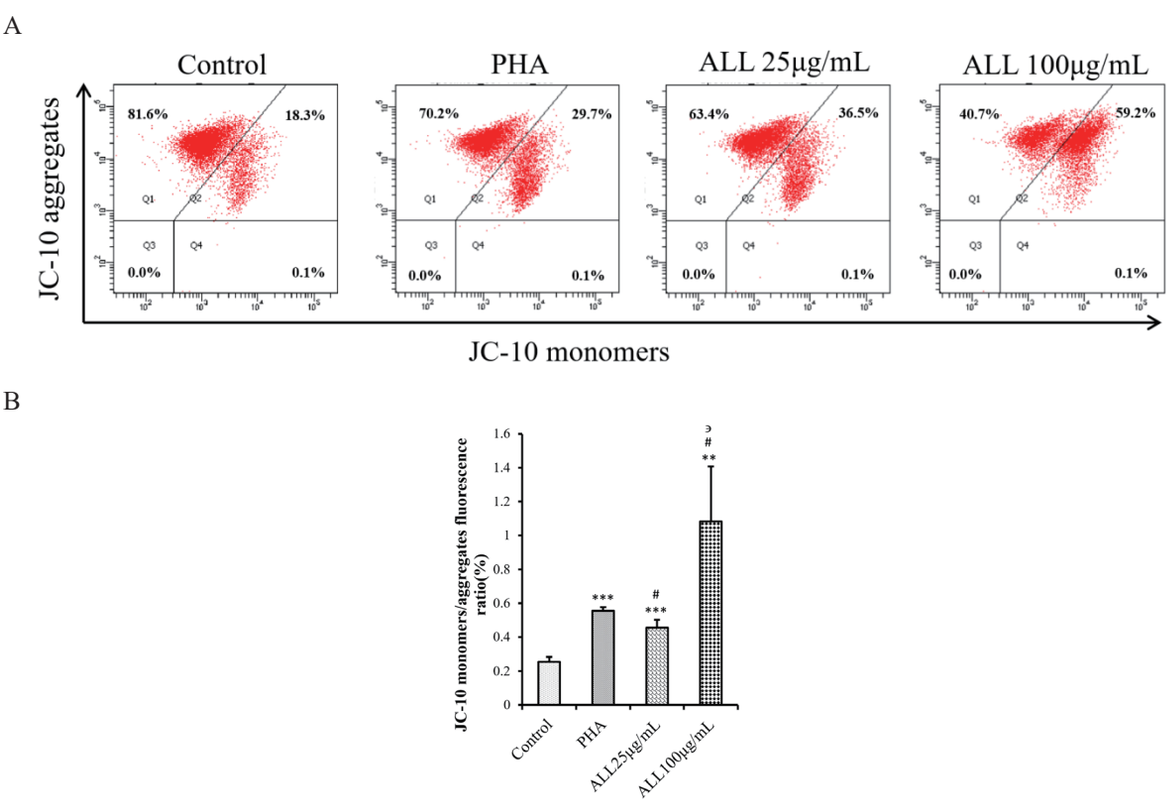
4.4 Detection of Intracellular Calcium Ion Concentration Discrepancy Using Fura-2 AM

As shown in Figure 4, compared to the Control group, the average intracellular free Ca^{2+} concentration in the PHA group, ALL 25μg/mL group, and ALL 100μg/mL group all significantly increased (P<0.001). The PHA group was significantly higher than the ALL 25μg/mL group (P<0.001) but significantly lower than the ALL 100μg/mL group (P<0.001), indicating a significant increase in the intracellular free calcium ion concentration of EL-4 cells under the influence of ALL. This may be related to the initiation of a signaling cascade by ALL, and the high dose of 100μg/mL of ALL resulted in a stronger elevation of intracellular free calcium ion concentration in EL-4 cells compared to the positive drug PHA at 100μg/mL.



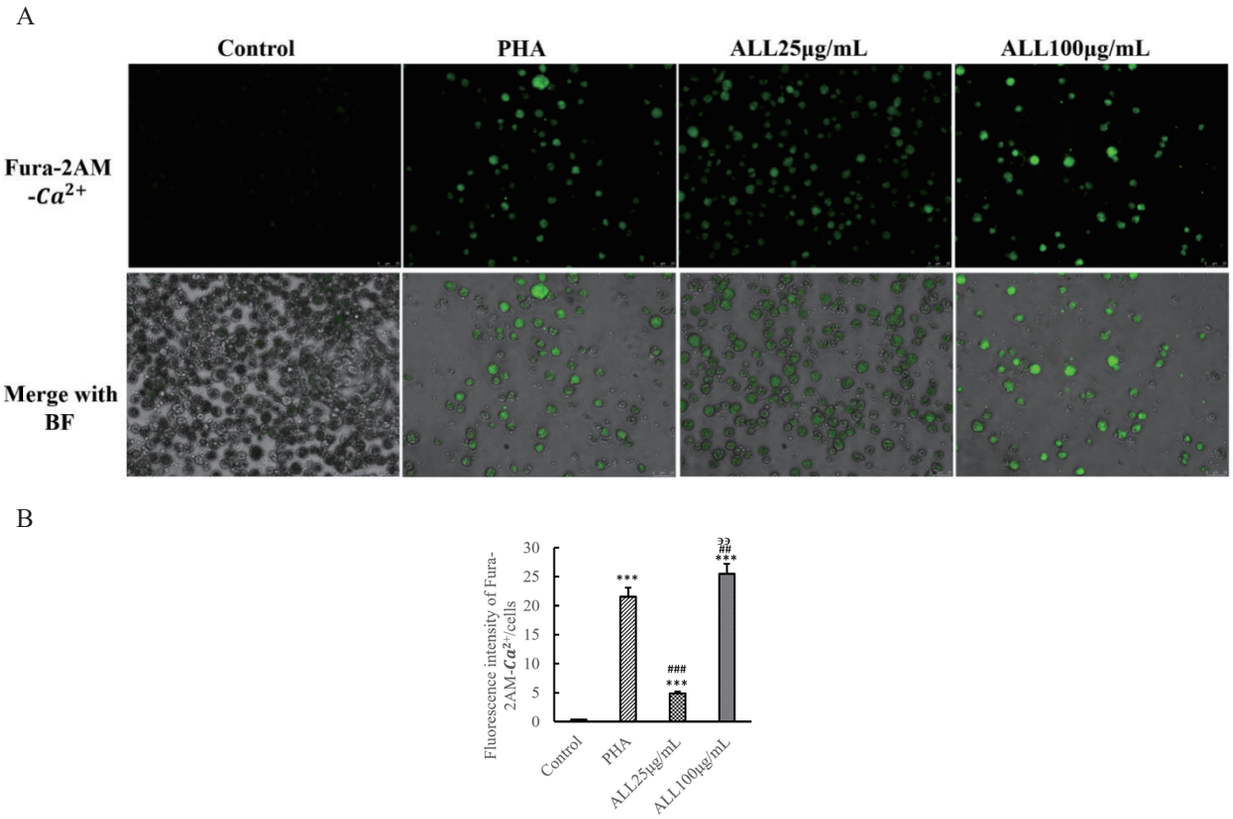
A: Flow cytometry assay to detect the apoptosis rate of each group of cells. B: Column chart of the apoptosis rate of each group of cells. Statistical significances were calculated via Student's t-test. ***P<0.001 compared with control, ###P<0.001 compared with PHA, ‴P<0.001compared with ALL 25μg/mL. N=3, biological raplicates.

Figure 2. Flow cytometry assay for detecting cellular apoptosis



A: Flow cytometry assay to detect the apoptosis rate of each group of cells. B: Column chart of the apoptosis rate of each group of cells. Statistical significances were calculated via Student's t-test. ***P<0.001 compared with control. #P<0.05 compared with PHA. ʾP<0.05 compared with ALL 25μg/mL. N=3, biological raplicates.

Figure 3. Flow cytometry detects the membrane potential of each cell population



A: Fura-2AM detect the free calcium ion concentrations of cells in each group. B: Column chart of calcium ion concentrations in cells of each group. Statistical significances were calculated via Student's t-test. ***P<0.001 compared with control. ###P<0.001, ##P<0.001 compared with PHA. *P<0.05 compared with ALL 25μg/mL. N=5, biological raplicates.

Figure 4. Detection of intracellular calcium ion concentration using calcium ion probe

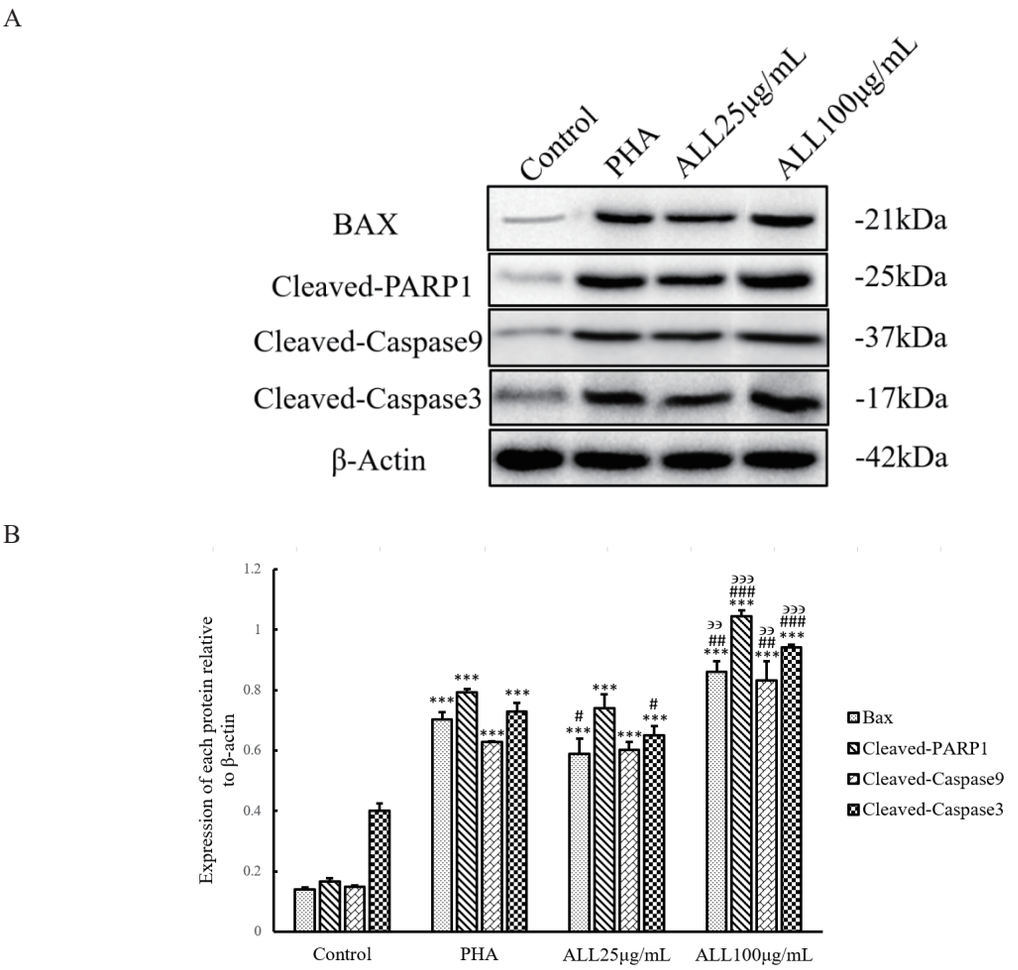
4.5 Western Blot Analysis of Apoptosis-Related Proteins in Caspase Pathway

As shown in Figure 5, compared with the Control group, the protein expression levels of BAX, Cleaved-Caspase-3, Cleaved-Caspase-9, and Cleaved-PARP1 were significantly increased in the PHA group, ALL 25μg/mL group, and ALL 100μg/mL group (P<0.001). When comparing with the PHA group, the expression levels of BAX and Cleaved-Caspase-3 in the ALL 25μg/mL group significantly decreased (P<0.05), while the levels of BAX and Cleaved-Caspase-3 in the ALL 100μg/mL group significantly increased (P<0.001). The levels of Cleaved-PARP1 and Cleaved-Caspase-3 were markedly elevated (P<0.001). When comparing between the ALL 25μg/mL group and the ALL 100μg/mL group, the levels of BAX and Cleaved-Caspase-9 in the ALL 100μg/mL group significantly increased (P<0.01), and Cleaved-PARP1 and Cleaved-Caspase-3 levels were highly elevated (P<0.001). The results suggest that ALL can increase the pro-apoptotic protein BAX on the mitochondrial membrane, activate the Caspase cascade amplification pathway, and the high concentration of 100μg/mL ALL activates the Caspase pathway more strongly than the positive control drug PHA at 100μg/mL.

5. Discussion

The cell membrane on the cell surface is composed of various glycoproteins, glycolipids, and polysaccharides, surrounded by oligosaccharide molecules. These glycoproteins play a crucial role in cell adhesion, nutrient absorption, and the reaction of cell membrane receptors to various active substances inside and outside the body. The composition of glycoproteins on the cell membrane surface varies depending on cell type, stage, or functional status, and changes occur in lectin receptors, i.e., changes in the surface glycoproteins and glycan structures during cell division, maturation, differentiation, and even malignant cell transformation[8].

Cancer is a genetic disease; gene mutations can lead to alterations in normal cell signaling pathways or indirectly reduce the cell's dependency on other cell signals, thereby affecting the normal physiological activity of cells[9]. Compared to their phenotypically normal neighbor cells, cancer cells can over-survive, grow excessively, and divide excessively, even usurping nutrients needed by other cells, ultimately forming tumor masses and endangering the patient's life[10]. Cancer cells exhibit



A: Electrophoresis image showing the expression of apoptosis-related proteins in cells of each group. B: Column chart of apoptosis-related protein expression in EL-4 cells. Statistical significances were calculated via Student's t-test. ***P<0.001 compared with control. ###P<0.001, ##P<0.01, #P<0.05, compared with PHA. ‴P<0.001, ″P<0.01, compared with ALL 25μg/mL. N=5, biological raplicates.

Figure 5.Western blot analysis of the effect of ALL on apoptosis-related protein expression in EL-4 cells

two heritable characteristics: 1) ignoring normal constraints on proliferation, 2) invading and colonizing territories reserved for other cells[11]. It is the combined operation of these two characteristics that poses a lethal risk to the body.

The glycocalyx layer on the cell membrane protects the cell surface from mechanical and chemical damage. Sugars on the cell surface not only protect and lubricate the cells but also play an essential role in the recognition and adhesion between cells[12]. Similar to how many proteins can recognize and bind to specific sites on another protein, certain proteins (such as lectin families) can specifically identify particular oligosaccharide side chains and bind to them. While the oligosaccharide side chains of glycoproteins and glycolipids are short, they exhibit immense diversity[13].

Current research indicates that the abnormal behavior of cancer cells is closely related to the expression of cancer proteins caused by mutated genes, especially those in the glycocalyx layer[14,15]. These mutated cancer proteins alter the types and quantities of sugars in the glycocalyx layer of cancer cells in many unknown ways. In conclusion, this variation in glycoproteins in the glycocalyx layer provides space for the specific sugar-binding ability of plant lectins, making it possible for plant lectins to target specific glycoproteins for recognition.

Due to the unique glycosylation processes of tumor cells compared to normal cells, based on the specific recognition ability of plant lectins for tumor cells, they play a crucial role in anti-tumor therapy or tumor recognition. Different types of plant lectins can selectively recognize various types of tumor cells and exert their unique anti-cancer effects. This exceptional recognition ability is due to the specific hydrogen and hydroxyl bonding of specific carbon atoms and glycosidic chains of plant lectins with monosaccharides or oligosaccharides on specific tumor cells[16]. Plant lectins can also mediate various biological effects of tumor cells by binding to tumor cell-related sugar receptors via signaling pathways. Additionally, based on the recognition ability of plant lectins to polysaccharide structures, they can distinguish between normal cells and

malignant tumor cells with abnormal glycosylation[17].

Cell apoptosis is a vital biological process in cancer development and tissue homeostasis, as well as a crucial indicator for evaluating the effectiveness of anti-cancer drugs[18,19]. Bax is an essential component of the mitochondrial apoptosis signaling pathway. When cells are stimulated with apoptosis signals, the expression of Bax increases, promoting the release of cytochrome C. Released cytochrome C binds with ATP, leading to the activation of initiator Caspase-9 and effector Caspase-3 in the execution phase of cell apoptosis[20,21]. Activated Caspase-3 cleaves PARP, causing loss of its DNA repair function, ultimately leading to DNA degradation between nucleosomes and cell apoptosis[22]. The analysis through flow cytometry in this study indicates that the enhanced toxicity of ALL on EL-4 cells is related to the induction of apoptosis. The results of Western blotting show that following ALL treatment, the expressions of Caspase-3, Caspase-9, BAX, and PARP significantly increased in EL-4 cells. The protein expressions of cleaved-Caspase-3, cleaved-Caspase-9, Bax, and cleaved-PARP also significantly increased after ALL treatment in cells. These results suggest that ALL may induce apoptosis in mouse lymphoma cells through the mitochondrial apoptosis pathway.

ALL exhibits a significant anti-tumor effect in mouse lymphoma, promoting cell apoptosis. ALL markedly upregulates the gene and protein expression levels of Caspase-3, Caspase-9, BAX, and PARP, indicating that its pro-apoptotic mechanism may be related to the mitochondrial apoptosis pathway. This study analyzed the effects of ALL on the apparent indicators of mouse lymphoma cells, providing a new source for developing anti-lymphoma protein drugs. However, the relevant mechanisms require further in-depth research.

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