• Basic Research •

Effect of *Andrographis paniculata* polysaccharide on human retinoblastoma Y79 cell proliferation and apoptosis

Bing Xu[1,4], Lei Li[2], Wei Zhang[1], Yue Li[3], Mao-Ren Wang[4], Jing-Chen Liu[4], Kai-Ye Dong[2], Ido Didi Fabian[5], Dong Qiu[4], Cai-Rui Li[2], Yi-Min Xiang[1]

[1]Department of Ophthalmology, Fuling Central Hospital, Chongqing 408000, China
[2]Department of Ophthalmology, the First Affiliated Hospital, Dali University, Dali 671000, Yunnan Province, China
[3]Department of Pathology, Fuling Central Hospital, Chongqing 408000, China
[4]Clinical Medicine, Dali University, Dali 671000, Yunnan Province, China
[5]Goldschleger Eye Institute, Sheba Medical Center, Tel Hashomer, Tel-Aviv University, Tel-Aviv 52621, Israel
Co-first authors: Bing Xu, Lei Li, and Wei Zhang
Correspondence to: Cai-Rui Li. Department of Ophthalmology, the First Affiliated Hospital, Dali University, Dali 671000, Yunnan Province, China. lcrbrett@163.com; Yi-Min Xiang. Department of Ophthalmology, Fuling Central Hospital, Chongqing 408000, China. 17661764@qq.com
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Abstract

● **AIM:** To explore the effect of the *Andrographis paniculata* (*A. paniculata*) polysaccharide on the proliferation and apoptosis of human retinoblastoma (RB) Y79 cells and its mechanism.

● **METHODS:** The refined *A. paniculata* polysaccharide was obtained using techniques such as water extraction, ethanol precipitation, and decompression concentration. The inhibition effect of the *A. paniculata* polysaccharide on the proliferation of Y79 cells was detected by cell proliferation assay. Flow cytometry was used for the detection of cell apoptosis rate and cycle change. Real-time qunatitative polymerase chain reaction (RT qPCR) and Western blotting were used to detect the expression of cell apoptosis signal pathway-related factors (caspase-3, caspase-8, and caspase-9) and cell cycle signal pathway-related factors (CDK1 and cyclinB1) at the transcriptional and translational levels.

● **RESULTS:** Infrared and ultraviolet spectrum scanning showed that the extracted drug was a polysaccharide with high purity. After being treated with different concentrations of *A. paniculata* polysaccharide for different periods of time, the Y79 cells showed different degrees of proliferation inhibition. Flow cytometric observations showed that the cell apoptosis rate and the proportion of cells blocked in the G2/M phase were significantly increased after *A. paniculata* polysaccharide treatment. Further analysis revealed that the mRNA and protein expression of caspase-3, caspase-8, and caspase-9 in the *A. paniculata* polysaccharide treatment groups increased significantly compared with that in the control groups, while the expression of CDK1 and cyclinB1 decreased significantly.

● **CONCLUSION:** The *A. paniculata* polysaccharide could inhibit the proliferation and induce apoptosis of Y79 cells. Its possible mechanism is *via* the upregulation of caspase-3, caspase-8, and caspase-9 expression in the cell apoptotic signaling pathway and the downregulation of CDK1 and cyclinB1 expression in the cell cycle signaling pathway.

● **KEYWORDS:** retinoblastoma; cell proliferation; apoptosis; cell cycle

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INTRODUCTION

Retinoblastoma (RB) is a malignant tumor originating from the developing retina. It usually occurs in children under 5 years old[1] and is the most common intraocular malignant tumor in infants[2-3]. At present, the global average annual incidence is approximately 1/15 000-1/20 000, and there are approximately 9000 annual new cases, among which approximately 1100 are reported in China. Although the incidence is low, it seriously affects the vision of children and even endangers their lives[4-5]. A large number of studies have shown that the pathogenesis of RB is mainly related to

the aberrance of autosomal 13q14 (*RB1* gene), and recent studies have shown that the alteration of the *MYCN* gene is also related to the occurrence of RB[1,6]. Clinically, it often causes red eyes, eye pain, strabismus, blindness, and can even be life-threatening. Due to its high degree of malignancy, the mortality rate of children patients is 100% without treatment[7]. Therefore, there is need for the development of effective treatment strategies for this disease. The present treatment options are varied and highly dependent on tumor stage, pathology, and genetic background[8]. Currently, chemotherapy, radiotherapy, local photocoagulation, cryotherapy, and enucleation are often used in the treatment of RB[9]. However, most chemotherapeutic drugs have serious side effects. Therefore, there is still a need for a novel, safer, and more effective treatment.

Traditional herbs are characterized by low side effects and low prices[10]. Ligustrazine, matrine, celastrol, morin, and andrographolide and genistein have been proved to have an anti-RB effect[10-16]. However, the study of the effects of the *Andrographis paniculata* (*A. paniculata*) polysaccharide against RB has not been reported.

Cell proliferation and apoptosis are common cellular processes, the imbalance between which can lead to the occurrence and development of tumors[17]. CDK1 and cyclinB1 are key proteins regulating the G2/M phase of the cell cycle, which can promote the transition from the G2 phase to the M phase and accelerate cell division and proliferation[18-19]. At present, it is believed that there are three apoptosis pathways in cells, namely the exogenous pathway, endogenous pathway, and endoplasmic reticulum pathway. These three pathways are carried out by the activation of caspase (cysteine-aspartate specific protease) proteins, followed by the involvement of mitochondria to perform apoptosis[20-21]. The endogenous pathway is the mitochondrial pathway, primarily comprising the activation of caspase-9. The exogenous pathway, the death receptor pathway, primarily comprises caspase-8 activation. The endoplasmic reticulum pathway involves caspase-12 activation. In these three pathways, caspase-3 and caspase-7 are the key proteases and executors[22]. For our study, we selected the human RB Y79 cell line to explore the specific mechanism of the *A. paniculata* polysaccharide in cell apoptosis and cell cycle signaling pathways.

MATERIALS AND METHODS

Extraction and Purification of *A. paniculata* Polysaccharide The extraction of *A. paniculata* polysaccharide was carried out as previously described[23-24]. A 4-fold increased volume of petroleum ether was used to reflux the *A. paniculata* powder for 4h. The filter residue was then dried at 40℃ and refluxed with 4 times the volume of anhydrous ethanol for 4h. Again, the filter residue was dried at 40℃, and 40 times deionized water was added, and the drug was extracted at 65℃ for 7.5h, with this step was repeated 4 times. The filtrate was collected and concentrated at low atmospheric pressure, and the protein was removed using the Sevage method. It was then precipitated with 75% ethanol overnight at 4℃. The precipitate was washed 3 times with anhydrous ethanol, acetone, and ether, in turn; then, freeze-dried to a constant mass and allowed to stand in the dryer. Finally, the composition and purity of the product were analyzed by infrared and ultraviolet spectra analysis.

Cell Culture and Grouping The human RB Y79 cell line (obtained from and authenticated by Beijing Beina Chuanglian Biotechnology Research Institute) was exposed in RPMI 1640 medium (Thermo Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, USA) and 1% penicillin-streptomycin in 37℃ humidified incubator with 5% CO_2. When the cell proliferation density reached approximately 80%, the cells were subcultured. The experiments were conducted on cells during logarithmic growth. Cells were divided into experimental groups (*A. paniculata* polysaccharide) and negative control groups (equal volume complete medium).

Cell Proliferation Assay Y79 cells were made into 1×10^5/mL single cell suspension and inoculated on 96-well plates with 100 μL of suspension per well. RPMI 1640 complete medium was added to the control groups with 100 μL added per well. In the experimental groups, 100 μL of complete medium containing *A. paniculata* polysaccharide was added per well. The final concentration was 100, 200, 300, and 400 μg/mL, and for each concentration, there were three repeat wells. After 24, 48, and 72h of culture, 20 μL of enhanced Cell Counting kit-8 (CCK8) reagent (Beyotime Biotechnology Co., Ltd.) was added to each well, and the chromogenic reaction was allowed for 4h at 37℃. Absorbance of each well at 450 nm was measured with a microplate reader. The experiments were repeated three times. Cell survival rate was calculated as: cell survival rate (%)=[(As-Ab)/(Ac-Ab)]×100%, where, As: experimental well (Y79 cell+drug+CCK8); Ac: control well (Y79 cell+CCK8); Ab: blank Well (medium+CCK8).

Detection of Cell Apoptosis Rate by Flow Cytometry The Y79 cells were treated with mock or 200 μg/mL *A. paniculata* polysaccharide for 48h first. Then, 1×10^6 cells were washed twice by precooled PBS and stained with 5 μL Annexin V-FITC and PI (Vazyme Biotech Co., Ltd.) at room temperature for 10min and were then analyzed using flow cytometry (ACEA NotoCyte™ flow cytometry, Essen Biosciences, USA). The experiments were repeated three times.

Detection of Cell Cycle by Flow Cytometry The Y79 cells in the experimental groups (200 μg/mL *A. paniculata* polysaccharide) and the control groups (equal volume complete medium) were first treated for 48h. Then, 1×10^6 cells

were washed twice by precooled PBS and fixed overnight with 75% ethanol at 4℃. After centrifugation, 100 μL of RNaseA was added, followed by placing in a water bath at 37℃ for 30min. Following this, 400 μL of PI (4A Biotech Co., Ltd.) was added and the mixture was incubated at 4℃ in the dark for 30min, and then measured on the enzyme-labeled instrument. The experiments were repeated three times.

RNA Isolation and Quantification In the experimental groups, the final *A. paniculata* polysaccharide concentration was 200 μg/mL, and in the control groups, the same volume of complete medium was added. After 48h of culture, Trizol reagent (Vazyme Biotechnology Co., Ltd.) was used to extract the total RNA, and then reverse transcription was performed with Hiscript® II Q RT Supermix for qPCR (vazyme Biotechnology Co., Ltd.). The amplification was carried out in triplicate using ChamQTM SYBR® qPCR Master MiX (Vazyme Biotech Co., Ltd.) on a 7900 H T RealTime PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the instructions. The expression of mRNA was normalized to β-actin. Primers were (Table 1) purchased from GenScript (Nanjing) Co., Ltd. The reaction conditions were as follows: 95℃ for 5min, and a total of 40 cycles at 95℃ for 15s, 55℃ for 15s, and 72℃ for 60s. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method from three independent experiments.

Western Blot Analysis The Y79 cells in the experimental groups (the final concentration of *A. paniculata* polysaccharide was 200 μg/mL) and the control groups (the same volume of complete medium was added) were collected after 48h of culture and then protein was extracted on ice. Gel electrophoresis was performed after protein quantification using the bicinchoninic acid (BCA) method and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in skimmed milk for 2h and then incubated overnight at 4℃ with primary antibodies (1:1000, Cell Signaling Technology, USA). Afterward, the membranes were incubated with secondary antibodies (1:1000, Beyotime Biotechnology Co., Ltd.) at room temperature for 2h. The protein bands were observed by miniChemi K4 (Beijing Sage Creation Technology Co., Ltd.) and analyzed by Image J. The data were derived from three independent experiments.

Statistical Analysis The data were analyzed by SPSS 21.0 and expressed as the mean±standard deviation. The one-way ANOVA method was used for the multiple-group comparison and the *t* test was used for the pairwise comparison. $P<0.05$ was considered to be statistically significant.

RESULTS

Components and Purity Identification of *A. paniculata* Polysaccharide Some studies have shown that the wave numbers of 3670-3220 cm^{-1}, 3000-2800 cm^{-1}, 1700-1400 cm^{-1}, and 1200-1000 cm^{-1} are typical absorption peaks of polysaccharides in the infrared spectrum analysis[25-26]. The infrared spectrum of the extracted compound is shown in Figure 1. It can be seen that there are obvious absorption peaks within the above-mentioned spectral bands; thus, confirming the identification of the compound as a polysaccharide. The ultraviolet spectra showed no obvious absorption peak at 260 nm and 280 nm. The results showed that the nucleic acid and protein from the *A. paniculata* polysaccharide extract had been substantially removed, resulting in a very high purity (Figure 2).

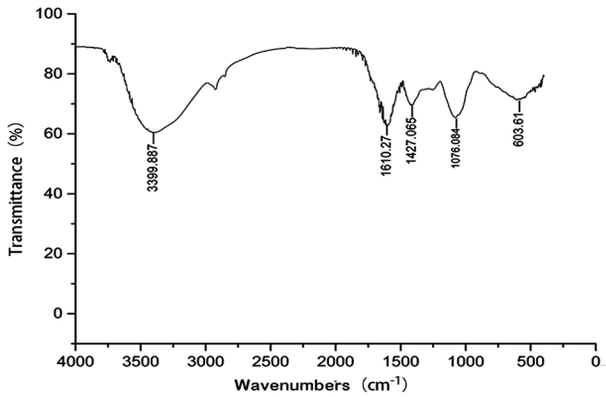

Figure 1 Infrared spectrum of *A. paniculata* polysaccharide.

Table 1 Quantitative polymerase chain reaction primer sequences

Primer name	Primer sequence
CDK1	F: 5’ GATTCTATCCCTCCTGGTCAGT 3’
	R: 5’ CAGCCAGTTTAATTGTTCCTTT 3’
Cyclin B1	F: 5’ GTCGGGAAGTCACTGGAAAC 3’
	R: 5’ AACCGATCAATAATGGAGACAG 3’
Caspase-3	F: 5’ GGTTCATCCAGTCGCTTTG 3’
	R: 5’ ATTCTGTTGCCACCTTTCG 3’
Caspase-8	F: 5’ TTCCTGAGCCTGGACTACATT 3’
	R: 5’ GAAGTTCCCTTTCCATCTCCT 3’
Caspase-9	F: 5’ ACTAACAGGCAAGCAGCAAA 3’
	R: 5’ CCAAATCCTCCAGAACCAAT 3’
β-actin	F: 5’ CTGGGACGACATGGAGAAA 3’
	R: 5’ GCACAGCCTGGATAGCAAC 3’

Effects of *A. paniculata* Polysaccharide on the Proliferation of Y79 Cells *A. paniculata* polysaccharide can significantly inhibit the proliferation of Y79 cells. The cell survival rates of the experimental groups were lower than that of the control groups, and the survival rates decreased with the increase in time and the concentration of *A. paniculata* polysaccharide ($P<0.01$; Figure 3 and Table 2).

Detection of Cell Apoptosis Rate by Flow Cytometry As can be seen from Figure 4, cell apoptosis rates increased after 200 μg/mL of *A. paniculata* polysaccharide treatment compared with the control groups. The cell apoptotic rates of control groups and experimental groups were 2.80%±0.30% and 10.10%±1.01%, respectively ($P<0.05$). This result showed that the *A. paniculata* polysaccharide can induce the apoptosis of Y79 cells.

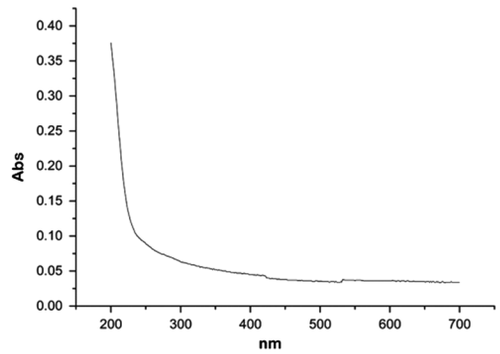

Figure 2 Ultraviolet scanning curve of *A. paniculata* polysaccharide.

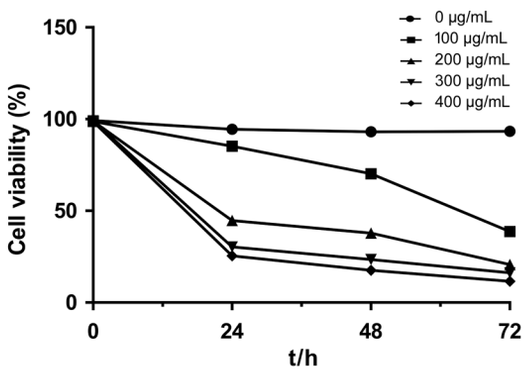

Figure 3 Effects of *A. paniculata* polysaccharide on the proliferation of Y79 cells.

Table 2 Survival rates of Y79 cells at different *A. paniculata* polysaccharide concentrations and time points n=3, mean±SD

Group (μg/mL)	Cell survival rate (%)		
	24h	48h	72h
Control	94.43±1.04	93.10±0.42	93.34±1.27
100	85.13±0.99[a]	70.19±0.85[a]	38.63±1.21[a]
200	44.69±2.54[a]	37.78±0.95[a]	20.68±0.97[a]
300	30.28±1.29[a]	23.48±0.90[a]	16.18±0.31[a]
400	25.49±0.86[a]	17.62±0.35[a]	11.58±1.14[a]
F	1391.119	5706.795	3129.480
P	<0.01	<0.01	<0.01

Compared with the control groups, [a]P<0.01.

Effect of *A. paniculata* Polysaccharide on Caspase-3, Caspase-8, and Caspase-9 mRNA Expression in Y79 Cells According to RT qPCR, the mRNA expression of caspase-3, caspase-8, and caspase-9 in the experimental groups was higher than that in the control groups (P<0.05 or 0.01). These results suggested that *A. paniculata* polysaccharide can upregulate the expression of caspase-3, caspase-8, and caspase-9 in Y79 cells at the transcriptional level (Figure 5).

Effect of *A. paniculata* Polysaccharide on Caspase-3, Caspase-8, and Caspase-9 Protein Expression in Y79 Cells The Western blot analysis results showed that the protein expression of caspase-3, caspase-8, and caspase-9 in the experimental groups was significantly higher than that in the control group (P<0.05). The results were consistent with the results of RT qPCR. These results meant that *A. paniculata* polysaccharide can upregulate the expression of caspase-3, caspase-8, and caspase-9 in Y79 cells at the translational level (Figure 6).

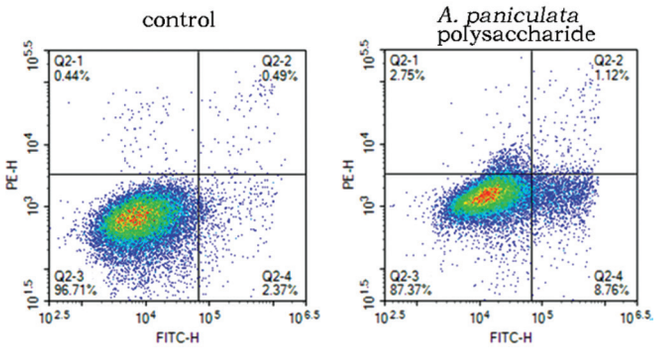

Figure 4 Detection of cell apoptosis rate by flow cytometry.

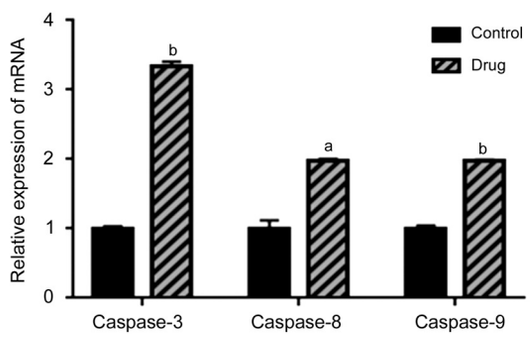

Figure 5 Effect of *A. paniculata* polysaccharide on caspase-3, caspase-8, and caspase-9 mRNA expression in Y79 cells Compared with the control groups, [a]P<0.05, [b]P<0.01.

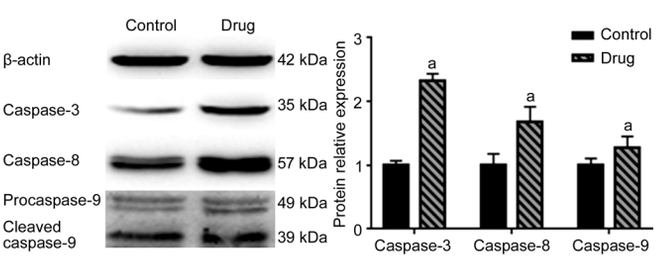

Figure 6 Effect of *A. paniculata* polysaccharide on caspase-3, caspase-8, and caspase-9 protein expression in Y79 cells Compared with the control groups, [a]P<0.05.

Detection of Cell Cycle by Flow Cytometry As depicted in Figure 7 and Table 3, compared with the control groups, the G2/M phase distribution proportion was increased after 200 μg/mL *A. paniculata* polysaccharide treatment, with values of 1.81%±0.54% in the control groups and 6.30%±0.75% in the experimental groups. These differences were statistically significant (P<0.05). The results showed that *A. paniculata* polysaccharide can induce Y79 cells to be blocked in the G2/M phase.

Effect of *A. paniculata* Polysaccharide on CDK1 and CyclinB1 mRNA Expression in Y79 Cells Compared with the control groups, the mRNA expression of CDK1 and cyclinB1 in the experimental groups was significantly lower than that in the control groups as determined by RT

qPCR (P<0.05). The results suggested that *A. paniculata* polysaccharide can downregulate the expression of CDK1 and cyclinB1 at the transcriptional level in Y79 cells (Figure 8).

Effect of *A. paniculata* Polysaccharide on CDK1 and CyclinB1 Protein Expression in Y79 Cells Western blotting results showed that the expression of CDK1 and cyclinB1 in the experimental groups was lower than that in the control groups (P<0.05). The results were consistent with the results of RT qPCR. These results meant that *A. paniculata* polysaccharide can downregulate the expression of CDK1 and cyclinB1 in Y79 cells (Figure 9).

DISCUSSION

In recent years, with the development of individualized comprehensive treatment and fundus screening, more children with RB can receive early diagnosis and treatment, greatly improving the survival rate among children. However, in many underdeveloped countries and regions, the survival rate is still low[27-28]. Currently, chemotherapy is still widely used in clinical practice, and most chemotherapeutic drugs, such as carboplatin, can cause gastrointestinal intolerance, temporary spinal cord inhibition, secondary leukemia, and other side effects[29]. Concurrent tumor resistance also limits its clinical application. Since the majority of the children with RB are less than 5 years old, this requires that chemotherapy drugs must be safe and effective. As a traditional herbal medicine, *A. paniculata* has been used in clinical treatment for many diseases for a long time, with the appealing characteristics of safety and affordability[30]. Many studies have confirmed the anti-tumor effect of polysaccharide drugs[31-34]. *A. paniculata* polysaccharide has been proven to have antioxidant activity and is capable of scavenging DPPH$^-$, O2$^-$, OH$^-$, *etc*[23-35]. Increased levels of oxidative stress in the body can lead to the uncontrolled growth and division of cells, invasion of adjacent tissues, and carcinogenesis[36]. Therefore, we explored the anti-RB activity and mechanism of *A. paniculata* polysaccharide.

Following the treatment with *A. paniculata* polysaccharide, the survival rate of Y79 cells was tested and it was found that *A. paniculata* polysaccharide can inhibit cell proliferation. From low to high concentrations of *A. paniculata* polysaccharide, the cell proliferation inhibition increased gradually; it also increased with an increase in time, showing evident dose- and time-dependent response. The apoptosis rates of Y79 cells in the experimental groups were significantly higher than those in the control groups and cell cycle detection showed that the G2/M phase ratios of the experimental groups were higher than those of the control groups. Therefore, the decrease of cell survival rate after *A. paniculata* polysaccharide treatment may be related to its promotion of cell apoptosis and cell cycle arrest. These prompted us to further explore the cell apoptotic signaling pathway and cell cycle signaling pathway.

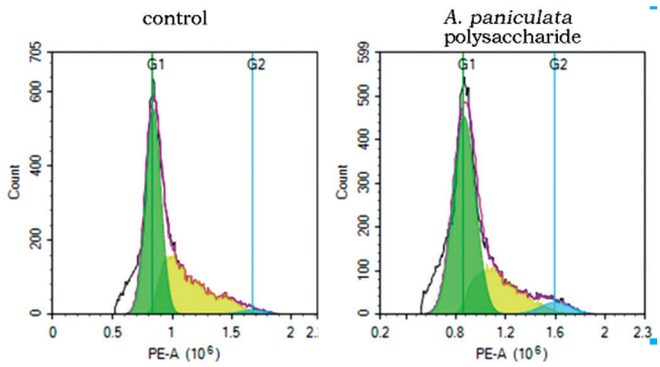

Figure 7 Detection of cell cycle by flow cytometry.

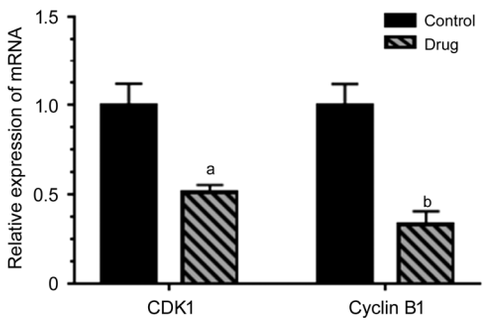

Figure 8 Effect of *A. paniculata* polysaccharide on CDK1, cyclinB1 mRNA expression in Y79 cells Compared with the control groups, ^aP<0.05, ^bP<0.01.

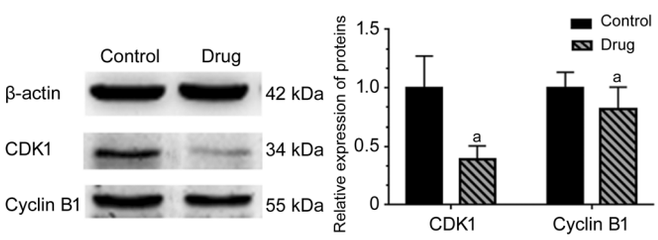

Figure 9 Effect of *A. paniculata* polysaccharide on CDK1 and cyclinB1 protein expression in Y79 cells Compared with the control groups, ^aP<0.05.

Table 3 Distribution of Y79 cell cycle detected by flow cytometry

n=3, mean±SD

Group	G0/G1 (%)	S (%)	G2/M (%)
Control group	55.46±2.82	40.59±3.37	1.81±0.54[a]
Experimental group	60.04±3.31	32.73±2.54	6.30±0.75[a]

Compared with the control groups, ^aP<0.05.

Ohata *et al*[37] suggested that abnormal cell apoptosis regulation plays a very important role in the development of malignant tumors, and the key to tumor prevention and treatment is the inhibition of cell proliferation and/or promotion of cell apoptosis. The caspase family is one of the important proteins inducing cell apoptosis, among which caspase-8 is an important factor to initiate apoptosis. The activated caspase-8 can cause caspase-mediated apoptosis cascade reaction, destroy the death structural proteins and related functional proteins in cells, and cause apoptosis[38-39]. Caspase-9 is an important

member of the cysteine protease family. Activated caspase-9 can lyse cell growth proteins and play an important role in cell apoptosis[40-41]. Caspase-3, known as the "killer protein", is the most important executor of cell apoptosis and is involved in almost all apoptosis processes. It is usually activated and cut into P17 and P12 fragments, causing apoptosis[42-44]. CyclinB1 is a cell cycle regulatory protein and an important member of the cyclin family and can induce phosphorylation of downstream RB proteins by forming complexes with the corresponding kinase CDK1. The phosphorylated RB protein loses its inhibitory effect on the transcription factor E2F, thus enabling E2F to resume transcriptional activity and participate in the regulation of cell G2/M phase transformation, accelerating cell cycle from G2 phase to M phase and continuously proliferating and dividing[16-17].

In our study, the expression levels of caspase-3, caspase-8, and caspase-9 in Y79 cells in *A. paniculata* polysaccharide treatment groups were higher than those in the control groups at the transcriptional and translational levels. These proteins are the key proteins in the apoptotic signaling pathway. This suggests that *A. paniculata* polysaccharide may induce Y79 cell apoptosis by activating the cell apoptotic signaling pathway. Further analysis showed that the mRNA and protein expression of CDK1 and cyclinB1 in Y79 cells treated with the *A. paniculata* polysaccharide was significantly downregulated compared with the control groups. CDK1 and cyclinB1 are key proteins regulating the G2/M phase, and we also found that the Y79 cells were blocked in the G2/M phase after the treatment of *A. paniculata* polysaccharide as determined by flow cytometry. Therefore, we speculated that the *A. paniculata* polysaccharide can induce apoptosis of Y79 cells by regulating the cell cycle signaling pathway.

This study has certain shortcomings. In the future, animal studies should also be conducted, using the RB xenograft model to evaluate the anti-tumor effect of *A. paniculata* polysaccharide and further explore other possible anti-tumor mechanisms. We will also invest in the further development of this drug. In conclusion, the *A. paniculata* polysaccharide has anti-RB activity. The possible mechanism is the regulation of cell apoptosis and of the cell cycle signaling pathway. As a new drug, *A. paniculata* polysaccharide is expected to be an effective drug or adjuvant drug in the treatment of RB.

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Conflicts of Interest: Xu B, None; **Li L,** None; **Zhang W,** None; **Li Y,** None; **Wang MR,** None; **Liu JC,** None; **Dong KY,** None; **Fabian ID,** None; **Qiu D,** None; **Li CR,** None; **Xiang YM,** None.

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