

# ***Ranunculus Japonicus* Induces Cell Cycle Arrest, Apoptosis, Autophagy and Targeting the Akt/Mapks Pathways in Human Neuroblastoma SH-SY5Y Cells**

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**Abstract:** *Objective: Ranunculus japonicus thumb. has been used as a traditional medicine for the treatment of asthma, malaria, tuberculosis, hepatitis, jaundice and so on. The aim of this study was to investigate the anti-cancer effect and action mechanism of the petroleum ether extract of Ranunculus japonicus Thumb. (RJTPPE) on human neuroblastoma SH-SY5Y cells. Methods: Cell viability was detected using MTT assay. FCM and Hoechst33258 staining was used to examine the cell cycle as well as apoptosis, and in addition, Western blot was also used to examine the expression of a range of related proteins. Results: MTT assay showed that RJTPPE could inhibit SH-SY5Y cells proliferation in concentration- and time-dependent manners. FCM analysis showed that RJTPPE could induce S and G2/M phase arrest, cell apoptosis, cell autophagy, the mitochondrial membrane potential loss ( $\Delta\Psi_m$ ) and increase the production of intracellular ROS of SH-SY5Y cells. Topical morphological changes of apoptotic body formation were also observed by Hoechst 33258 staining. Western blot analysis indicated that the expressions of cell cycle-related protein Cyclin B1/D1 and CDK1/2/4/6 were obviously decreased. Meanwhile, obvious increase of apoptosis-related protein expressions, such as Bax, cytochrome c, caspase-3, caspase-9, PARP-1, and decrease of Bcl-2 protein expression were observed. Furthermore, Akt/MAPKs pathway-related proteins were also detected. Moreover, the decreased expression of autophagy-related proteins LC3 I protein indicated that RJTPPE could also enhance the autophagy ability of cells. Conclusion: RJTPPE could exert the antitumor activity on SH-SY5Y cells by inducing cell cycle arrest, cell autophagy, the mitochondrion-dependent cell apoptosis and targeting the Akt/MAPKs signaling pathway. RJTPPE has a promising therapeutic potential for neuroblastoma.*

**Keywords:** *Ranunculus japonicus thumb; Neuroblastoma; Cell cycle; Apoptosis; Autophagy; ROS*

## **1. Introduction**

Neuroblastoma is the most common solid extracranial tumor in pediatrics and can regress spontaneously or grow and metastasize through resistance to a variety of therapeutic approaches<sup>[1]</sup>. At present, a large number of basic and clinical studies that has been conducted on the subject of this disease entity, but patients with adverse prognostic factors have a poor prognosis. Especially, those with high-risk disease that are difficult to cure<sup>[2]</sup>.

Cell cycle control is one of the major regulatory mechanisms of cell growth. Many anticancer agents have been reported to arrest the cell cycle at a specific checkpoint, thereby inducing apoptotic cell death<sup>[3]</sup>. The cell cycle is an irreversible process, its progression is orchestrated through sequential activation of cyclin-dependent kinases (CDKs) by their proper cyclin partner<sup>[4]</sup>. Among of them, CDK4 and CDK6 mainly control cell growth in G1 phase, CDK2 regulates chromosomal replication, and CDK1 regulates mitosis and meiosis<sup>[5]</sup>. Therefore, tumor cell growth is inhibited by arrest of the cell cycle. In addition, PI3K/Akt signaling pathway is abnormal in cancer cells and plays a pivotal role in neoplastic transformation. According to many studies, Akt should be a key mediator of the PI3K pathway, and the inhibition of Akt is effective in reducing tumor growth<sup>[6]</sup>.

Autophagy and apoptosis are two pathways for cell death. These two pathways occur during cellular damage or stress and are also present during normal development and morphogenesis<sup>[7]</sup>. Autophagy is a multi-step catabolic and also is a highly conserved pathway<sup>[8]</sup>. It plays a vital role in combating certain diseases, such as cancer, infection and neurodegeneration. Autophagy is also general known as type II programmed cell death<sup>[9]</sup>. In apoptosis, internal and/or external stimuli trigger a series of highly controlled reactions that ultimately lead to cell death. The two most important proteomes involved in

apoptosis, the caspase and Bcl-2 protein family are involved in all pathways of apoptotic cell death. In addition, Caspase-9 and Caspase-3 cleave their target proteins, leading to the emergence of classical biochemical and morphological phenotypes of apoptosis <sup>[10]</sup>.

Natural products have been shown to be excellent and reliable sources for pharmaceutical development of anticancer drugs <sup>[11]</sup>. *Ranunculus japonicus* Thunb. has been used as a traditional medicine for the treatment of asthma, malaria, tuberculosis, hepatitis, jaundice and so on. To best of our knowledge, it has many pharmacological activities such as anti-tumor, anti-inflammatory, anti-aging, anti-bacterial, anti-cardiovascular diseases and so on <sup>[12]</sup>. But its anticancer effect on human neuroblastoma is still unclear. The aim of this study was to investigate the anti-cancer effect and detailed mechanism of petroleum ether extract of *Ranunculus japonicus* Thunb. (RJTPEE) on human neuroblastoma SH-SY5Y cells.

## 2. Materials and methods

### 2.1. Materials

The whole herb of *Ranunculus japonicus* Thunb was obtained from Chinese herbal medicine company in Bozhou City (Anhui, China), and identified by Prof. Guangtong Chen, Nantong University, Jiangsu, PR China. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (USA). ROS detection kit and Rhodamine 123 were purchased from Beyotime Biotechnology Research Institute (China). Dansylcadaverine (MDC), Hoechst33258, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). Annexin V-FITC/PI apoptosis detection kit was purchased from KeyGEN Bio TECH (China). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Beckman Coulter (USA). In addition, some antibodies such as Bcl-2, Bax, cytochrome c and procaspase-9 were obtained from Santa Cruz. Part of the antibody which are CDK2, CDK4, caspase-3, CDK6, PARP-1 and  $\beta$ -actin were obtained from Abcam. Most antibodies that example as LC3I, p-JNK, JNK, p-Akt, Akt, cyclin D1, p-p38, p38, cyclin B1, p-ERK, ERK, CDK1 were purchased from Beyotime.

### 2.2. Preparation of RJTPEE extract

The dried powder of *Ranunculus japonicus* Thunb. whole herb (1 kg) were extracted three times under reflux with 70% ethanol, filtered, evaporated, and fractioned with petroleum ether, methylene dichloride, ethyl acetate, and *n*-BuOH saturated with H<sub>2</sub>O. The dried petroleum ether extract (RJTPEE, 11.2 g) was found to be the most effective using MTT assay.

### 2.3. Cell culture

The human neuroblastoma SH-SY5Y cells were cultured in DMEM media including 10% heat inactivated FBS or NBS and 1% penicillin/streptomycin at 37 °C in a humidified incubator supplemented with 5% CO<sub>2</sub> atmosphere.

### 2.4. Cell viability assay (MTT assay)

SH-SY5Y cells were seeded into 96-well culture plates and incubated in the presence of RJTPEE (30, 60, 120  $\mu$ g/mL) for 24, 48 or 72 h with the densities of  $8 \times 10^4$ ,  $4 \times 10^4$  or  $2 \times 10^4$  cells/well (0.2 mL), respectively. Then, cell viability was monitored using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The absorbance was surveyed at 570 and 630 nm on a microplate reader (SpectraMax M5, Molecular Devices, USA). The percentage of cell viability was computed relative to the absorbance intensity of control cells.

### 2.5. Cell cycle analysis and Annexin V-FITC/PI assay

The SH-SY5Y cells at a density of  $3 \times 10^5$  cells/well were plated in 6-well culture plates and incubated overnight. Subsequently, the cells were treated with different concentration of RJTPEE (30, 60, 120  $\mu$ g/mL) for 48 h. Then the cells were trypsinized and harvested in PBS. Followed by washing twice with PBS, the collected cells were stained with 10 nM of DAPI for 30 min at room temperature. Approximately  $1 \times 10^5$  cells were incubated with the apoptosis detection kit according to the manufacturer's instructions. The cell cycle or apoptosis was analyzed by way of flow cytometer

(Beckman Coulter) employing CXP analysis software.

## **2.6. Morphological analysis of Hoechst 33258 stained cells**

SH-SY5Y cells which have a density of  $1 \times 10^5$  cells/well were seeded into 24-well culture plates and treated under the same conditions as described above. The cells were stained with 1  $\mu\text{g/mL}$  of Hoechst 33258 for 15 min, and this process needs to be carried out in the dark, followed by washing twice with PBS. Subsequently, it is required to use fluorescence microscope with 20 X objective lenses to observe a morphological change.

## **2.7. Intracellular ROS assay**

The production of intracellular ROS was calculated according to the manufacturer's protocol. The cells were treated under the same conditions as described above. Rosup was used as a positive control. Then, they were incubated in 1 mL of DCFH-DA (10  $\mu\text{M}$ ) for 30 min, and this process needs to be carried out in the dark, followed by washing twice with PBS. The ROS production was calculated to fluorescence intensity by way of flow cytometry using CXP analysis software.

## **2.8. Measurement of mitochondrial membrane potential ( $\Delta\Psi\text{m}$ )**

SH-SY5Y cells which have a density of  $1 \times 10^5$  cells/well were seeded into 6-well culture plates and incubated in the presence of RJTPEE (30, 60, 120  $\mu\text{g/mL}$ ) for 48 h. Subsequently, the cells were washed twice with PBS. Furthermore, the cells were gathered with Trypsin and resuspended in 1 mL of media. Cell pellet was collected through centrifugation at 1500 rpm for 3 min and washed with PBS. Finally, the cells were resuspended in 1 mL of Rhodamine123 (5  $\mu\text{M}$ ) without light for 30 min. These samples were analyzed after using CXP analysis software by flow cytometry. At least 10000 events were recorded for each analysis performed at least three times independently.

## **2.9. Autophagy detection**

Quantitative analysis of autophagosome formation was detected. First of all, SH-SY5Y cells were treated with different concentrations of RJTPEE (30, 60, 120  $\mu\text{g/mL}$ ) for 8 h. Next, they are gathered and incubated with MDC (50  $\mu\text{M}$ ) for 60 min at 37 °C. Finally, they were analyzed by flow cytometry using CXP analysis software.

## **2.10. Western blot analysis**

SH-SY5Y cells were harvested and lysed with Radio-Immunoprecipitation Assay (RIPA) buffer for 20 min to extract total protein. After quantifying the protein concentration, proteins (40  $\mu\text{g/lane}$ ) of different molecular weights were separated by a 6%-15% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody after reaction with the primary antibody (caspase-3, caspase-9, p-Akt, Akt, PARP-1, Bcl-2, Bax, cytochrome c, LC3 I, CDK1/2/4/6, cyclin D1, cyclin B1, p-ERK, ERK, p-JNK, JNK, p38 and p-p38) overnight at 4 °C. Band intensity was measured using chemiluminescence western blot detection system.

# **3. Results**

## **3.1. Effect of RJTPEE on the cell growth**

To evaluate the cytotoxicity induced by RJTPEE, SH-SY5Y cells were treated with 30, 60, 120  $\mu\text{g/mL}$  of RJTPEE at different time points (24, 48 or 72 h). Furthermore, MTT assay was carried out to determine their cell viability. As shown in Fig. 1, RJTPEE demonstrated significantly inhibitory activity against SH-SY5Y cells at a dose-dependent manner, and the  $\text{IC}_{50}$  value reached 75.04  $\mu\text{g/mL}$  when cells were treated with RJTPEE for 72 h. By the way, the time-dependent inhibitory curve of RJTPEE on SH-SY5Y cells was also observed.

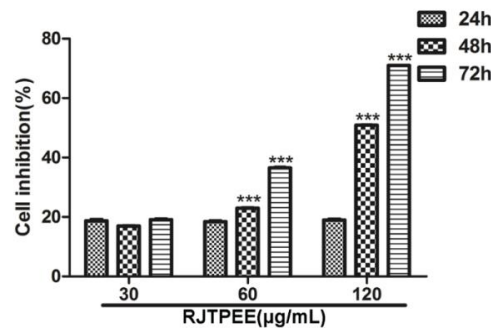


Figure 1: RJTPEE-induced cytotoxicity in SH-SY5Y cells.

MTT assay was used to detect the cell viability after treatment with different concentrations of RJTPEE for 24, 48 and 72 h, respectively. Data shown are the mean from three parallel experiments. \*\*\* $p < 0.001$  as compared to the untreated cells as control.

### 3.2. Effect of RJTPEE on cell cycle by flow cytometry

We investigated whether the inhibition of proliferation of SH-SY5Y cells by RJTPEE is associated with cell cycle arrest. After 48 h of treatment with RJTPEE, the cells showed S phase and G2/M phase arrest by way of a concentration-dependent manner (Fig. 2A and B). Therefore, RJTPEE can inhibit cell proliferation by blocking the S phase and G2/M phase of the SH-SY5Y cell cycle.

### 3.3. Effects of RJTPEE on cell cycle-related proteins

After analyzing the results of the cell cycle, the action mechanism of RJTPEE on the cell cycle of SH-SY5Y was further studied. Cell cycle-related protein CDK family proteins were evaluated by western blot assay. As can be seen from Fig. 2C-F, the expressions of Cyclin B1, CDK1/2/4/6, and Cyclin D1 proteins associated with S phase and G2/M phase were all decreased and showed a dose-dependent manner. This indicates that RJTPEE inhibits the expression of CDK protein and causes cell cycle arrest. The results are consistent with the results of flow cytometry.

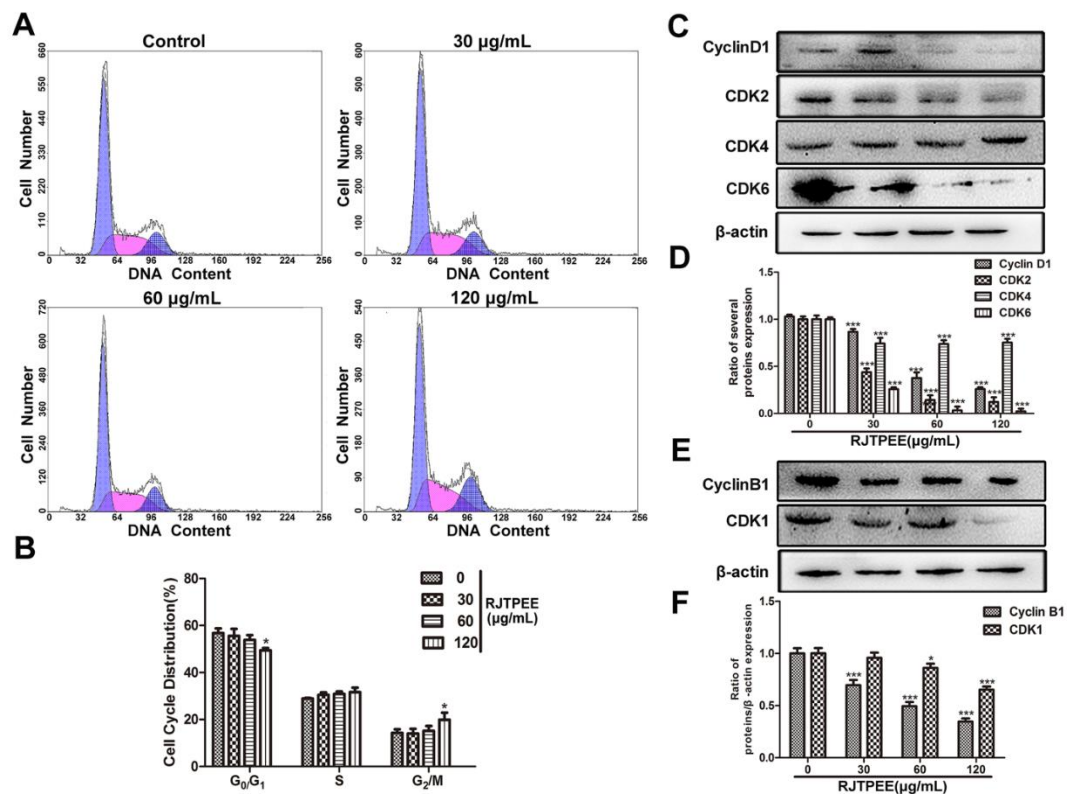


Figure 2: Effect of RJTPEE on the cell cycle of SH-SY5Y cells.

(A and B) SH-SY5Y cells were treated with RJTPEE (30, 60, 120  $\mu\text{g/mL}$ ) for 48 h, and the DNA content of 10,000 events were analyzed by flow cytometry. The profiles showed the cell cycle (A) and the proportions (%) in each phase (B) of SH-SY5Y cells. (C and D) Expression of Cyclin D1, CDK2, CDK4, CDK6 proteins and the ratio of several protein expressions using densitometric analysis in SH-SY5Y cells treated with RJTPEE for 48 h. (E and F) Expression of Cyclin B1, CDK1 proteins and the ratio of several protein expressions using densitometric analysis in SH-SY5Y cells treated with RJTPEE for 48 h. Take  $\beta$ -actin as an internal reference. \* $p < 0.05$ , \*\*\* $p < 0.001$  as compared to the untreated cells as control.

### 3.4. Effect of RJTPEE on the cell Apoptosis

Effect of RJTPEE on apoptosis of the cells was decided with Annexin-V FITC/PI fluorescent double staining kit by flow cytometry. As can be seen from Fig. 3A and B, compared to the control group, the RJTPEE-treated group significantly increased the cell apoptosis, and the proportion of apoptotic cells also increases, showing a concentration-dependent manner.

The Hoechst 33258 staining method is commonly used to detect changes in cell morphology. As shown in Fig. 3C, SH-SY5Y cell nuclei of the control group were uniformly stained and the edge contours were clear by inverted fluorescence microscopy. However, after 48 h of treatment with RJTPEE, the nuclear margin of SH-SY5Y cells became unclear and sleek, the chromatin gradually shrunk, the marginalization was high, and apoptotic bodies appeared. From this, it was revealed that RJTPEE could induce apoptosis of SH-SY5Y cells.

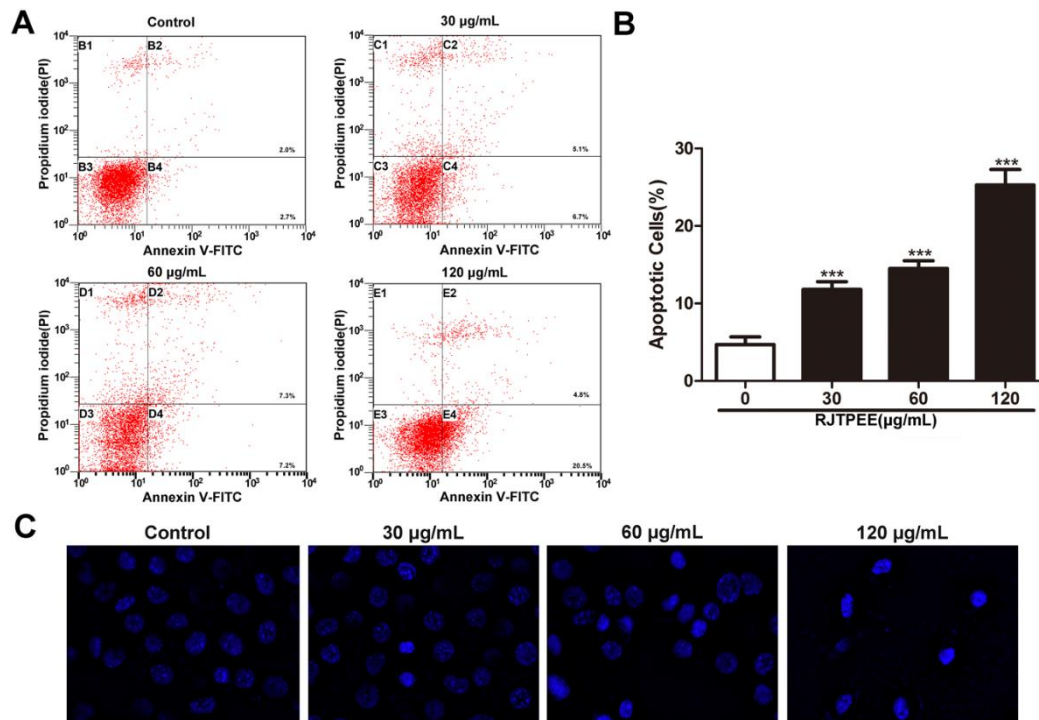


Figure 3: Effect of RJTPEE on the cell apoptosis of SH-SY5Y cells.

(A) Apoptosis distribution of SH-SY5Y cells treated with RJTPEE for 48 h. (B) The apoptosis rate of SH-SY5Y treated by RJTPEE. (C) Cell morphology was observed under an inverted phase contrast fluorescence microscope after treated with RJTPEE for 48 h. \*\*\* $p < 0.001$  as compared to the untreated cells as control.

### 3.5. Effect of RJTPEE on the mitochondrial membrane potential and mitochondrial pathway-associated proteins expressions in SH-SY5Y cells

To determine whether RJTPEE induces apoptosis of SH-SY5Y cells through the mitochondrial pathway, the effect of RJTPEE on the mitochondrial membrane potential was analyzed after the cells were stained with Rhodamine123. It can be seen from Fig. 4A and B that after treatment with not the same concentrations of RJTPEE (30, 60, 120  $\mu\text{g/mL}$ ) for 24 h, the mitochondrial membrane potential

decreased gradually with increasing drug concentration, showing a certain concentration dependence.

To further certify that RJTPEE induced apoptosis of the cells is dependent on the mitochondrial pathway. Therefore, it is required to detect the effect of RJTPEE on the expressions of mitochondrial pathway-associated apoptotic protein in the cells. The results illustrated that when different concentrations of RJTPEE acted on the cells for 48 h, the expression of protein Bcl-2 decreased obviously, while the expression of protein Bax increased gradually, and the ratio of Bax/Bcl-2 increased (Fig. 4C and D). This result suggests that RJTPEE targets Bcl-2 family proteins to induce apoptosis of the cells. Moreover, the expression of cleaved caspase-9, Cyto c and cleaved caspase-3 proteins was discovered by western blot to analyze whether RJTPEE induces apoptosis of the cells through a caspase-dependent mitochondrial pathway. As shown in Fig. 4E and F, the expression of the three proteins increased after 48 h of treatment with RJTPEE showing a concentration-dependent manner. PARP 1 was used as a cleavage substrate for caspase, and its expression level was also increased. This result indicates that RJTPEE leads to apoptosis in the cells by way of a mitochondrial pathway that is dependent on caspase.

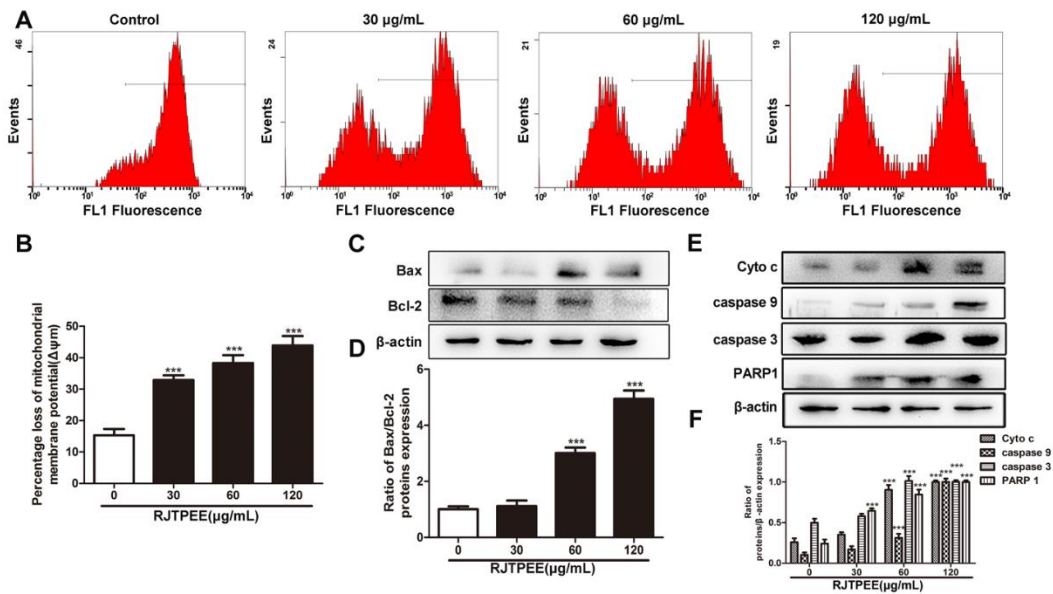


Figure 4: Effect of RJTPEE on the mitochondria-dependent apoptosis pathway of SH-SY5Y cells.

(A and B) Effect of RJTPEE on mitochondrial membrane potential in SH-SY5Y cells. Cells treated with RJTPEE for 24 h were incubated with Rhodamine 123 and measured by flow cytometry. (A) The percentages of cells in right section of fluorocytogram indicate the number of  $\Delta\Psi_m$  collapsed cells. (B) Percentage loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) in SH-SY5Y cells induced by RJTPEE. (C) Expression of Bax and Bcl-2 proteins in cells treated with RJTPEE for 48 h. (D) Ratio of Bax/Bcl-2 protein expressions using densitometric analysis. (E) Expression of Cyto c, cleaved caspase-9/-3 and PARP 1 proteins in SH-SY5Y cells after 48 h of treatment with RJTPEE. (F) Ratio of several protein expressions using densitometric analysis. Take  $\beta$ -actin as an internal reference. \*\*\* $p < 0.001$  as compared to the untreated cells as control.

### 3.6. Effect of RJTPEE on the ROS production in SH-SY5Y cells

Next, we made use of the ROS detection kit to determine the effect of RJTPEE on reactive oxygen species in the cells, and analyzed the changes of intracellular ROS content. As shown in Fig. 5A, after treatment with RJTPEE (30, 60, 120  $\mu\text{g/mL}$ ) for 48 h, ROS obviously increased in the cells at a certain concentration- dependent manner.



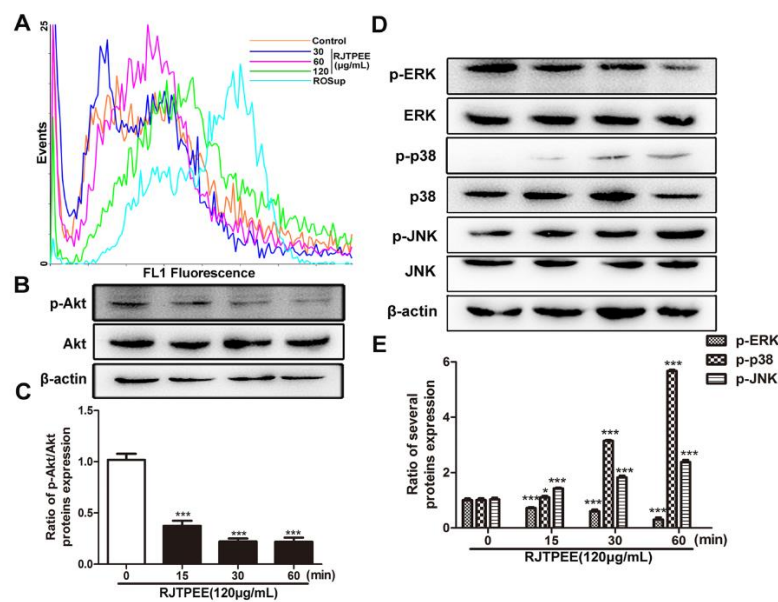


Figure 5: Effect of RJTPEE on intracellular ROS production and Akt/MAPKs pathway-related proteins expressions.

(A) Effect of RJTPEE on intracellular ROS production. Intracellular ROS levels were detected by flow cytometry using a fluorescent probe DCFH-DA label, after SH-SY5Y cells were treated with RJTPEE for 48 h. (B) Expression of p-Akt and Akt proteins in SH-SY5Y cells treated with RJTPEE for 48 h. (C) Ratio of p-Akt/Akt protein expressions using densitometric analysis. (D) Expression of MAPKs family proteins (p-ERK, ERK, p-JNK, JNK, p-p38, p38) in SH-SY5Y cells treated with RJTPEE for 15 min, 30 min and 60 min. (E) Ratio of MAPKs/β-actin protein expressions using densitometric analysis. Take β-actin as an internal reference. \* $p < 0.05$  and \*\*\* $p < 0.001$  as compared to the untreated cells as control.

### 3.7. Effect of RJTPEE on the expressions of the Akt/MAPKs signaling pathway-related proteins

To investigate whether RJTPEE is targeted to Akt, we examined its effect on the proportion of p-Akt/Akt in SH-SY5Y cells. The consequences displayed that after 48 h of administration, the expression of p-Akt was decreased, the expression of Akt protein was almost unchanged, and the proportion of p-Akt/Akt was decreased in a concentration-dependent manner (Fig. 5B and C). This suggests that RJTPEE may act on the PI3K/Akt signaling pathway and regulate apoptosis in conjunction with the mitochondrial pathway.

The cells were treated with 120 µg/mL of RJTPEE for 15 min, 30 min and 60 min, separately, and then the expression of MAPKs family proteins in SH-SY5Y cells was detected. The consequences displayed that RJTPEE can down-regulate the expression of p-ERK and increase the expression of p-JNK and p-p38. (Fig. 5D and E)

### 3.8. Effects of RJTPEE on the cell autophagy

The autophagosome formed by autophagy in the cells is acidic, and MDC is a weakly basic green fluorescent substance capable of binding to autophagosomes. Therefore, cells that are autophagic with MDC markers usually show strong green fluorescence. In this experiment, to investigate whether RJTPEE can induce autophagy in the cells, MDC staining was employed to detect the changes in fluorescence intensity by flow cytometry. As can be seen from Fig. 6A and B, after treating them with different concentrations of RJTPEE (30, 60, 120 µg/mL) for 8 h, the fluorescence intensity increased with increasing concentration and was dose-dependent.

In order to further investigate whether autophagy-related proteins are involved in RJTPEE-induced autophagy in the cells, we used western blot to detect the change of LC3 protein expression. As shown in Fig. 6C and D, the expression of LC3 I protein was decreased and showed a concentration-dependent manner, when SH-SY5Y cells were treated with different concentrations of RJTPEE (30, 60, 120 µg/mL) for 8 h. The consequences displayed that RJTPEE can lead to autophagy in the cells may be relevant to LC3 protein.

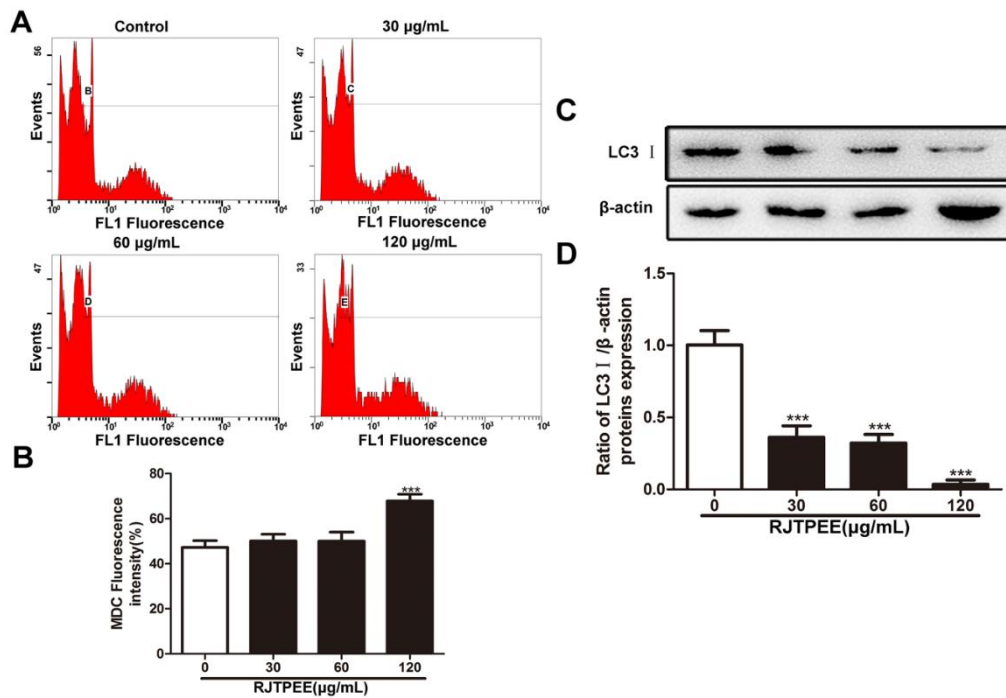


Figure 6: Effect of RJTPEE on cell autophagy in SH-SY5Y cells.

(A) The fluorescence intensity of MDC dye in SH-SY5Y cells was changed after treatment with RJTPEE for 8 h. (B) Proportion (%) of autophagy in SH-SY5Y cells induced by RJTPEE. (C) Expression of LC3I protein in SH-SY5Y cells treated with RJTPEE for 8 h. (D) Ratio of LC3I/β-actin protein expression using densitometric analysis. Take β-actin as an internal reference. \*\*\* $p < 0.001$  as compared to the untreated cells as control.

#### 4. Discussion

In the United States, children with extracranial tumors account for approximately one-twelfth of all pediatric cancers and approximately one-sixth of childhood cancer deaths<sup>[13]</sup>. Currently, there are various treatments such as myeloablative chemotherapy, radiotherapy, immunotherapy and active surgery, however, the curative outcome is disappointing<sup>[14]</sup>.

To best of our knowledge, *Ranunculus japonicus* thumb., as a traditional medicine, has many pharmacological activities such as anti-tumor, anti-inflammatory, anti-aging, anti-bacterial, anti-cardiovascular diseases and so on for the treatment of asthma, malaria, tuberculosis, hepatitis, jaundice and so on. But its anticancer effect on human neuroblastoma is still unclear. Based on this, bioassay-guided fractionation of *Ranunculus japonicus* Thunb. whole herb was carried out. And MTT assay was used to determine their cytotoxicity on human neuroblastoma SH-SY5Y cells. The results showed that RJTPEE had strong cell proliferation inhibition and antitumor activity in vitro in the SH-SY5Y cells. Next, with the increase of the concentration of RJTPEE and the prolongation of the drug action time, the inhibitory effect of RJTPEE on SH-SY5Y cell proliferation was stronger. When RJTPEE treated the cells for 72 h, the  $IC_{50}$  value was 75.04 µg/mL. In the following experiments, the anti-cancer mechanism of RJTPEE on human neuroblastoma SH-SY5Y cell was explored in detail.

The dysregulation of cell cycle progression affects regulatory pathways and cellular processes, which are important features of many human cancers, such as proliferation, differentiation and apoptosis, leading to genetic instability, tumorigenesis<sup>[15]</sup>.

Apoptosis typically occurs during development and aging and as a homeostatic mechanism that maintains cell populations in tissues. The cell apoptosis is characterized by Caspase-dependent, cell contraction and apoptotic body formation<sup>[16]</sup>. SH-SY5Y cells were stained with Hoechst 33258, then we need to use an inverted fluorescence microscope to observe them in this experiment. We found that they were handled with RJTPEE for 48 h, the chromatin of the cells became shrunk and apoptotic bodies appeared. Moreover, Annexin V-FITC/PI fluorescent double staining showed that RJTPEE could induce cell apoptosis in a concentration-dependent manner. The mitochondrial apoptosis pathway is an



important apoptotic pathway<sup>[17]</sup>. Therefore, we wanted to confirm whether RJTPEE induces apoptosis in SH-SY5Y cells and is related to the mitochondrial pathway. The cells were stained with Rhodamine123, and then the mitochondrial membrane potential ( $\Delta\Psi_m$ ) was detected by employing a flow cytometer. The results showed that  $\Delta\Psi_m$  obviously decreased after the cells were handled with RJTPEE for 24 h. Therefore, this displays that the cells apoptosis induced by way of RJTPEE is related to the mitochondrial pathway.

When the mitochondrial pathway is activated, Cyto c is released and forms a complex with the cytoplasmic apoptotic protease activator Apaf-1, which recruits and activates procaspase 9, activates caspase 3, and shears PARP. After these changes, the cells are allowed to enter apoptosis<sup>[18]</sup>. Furthermore, we detected the expression of mitochondrial apoptosis-related proteins by way of employing Western blot. It was found that RJTPEE could increase the expression of Bax protein, decrease the expression of Bcl-2 protein and increase the proportion of Bax/Bcl-2 in the cells. At the same time, the expression of Cyto c, cleaved-caspase9/3, and PARP 1 increased, indicating that RJTPEE can induce apoptosis of SH-SY5Y cells through caspase-dependent mitochondrial apoptosis pathway.

Reactive oxygen species (ROS) are mainly produced in the mitochondria and have close contact to the development, growth, differentiation and proliferation of multicellular organisms. Currently, elevated levels of ROS are thought to cause DNA and protein damage, promoting genetic instability and tumorigenesis<sup>[19]</sup>. In this regard, ROS assay kit was used to detect ROS levels by flow cytometry after the cells were administrated for 48 h. Therefrom, it can be seen that as the concentration of RJTPEE increased, the ROS level also increased and gradually approached the positive control group. The results showed that RJTPEE induced an increase in ROS levels during apoptosis occurrence of SH-SY5Y cells, which stimulated cell death or autophagy.

The PI3K/Akt signaling pathway is also one of the most important intracellular pathways that regulate cell survival, cell growth, differentiation, and the like<sup>[20]</sup>. It is closely related to apoptosis. It is also a good method to treat cancer by inhibiting the PI3K/Akt signaling pathway to induce cell apoptosis. Our results showed that after 48 h of treatment with RJTPEE, the expression of p-Akt protein was decreased in SH-SY5Y cells, the expression of Akt protein was almost unchanged, and the ratio of p-Akt/Akt was decreased.

In addition, mitogen-activated protein kinases (MAPKs) signal transduction pathways are ubiquitous and highly evolutionarily conserved mechanisms of eukaryotic cell regulation. Numerous studies have proven that MAPK family can control and regulate numerous physiological activities such as cell differentiation, cell proliferation and cell apoptosis<sup>[21]</sup>. We used western blot to detect the expression of MAPKs protein. RJTPEE could down-regulate the expression of p-ERK and increase the expression of p-JNK and p-p38. These consequences indicated that the cells apoptosis induced by RJTPEE was involved in the Akt/MAPKs signaling pathway.

LC3 is a biochemical marker of the autophagosome. The p62 is a stress-inducible intracellular protein known, which can regulate diverse signal transduction pathways involved in cell survival and death. Most importantly, autophagy flux can be detected by total cellular expression of p62<sup>[22]</sup>. In this experiment, the intensity of the fluorescent signal in the cells was detected by flow cytometry after MDC staining. As shown in Fig. 6A and B, RJTPEE induced autophagy in the cells in a concentration-dependent manner. Next, we used western blot to detect the expression of autophagy-related proteins LC3 I, and found that RJTPEE could inhibit the expression of LC3 I protein. The above results indicated that RJTPEE could enhance the autophagy level of SH-SY5Y cells.

In summary, the current research demonstrated that RJTPEE could induce cell cycle arrest, mitochondrial pathway-dependent apoptosis and autophagy to exert the cell proliferative inhibitory effect in SH-SY5Y cells, and targeting the Akt/MAPKs pathway. Our findings provided preliminary experimental evidences for supporting the possibility of RJTPEE to be considered as one of the novel pharmacological treatment strategies in human neuroblastoma.

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