

# Effects of *Rehmannia glutinosa* polysaccharides on the proliferation and apoptosis of K562 cells

Qilan Fan<sup>1\*</sup>, Jialin Li<sup>2</sup>, Chunjuan Xu<sup>1</sup> and Suzhen Wu<sup>1</sup>

<sup>1</sup>School of Basic Medicine, Gannan Medical University, Ganzhou, China

<sup>2</sup>College of Pharmacy, Gannan Medical University, Ganzhou, China

**Abstract:** Leukemia is one of the malignant tumors of hemopoietic system which is characterized by the block of hemopoietic cell differentiation and maturation, increased proliferation of undifferentiated cells and impairment of normal apoptosis process. The proliferating leukemia cells accumulated in bone marrow and other hemopoietic tissue, infiltrated other organs and tissues, so that the normal hematopoiesis will be inhibited. Chemotherapy is currently the most effective means of treatment of leukemia which has greatly improved survival rate, but it also induces undesirable adverse effects and high recurrence rate. Therefore, it is necessary to search for new drugs with low toxicity for the treatment of leukemia. *Rehmannia glutinosa* is a traditional Chinese medicine and polysaccharides of *Rehmannia glutinosa* (RPS) were reported to have the immune stimulatory effect and anti-tumor effect both *in vivo* and *in vitro*. After incubating K562 cells (a kind of chronic myelogenous leukemia cell model) with different concentration of RPS, we found that RPS could induce cells arrest in G0/G1 phase and apoptosis, meanwhile, the cell proliferation was also inhibited. Moreover, the expression of Bcl2 was downregulated, and the activity of Caspase3 was improved after incubating K562 cells with RPS for 48 h.

**Keywords:** *Rehmannia glutinosa* polysaccharides, K562 cells, proliferation, apoptosis.

## INTRODUCTION

Chronic myelogenous leukemia (CML) is the third most common type of leukemia, which is one of the tumor of hemopoietic system characterized by the block of hemopoietic cell differentiation and maturation, the proliferation of undifferentiated cells and impairment of normal apoptosis process (Chen *et al.*, 2015; Srdic-Rajic *et al.*, 2016). The over proliferative leukemia cells accumulated in bone marrow and other hemopoietic tissue, infiltrated other organs and tissues, and then caused the inhibition of normal hematopoiesis. Every year, many people died from leukemia (Gong *et al.*, 2017). Chemotherapy is currently the most effective means for the treatment of leukemia which has greatly improved survival rate, but it also induces undesirable adverse effects such as gastrointestinal bleeding, ulcers, diarrhea, and especially have great damage to the body's immune system and hemopoietic systems (Krishnan *et al.*, 2014). Therefore, it is necessary to search for new drugs with low toxicity for the treatment of leukemia.

*Rehmannia glutinosa*, a traditional Chinese medicinal herb, has exhibited an immune stimulatory effect, such as dendritic cell activation and hematopoiesis (Xu *et al.*, 2017a; Huang *et al.*, 2016). Studies indicated that polysaccharides from Chinese medicines could induce apoptosis and could inhibit the proliferation of tumor cells (Yang *et al.*, 2016; Yang *et al.*, 2013). Polysaccharides from *Rehmannia glutinosa* had shown an activation of

immune cells *in vitro* and could be a useful adjuvant molecule for immunotherapy against cancer (Xu *et al.*, 2017b). One of our previous research found that RPS could down regulate BCR-ABL fusion gene expression in K562 cells (Su-Zhen *et al.*, 2017). Herein, we used different concentrations of RPS to incubate with K562 cells for 48 h to investigate whether RPS could inhibit the proliferation and promote the apoptosis of K562 cells.

## MATERIALS AND METHODS

### *Extraction and purification of RPS from Rehmannia glutinosa*

*Rehmannia glutinosa* was purchased from Youbang Pharmaceutical Group Co., Ltd. (Ganzhou, China) and identified by Professor Jialin Li from Gannan Medical University. Powdered the *Rehmannia glutinosa* with a pulverizer, extracted the powder with ultra pure water at 90±1°C for 2 h, then filtered. The residue was extracted with ultra-pure water at 90±1°C for 2 h again, collected the filtrate, concentrated with reduced pressure and then added 4 times the volume ethanol to the filtrate to precipitated the polysaccharides at 4°C for 24 h. Remove the proteins by Sevag method, dialyzed the polysaccharide solution extensively against distilled water and then lyophilized and weighted it. Prepared different concentration of RPS with PBS for the treatment of K562 cells.

\*Corresponding author: e-mails: fanql.sh@163.com

Fan Qilan and Li Jialin are co-first authors of this article

### Cell culture and treatments

K562 cells were reported to derive from a 53-year-old female patient with chronic myelogenous leukemia in blast crisis and could be used as a cell model system of chronic myelogenous leukemia (Aydın *et al.*, 2013). The K562 cell line was purchased from Land Unicomed (Guangzhou, China) and cultured in RPMI 1640 media (Invitrogen, Carlsbad, USA), 10% fetal bovine serum, 100mg/mL streptomycin plus 100units/mL penicillin were added to the medium. Cells were cultured at 37°C in a humidified atmosphere, containing 5% CO<sub>2</sub> and 95% air, the medium were refreshed every 2-3 days. RPS was dissolved in RPMI 1640 medium. The K562 cells were made quiescent by incubation in serum-free medium for 24 h prior to be incubated with RPS.

### Protein extraction and Western Blotting

After treatment with or without RPS for 48 h, cells were harvested, proteins were extracted with RIPA, centrifuged the cell lysates at 12,000 g, 4°C for 15 min to remove cellular debris. The protein concentration was tested with BCA method, and the proteins were separated by SDS-PAGE, each lane contained equal amounts of protein. The following primary antibodies included monoclonal β-actin antibody (1:1000, Sigma, St. Louis, USA), polyclonal caspase3 antibody (1:1000, Sigma, St. Louis, USA), polyclonal Bcl2 antibody (1:1000, Proteintech, USA) were incubated overnight at 4°C.

### Effects of RPS on the proliferation of K562 cells

Cells at log phase were placed into 96-well plate (1×10<sup>4</sup> cells/well) and then dosed different concentrations of RPS for indicated time. Then, CCK-8 reagent (Beyotime Institute of Biotechnology, China) was added to each well (vol/vol=1:10) and mixed gently by shaking, the cells were further incubated in the incubator for 4 h. Then used a microplate reader (Thermo Scientific, USA) to measure the absorbance (A450). The proliferation of K562 cells inhibited by RPS was calculated with the following equation: The inhibition rate (%) = (A450<sub>blank</sub>-A450<sub>sample</sub>)/A450<sub>blank</sub>×100%.

### Flow cytometric analysis of cell cycle phase distribution by PI staining

Plated 2×10<sup>5</sup> cells on petri dish, dosed different concentrations of RPS for indicated time. Collected the cells by centrifugation, washed twice with cold PBS, fixed the cells overnight at 4°C with 70% ethanol. Washed again with PBS, and incubated with 500 μL PBS which containing 100 μg/mL RNase A, 0.2% Triton X-100, 50 μg/mL PI (Sigma-Aldrich) for 30 min away from light in room temperature. Cell cycle phase distribution was analyzed using a FACS-Calibur cytometer and Cell Quest computer software.

### Analysis of gene expression by Real-time PCR

Total RNA was extract by Trizol reagent (Invitrogen,

Carlsbad, USA) from K562 cells according to the manufacturers' instructions. The first strand DNA was synthesized by reverse transcription kit (Thermo, USA). Quantitative PCR was performed in triplicate using qPCR Kit (Thermo, USA) and the gene amplification was performed with ABI 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA). The primer sequence of Bcl2 was shown as: Forward-5'-CCTCTGTCCCACATAAG -3'; Reverse-5'-GTACCCATTGGGGTAATT -3'. A 2<sup>-ΔΔCT</sup> method was used to calculate the relative gene expression level by using β-actin for normalization as an internal control. The β-actin primer was gotten from the primer bank, the forward primer was: -5'-TGGATCAGCAAGCAGGAGT A-3'; The reverse β-actin primer was: -5'-TCGGCCACA TTGTGAACTTT-3'.

### Caspase 3 activity assay

Treated the cells with different dosages of RPS for 48 h, centrifuged at 600 g for 5 min to collect the cells, washed 3 times with ice-cold PBS, collected the cell debris, sat it on ice for 10 min after adding cell lysis buffer to it. Centrifuged at 20,000 g at 4°C for 10 min to collect the supernatant. Then tested the caspase 3 activity of the K562 cells with a kit (Beyotime Institute of Biotechnology, China) following manual instructions. 2 mmol/L Ac-DEVD-p-NA was added as the substrate of caspase3, incubated them at 37°C for 4 h, then the samples were measured at an absorbance of 405 nm.

### Hoechst 33258 staining

Incubated the K562 cells with or without RPS for 48 h, washed with PBS, then fixed the cells with 4% paraformaldehyde for 15 min. Washed again with PBS and then added 0.1% Triton X-100, incubated for 5 min at 4°C, stained with 5μg/mL Hoechst 33258 (Beyotime Institute of Biothechnology, China) at 37°C for 30 min, washed with PBS, observed them under a fluorescence microscope.

### Analysis of phosphorylated protein tyrosine kinase (P-PTK) by ELISA

Treated the K562 cells with indicated concentrations of RPS for 48 h, lysed the cells with lysis buffer and then collected the supernatants. ELISA kits were used to detect the P-PTK in the collected supernatants according to the manufacture's instruction. The absorbance at 450 nm was measured by Microplate Reader (Thermo Scientific, USA).The data was shown as picogram per milligram protein (pg/mg protein).

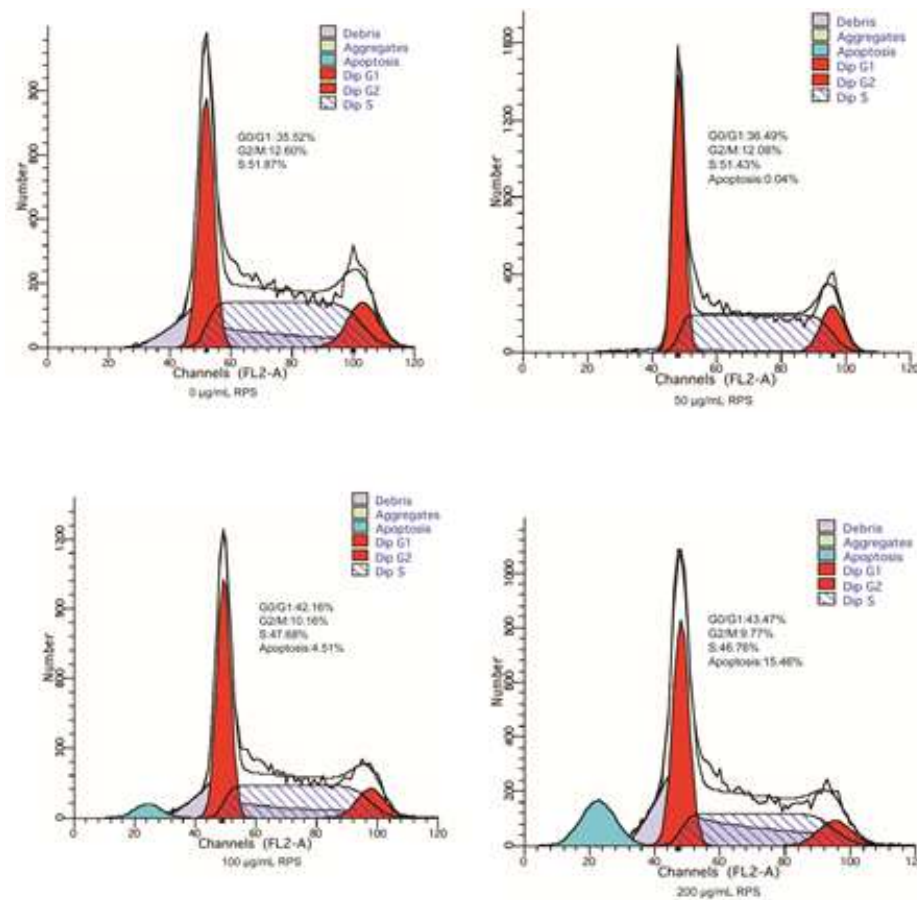
## STATISTICAL ANALYSIS

SPSS software version 21.0 was used to analyze the data by one-way ANOVA method. P<0.05 was considered to have significant differences.

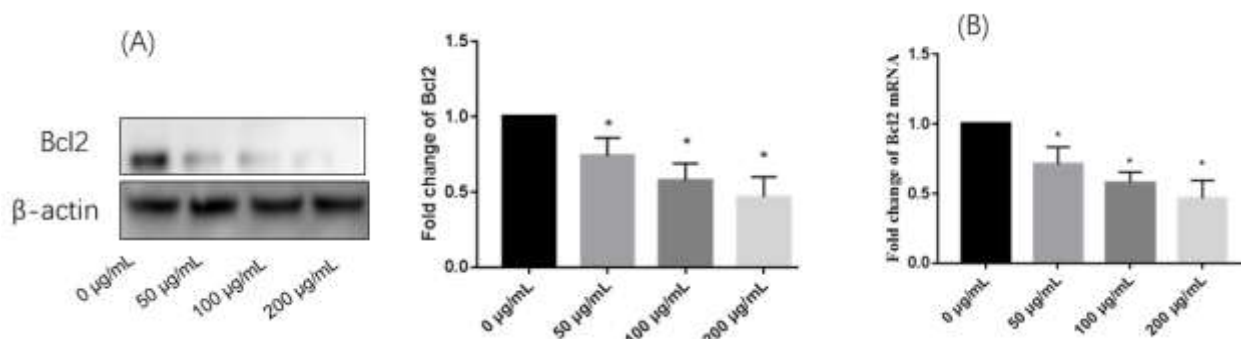
**Table 1:** Inhibition of RPS on the proliferation of K562 cells ( $x \pm s, n=3$ )

RPS ( $\mu\text{g/mL}$ )	48 h	
	OD	inhibitory rate
0	$0.995 \pm 0.011$	-
25	$0.967 \pm 0.003^*$	2.88%
50	$0.934 \pm 0.006^{**}$	6.16%
100	$0.904 \pm 0.008^{**}$	9.18%
200	$0.857 \pm 0.006^{**}$	13.90%
400	$0.800 \pm 0.019^{**}$	19.62%

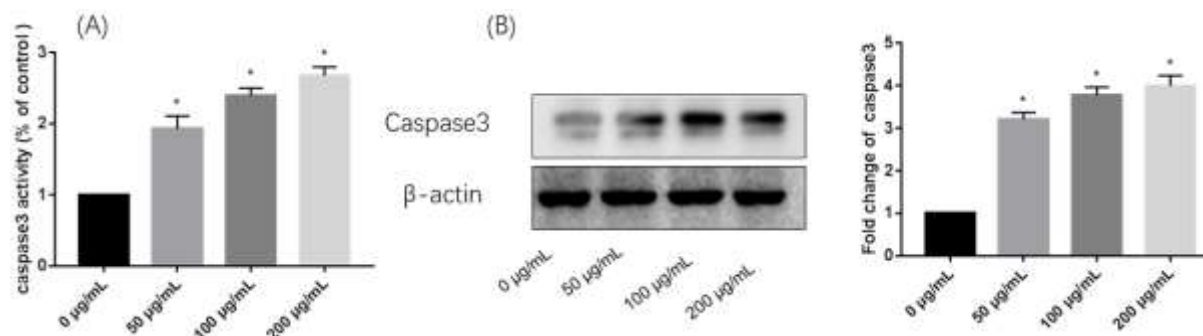
Note:  $*P < 0.05$ ,  $**P < 0.01$  vs. 0  $\mu\text{g/mL}$  RPS group.



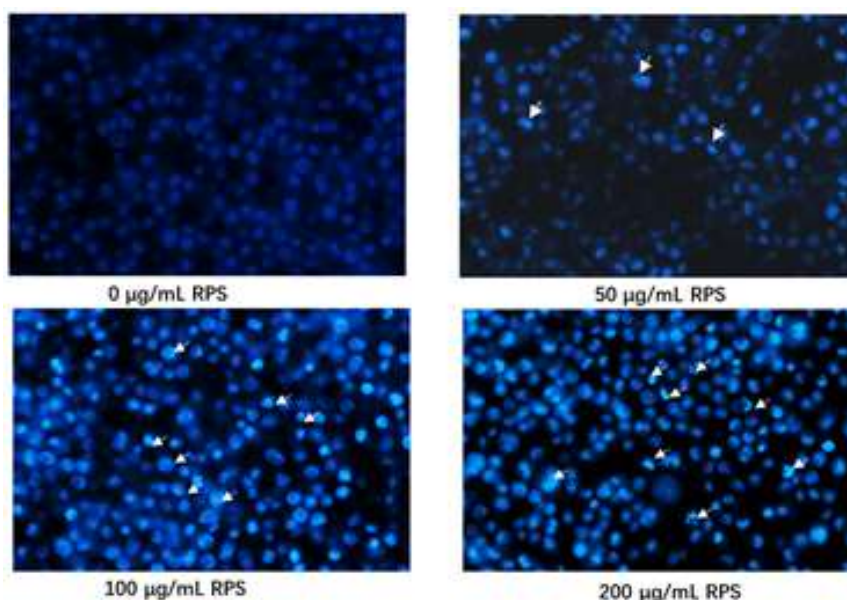
**Fig. 1:** Effects of RPS on the cell-cycle distribution of K562 cells



**Fig. 2:** (A) Effects of RPS on the protein expression of Bcl2 in K562 cells analyzed by Western Blotting. (B) Gene expression of Bcl2 by RT-qPCR. The error bars represent  $\pm$ SD of triplicate data,  $*p < 0.05$  as compared with 0  $\mu\text{g/mL}$  group.



**Fig. 3:** Effects of RPS on the activity of caspase3 tested by ELISA (A). The protein expression of caspase3 determined by Western Blotting in K562 cells (B). The error bars represent  $\pm$ SD of triplicate data, \* $p < 0.05$  as compared with 0  $\mu\text{g/mL}$  group.



**Fig. 4:** Incubated K562 cells with different concentrations of RPS for 48 h, and then analyzed the effect of RPS on apoptosis of K562 cells by Hoechst 33258 staining. Apoptosis was marked by white arrows. ( $\times 400$ )

## RESULTS

### *Effects of RPS on K562 cells proliferation*

After treating the K562 cells with different doses of RPS for 48h, we used CCK-8 kit to assay the cell proliferation. Results showed that RPS has antiproliferative effects on K562 cells in a dose-dependent manner at 48 h. (table 1).

### *Effects of RPS on the cell cycle of K562 cells*

After incubating the K562 cells with 0  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$  and 200  $\mu\text{g/mL}$  RPS for 48 h, the samples were stained with propidium iodide and analyzed by flow cytometry. table 2 showed that RPS could induce the K562 cells accumulated in G0/G1 phase in a dose dependent manner. Cells in S phase and G2/M phase were obviously decreased compared with the 0  $\mu\text{g/mL}$  RPS group. It was inferred that RPS could inhibit K562 cells proliferation by inducing cell arrest in G0/G1 phase. Moreover, we found that typical sub-G1 phase peaks

appeared on the cell cycle distribution after treating the K562 cells with 100  $\mu\text{g/mL}$  and 200  $\mu\text{g/mL}$  RPS for 48 h. This phenomenon indicated that RPS could induce the K562 cells apoptosis at 48 h, and the ratio of apoptotic cells increased from 0.04% to 15.46% with the increase of RPS concentration from 50  $\mu\text{g/mL}$  to 200  $\mu\text{g/mL}$  (fig. 1).

### *Effects of RPS on the expression of Bcl2 in K562 cells*

Treated K562 cells with different concentrations of RPS for 48 h, the protein and gene expression level of Bcl2 was displayed by Western blot and RT-qPCR. Results showed that the Bcl2 expression was down regulated both in gene and protein level after treatment of K562 cells with RPS (fig. 2A, fig. 2B).

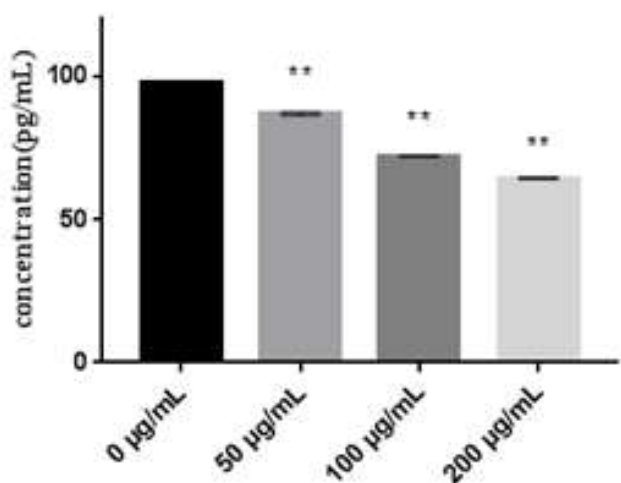
### *Effects of RPS on caspase3 activity and the protein expression in K562 cells*

The K562 cells were incubated with 0  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$  and 200  $\mu\text{g/mL}$  RPS for 48 h and then

caspase3 activity was tested using a caspase3 activity assay kit. Results showed that different dosages of RPS could increase the caspase3 activity in K562 cells compared with 0  $\mu\text{g/mL}$  RPS group and the protein expression of caspase3 are similar to those of caspase3 activity (fig. 3A, fig. 3B).

#### **Apoptosis induced by RPS was determined by Hoechst 33258 staining**

Apoptotic cells were characterized by the features of DNA condensation and nuclear fragmentation (Xue *et al.*, 2016), we found that RPS could significantly induce nuclear condensation compared with 0  $\mu\text{g/mL}$  group (Fig. 4). These results indicated that RPS treatment could induce the apoptosis of K562 cells.



**Fig. 5:** Effects of RPS on the activity of PTK tested by ELISA kit. The error bars represent  $\pm\text{SD}$  of triplicate data, \*\*  $P < 0.01$  as compared with 0  $\mu\text{g/mL}$  group.

#### **Effects of RPS on the phosphorylation level of PTK**

The K562 cells were stimulated with 0  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , 200  $\mu\text{g/mL}$  RPS for 48 h, the cells were collected and the level of phosphorylation of PTK was tested using ELISA kit following manufacturer's instruction. We found that after treating the K562 cells with RPS, the phosphorylation level of PTK down regulated compared to 0  $\mu\text{g/mL}$  group (fig. 5).

## **DISCUSSION**

Leukemia is one of the most serious malignant tumors of hemopoietic system to human health, it is characterized by loss control of cell division and cell growth. Many studies showed that the uncontrolled proliferation and inhibited apoptosis of leukemia cells will develop into leukemia (Duncan *et al.*, 2016; Rodrigues *et al.*, 2016; Tuo *et al.*, 2015; Ahmad *et al.*, 2017).

As we all know *Rehmannia glutinosa* is a traditional Chinese medicinal herb. RPS is one of the most important

medicinal ingredient of *Rehmannia glutinosa*, it can improve the body's immunity (Huang *et al.*, 2016), and remarkably inhibit the growth of H22 and S180 tumor in vivo (Bo-Yan *et al.*, 2012). One of our previous research showed that PRS could inhibit the proliferation of K562 cells via down regulated BCR-ABL fusion gene expression both at mRNA and protein level (Su-Zhen *et al.*, 2017).

Many life processes such as cell proliferation/apoptosis and cell growth/differentiation played crucial role in formation and inhibition of tumor (Mogavero *et al.*, 2017; Pei *et al.*, 2018; Chen *et al.*, 2017; Fang *et al.*, 2017). In this study, we indicated that RPS could obviously inhibit the proliferation of K562 cells. The inhibitory rate reached 13.9% when incubated K562 cells with 200  $\mu\text{g/mL}$  RPS for 48 h. Flow cytometry indicated that K562 cells responded to RPS with a G0/G1 arrest. RPS could induce the K562 cells accumulated in G0/G1 phase in a dose dependent manner. A typical sub-G1 phase peaks appeared on the cell cycle distribution after treated the K562 cells with different concentration of RPS for 48 h. Results of Western Blotting and Real-time PCR also show that RPS could inhibit Bcl2 expression in gene and protein level and up regulate the activity and protein expression of caspase3 in K562 cells compared to the control group. Taken together, we can conclude that RPS can not only induce the K562 cells accumulated in G0/G1 phase but also can induce K562 cells apoptosis.

Many studies showed that sustained activation of protein tyrosine kinases (PTKs) are involved in tumor cell proliferation, differentiation, migration and then result in uncontrollable proliferation of cells, it played a vital role in the pathogenesis of CML. Imatinib (IM), a PTK inhibitor was used to treat CML and it was likely to be curative (Herbst *et al.*, 2002; Levitzki *et al.*, 2006; Ferguson *et al.*, 2018). In the present study we found that RPS could down regulate the phosphorylated level of PTKs. Therefore, we speculated that RPS could inhibit the proliferation and induce the apoptosis of K562 cells by inhibiting the activity of PTKs. We will continue to further study the mechanisms of this phenomenon.

## **CONCLUSION**

Our results showed that RPS could suppress the proliferation and induce the apoptosis of K562 cells in vitro. It may be related to the reduced expression of Bcl2 and raised expression of caspase3 and improved activity of caspase3 as well as the inhibition of protein tyrosine kinases activity induced by RPS.

## **ACKNOWLEDGEMENT**

This work received funds from the Provincial Education Department of Jiangxi (GJJ12542).

## REFERENCES

- Ahmad HM, Muiwo P, Muthuswami R and Bhattacharya A (2017). FosB regulates expression of miR-22 during PMA induced differentiation of K562 cells to megakaryocytes. *Biochimie.*, **133**: 1-6.
- Alves A, Machado-Neto J, Scheucher P, Paiva, H, Simões, B and Traina F (2016). Reversine triggers mitotic catastrophe and apoptosis in K562 cells. *Leuk. Res.*, **48**: 26-31.
- Aydin B, Kan B and Cabadak H (2013). The role of intracellular pathways in the proliferation of human K562 cells mediated by muscarinic receptors. *Leuk. Res.*, **37**(9): 1144-1149.
- Bo-Yan WU, Wang X, Wang JL, Shao-Na XU and Liang Y (2012). Effects of Anti-tumor Function and Survival Time of RPS on Tumor-bearing Mice. *Inf. Tradit. Chin. Med.*, **29**(6): 9.
- Chen G, Qian W, Li J, Xu Y and Chen K (2015). Exopolysaccharide of Antarctic bacterium *Pseudoaltermonas* sp. S-5 induces apoptosis in K562 cells. *Carbohydr. Polym.*, **121**: 107-114.
- Chen S, Yong T, Zhang Y, Su J, Jiao C and Xie Y (2017). Anti-tumor and Anti-angiogenic Ergosterols from *Ganoderma lucidum*. *Front. Chem.*, **5**: 85.
- Duncan MT, DeLuca TA, Kuo HY, Yi M, Mrksich M and Mille WM (2016). SIRT1 is a critical regulator of K562 cell growth, survival, and differentiation. *Exp. Cell Res.*, **344**(1): 40-52.
- Fang J, Ying H, Mao T, Fang Y, Lu Y, Wang H and Luo X (2017). Up regulation of CD11b and CD86 through LSD1 inhibition promotes myeloid differentiation and suppresses cell proliferation in human monocytic leukemia cells. *Oncotarget.*, **8**(49): 85085-85101.
- Ferguson P, Vincent M and Koropatnick J (2018). Synergistic antiproliferative activity of the RAD51 inhibitor IBR2 with inhibitors of receptor tyrosine kinases and microtubule protein. *J. Pharmacol. Exp. Ther.*, **364**(1): 46-54.
- Gong J, Zheng S, Zhang L, Wang Y and Meng J (2017). Induction of K562 Cell Apoptosis by As4S4 via Down-Regulating miR181. *Med. Sci. Monit.*, **23**: 144-150.
- Herbst R (2002). Targeted therapy in non-small-cell lung cancer. *Oncology* (Williston Park, N.Y.) **16**(Suppl 9): 19-24.
- Huang Y, Liu Z, Bo R, Xing J, Luo L, Zhen S and Wang D (2016). The enhanced immune response of PCV-2 vaccine using *Rehmannia glutinosa* polysaccharide liposome as an adjuvant. *Int. J. Biol. Macromol.*, **86**: 929-936.
- Krishnan V and Rajasekaran AK (2014). Clinical nanomedicine: A solution to the chemotherapy conundrum in pediatric leukemia therapy. *Clin. Pharmacol. Ther.*, **95**(2): 168-178.
- Levitzi A and Mishani E (2006). Tyrosine kinases and other tyrosine kinase inhibitors. *Annu. Rev. Biochem.*, **75**: 93-109.
- Mogavero A, Maiorana MV, Zanutto S, Varinelli L, Bozzi F, Belfiore A and Garibold M (2017). Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. *Sci. Rep.*, **7**(1): 15992.
- Pei R, Si T, Lu Y, Zhou J and Jiang L (2018). Salvianolic acid A, a novel PI3K/Akt inhibitor, induces cell apoptosis and suppresses tumor growth in acute myeloid leukemia. *Leuk. Lymphoma.*, **59**(8): 1959-167.
- Srdic-Rajic T, Nikolic K, Cavic M, Djokic I, Gemovic B, Perovic V and Veljkovic N (2016). Rilmenidine suppresses proliferation and promotes apoptosis via the mitochondrial pathway in human leukemic K562 cells. *Eur. J. Pharm. Sci.*, **81**: 172-180.
- Su-Zhen WU, Chun-Juan XU, Jia-Lin LI and Fan QL (2017). Effects of *Rehmannia glutinosa* polysaccharides on K562 cell proliferation and Bcr/Abl fusion gene expression. *J. China Pharm.*, **37**(8): 698-701.
- Tuo Y, Jiang S, Qian F, Mu G, Liu P, Guo Y and Ma C (2015). Short communication: Antiproliferative effect of 8 different *Lactobacillus* strains on K562 cells. *J. Dairy Sci.*, **98**(1):106-110.
- Xu L, Kwak M, Zhang W, Zeng L, Lee P and Jin J (2017b). *Rehmannia glutinosa* polysaccharide induces toll-like receptor 4 dependent spleen dendritic cell maturation and anti-cancer immunity. *Oncimmunology*, **6**(7): e1325981.
- Xu L, Zhang W, Zeng L, and Jin J (2017a). *Rehmannia glutinosa* polysaccharide induced an anti-cancer effect by activating natural killer cells. *Int. J. Biol. Macromol.*, **105**:680-685.
- Xue H, Ji Y, Wei S, Yu Y, Yan X, Liu S and Chen L (2016). HGSD attenuates neuronal apoptosis through enhancing neuronal autophagy in the brain of diabetic mice: The role of AMP-activated protein kinase. *Life Sci.*, **153**: 23-34.
- Yang C, Cai H and Meng X (2016). Polyphyllin D induces apoptosis and differentiation in K562 human leukemia cells. *Int. Immunopharmacol.*, **36**: 17-22.
- Yang C, Yang J, Sun M, Yan J, Meng X and Ma T (2013). Alantolactone inhibits growth of K562/adriamycin cells by downregulating Bcr/Abl and P-glycoprotein expression. *IUBMB Life*, **65**(5): 435-444.