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An exopolysaccharide from *Trichoderma pseudokoningii* and its apoptotic activity on human leukemia K562 cells

Taotao Huang[a], Jun Lin[b], Jianfeng Cao[a], Pengying Zhang[a,c], Yungui Bai[a], Guochuang Chen[a], Kaoshan Chen[a,b,c,*]

[a] School of Life Sciences, Shandong University, Jinan 250100, PR China
[b] Department of Pharmacy, Wannan Medical college, Wuhu 241000, PR China
[c] National Glycoengineering Research Center, School of Life Science, Shandong University, Jinan 250100, PR China

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ABSTRACT

In this study, a novel exopolysaccharide (EPS) was isolated from the fermentation broth of *Trichoderma pseudokoningii* and its anticancer activities on human leukemia K562 cells were studied. EPS could significantly inhibited K562 cells proliferation in a time- and concentration-dependent manner. Meanwhile, characteristic of apoptosis, including apoptotic morphological features and the apoptosis rate were obtained. Sequentially, the dissipation of mitochondrial membrane potential, increase production of Reactive oxygen species (ROS), enhancement of the concentration of intracellular, up-regulation of Bax and p53 mRNA, down-regulation of Bcl-2 mRNA were also detected. The results indicate that the EPS could induce of K562 cells apoptosis, primarily in involved the mitochondrial pathways. The present studies suggest that EPS could be a new potential adjuvant chemotherapeutic and chemo preventive agent against human leukemia.

1. Introduction

Leukemia is a malignant disease of blood cell-forming tissue, also known as "cancer of the blood". It occurs when white cells multiply in an abnormal manner in the bone marrow, being reported as the cause of death in 82% of diagnosed subjects in 2000 (Mathers, Boschi-Pinto, Lopez, & Murray, 2001). Chemotherapy combined with pharmaceutical medication are the typically strategy to treat leukemia because unlike solid tumor, hematological malignant tumor cannot be cured by surgical treatment or radiation therapy (Singh, Dilnawaz, & Sahoo, 2011). However, chemotherapy causes a number of undesirable side effects in the host (Ehrke, 2003; Rosenberg et al., 1994). Therefore, new anti-cancer drugs with higher bioactivities and less or without side effects from nature appear to be a great significance.

Recently, many polysaccharides have been isolated from fungi, yeasts, plants, lichens and algae (Liu et al., 2008). Amounts of attention have focused on the biological activities of polysaccharides due to their immunomodulatory and antioxidant effects in the biochemical and medical areas (Liu et al., 2010; Ooi & Liu, 2000; Wang et al., 2008). Increasing lines of evidence demonstrate that the polysaccharides could induce cancer cells apoptosis, whereas less toxic to normal cells (Chow, Lo, Loo, Hu, & Sham, 2003; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Lin et al., 2003). Such as *Lycium barbarum* polysaccharide (LBP) induces apoptosis by increasing intracellular calcium in QGY7703 cells (Zhang et al., 2005), *Grifola frondosa* polysaccharide-peptide (GFPS) induces apoptosis associated with drop in mitochondrial membrane potential, increase the ratio of Bax to Bcl-2 protein, and activation of caspase-3 (Cui et al., 2007). Drugs promoting cancer cells apoptosis may be an effective and important strategy to counteract cancer (Fesik, 2005). Therefore, it is great meaningful to find the polysaccharide which possesses the splendid activity of inducing apoptosis in oncotherapy and adjuvant therapy.

Our group has isolated an exopolysaccharide (EPS) from the fermentation broth of *Trichoderma pseudokoningii*, and EPS was showed to enhance the immunity system. However, the antiproliferative activity on tumor cells of EPS has not yet been elucidated. In the current research, we have investigated the anticancer activity of EPS against K562 cells, and possible apoptosis mechanism was also studied.

2. Materials and methods

2.1. Materials

RPMI 1640, fetal bovine serum, penicillin, streptomycin and TRIzol reagent were purchased from GIBCO BRL. Sulforhodamine

* Corresponding author at: National Glycoengineering Research Center, School of Life Science, Shandong University, 27 Shanda Nanlu, Jinan 250100, PR China. Tel.: +86 531 8836 5311; fax: +86 531 8836 5311.
E-mail address: ksc313@126.com (K. Chen).

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B, 2′,7′-dichlorofluorescein diacetate and Hoechst 33258 were obtained from Sigma–Aldrich Chemical. JC-1 mitochondrial membrane potential detection assay kit and Annexin V-FITC staining kit were from Beyotime Institute of Biotechnology (Haimen, China).

2.2. Isolation and purification of exopolysaccharide

EPS was isolated and purified according to the reported method with some modifications (Jin et al., 2010). Briefly, *T. pseudokoningii* was obtained from the Laboratory of Biomass Resources, Shandong University (Jinan, China), and incubated in a 1000 mL flask containing 600 mL Potato Dextrose Agar (PDA) at 28 °C for 10 days at 180 rpm. The supernatants was collected, concentrated to a proper volume, deproteinated by the method of Sevag (1938) and mixed with three times volume of 95% ethanol. The mixture was kept overnight at 4 °C. Then precipitate was collected, resolved in distilled water and centrifugated at 12000 rpm. The results supernatant was applied to a DEAE Sepharose Fast flow column (1.6 cm × 20 cm), eluting successively with distilled water and a linear gradient of 0–0.5 M Nacl with a flow rate of 0.5 mL/min. The main peak obtained was concentrated, dialyzed and further fractionated on a Sephadex G-75 column (1.6 cm × 60 cm) eluted with distilled water. The main fraction was collected and lyophilized to obtain a white purified EPS. The average molecular weight was 31.9 kDa measured by gel permeation chromatography (GPC). The component sugars of EPS, determined by GC, were rhamnose, xylose, fucose, mannose, glucose, and galactose and with molar ratios of 16.2:14.4:1:25.8:23.6:48.1. The data are the same to our previous results.

2.3. Cell culture

Human Leukemia cell line K562 was obtained from Shanghai Cell Biology Institute, China. Cells were maintained in RPMI the 1640 mediums supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.4. Cell proliferation assay

Viability of cells was evaluated by the Sulforhodamine B (SRB) assay as described previously (Skehan et al., 1990). This method was used for cell density determination, based on the measurement of cellular protein content. K562 cells were seeded in a 96-well plate at a density of 3×10^4 cells in 180 μL RPMI-1640 culture medium containing 10% fetal bovine serum per well and incubated for 24 h. In the experiments, six replicate wells were used in each point. Subsequently, cells were incubated with increasing concentration of EPS for 24, 48, or 72 h. Cells then were fixed with 50 μL of ice-cold 50% trichloroacetic acid (TCA) and fixed 1 h at 4 °C. The cells were washed five times with deionized water and air dried, then stained with 0.4% SRB for 30 min at 37 °C Unbound dye was removed by washing five times with 1% acetic acid. After air drying, the bound dye was solubilized with 150 μL Tris base (10 mM, pH 10.5), and then the optical density determination at 510 nm with a microplate reader. All experiments were performed at least three times.

$$\text{Inhibition rate}\,(\%) = \left(\frac{1-\text{OD}_{sample}}{\text{OD}_{control}}\right) \times 100$$

2.5. Hoechst 33258 staining

Hoechst 33258 staining was conducted as previously described (Ramoneda & Perez-Tomas, 2002). Briefly, K562 cells were treated with 0, 0.2, 0.6 and 1 mg/mL EPS for 48 h, then cells were collected, fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C, and stained with Hoechst 33258 for 10 min in a dark at room temperature. The cells were washed with PBS and smeared onto cover slides. Nuclear morphology was observed and immediately photographed under a fluorescence microscope.

2.6. Annexin V-FITC and PI double staining

The cells were grown in the six-well flask for 24 h. Then cells were incubated with various concentrations of EPS (0, 0.2, 0.6 and 1 mg/mL). After 48 h, cells were collected and washed twice with PBS, and re-suspended in 195 μL Annexin V-FITC binding buffer. Five microliters of Annexin V-FITC was added to the tubes. Mixtures were gently vortexed and incubated for 10 min in dark at room temperature. PI was added before a final wash with PBS and analyzed by flow cytometry immediately.

2.7. Mitochondrial potential analysis

A fluorescent carbocyanine dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolocarbocyanine iodide, also called JC-1, was used as a probe to measure the mitochondrial membrane potential ($\Delta\psi m$) during the early stages of apoptosis (Troyan, Gilman, & Gay, 1997). After treated with EPS (0, 0.2, 0.6, 1 mg/mL) for 24 h, cells were incubated with 5 μg/mL JC-1 at 37 °C for 30 min in complete medium. Then washed with PBS, the stained cells were analyzed using laser confocal scanning microscopy (LSCM). The cells were scanned by dual excitation of 488 nm (green) for detect JC-1 monomers and 568 nm (red) for detect JC-1 aggregates. The green/red fluorescence intensity ratio was represented $\Delta\Psi m$ of K562 cells.

2.8. Reactive oxygen species (ROS) measurement

ROS was determined with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as previously described (Tedesco, Luigi Russo, Nazzaro, Russo, & Palumbo, 2001). K562 cells were seeded into 6-well plates, incubated for 24 h and treated with EPS in different concentrations (0, 0.2, 0.4, 0.6, 0.8, 1 mg/mL) for another 24 h. After removed the culture, cells were incubated with 10 mM DCFH-DA at 37 °C for 20 min and washed three times with PBS. Then, the cells were collected and further analyzed using fluorospectro-photometer (Genios, TECAN) at an excitation wave length of 488 nm and an emission wave length of 535 nm. Images of the cells treated with EPS at the concentration of 0.4 mg/mL, were also captured by LSCM at 488 nm for excitation.

2.9. Intracellular calcium measurements

K562 cells were cultured for 24 h with the medium containing 0.2, 0.6 and 1 mg/mL EPS. Then the fluo-3/AM in a nominally Ca^{2+}-free buffer was loaded to cells for 30 min at 37 °C. Finally, the cells were washed by Ca^{2+}-free buffer for three times and examined by LSCM.

2.10. Reverse transcription-PCR analysis

The expression of mRNA for Bax, Bcl-2 and P53 were determined by RT-PCR. K562 cells were seeded in 6-well plate with different concentrations of EPS (0, 0.2, 0.6 and 1 mg/mL) and incubated at 37 °C for 48 h. Then the total RNA was extracted from the K562 cells using the TRIzol agent according the instructions supplied by manufacturer. cDNA was synthesized following the program: 25 °C for 10 min, 42 °C for 30 min and 85 °C for 5 min. The 20 μL reaction mixture contained 100 ng of RNA sample, 0.1 μg of Random Primer, 10 μL 2× reaction mixes and 1 μL reverse transcriptase. The cDNA obtained was used as the template for PCR. PCR was carried out in a 20 μL reaction volume

containing 0.2 mM each dNTP, 0.5U Taq DNA Polymerase, 10 μM each primer mixture, 2 μL of 10× PCR Buffer (with Mg^{2+}) and 1 μL of cDNA solution. The primer sequences were; GADPH (Dias et al., 2011) (Forward: 5′-ATGGCACCGTCAAGGCTGAG-3′, Reverse: 5′-GCAGTGATGGCATGGACTGT-3′), Bax (Agarwal et al., 1999) (Forward: 5′-ACCAAGAAGCTGAGCGAGTGT-3′, Reverse: 5′-ACAAACATGGTCACGGTCTGC-3′), Bcl-2 (Dias et al., 2011) (Forward: 5′-GACTTCGCCGAGATGTCCAG-3′, Reverse: 5′-CAGGTGCCGGTTCAGGTACT-3′), P53 (Zhang & Huang, 2006) (Forward: 5′-AAGGAAATTTGCGTGTGGAG-3′, Reverse: 5′-TTCTGACGCACACCTATTGC-3′). The expected sizes for the PCR products: 379 bp for GADPH, 332 bp for Bax, 225 bp for bcl-2, 702 bp for p53. The reaction was incubated at 95 °C for 2 min followed by 28 (GADPH), 35 (P53, Bax), 40 (Bcl-2) cycles of 30 s at 94 °C, annealing at 56 °C for 30 s (GADPH), 53 °C for 60 s (P53, Bax), 65 °C for 30 s (Bcl-2), and extension at 72 °C for 30 s (GADPH, Bcl-2), 72 °C for 2 min (P53, Bax), and with an final extension step of 10 min at 72 °C The PCR products were separated by electrophoresis on a 1% agarose gel and stained with 0.5 μg/mL ethidium bromide. The densitometry of each band was performed with Quantity One Software.

2.11. Statistical analysis

Significant differences were evaluated by using Student's *t*-test and all the results were expressed as Mean ± SD. Statistical significance was taken into consideration when $p < 0.05$.

3. Results and discussion

3.1. Effect of EPS on K562 cell proliferation

In general, polysaccharides have no direct cytotoxicity on cancer cells. Recently, some polysaccharides exhibit cytotoxic activity on human tumor cells, and some of them show significant apoptosis-inducing activities. For instance, Protein-bound polysaccharide K (PSK), *Angelica sinensis* polysaccharide (ASP), and *Phellinus linteus* polysaccharide (PLP) had been revealed to show directly cytotoxic effects on human cancer cells by inducing the cell apoptosis (Cao et al., 2010; Hattori, Komatsu, Shichijo, & Itoh, 2004; Li et al., 2004).

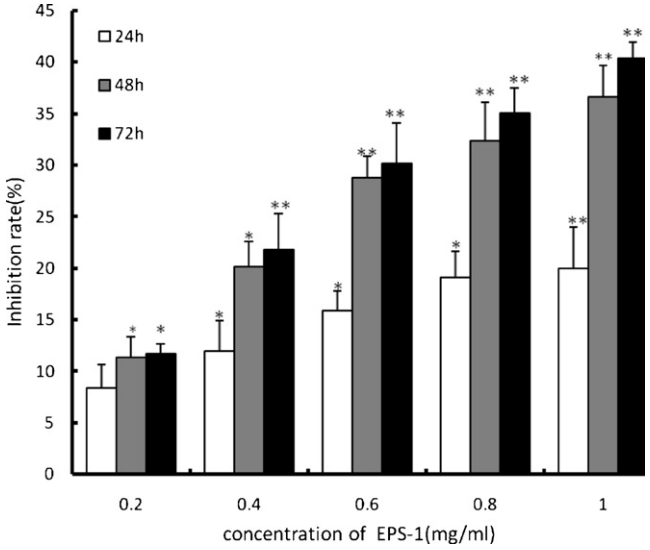

Fig. 1. Concentration- and time-dependent cytotoxic effects of EPS on K562 cells. Cells were cultured in 96-well plates for 24 h and then treated with EPS at increasing concentrations for 24, 48, 72 h followed by the SRB assay. Values are means ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$.

To evaluate the growth inhibitory by EPS, the cells were treated with increasing concentrations of EPS for 24, 48, 72 h and cell viability was measured by SRB assay. Fig. 1 showed that EPS-1 inhibited the growth of K562 cells in a time- and concentration-dependent relative to control cells. After 48 h treatment, inhibition rates with rates of 11.35–36.7% at the concentrations ranging from 0.2 to 1 mg/mL, whereas the inhibitory effects for 24 h treatment were not significant. Treatment with 1 mg/mL EPS for 72 h, the inhibition rate approximately to 41%.

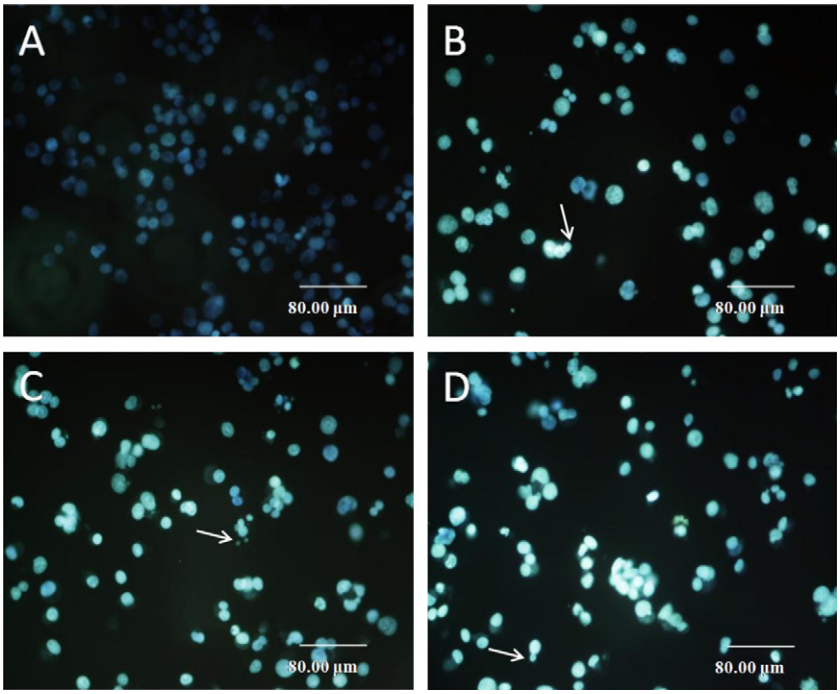

Fig. 2. Fluorescence photomicrograph of K562 cells stained with Hoechst 33258. K562 cells were treated with 0, 0.2, 0.6 and 1 mg/mL EPS for 48 h. (A) Control (200×): the nuclei were stained homogeneous and less bright. (B–D) Treated cells (200×): chromatin condensed and apoptotic bodies formed. Arrows point the apoptotic bodies.

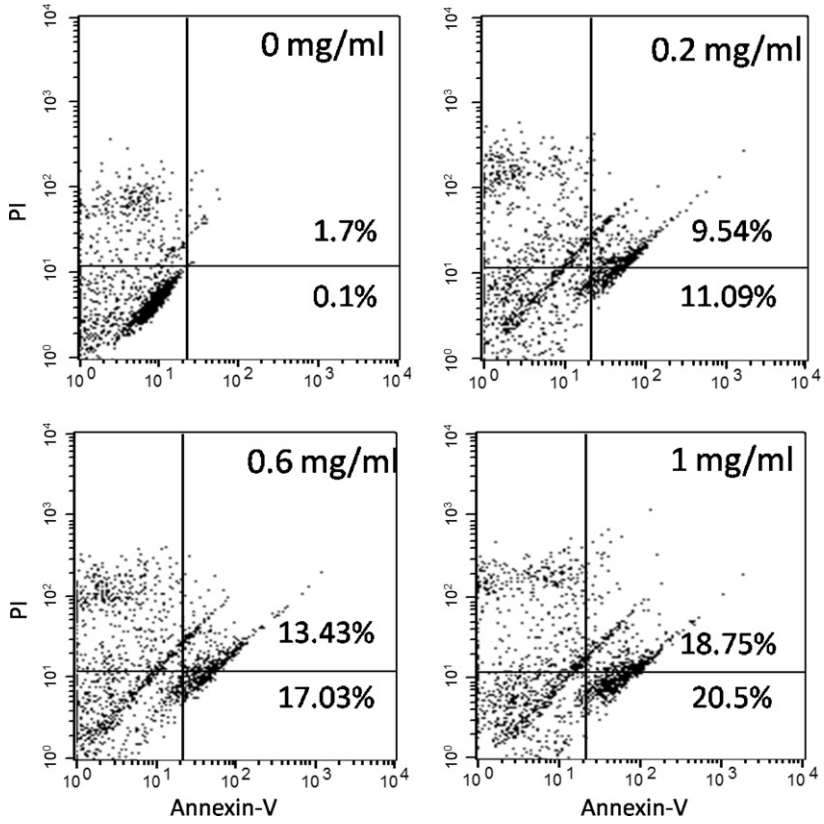

Fig. 3. Apoptosis effect of EPS on K562 cells discriminated by Annexin-V/PI double stain. K562 cells were treated for 0, 0.2, 0.6 and 1 mg/mL of EPS with 48 h. As shown, the cell populations shown in the lower right (Annexin V+/PI−) represents apoptosis cells, upper right (Annexin V+/PI+) represents necrotic cells or post-apoptotic.

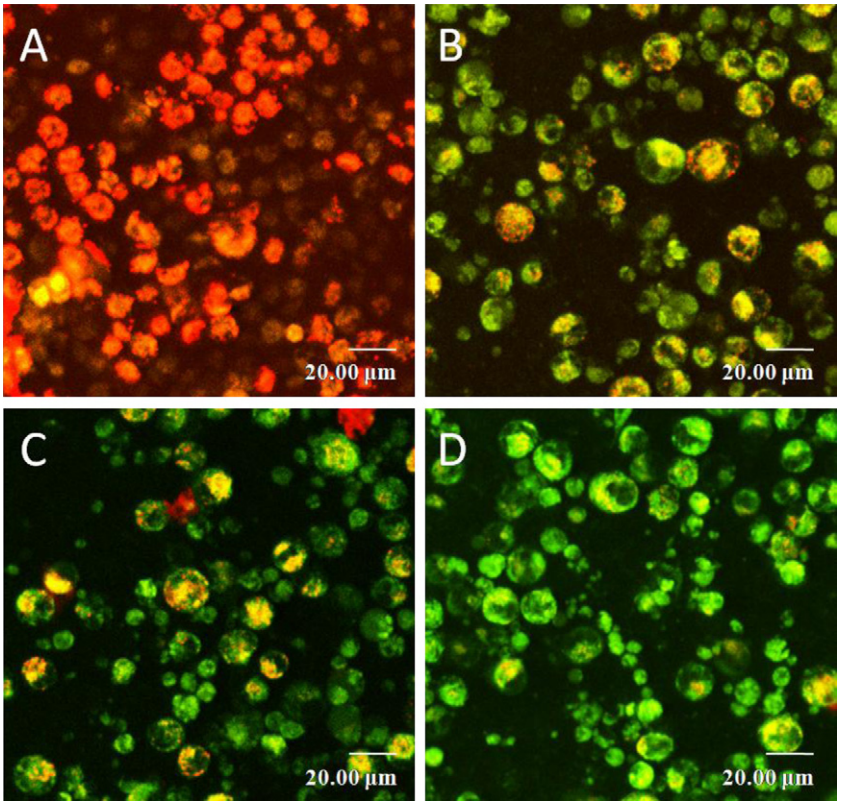

Fig. 4. Effect of EPS on mitochondrial membrane potential in K562 cells loaded with JC-1. K562 cells were treated for 0, 0.2, 0.6 and 1 mg/mL of EPS with 48 h. Images were taken by LSCM. As shown, the green fluorescence intensity grows and red fluorescence intensity decreases accompanied with the increasing concentration of EPS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

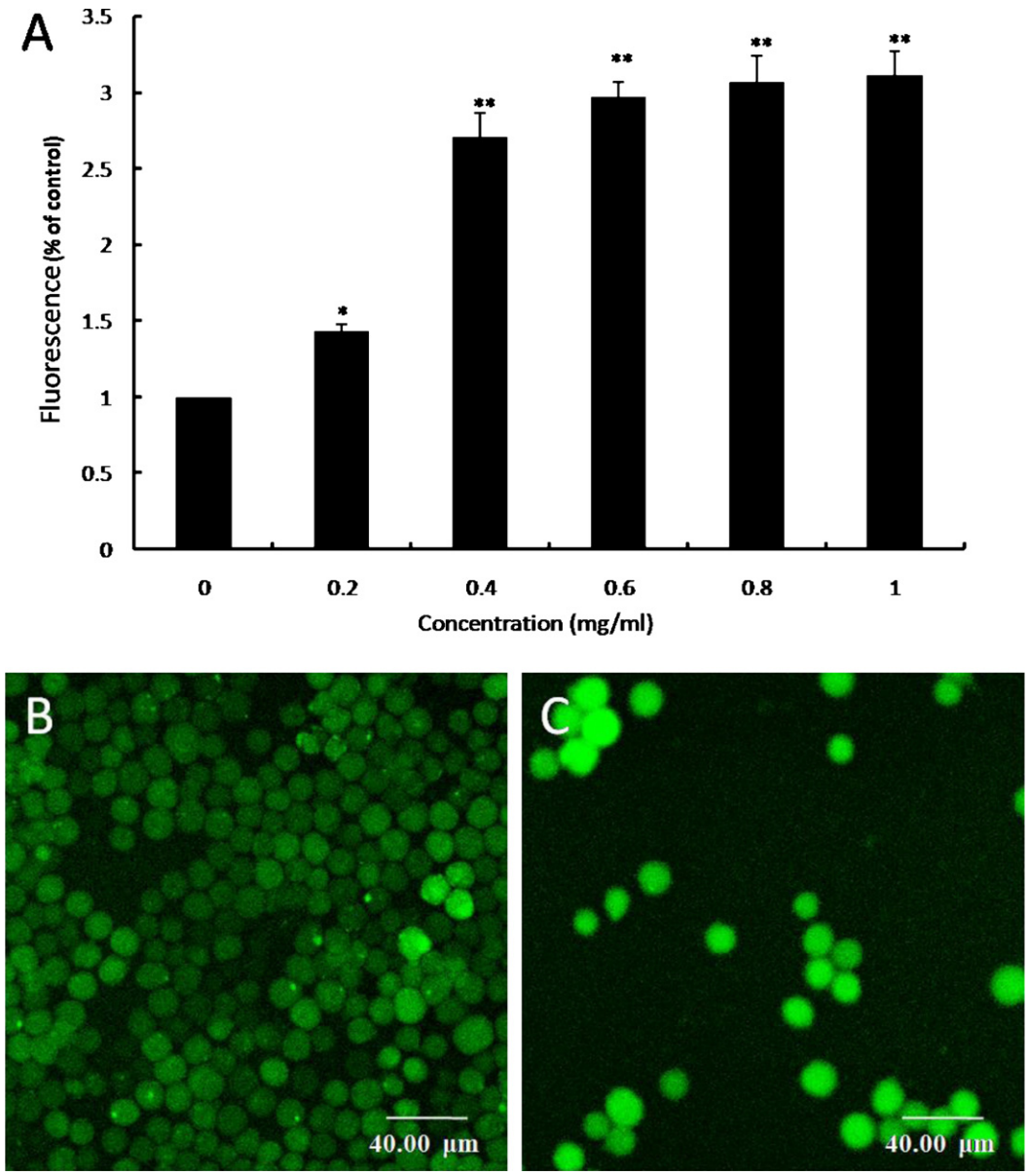

Fig. 5. Effect of EPS treatment on ROS production in K562 cells loaded with DCFH-DA. Changes of ROS production in cells at the increasing concentration of EPS were measured by fluorospectro-photometer (A). Data are represented as the means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. Photographs about the cells cultured without (B) and with 0.4 mg/ml EPS (C) for 24 h were taken by LSCM.

3.2. Morphological characterization of K562 apoptosis by Hoechst 33258 staining

To investigate whether the growth-inhibitory effect is related to the induction of apoptosis, K562 cells were treated with 0.2, 0.6, 1 mg/mL EPS for 48 h and stained with membrane permeable blue Hoechst 33258. Fig. 2 showed the significant morphological changes induced by EPS in the nuclear chromatin, expressed cell shrinkage, chromatin compaction, condensation of cytoplasm and nuclear fragmentation. In the untreated groups, the nuclei have similarly sized, regularly shaped with control, and evenly stained (lightly stained). However, condensed chromatin (brightly stained) could be observed in treated cells, and some of them formed the structure of apoptotic bodies, which is one of the typical characteristics of apoptotic cells. Further confirmation of EPS induced apoptosis was obtained by using flow cytometry with Annexin V–PI staining.

3.3. Detection of apoptosis by flow cytometry

Apoptosis results in membrane changes, with translocation of the phosphatidylserine (PS) from the inner to the outer layer of the cell membrane, thereby exposing PS to the external cellular environment (Fadok et al., 1992). Annexin V is a calcium dependent phospholipid-binding protein that has a high affinity for PS, can be used as a probe for the PS on the outer leaf cell membrane and hence as a marker of apoptosis.

Based on the morphological changes of K562 cells with the treatment of EPS, we further showed the occurrence of EPS-induced apoptosis by Annexin V-PI staining methods. The sets of cell population in different quadrants were analyzed using quadrant statistics. Annexin V−/PI− (Lower Left) cells were represented survivals, Annexin V+/PI− (lower right) cells were defined as apoptosis cells, Annexin V+/PI+ (Upper Right) cells were recognized as necrosis or post-apoptotic.

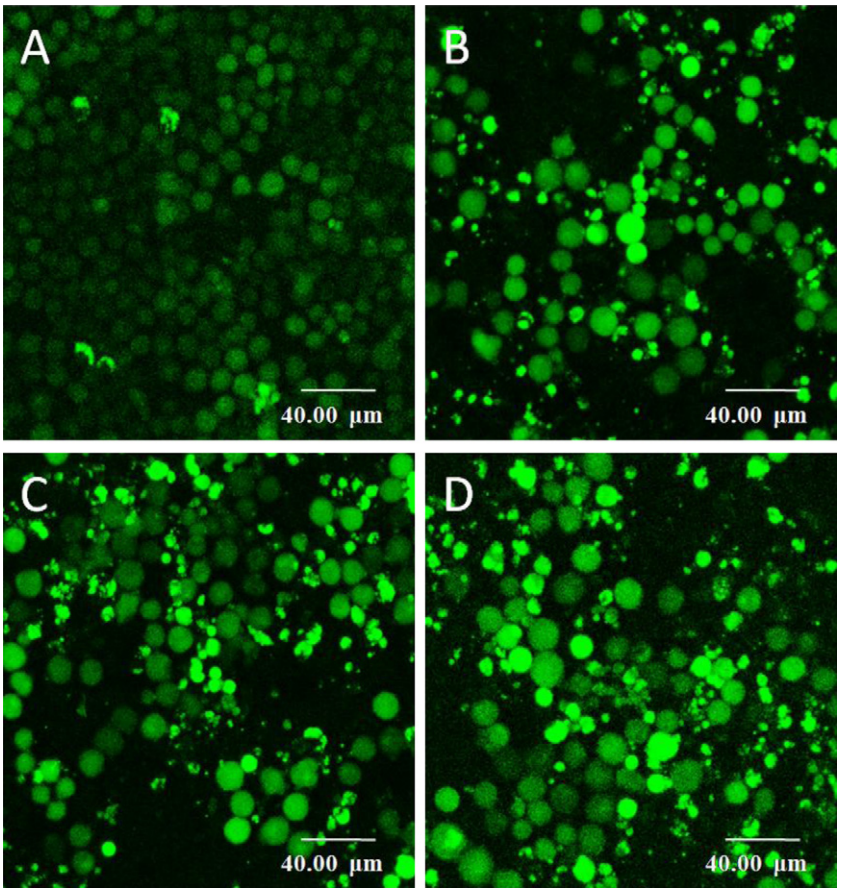

Fig. 6. Effects of EPS on intracellular Ca^{2+} distribution in K562 cells. Cells were treated with EFS (0, 0.2, 0.6, 1 mg/mL) for 24 h and labeled with Fluo-3/AM. (A) Calcium distribution in control cells. (B–D) Calcium distribution in EPS treated cells.

Fig. 3 showed that the population of pro-apoptotic cells (lower right) significantly elevated in EPS (0, 0.2, 0.6, 1 mg/mL)-treated cells from 0.1% in untreated cells to 20.5%. The later apoptotic and necrotic cells increase from 1.7% to 18.75%. The results indicated that PS externalization and necrotic cells were evident in K562 cells treated with EPS. These data provided support for EPS-induced apoptosis contributes to the growth inhibition of K562 cells.

3.4. Measurement of changes in mitochondrial membrane potential

Mitochondrion has been shown to play an important role in the regulation of cell death (Gil, Almeida, Oliveira, & Rego, 2003) and the collapse of the mitochondrial membrane potential was considered an irreversible step in the death cascade (Zamzami et al., 1995). Disruption of mitochondrial membrane potential is thought to be a common event in the induction of apoptosis (Barbu, Welsh, & Saldeen, 2002). To identify whether cell apoptosis induced by EPS was involved in mitochondrial pathways, the effect of EPS on mitochondrial membrane potential was observed by LSCM. According to studies of Troyan, red fluorescence suggested the membrane potential in mitochondria was highly negative, green fluorescence indicated the potential becomes reduced by JC-1 stain (Troyan et al., 1997). Fig. 4 was representative control and treated K562 cells. In Fig. 4a, most K562 cells showed red fluorescence, which indicated that they were live cells. However, in Fig. 4d, most cells showed green fluorescence, suggesting EPS induced K562 cell apoptosis. And the green fluorescence intensity growing accompanied with the increasing concentration of EPS.

3.5. Generation of ROS by EPS in K562 cells

ROS including superoxide, hydrogen peroxide and highly toxic hydroxyl radical, was generated as natural byproduct during aerobic metabolism. Growing evidences suggested that ROS played an important role in cell signaling. It can activate diverse downstream signaling molecules (Rosenberger, Petrovics, & Buzas, 2001). Mitochondria are the predominate source of ROS. When the mitochondria are damaged, excessive ROS may be released from mitochondria. The excessive ROS result in the loss of the membrane potential, and inducing the execution of apoptosis finally (Ricci, Gottlieb, & Green, 2003; Simizu, Takada, Umezawa, & Imoto, 1998).

Our previous data indicated that EPS effect the dissipation of $\Delta\Psi m$. Thus, we next determined the intracellular ROS generation induced by EPS in K562 cells. The DCFH-DA fluorescence intensity was measured by fluorospectro-photometer. As shown in Fig. 5A, a significant increase in intracellular ROS generation was observed upon exposure to the increasing concentration of EPS, as determined with the fluorescence intensity of DCFH-DA. We also observed the cells after treatment 24 h with 0.4 mg/mL EPS by LSCM (Fig. 5B). The results showed that EPS boosted generation of ROS, which implied that EPS was an apoptotic stimulus and could impact the mitochondria of the cells.

3.6. Effect of EPS on intracellular-free calcium

Apoptosis is a tightly regulated cell death progress. It is a complicated networking involving many pathways, regulators and

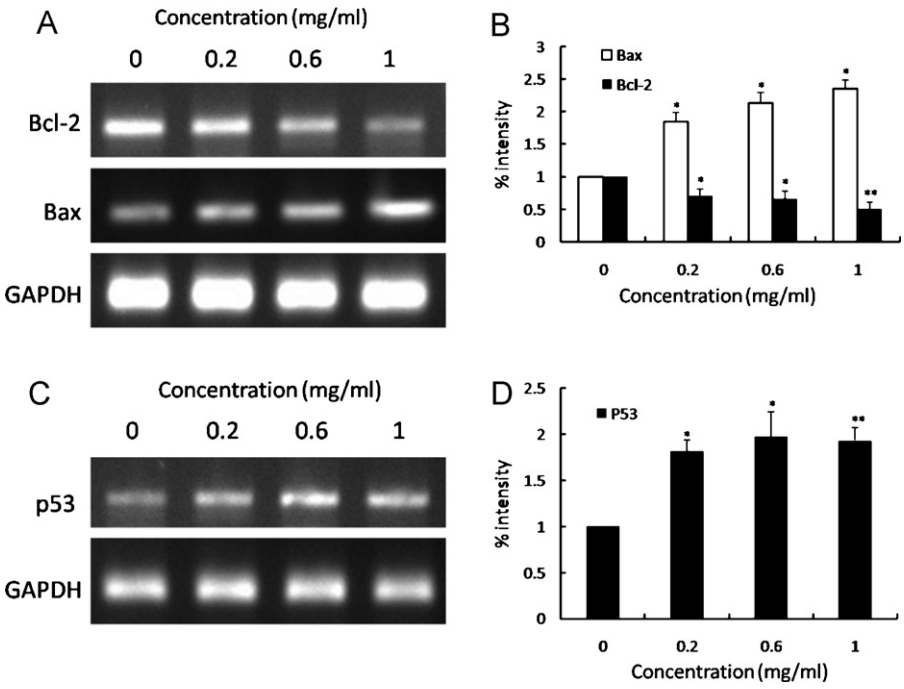

Fig. 7. Apoptotic effect of EPS in apoptosis related genes. The expression of p53, Bcl-2 and Bax mRNA were detected by RT-PCR. GAPDH was used as an internal standard. Data are represented as the means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

effectors. Intracellular Calcium (Ca^{2+}), as one of the most important signal elements, plays a significant role in induction of apoptosis and regulation of the apoptotic signaling pathways. Ca^{2+} signaling is potentially involved in all types of cell death (Berridge, Lipp, & Bootman, 2000). It regulated many steps in the cell death initiation, in the downstream effects of cell death and in cell death recognition by macrophages. It has been suggested that Ca^{2+} can activate caspase-12 and dimerize pro-apoptotic factors (e.g. Bax-Bax, Bax-Bad, Bax-Bid) on or near mitochondria, induce the pores open in the outer membrane, and release several factors (Smac/DIABLO, cytochrome c, AIF) into the cytosol. These factors can activate caspase or caspase-independent effectors of apoptosis and produce the apoptotic phenotype (Dispersyn & Borgers, 2001; Galluzzi et al., 2008; Goldspink, Burniston, & Tan, 2003).

Many studies have demonstrated that apoptosis was accompanied by an enhancement of intracellular Ca^{2+} (Kaneko & Tsukamoto, 1994; Nègre-Salvayre & Salvayre, 1992). It is interesting to know whether EPS induce K562 cells apoptosis with an increase of intracellular-free calcium concentration ($[Ca^{2+}]_i$). Therefore, the cells were stained by Ca^{2+}-sensitive fluorescence probe Fluo-3/AM and examined by LSCM. EPS promoted the increase of $[Ca^{2+}]_i$ in K562 cells after 24 h treatment (Fig. 6). As the extracellular buffer without Ca^{2+}, the results suggested that the increase of intracellular calcium was from the intracellular Ca^{2+} pool.

3.7. Effect of EPS on mRNA expression of p53, Bcl-2 and Bax in K562 cell

Apoptosis is a tightly regulated process, which involves changes of a distinct gene expression. Among the numerous factors known to modulate cancer-related apoptosis, several pro- and anti-apoptotic genes can be used as markers of apoptosis, such as p53, Bcl-2 and Bax. The tumor suppressor gene p53 acts as both an inducer (Clarke et al., 1993; Lowe, Schmitt, Smith, Osborne, & Jacks, 1993) and mediator of apoptosis. It is involved in the control of the DNA repair, cell cycle, apoptosis induced by toxic agents (Haunstetter & Izumo, 1998). However, it also induced apoptosis as a mediator through expression of Fas, Bax (Sleight et al., 1995), and involves oxidative stress (Polyak, Xia, Zweier, Kinzler, & Vogelstein, 1997). Increasing evidence demonstrated that Bcl-2 family proteins are important regulators of the apoptosis (Burlacu, 2003). The family includes anti-apoptotic (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic (e.g. Bax and Bid) members. The ratio of pro- and anti-apoptotic components, such as the Bcl-2/Bax ratio, appears to be a critical determinant whether cells continue to exist or undergo apoptosis (Allsopp, Wyatt, Paterson, & Davies, 1993; Kuang & Chen, 2004; Shen & White, 2001).

To gain further information on the mechanism of apoptosis induced by EPS, the expression of p53, Bcl-2, Bax mRNAs were examined in K562 cells. By studying the results of RT-PCR, we observed that the expression of Bcl-2 was down-regulated, but the level of p53 and Bax mRNA were up-regulated, compared to the control (see Fig. 7). Therefore, we postulated that the possibly mechanism of EPS-induced apoptosis in K562 cells by the increase of p53 and the low ratio of Bcl-2 to Bax modulated. This conclusion was supported by the cell morphological changes (Fig. 2).

4. Conclusion

In this study, EPS was extracted and purified from *T. pseudokoningii* and the anti-proliferative effect was evaluated in human leucocythemia K562 cells. The inhibitory action, in a time- and concentration-dependent manner, was mainly caused by apoptosis. And the apoptosis induced by EPS-1 was associated with drop in mitochondrial membrane potential, increase production of ROS, enhancement of $[Ca^{2+}]_i$, up-regulation of Bax and p53 mRNA, down-regulation of Bcl-2 mRNA. The results suggested the mechanism of cell apoptosis induced by EPS was mainly mediated by the intrinsic mitochondrial apoptotic pathway. Nevertheless, further studies are still necessary to elucidate the mechanisms of EPS induced apoptosis in K562 cells. Therefore, the present studies demonstrated that EPS would be a new adjuvant chemotherapeutic and chemo preventive strategy against human leukemia.

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References

Agarwal, R., Talati, M., Lambert, W., Clark, A. F., Wilson, S. E., Agarwal, N., et al. (1999). Fas-activated apoptosis and apoptosis mediators in human trabecular meshwork cells. *Experimental Eye Research*, *68*(5), 583–590.

Allsopp, T. E., Wyatt, S., Paterson, H. F., & Davies, A. M. (1993). The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell*, *73*(2), 295–307.

Barbu, A., Welsh, N., & Saldeen, J. (2002). Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential (Deltapsi(m)) in pancreatic RINm5F cells: Prevention by Bcl-2. *Molecular and Cellular Endocrinology*, *190*(1–2), 75–82.

Berridge, M. J., Lipp, P., & Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nature Reviews. Molecular Cell Biology*, *1*(1), 11–21.

Burlacu, A. (2003). Regulation of apoptosis by Bcl-2 family proteins. *Journal of Cellular and Molecular Medicine*, *7*(3), 249–257.

Cao, W., Li, X. Q., Wang, X., Fan, H. T., Zhang, X. N., Hou, Y., et al. (2010). A novel polysaccharide, isolated from *Angelica sinensis* (Oliv.) Diels induces the apoptosis of cervical cancer HeLa cells through an intrinsic apoptotic pathway. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, *17*(8–9), 598–605.

Chow, L. W., Lo, C. S., Loo, W. T., Hu, X. C., & Sham, J. S. (2003). Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D1 gene. *The American Journal of Chinese Medicine*, *31*(1), 1–9.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., et al. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, *362*(6423), 849–852.

Cui, F. J., Li, Y., Xu, Y. Y., Liu, Z. Q., Huang, D. M., Zhang, Z. C., et al. (2007). Induction of apoptosis in SGC-7901 cells by polysaccharide-peptide GFPS1b from the cultured mycelia of *Grifola frondosa* GF9801. *Toxicology In Vitro: An International Journal Published in Association with BIBRA*, *21*(3), 417–427.

Dias, M. C., Votto, A. P., Filgueira, D. D., Almeida, D. V., Vallochi, A. L., D OCa, M. G., et al. (2011). Anti-MDR and antitumoral action of acetylsalicylic acid on leukemic cells. *Bioscience Reports*.

Dispersyn, G. D., & Borgers, M. (2001). Apoptosis in the heart: About programmed cell death and survival. *News in Physiological Sciences: An International Journal of Physiology Produced Jointly by the International Union of Physiological Sciences and the American Physiological Society*, *16*, 41–47.

Ehrke, M. J. (2003). Immunomodulation in cancer therapeutics. *International Immunopharmacology*, *3*(8), 1105–1119.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., & Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology*, *148*(7), 2207–2216.

Fesik, S. W. (2005). Promoting apoptosis as a strategy for cancer drug discovery. *Nature Reviews. Cancer*, *5*(11), 876–885.

Galluzzi, L., Vitale, I., Kepp, O., Seror, C., Hangen, E., Perfettini, J. L., et al. (2008). Methods to dissect mitochondrial membrane permeabilization in the course of apoptosis. *Methods in Enzymology*, *442*, 355–374.

Gil, J., Almeida, S., Oliveira, C. R., & Rego, A. C. (2003). Cytosolic and mitochondrial ROS in staurosporine-induced retinal cell apoptosis. *Free Radical Biology and Medicine*, *35*(11), 1500–1514.

Goldspink, D. F., Burniston, J. G., & Tan, L. B. (2003). Cardiomyocyte death and the ageing and failing heart. *Experimental Physiology*, *88*(3), 447–458.

Hattori, T. S., Komatsu, N., Shichijo, S., & Itoh, K. (2004). Protein-bound polysaccharide K induced apoptosis of the human Burkitt lymphoma cell line, Namalwa. *Biomedicine and Pharmacotherapy = Biomedecine and Pharmacotherapie*, *58*(4), 226–230.

Haunstetter, A., & Izumo, S. (1998). Apoptosis: Basic mechanisms and implications for cardiovascular disease. *Circulation Research*, *82*(11), 1111–1129.

Jin, M., Wang, Y., Xu, C., Lu, Z., Huang, M., & Wang, Y. (2010). Preparation and biological activities of an exopolysaccharide produced by Enterobacter cloacae Z0206. *Carbohydrate Polymers*, *81*(3), 607–611.

Kaneko, Y., & Tsukamoto, A. (1994). Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells. *Cancer Letters*, *79*(2), 147–155.

Kuang, Y. F., & Chen, Y. H. (2004). Induction of apoptosis in a non-small cell human lung cancer cell line by isothiocyanates is associated with P53 and P21. *Food and Chemical Toxicology: An International Journal published for the British Industrial Biological Research Association*, *42*(10), 1711–1718.

Lavi, I., Friesem, D., Geresh, S., Hadar, Y., & Schwartz, B. (2006). An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. *Cancer Letters*, *244*(1), 61–70.

Li, G., Kim, D. H., Kim, T. D., Park, B. J., Park, H. D., Park, J. I., et al. (2004). Protein-bound polysaccharide from *Phellinus linteus* induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters*, *216*(2), 175–181.

Lin, X., Cai, Y. J., Li, Z. X., Chen, Q., Liu, Z. L., & Wang, R. (2003). Structure determination, apoptosis induction, and telomerase inhibition of CFP-2, a novel lichenin from Cladonia furcata. *Biochimica et Biophysica Acta*, *1622*(2), 99–108.

Liu, C., Lu, J., Lu, L., Liu, Y., Wang, F., & Xiao, M. (2010). Isolation, structural characterization and immunological activity of an exopolysaccharide produced by Bacillus licheniformis 8-37-0-1. *Bioresource Technology*, *101*(14), 5528–5533.

Liu, J., Guo, L., Yin, F., Zheng, X., Chen, G., & Wang, Y. (2008). Characterization and antitumor activity of triethylene tetramine, a novel telomerase inhibitor. *Biomedicine and Pharmacotherapy = Biomedecine and Pharmacotherapie*, *62*(7), 480–485.

Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., & Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, *362*(6423), 847–849.

Mathers, C. D., Boschi-Pinto, C., Lopez, A. D., & Murray, C. J. L. (2001). Cancer incidence, mortality and survival by site for 14 regions of the world. Global Programme on Evidence for Health Policy Discussion Paper No. 13.

Nègre-Salvayre, A., & Salvayre, R. (1992). UV-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. 4. Calcium is involved in the cytotoxicity of UV-treated LDL on lymphoid cell lines. *Biochimica et Biophysica Acta (BBA) – Lipids and Lipid Metabolism*, *1123*(2), 207–215.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*(7), 715–729.

Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., & Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature*, *389*(6648), 300–305.

Ramoneda, B. M., & Perez-Tomas, R. (2002). Activation of protein kinase C for protection of cells against apoptosis induced by the immunosuppressor prodigiosin. *Biochemical Pharmacology*, *63*(3), 463–469.

Ricci, J. E., Gottlieb, R. A., & Green, D. R. (2003). Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *The Journal of Cell Biology*, *160*(1), 65–75.

Rosenberg, S. A., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., et al. (1994). Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA: The Journal of the American Medical Association*, *271*(12), 907–913.

Rosenberger, J., Petrovics, G., & Buzas, B. (2001). Oxidative stress induces proorphanin FQ and proenkephalin gene expression in astrocytes through p38- and ERK-MAP kinases and NF-kappaB. *Journal of Neurochemistry*, *79*(1), 35–44.

Sevag, M. G. (1938). The presence of a type-and species-specific conjugated polysaccharide in Type I pneumococcus. *Science*, *87*(2257), 304–305.

Shen, Y., & White, E. (2001). p53-dependent apoptosis pathways. *Advances in Cancer Research*, *82*, 55–84.

Simizu, S., Takada, M., Umezawa, K., & Imoto, M. (1998). Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. *The Journal of Biological Chemistry*, *273*(41), 26900–26907.

Singh, A., Dilnawaz, F., & Sahoo, S. K. (2011). Long circulating lectin conjugated Paclitaxel loaded magnetic nanoparticles: A new theranostic avenue for leukemia therapy. *PloS One*, *6*(11), e26803.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., et al. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute*, *82*(13), 1107–1112.

Sleight, J. W., Aggarwal, R. J., Duncan, W., Kao, Y., Tsai, H. L., Frensley, W. R., et al. (1995). Spectroscopic study of intraminiband and interminiband tunneling in finite superlattices. *Physical Review B: Condensed Matter*, *51*(16), 10701–10708.

Tedesco, I., Luigi Russo, G., Nazzaro, F., Russo, M., & Palumbo, R. (2001). Antioxidant effect of red wine anthocyanins in normal and catalase-inactive human erythrocytes. *The Journal of Nutritional Biochemistry*, *12*(9), 505–511.

Troyan, M. B., Gilman, V. R., & Gay, C. V. (1997). Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. *Experimental Cell Research*, *233*(2), 274–280.

Wang, M., Xie, C., Cai, R. L., Li, X. H., Luo, X. Z., & Qi, Y. (2008). Studies on antioxidant activities of breviscapine in the cell-free system. *The American Journal of Chinese Medicine*, *36*(6), 1199–1207.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X., et al. (1995). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *The Journal of Experimental Medicine*, *181*(5), 1661–1672.

Zhang, C. X., & Huang, K. X. (2006). Mechanism of apoptosis induced by a polysaccharide, from the loach Misgurnus anguillicaudatus (MAP) in human hepatocellular carcinoma cells. *Toxicology and Applied Pharmacology*, *210*(3), 236–245.

Zhang, M., Chen, H., Huang, J., Li, Z., Zhu, C., & Zhang, S. (2005). Effect of *Lycium barbarum* polysaccharide on human hepatoma QGY7703 cells: Inhibition of proliferation and induction of apoptosis. *Life Sciences*, *76*(18), 2115–2124.