Carbohydrate Polymers 121 (2015) 107–114

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Exopolysaccharide of Antarctic bacterium *Pseudoaltermonas* sp. S-5 induces apoptosis in K562 cells

Guochuang Chen[b,1], Wen Qian[a,d,1], Jing Li[a], Yanghui Xu[a], Kaoshan Chen[a,b,c,*]

[a] *Anhui Provincial Engineering Research Center for Polysaccharide Drugs, Anhui Province Key Laboratory of active biological macro-molecules, Department of Pharmacy, Wannan Medical College, Wuhu 241000, China*
[b] *School of Life Science and National Glycoengineering Research Center, Shandong University, Jinan 250100, China*
[c] *State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China*
[d] *Department of Pharmacy, Huaibei People's Hospital, Huaibei 235000, China*

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form
19 November 2014
Accepted 12 December 2014
Available online 3 January 2015

Chemical compounds studied in this article:
Sulforhodamine B (PubChem CID: 9916275)
Hoechst 33258 (PubChem CID: 5360743)
JC-1 (PubChem CID: 5492929)
Flou-3 AM (PubChem CID: 16213075)
BAPTA-AM (PubChem CID: 2293)

Keywords:
Antarctic bacterium
Exopolysaccharide
Apoptosis
Intrinsic pathway
Human leukemia
K562 cells

ABSTRACT

The aim of this study was to investigate the anticancer activity of exopolysaccharide (PEP) of Antarctic bacterium *Pseudoaltermonas* sp. S-5 and elucidate the underlying molecular mechanisms. PEP significantly inhibited the growth of human leukemia K562 cells. Results of morphological characterization showed that PEP-treated cells displayed typical morphological characteristics of apoptosis such as condensation of chromatin and formation of apoptotic bodies. Flow cytometry analyses and colorimetric assay demonstrated that PEP induced collapse of mitochondrial membrane potential and activation of caspase-9, which indicated that intrinsic apoptotic signaling pathway was involved in apoptosis induced by PEP in K562 cells. Western blot analysis showed that PEP increased the ratio of Bax/Bcl-2. In addition, calcium signal might contribute to the cytotoxicity of PEP against K562 cells. These findings suggest that PEP may be potentially effective drug against human leukemia.

1. Introduction

Leukemia is a hematopoietic malignancy which starts in blood-forming tissue and causes large numbers of abnormal blood cells. In 2014, about 24,090 Americans will die of Leukemia (Siegel, Ma, Zou, & Jemal, 2014). Although chemotherapy-based treatment of leukemia has tremendously improved survival rates, current strategies also induce significant undesirable side effects, including ulcers, diarrhea and gastrointestinal bleeding. It is critical to develop novel drugs with low toxicity for leukemia.

Apoptosis, or type I programmed cell death, is a finely regulated process of physiological cell death. It plays an essential role in the development and maintenance of multi-cellular organism. Compelling functional studies have established the concept that apoptotic programmed cell death serves as a natural barrier to cancer development (Adams & Cory, 2007; Lowe, Cepero, & Evan, 2004). Abnormalities in cell death regulation are important components of neoplastic diseases. It presents an obvious drug target for therapeutic intervention in cancers. Thereby, promoting apoptosis may be effective to block the neoplastic progression.

Natural polysaccharides possess various biological and pharmacological activities, such as immunostimulation and antitumor (Lee, Ryu, Je, & Kim, 2011; Thangam et al., 2014; Wijesekara, Pangestuti, & Kim, 2011). As biological response modifiers (BRMs), many of them (Lentinan, Grifolan and Protein-bound polysaccharide-K) are widely used in the treatment of various types of malignancy. Attractive studies have reported that some novel polysaccharides induced apoptosis to exert inhibition of tumor cell proliferation (Chen et al.,

* Corresponding author at: School of Life Science and National Glycoengineering Research Center, Shandong University, No. 27 South Shanda Road, Jinan 250100, China. Tel.: +86 531 8836 5311; fax: +86 531 8836 5311.
E-mail address: ksc313@126.com (K. Chen).
[1] Both authors contributed equally to this work, thus share the first authorship.

http://dx.doi.org/10.1016/j.carbpol.2014.12.045
0144-8617/

2013; Hirahara et al., 2011). Because of the unique activity of apoptosis induction, these substances might be developed as novel anti-tumor agents.

Psychrophiles are the predominant microorganism in the Antarctica. These organisms can produce abundant extracellular polymeric substances (especially exopolysaccharides) which possessed special physiological and biochemical characteristics under low temperature and limited nutrient condition (Fendrihan & Negoiţă, 2012). Exopolysaccharides (EPSs) play an important role in cell adhesion, nutrition metabolism and cryoprotection for the psychrophiles in extreme habitats (Junge, Eicken, & Deming, 2004; Nichols, Guezennec, & Bowman, 2005). These substances possess a great diversity of biological activities, including immunoregulatory, antiviral, anticancer and antioxidant, and are widely used in pharmaceutical industries. We recently isolated and purified an exopolysaccharide from the fermentation liquor of Antarctic bacterium *Pseudoaltermonas* sp. S-5, named as PEP (Bai et al., 2012). PEP exhibits immunomodulatory activity which was evidenced by the enhanced phagocytosis and production of NO and cytokines in mouse peritoneal macrophage (Bai et al., 2012). However, the antiproliferative activity on tumor cells of PEP has not yet been elucidated. This study was designed to evaluate the anti-tumor effects of PEP against human leukemia K562 cells and further investigate the underlying mechanisms of the cytotoxic activity of PEP.

2. Materials and methods

2.1. Materials

Fetal bovine serum, RPMI-1640 medium, penicillin and streptomycin were provided by Gibco (Gaithersburg, USA). Fluo-3 acetoxymethyl ester (Fluo-3 AM), sulforhodamine B (SRB), Hoechst 33258, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) were purchased from Sigma–Aldrich (St. Louis, USA). MitoProbe™ JC-1 Assay Kit was obtained from Molecular Probes (Eugene, USA). Antibodies against Bax, Bcl-2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). BCA protein assay kit was obtained from Thermo Fisher Scientific (Rockford, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK). Annexin V-FITC apoptosis detection kit and caspase activity assay kits were supplied by Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Organism and growth conditions

Pseudoaltermonas sp. S-5 (CGMCC 3433), isolated from the sea ice samples which were collected by the 19th Antarctic Scientific Research Expedition of China during 2002–2003, was preserved in China General Microbiological Culture Collection Center. It was cultured in shaking flask containing liquid 2216E medium which was comprised of 10 g/l glucose, 5.0 g/l tryptone and 1.0 g/l yeast extract, dissolved in artificial seawater. The flasks were incubated on a rotary shaker under the conditions of 140 rpm at 8 °C for 60 h.

2.3. Isolation and purification of the exopolysaccharide

The exopolysaccharide from the fermentation liquor of Antarctic bacterium *Pseudoaltermonas* sp. S-5 was isolated and purified as described previously (Bai et al., 2012). Briefly, the supernatant fermentation was obtained by centrifuging at 10,000 rpm for 10 min, and then concentrated by rotary evaporator at a reduced pressure below 60 °C. Alcohol was added into the concentrated solution until the concentration of the alcohol reached 75% and the mixture was kept at 4 °C overnight. The precipitate was dissolved in distilled water and deproteinated according to the method of Sevag (1938). The resulting polysaccharide solution was treated with D301R resin to decolorize, then dialyzed and lyophilized to obtain colorless powder. The crude exopolysaccharides were dissolved in start buffer (20 mmol/l Tris–HCl, pH 8.2) and fractionated using DEAE Sepharose Fast Flow column (1.6 cm × 20 cm) equilibrated with start buffer. The unabsorbed fraction was collected and further purified using Sephadex G-75 column (1.6 cm × 60 cm). The major peak was collected, dialyzed and lyophilized to obtain a purified exopolysaccharide termed as PEP.

2.4. Characterization of PEP

Spectroscopic and chromatographic methods were performed to analyze the physicochemical properties of the polysaccharide PEP. PEP appeared as a white powder and showed negative triiodide reaction, indicating the absence of starch-type polysaccharide. Ultraviolet spectroscopy assay showed no absorption at 280 nm and 260 nm, which indicated that PEP did not contain protein and nucleic acid (Fig. S1). The Fourier transform infrared spectrum of PEP displayed the characteristic peaks of polysaccharide (Fig. S2). As shown in supplementary data, the average molar masses of PEP was estimated to be 397 kDa and monosaccharide components of PEP consist of mannose, glucose and galactose in a molar ratio of 4.8:50.9:44.3 (Bai et al., 2012).

2.5. Cell culture

Human leukemia cell line K562 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2.

2.6. Cell proliferation viability assay

Viability of K562 cells was measured by sulforhodamine B colorimetric assay (Skehan et al., 1990). Briefly, exponentially growing cells were placed into 96-well plate and treated with different concentrations of PEP for indicated time. After incubation, cells were centrifuged at 450 × *g* for 10 min to obtain cell layer. The cell pellets were fixed with 10% (wt/vol) trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid for 1 h. The excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid and the protein-bound dye was dissolved with 10 mM Tris base solution. Absorbance was recorded at 510 nm on an automatic microplate reader.

2.7. Hoechst 33258 staining

Cells were seeded in 12-well plate and treated with PEP for 48 h. The cells were fixed with methanol/acetic acid (3:1) for 15 min, washed twice with phosphate-buffered saline (PBS) and stained with Hoechst 33258 solution for 10 min at room temperature in the dark. Samples were observed under fluorescence microscopy (Olympus, Japan).

2.8. Flow cytometric analysis of apoptosis

Detection of PEP-induced apoptosis was performed by flow cytometry using a commercially available Annexin V-FITC/PI apoptosis detection kit. Cells were plated in 6-well plate and incubated with PEP for 48 h. The treated cells were harvested and washed with ice-cold PBS. The cell pellets were resuspended in 195 μl binding buffer, followed by added 5 μl of FITC-labeled Annexin V and propidium iodide. After incubated at room temperature for

20 min in the dark, stained cells were analyzed by flow cytometry (BD Biosciences, USA). The results were shown as scatter plots and the early apoptotic cells were presented in right lower quadrant.

2.9. Analysis of the mitochondrial membrane potential ($\Delta\psi m$)

Changes of mitochondrial transmembrane potential were monitored by JC-1. Briefly, K562 cells (1×10^6) were seeded in 6-well plate and incubated in the absence or presence of PEP for 24 h. The cells were collected and stained with JC-1 for 20 min at 37 °C in the dark. Finally, the cell pellets were washed twice with PBS and suspended in pre-warmed assay buffer. The fluorescence density variation of sample was analyzed by flow cytometry using 488 nm excitation equipped with 530 nm and 585 nm bandpass emission filters (BD Biosciences, USA). The reduced ratio of red to green fluorescence indicated the collapse of mitochondrial membrane potential.

2.10. Measurements of cytosolic calcium concentration ($[Ca^{2+}]c$)

Fluo-3 AM was used to probe the cytosolic calcium concentration ($[Ca^{2+}]c$) in K562 cells. After treated with indicated concentrations of PEP for 24 h, cells were loaded with 1 μM Fluo-3 AM at 37 °C for 20 min, followed by analyzed with fluorescence microscopy.

2.11. Measurements of caspase-3 and caspase-9 activities

The enzymatic activities of caspase-3 and caspase-9 were determined by colorimetric caspase activity detection kits according to the manufacturer's protocol. Briefly, after exposure of the cells to PEP for 48 h, cells were collected and incubated with lysis buffer for 30 min in ice. The lysates containing 100 μg of protein were obtained and incubated with Ac-DEVD-*p*NA (caspase-3 substrate) and Ac-LEHD-*p*NA (caspase-9 substrate) at 37 °C for 1 h. Absorbance of the produced chromophore *p*NA was determined at 405 nm using microplate reader. The results were presented as folds-increase compared with the untreated group.

2.12. Western blot

After treatment in the absence or presence of PEP for 48 h, cells were washed twice with ice-cold PBS, and the total cell lysates were prepared in lysis buffer. The total cell lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C to remove cellular debris. The protein concentration was determined using BCA protein assay kit according to the manufacturer's protocol. Cell lysates containing equal amounts of protein were separated by 10–12% SDS-PAGE and then transferred onto polyvinylidene fluoride member (Millipore, USA). The membrane was blocked with solution containing 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature, and incubated with primary antibodies at 4 °C overnight. The member was washed 3 times with TBST and further probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The visualization of the immune complexes was performed using an enhanced chemiluminescence detection system.

2.13. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA). All data were presented as the mean ± standard deviation of at least three independent experiments. *P* values of less than 0.05 were considered statistically significant.

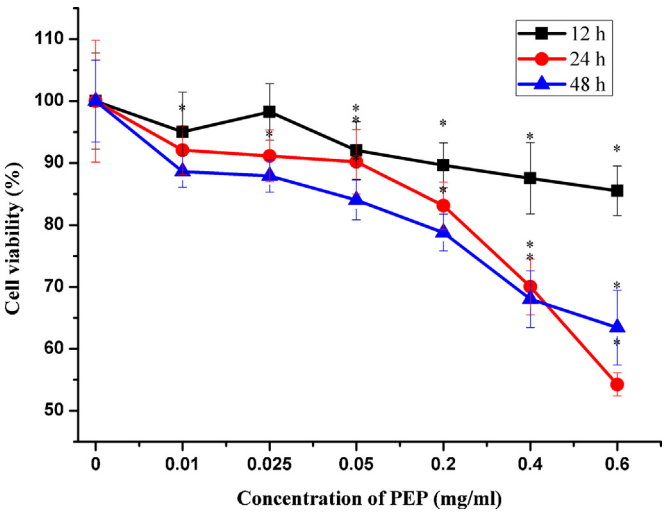

Fig. 1. Effects of PEP on cell viability of K562 cells. The exponentially growing K562 cells were cultured in 96-well plate and treated with different doses of PEP (0–0.6 mg/ml) for 12 h, 24 h and 48 h. The cell viability was analyzed by SRB assay. Results were expressed as mean values ± SD of three independent experiments (*$p \leq 0.05$ versus control group).

3. Results

3.1. PEP inhibited the proliferation of K562 cells

In order to identify the cytotoxic effects of PEP on human leukemia cells, K562 cells were exposed to indicated concentrations of PEP for 12 h, 24 h and 48 h, followed by SRB assay. As shown in Fig. 1, PEP treatment significantly inhibited the growth of K562 cells with a 45.75% decrease of cell viability at concentration of 0.6 mg/ml.

3.2. PEP induced apoptosis in K562 cells

To ascertain whether PEP treatment induced apoptosis, K562 cells were treated with PEP at concentrations of 0.2, 0.4 and 0.6 mg/ml for 48 h and stained with Hoechst 33258. The samples were visualized using fluorescence microscopy. Data from morphological characterization showed that K562 cells treated by PEP presented typical morphological characteristics of apoptosis, including nuclear pyknosis, condensation of chromatin and formation of apoptotic bodies (Fig. 2). To validate the results of morphological analysis and quantify apoptosis induced by PEP, we conducted flow cytometry based on Annexin V-FITC/PI double labeling to determine the percentages of early apoptotic cells (Annexin V-positive/PI-negative), late apoptotic cells and necrotic cells (Annexin V-positive/PI-positive). The percentage of early apoptotic cells was significantly increased from 0.012% to 15.6% by PEP treatment for 48 h (Fig. 3). These results collectively indicated that PEP induced apoptosis in K562 cells.

3.3. PEP induced loss of mitochondrial membrane potential (MMP)

There are multiple pathways triggering apoptosis, two of which are well characterized, the extrinsic pathway and intrinsic pathway. Loss of mitochondrial membrane potential is one of the key events in the intrinsic apoptosis pathway. To investigate the role of mitochondria in PEP-induced apoptosis, K562 cells were probed with JC-1 after the treatment with PEP for 24 h. As compared with the control group, cells emitted green fluorescence were remarkably increased and a paralleled decrease of cells emitted red

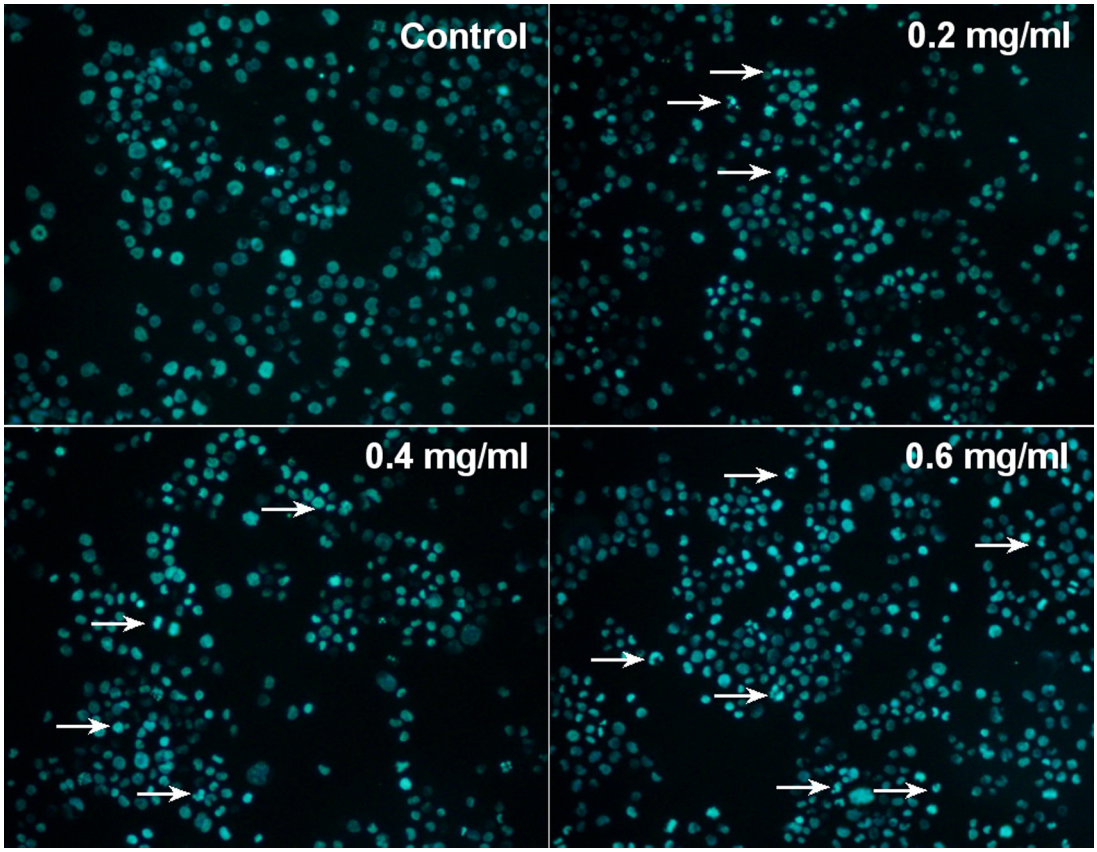

Fig. 2. Effects of PEP on morphology of K562 cells. After treated by PEP for 48 h, cells were stained with Hoechst 33258 solution and visualized using fluorescence microscopy. White arrows indicated the condensation and fragmentation of chromatin.

fluorescence was also observed, which indicated the loss of mitochondrial membrane potential (Fig. 4). These data suggested that the loss of mitochondrial membrane potential might involve in the apoptosis induced by PEP.

3.4. PEP induced activation of caspase-9 and caspase-3 in K562 cells

Caspase activation is one of the major mechanisms of apoptotic process. Caspase-9 is the apical protease in the intrinsic apoptosis pathway. In order to confirm whether intrinsic apoptosis pathway was associated with PEP-induced apoptosis in K562 cells, we examined the effect of PEP on caspase-9 and caspase-3 activation. As shown in Fig. 5, the activities of caspase-9 and caspase-3 were significantly increased in a concentration-dependent manner after exposed to PEP for 48 h. Our findings suggested that PEP induced apoptosis through intrinsic apoptosis pathway in K562 cells.

3.5. PEP regulated the expression of Bcl-2 family proteins

Bcl-2 family members are important regulators of mitochondrial permeability transition (MPT) and play a pivotal role in intrinsic apoptosis. In order to decipher the mechanism of PEP induced apoptosis, we measured the expression of Bax and Bcl-2 in K562 cells upon PEP treatment for 48 h. As shown in Fig. 6, western blot analysis revealed that the level of pro-apoptotic protein Bax was increased, whereas the level of anti-apoptotic protein Bcl-2 notably decreased compared with the control group in K562 cells.

3.6. PEP induced elevation of cytosolic calcium concentration ($[Ca^{2+}]c$)

It is well known that alteration in cytosolic Ca^{2+} homeostasis has a major function in the regulation of cell death (Mattson & Chan, 2003). To explore the role of calcium signal in the cytotoxicity of PEP, K562 cells were loaded with fluorescent probe Fluo-3 AM to detect the variation of cytosolic calcium. As shown in Fig. 7A, PEP treatment for 24 h markedly increased the fluorescence intensity, which indicated the elevation of cytosolic calcium in K562 cells. Furthermore, BAPTA-AM, a selective intracellular Ca^{2+} chelator, was used to block the calcium waves. As shown in Fig. 7B, the blockage of calcium waves alleviated the cell death induced by PEP, which indicated that calcium signal might contribute to the cytotoxicity of PEP against K562 cells.

4. Discussion

Chemotherapy is widely used for the treatment of leukemia. However, chemotherapeutics have poor specificity and cause severe damage to healthy cells in patients. The optimal therapeutic goal in cancer treatment is to trigger tumor-selective cell death. Many natural polysaccharides with low toxicity can selectively induce tumor cells apoptosis. Due to the unique biological properties, exopolysaccharides from extreme marine habitats have attracted the interest of academic and industrial scientists. Several exopolysaccharides have been isolated from the cultured supernatant of Antarctic psychrophiles and possess immunoregulatory activity and cytotoxicity on cancer cells. Most of EPSs are often linear heteropolysaccharides and generally possess a larger molecular weight (100–500 kDa). These substances have been the good

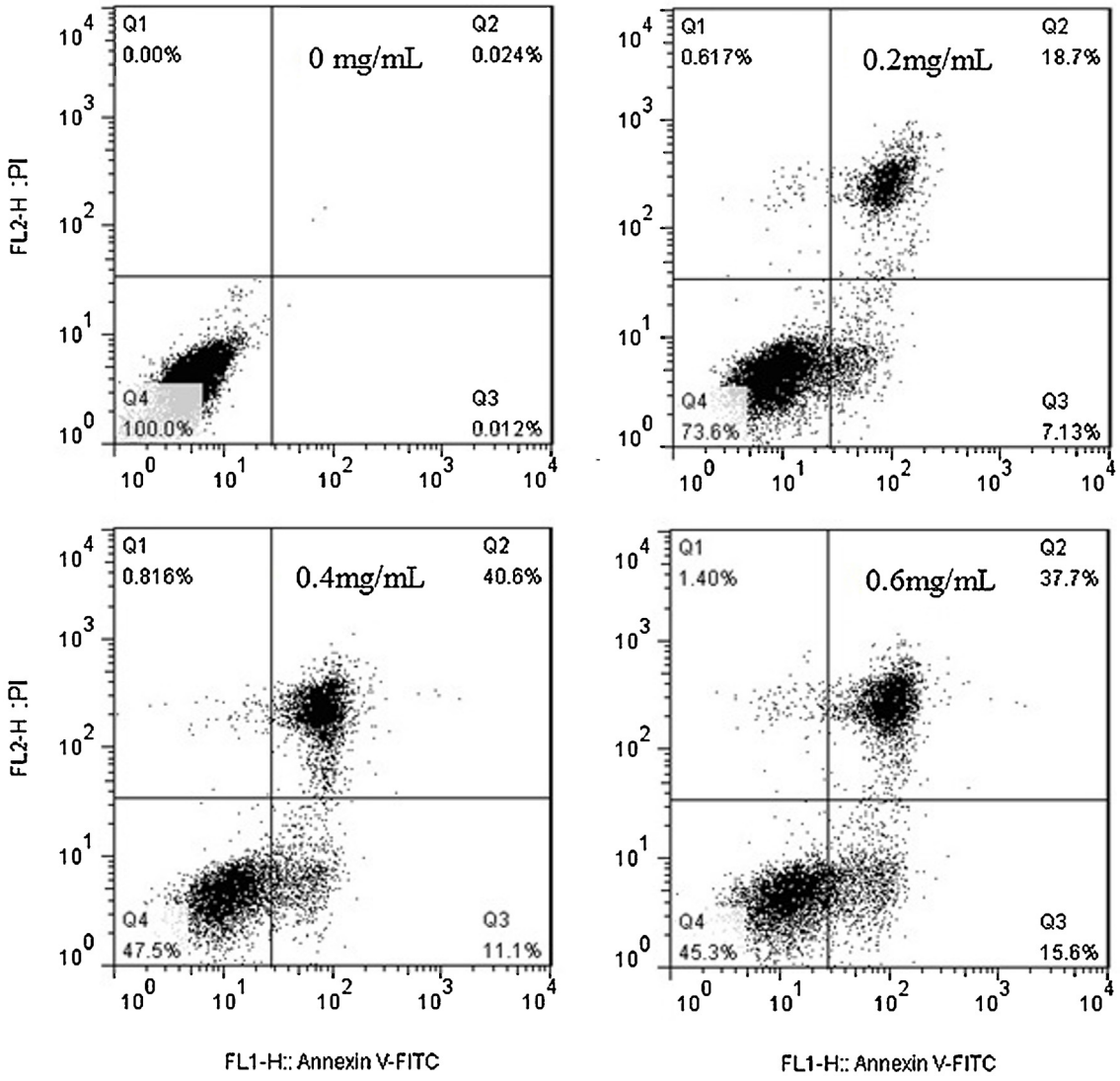

Fig. 3. Apoptosis detection in K562 cells treated with PEP. Cells were treated with the indicated concentrations of PEP for 48 h. Treated cells were stained with Annexin V-FITC and propidium iodide, followed by analyzed using flow cytometry.

source for the development of potential immune activator and cancer therapy drugs. In this paper, we first reported that PEP presented cytotoxicity against human leukemia K562 cells.

Uncontrolled cell proliferation and resistant to cell death are the typical characters of cancer. The defects in the apoptotic machinery of abnormal cells are central to the development of cancer (Brown & Attardi, 2005; Lowe & Lin, 2000). Apoptosis is a finely tuned cellular self-destruction mechanism to remove unwanted cells. It is now well documented that most cytotoxic anticancer agents target the apoptotic machinery (Ghobrial, Witzig, & Adjei, 2005). A branched (1 → 3)-linked β-D-glucan with an average molecular mass of 500 kDa derived from *Thelebolus* sp. IITKGP-BT12 inhibited the proliferation of mouse melanoma B16-F0 cells by triggering apoptosis and cell cycle arrest (Mukhopadhyay et al., 2014). PEP is neutral heteropolysaccharide, having complex structures. The main chain of core fragment of PEP contains branched (1 → 3)-linked β-D-glucopyranose and (1 → 4)-linked α-D-galactopyranose (unpublished results). The presence of β-(1 → 3)-glucopyranose linkages is proposed to be involved in the apoptosis induction of polysaccharides (Kim et al., 2009; Zhang, Chiu, Cheung, & Ooi, 2006). In this paper, we showed that PEP inhibited the proliferation of human leukemia K562 cells by triggering apoptosis, which was confirmed by the condensation of chromatin and externalization of phosphatidylserine (Figs. 2 and 3).

Mitochondria are essential organelles because they provide a myriad of services to the cell, including energy production, calcium buffering, oxidative stress and regulation of apoptosis (Camello-Almaraz, Gomez-Pinilla, Pozo, & Camello, 2006; McBride, Neuspiel, & Wasiak, 2006). Mitochondria participate in intrinsic pathway of apoptosis where they release pro-apoptotic factors (e.g., cytochrome *c*, Smac/DIABLO, Omi/Htra2, endonuclease G and apoptosis-inducing factor) from the intermembrane space to initiate apoptosis (Du, Fang, Li, Li, & Wang, 2000; Li, Luo, & Wang, 2001; Lorenzo, Susin, Penninger, & Kroemer, 1999; Yang, Church-Hajduk, Ren, Newton, & Du, 2003). As shown in Fig. 4, PEP treatment induced collapse of mitochondrial membrane potential, which indicated the integrity of the mitochondrial outer membrane (OMM) is compromised, also called mitochondrial outer membrane permeabilization (MOMP). The loss of MMP is associated with the generation of cellular ROS (Park, Lee, & Choi, 2011). Accumulating studies have suggested that ROS are also essential mediators in for cell death (Circu & Aw, 2010). The oxidative modifications on components of the mitochondrial permeability transition complex will significantly impact mitochondrial function and trigger opening of

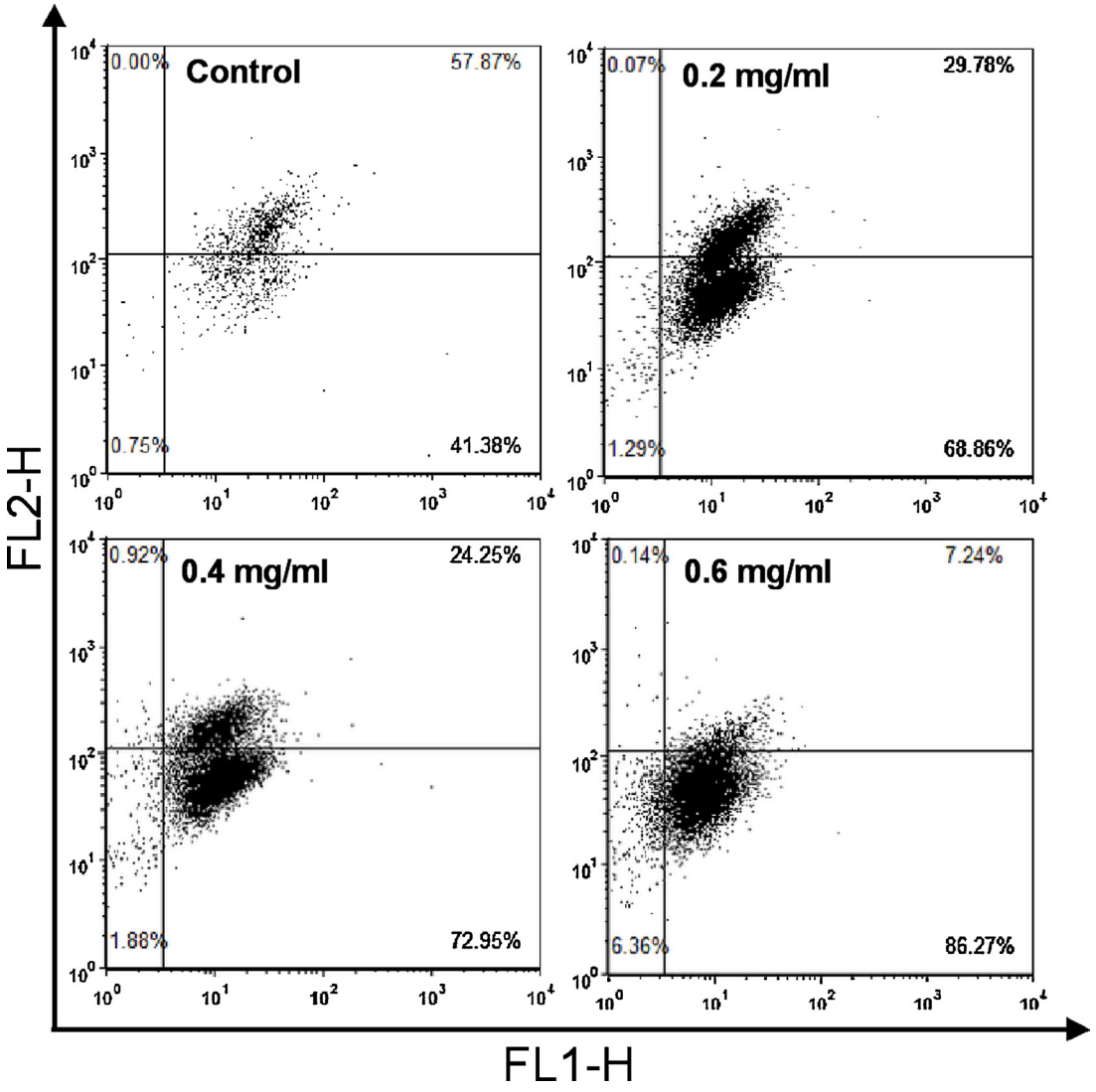

Fig. 4. PEP induced loss of mitochondrial membrane potential in K562 cells. Cells were treated by variety concentrations of PEP for 24 h. Treated cells were harvested, stained with JC-1, and assayed by flow cytometry.

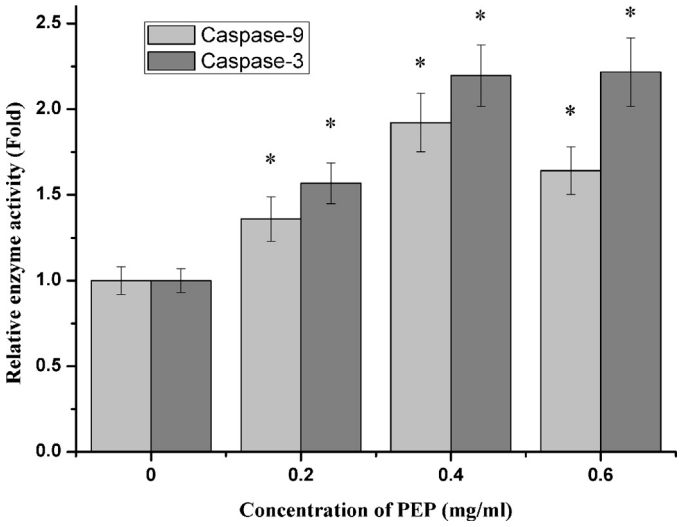

Fig. 5. PEP induced activation of caspase-9 and caspase-3 in K562 cells. The total cytosolic fraction of the cells was prepared after treatment with PEP for 48 h. The activities of caspase-9 and caspase-3 were assayed by colorimetric analysis and shown as fold-increase compared with control group ($^{*}p \leq 0.05$ versus control group).

mitochondrial permeability transition pore which results in apoptotic initiation (Tsujimoto & Shimizu, 2007). Further studies should be performed to evaluate the production of intracellular ROS and investigate their roles in the PEP-induced apoptosis.

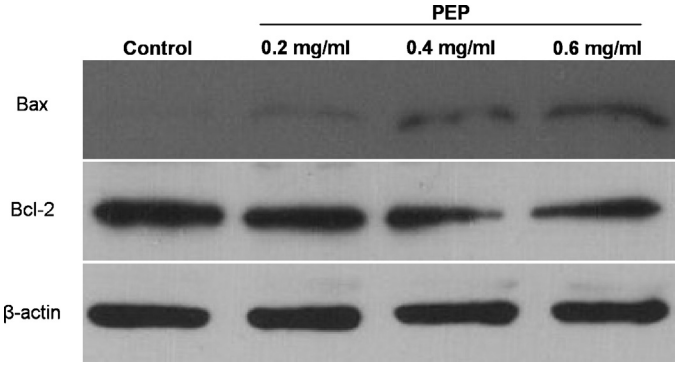

Fig. 6. Effects of PEP on the expression of Bax and Bcl-2 in K562 cells. Cells were treated with indicated concentrations of PEP for 48 h, and the expression of Bax and Bcl-2 was evaluated by western blot analysis. β-Actin was used as an equal loading control.

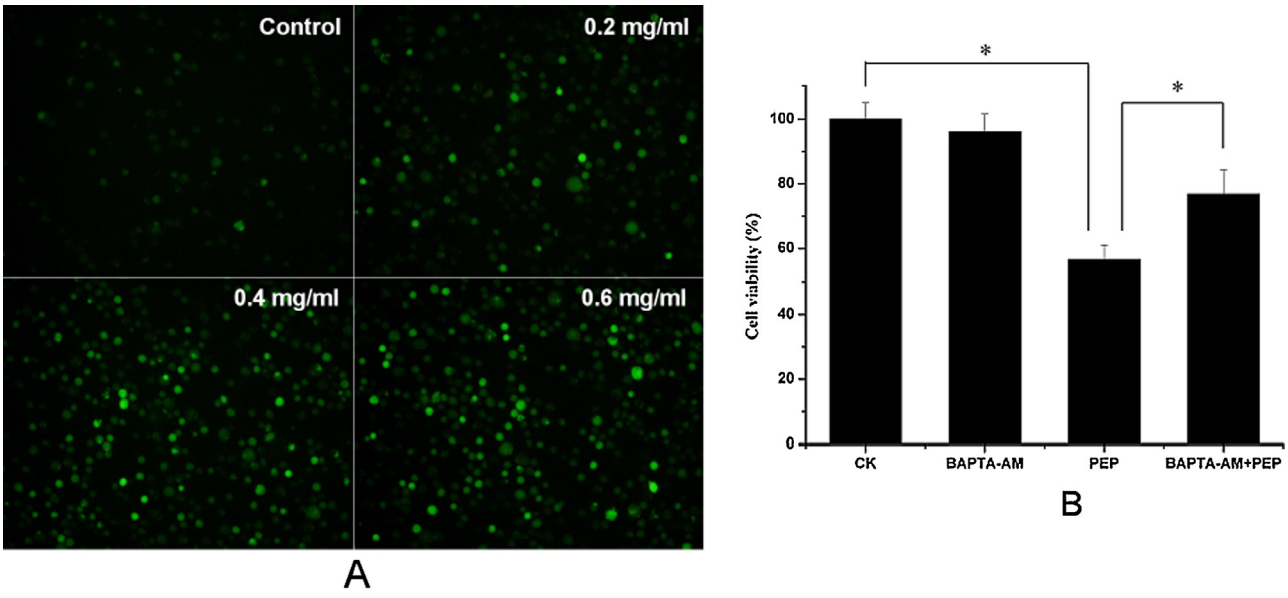

Fig. 7. PEP induced elevation of cytosolic calcium concentration in K562 cells. (A) Cells were incubated with PEP for 24 h and probed with Fluo-3 AM for 30 min, followed by analyzed with fluorescence microscope. (B) Cells were pretreated with BAPTA-AM (5 μM) for1 h, further treated with PEP (0.6 mg/ml) for 48 h, and then assayed by SRB assay. Results were expressed as mean values ± SD of three independent experiments ($^*p \leq 0.05$ versus control group).

MOMP is thought to be tightly regulated by Bcl-2 family members. Based on their pro- or anti-apoptotic action and the Bcl-2 Homology (BH) domains, Bcl-2 family proteins are classified as anti-apoptotic Bcl-2-like proteins (e.g., Bcl-2, Bcl-xL, Bcl-w and Mcl-1), proapoptotic Bax-like proteins (e.g., Bax and Bak) and proapoptotic BH3-only proteins (e.g., Bid, Bim/Bod, Bad, Noxa and Puma/Bbc3) (Schinzel, Kaufmann, & Borner, 2004; Youle & Strasser, 2008). The apoptosis signal promotes translocation of Bax from the cytosol to the OMM where it oligomerizes and inserts, resulting in MOMP (Harris & Thompson, 2000; Kuwana et al., 2005). The ratio of Bax/Bcl-2 is a major checkpoint in the intrinsic apoptosis pathway (Gross, McDonnell, & Korsmeyer, 1999). Bcl-2-like proteins can sequester Bax-like proteins in an inactive protein complex (Cheng et al., 2001). We conducted western blot analysis to examine the expression of Bax and Bcl-2 in K562 cells. Our results showed that the treatment by PEP for 48 h increased the expression of Bax and decreased the expression of Bcl-2 (Fig. 6). The release of pro-apoptotic factors subsequently triggers activation of the caspase-dependent and caspase-independent apoptosis pathways. The caspase–cascade system is initiated by the apoptosome assembly of cytochrome *c*, apoptotic protease-activating factor-1 (Apaf-1), ATP, and procaspase-9 (Acehan et al., 2002). The active form of caspase-9 activates procaspase-3 and procaspase-7, leading to oligonucleosomal DNA fragmentation and disassembly of cytoskeleton (Wolf & Green, 1999). As shown in Fig. 5, treatment with PEP significantly increased the activities of caspase-9 and caspase-3 in a concentration-dependent manner, which indicated that PEP induced apoptosis via intrinsic pathway.

Mitochondria participate actively in intracellular Ca^{2+} compartmentalization (Carafoli, 2002). Ca^{2+} is taken up electrophoretically from the cytosol by a uniport transporter of mitochondria. It has long been known that Ca^{2+} was involved in regulating mitochondrial apoptosis (Orrenius, Zhivotovsky, & Nicotera, 2003). We observed a notably elevation of cytosolic calcium concentration in PEP treated K562 cells and the blockage of calcium signal alleviated the PEP induced cell death (Fig. 7). Ca^{2+} seems to be responsible for OMM permeabilization and the release of proapoptotic effectors, which involves the opening of mitochondria a permeability transition pore (MPTP) (Szalai, Krishnamurthy, & Hajnoczky, 1999). Due to the proximity of the mitochondria to the ER, a rapid and large accumulation of the calcium occurred in the mitochondria matrix response to cell stress. However, the high concentration of Ca^{2+} in the mitochondrial matrix will lead to mitochondrial swelling and fragmentation as well as paralleled release of cytochrome *c*. In addition, elevation of intracellular calcium can activated calpains which participate in caspase cascade and serve as upstream triggers of mitochondrial membrane permeabilization (Wu, Liu, Xie, Xin, & Guo, 2006).

In conclusion, these studies demonstrated that PEP inhibited the proliferation of human leukemia K562 cells by inducing apoptosis via intrinsic pathway. The calcium signaling was also involved in the cytotoxicity of PEP. Our findings will benefit the future studies and drug development of PEP in the treatment of leukemia.

Acknowledgements

This study was supported by “National Basic Research Program of China” (No. 2012CB822102), Anhui Provincial Natural Science Foundation (No. 1408085MH197) and the Key Program for the Excellent Young Talents in College of Anhui Province (No. 2013SQRL054ZD).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.12.045.

References

Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., & Akey, C. W. (2002). Three-dimensional structure of the apoptosome: Implications for assembly, procaspase-9 binding, and activation. *Molecular Cell*, *9*(2), 423–432.

Adams, J. M., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*, *26*(9), 1324–1337.

Bai, Y., Zhang, P., Chen, G., Cao, J., Huang, T., & Chen, K. (2012). Macrophage immunomodulatory activity of extracellular polysaccharide (PEP) of Antarctic bacterium *Pseudoaltermonas* sp. S-5. *International Immunopharmacology*, *12*(4), 611–617.

Brown, J. M., & Attardi, L. D. (2005). The role of apoptosis in cancer development and treatment response. *Nature Reviews Cancer*, *5*(3), 231–237.

Camello-Almaraz, C., Gomez-Pinilla, P. J., Pozo, M. J., & Camello, P. J. (2006). Mitochondrial reactive oxygen species and Ca^{2+} signaling. *American Journal of Physiology – Cell Physiology*, *291*(5), C1082–C1088.

Carafoli, E. (2002). Calcium signaling: A tale for all seasons. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(3), 1115–1122.

Chen, G., Zhang, P., Huang, T., Yu, W., Lin, J., Li, P., et al. (2013). Polysaccharides from *Rhizopus nigricans* mycelia induced apoptosis and G2/M arrest in BGC-823 cells. *Carbohydrate Polymers*, *97*(2), 800–808.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., et al. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell*, *8*(3), 705–711.

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radical Biology and Medicine*, *48*(6), 749–762.

Du, C., Fang, M., Li, Y., Li, L., & Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, *102*(1), 33–42.

Fendrihan, S., & Negoiţă, T. G. (2012). Psychrophilic microorganisms as important source for biotechnological processes. In H. Stan-Lotter, & S. Fendrihan (Eds.), *Adaption of Microbial Life to Environmental Extremes* (pp. 133–172). Vienna: Springer.

Ghobrial, I. M., Witzig, T. E., & Adjei, A. A. (2005). Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians*, *55*(3), 178–194.

Gross, A., McDonnell, J. M., & Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes & Development*, *13*(15), 1899–1911.

Harris, M. H., & Thompson, C. B. (2000). The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. *Cell Death and Differentiation*, *7*(12), 1182–1191.

Hirahara, N., Fujioka, M., Edamatsu, T., Fujieda, A., Sekine, F., Wada, T., et al. (2011). Protein-bound polysaccharide-K (PSK) Induces apoptosis and inhibits proliferation of promyelomonocytic leukemia HL-60 cells. *Anticancer Research*, *31*(9), 2733–2738.

Junge, K., Eicken, H., & Deming, J. W. (2004). Bacterial activity at −2 to −20 °C in Arctic Wintertime Sea Ice. *Applied and Environmental Microbiology*, *70*(1), 550–557.

Kim, M. J., Hong, S. Y., Kim, S. K., Cheong, C., Park, H. J., Chun, H. K., et al. (2009). Beta-glucan enhanced apoptosis in human colon cancer cells SNU-C4. *Nutrition Research and Practice*, *3*(3), 180–184.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., et al. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Molecular Cell*, *17*(4), 525–535.

Lee, S.-H., Ryu, B., Je, J.-Y., & Kim, S.-K. (2011). Diethylaminoethyl chitosan induces apoptosis in HeLa cells via activation of caspase-3 and p53 expression. *Carbohydrate Polymers*, *84*(1), 571–578.

Li, L. Y., Luo, X., & Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, *412*(6842), 95–99.

Lorenzo, H. K., Susin, S. A., Penninger, J., & Kroemer, G. (1999). Apoptosis inducing factor (AIF), a phylogenetically old, caspase-independent effector of cell death. *Cell Death and Differentiation*, *6*(6), 516–524.

Lowe, S. W., Cepero, E., & Evan, G. (2004). Intrinsic tumour suppression. *Nature*, *432*(7015), 307–315.

Lowe, S. W., & Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis*, *21*(3), 485–495.

Mattson, M. P., & Chan, S. L. (2003). Calcium orchestrates apoptosis. *Nature Cell Biology*, *5*(12), 1041–1043.

McBride, H. M., Neuspiel, M., & Wasiak, S. (2006). Mitochondria: More than just a powerhouse. *Current Biology*, *16*(14), R551–R560.

Mukhopadhyay, S. K., Chatterjee, S., Gauri, S. S., Das, S. S., Mishra, A., Patra, M., et al. (2014). Isolation and characterization of extracellular polysaccharide Thelebolan produced by a newly isolated psychrophilic Antarctic fungus *Thelebolus*. *Carbohydrate Polymers*, *104*, 204–212.

Nichols, C. A., Guezennec, J., & Bowman, J. P. (2005). Bacterial exopolysaccharides from extreme marine environments with special consideration of the southern ocean, sea ice, and deep-sea hydrothermal vents: A review. *Marine Biotechnology*, *7*(4), 253–271.

Orrenius, S., Zhivotovsky, B., & Nicotera, P. (2003). Regulation of cell death: The calcium-apoptosis link. *Nature Reviews Molecular Cell Biology*, *4*(7), 552–565.

Park, J., Lee, J., & Choi, C. (2011). Mitochondrial network determines intracellular ROS dynamics and sensitivity to oxidative stress through switching inter-mitochondrial messengers. *PLoS ONE*, *6*(8), e23211.

Schinzel, A., Kaufmann, T., & Borner, C. (2004). Bcl-2 family members: Integrators of survival and death signals in physiology and pathology [corrected]. *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research*, *1644*(2–3), 95–105.

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

Siegel, R., Ma, J., Zou, Z., & Jemal, A. (2014). Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, *64*(1), 9–29.

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 Insitiute*, *82*(13), 1107–1112.

Szalai, G., Krishnamurthy, R., & Hajnoczky, G. (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO Journal*, *18*(22), 6349–6361.

Thangam, R., Sathuvan, M., Poongodi, A., Suresh, V., Pazhanichamy, K., Sivasubramanian, S., et al. (2014). Activation of intrinsic apoptotic signaling pathway in cancer cells by *Cymbopogon citratus* polysaccharide fractions. *Carbohydrate Polymers*, *107*, 138–150.

Tsujimoto, Y., & Shimizu, S. (2007). Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis*, *12*(5), 835–840.

Wijesekara, I., Pangestuti, R., & Kim, S.-K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydrate Polymers*, *84*(1), 14–21.

Wolf, B. B., & Green, D. R. (1999). Suicidal tendencies: Apoptosis by caspase family proteinases. *Journal of Biological Chemistry*, *274*(29), 20049–20052.

Wu, J., Liu, T., Xie, J., Xin, F., & Guo, L. (2006). Mitochondria and calpains mediate caspase-dependent apoptosis induced by doxycycline in HeLa cells. *Cellular and Molecular Life Sciences*, *63*(7-8), 949–957.

Yang, Q. H., Church-Hajduk, R., Ren, J., Newton, M. L., & Du, C. (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes & Development*, *17*(12), 1487–1496.

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology*, *9*(1), 47–59.

Zhang, M., Chiu, L. C., Cheung, P. C., & Ooi, V. E. (2006). Growth-inhibitory effects of a beta-glucan from the mycelium of Poria cocos on human breast carcinoma MCF-7 cells: Cell-cycle arrest and apoptosis induction. *Oncology Reports*, *15*(3), 637–643.