Carbohydrate Polymers 97 (2013) 800–808

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

# Polysaccharides from *Rhizopus nigricans* mycelia induced apoptosis and G2/M arrest in BGC-823 cells

Guochuang Chen [a], Pengying Zhang [a,b], Taotao Huang [a], Wenqian Yu [a], Jun Lin [c], Ping Li [c], Kaoshan Chen [a,b,c,*]

[a] School of Life Science and National Glycoengineering Research Center, Shandong University, Jinan 250100, China
[b] State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China
[c] Department of Pharmacy, Wannan Medical College, Wuhu 241000, China

ARTICLE INFO

*Article history:*
Received 25 January 2013
Received in revised form 28 April 2013
Accepted 24 May 2013
Available online 1 June 2013

*Keywords:*
*Rhizopus nigricans*
Mycelia polysaccharides
Apoptosis
Cell-cycle arrest
Human gastric cancer

ABSTRACT

In this study, we reported that polysaccharides (RPS) from the mycelia of liquid-cultured *Rhizopus nigricans* inhibited the proliferation and clonogenic potential of human gastric cancer BGC-823 cells. Results of acridine orange/ethidium bromide and Hoechst 33258 staining showed that treated cells displayed typical morphological characteristics of apoptosis such as condensation of chromatin, nuclear pyknosis and formation of apoptotic bodies. Flow cytometry analyses and colorimetric assay indicated that RPS induced mitochondria-mediated apoptosis which was associated with collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, generation of intracellular reactive oxygen species and elevation of intracellular calcium in BGC-823 cells. Moreover, cell-cycle analysis revealed that RPS arrested cells in the G2/M phase of the cell cycle. These results provided further insights into the potential use of RPS as an anti-cancer agent against human gastric cancer.

© 2013 Elsevier Ltd. All rights reserved.

## 1. Introduction

In spite of recent advances in diagnostic and therapeutic techniques, gastric cancer is still a major life-threatening disease (Jemal et al., 2011). Chemotherapy in conjunction with surgical resection is often implemented for gastric cancer patients. However, some undesirable effects, including nausea, vomiting and fatigue, declined the quality of patients' lives (Adamsen et al., 2006; Wagner et al., 2006).

Apoptosis is a genetically determined process of cell death for eliminating unwanted cells such as damaged cells and those with mutation or aberrant substratum attachments. Apoptotic cell death plays a major role in maintaining tissue homeostasis and serves as a natural barrier against cancer. The evasion of apoptosis, together with deregulated cell proliferation, can perturb delicate balance between cell proliferation and cell death, which resulting in cancer development (Lowe & Lin, 2000). Drugs that could promote apoptosis and prevent uncontrolled cell division might be effective in controlling many cancers (Fesik, 2005; Nicholson, 2000).

It has been reported that polysaccharides possess a broad spectrum of biological effects, such as anti-tumor, immunostimulation, antibiotic and antioxidant (Lee, Ryu, Je, & Kim, 2011; Wijesekara, Pangestuti, & Kim, 2011; Yu, Yin, Qian, & Yan, 2009). The anti-tumor effects of these polysaccharides were primarily produced by modulating the immunological functions of the host organism (Wasser & Weis, 1999; Zaidman, Yassin, Mahajna, & Wasser, 2005). However, direct inhibition on the tumor cells also conferred the anti-tumor activities of some novel polysaccharides. Increasing evidence has indicated that polysaccharides derived from fungus were effective to kill or incapacitate cancer cells with less toxicity to normal cells (Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Taraphdar, Roy, & Bhattacharya, 2001). These substances initiated cell stress responses and deranged the expression of signal molecules in tumor cells, thus induced apoptosis, cell cycle arrest and autophagy (Huang et al., 2012; Park, Kim, Nam, Deuk Kim, & Hyun Choi, 2011; Zeng et al., 2012). Therefore, fungus polysaccharides that could induce apoptosis in cancer cells would be potential anti-tumor medicines.

*Rhizopus nigricans* is widely used in pharmaceutical industry, such as biotransformation and production of organic acids (Alarcón, Águila, Cornejo, & Alderete, 2007; Guillermo, Carlos, Marcela, Jaime, & Cristina, 2007). In this study, we obtained aqueous polysaccharides (RPS, hereafter) from fermentation mycelia of *R. nigricans* and investigated the anti-tumor effects with BGC-823 cells. Our data indicated that RPS inhibited the growth of BGC-823 cells by

* 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).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.05.068

inducing apoptotic cell death and cell cycle arrest, which suggested that RPS was a potential anti-tumor agent against gastric cancer.

## 2. Materials and methods

### 2.1. Materials

RPS was derived from fermentation mycelia of *R. nigricans*. Sephadex G-100 was obtained from General Electric Healthcare Life Sciences. Fetal bovine serum, RPMI-1640 medium, penicillin and streptomycin were provided by GIBCO BRL. Monosaccharides (glucose, mannose, galactose, glucuronic acid, galacturonic acid, glucosamine, galactosamine, arabinose, xylose, rhamnose and fucose), acridine orange, ethidium bromide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, propidium iodide, crystal violet and ribonuclease were purchased from Sigma–Aldrich. 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from USA Acros Organics Co. JC-1 mitochondrial membrane potential detection assay kit, Annexin V-FITC apoptosis detection Kit, reactive oxygen species assay kit and Fluo-3 AM were supplied by Beyotime Institute of Biotechnology (Shanghai, China).

### 2.2. Organism and growth conditions

*R. nigricans*, isolated from straw, was preserved in Laboratory of Biomass Resources, Shandong University (Jinan, China). *R. nigricans* was cultured in shaking flask containing potato dextrose broth under controlled conditions (28 ± 1 °C, 130 rpm) for 10 d.

### 2.3. Isolation of the R. nigricans polysaccharides

Fungal mycelia were defatted with 95% ethanol for 8 h at 75 °C under reflux and extracted in boiling water for 2 h. The extract was concentrated for 3 folds and treated with 3 volumes of ethanol at 4 °C overnight. The precipitate was dissolved in distilled water and deproteinated according to the method of Sevag (Sevag, 1938), followed by dialyzed and lyophilized. The crude polysaccharides were fractionated using Sephadex G-100 column chromatography (1.6 cm × 60 cm) eluted with distilled water with a flow rate of 0.6 ml/min. The fraction of major peak was collected and lyophilized to powder named as RPS.

### 2.4. Monosaccharide analysis

Monosaccharide analysis was performed as described previously (Dai et al., 2010) with some modifications. RPS was hydrolyzed with 2.0 M $CF_3COOH$ at 120 °C for 2 h in a sealed ampoule bottle. After removed the TFA, hydrolyzed sample was dissolved in 100 μl of double distilled water. 100 μl of 0.3 M sodium hydroxide and 120 μl of 0.5 M PMP (dissolved in methanol) were added and mixed by a vortex mixer. The mixture was incubated at 70 °C for 1 h and neutralized with 100 μl 0.3 M hydrochloric acid. After extracted by 500 μl of chloroform for 3 times, the aqueous layer was collected and filtered through a 0.22 μm pore membrane filter for HPLC analysis. The derivatization of standard monosaccharide was carried under the same condition. The derivatives were analyzed by HPLC (UltiMate 3000) equipped with Kromasil C18 HPLC column (4.6 mm × 150 mm, 5 μm) and ultraviolet detector. The flow rate was 0.8 ml/min and the column temperature was controlled at 30 °C. The mobile phase was composed of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 82:18 (v/v).

### 2.5. Cell culture

Human gastric cancer cell line BGC-823 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were incubated in humidified atmosphere of 5% $CO_2$ at 37 °C.

### 2.6. Cell proliferation viability and colony formation assay

Viability of BGC-823 cells was measured by MTT assay as described previously (Mosmann, 1983). Logarithmically growing BGC-823 cells were trypsinized from culture bottles. A 100 μl aliquot of the cell suspension was gently subjected into 96-well plate and incubated overnight. Cells were treated with serial concentrations of RPS for 12 h, 24 h and 48 h, and then 10 μl of MTT solution was added to each well. After 4 h of incubation, the medium was discarded, and 100 μl of DMSO was applied to solubilize the formazan with gentle shaking for 5 min. Absorbance was detected at 570 nm by an automatic microplate reader. Relative cell viability was expressed as a percentage relative to the untreated control group. The long-term effects of RPS on BGC-823 cells were tested by clonogenic assay. Briefly, $1 \times 10^2$ cells were seeded in 24-well plate and treated with RPS for 24 h. After rinsed with fresh medium, cells were allowed to form colonies for 10 d and dyed with crystal violet.

### 2.7. Acridine orange/ethidium bromide (AO/EB) staining and Hoechst 33258 staining

Cells were seeded in 12-well plate, followed by incubation with RPS (0, 200, 400, 600 μg/ml) for 12 h. The cells were stained with mixture of 100 μg/ml AO and 100 μg/ml EB for 5 min, and then washed with phosphate buffer. For Hoechst 33258 staining, cells were fixed with methanol/acetic acid (3:1) for 15 min and loaded with Hoechst 33258 for 10 min at room temperature in the dark. Samples were visualized using fluorescence microscopy (Olympus).

### 2.8. Annexin V-FITC and propidium iodide staining

Cells ($1 \times 10^6$) were plated in 6-well plate and treated with indicated concentrations of RPS. After treated for 12 h, cells were stained with Annexin V-FITC and propidium iodide according to the manufacturer's manual. Briefly, cells were incubated with solution containing 195 μl Annexin V-FITC binding buffer and 5 μl of FITC-labeled Annexin V at room temperature for 20 min in the dark. Samples were centrifuged to remove the supernatants, followed by added propidium iodide. Stained cells were analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

### 2.9. Assays of mitochondrial membrane potential (MMP), reactive oxygen species (ROS), intracellular calcium ion ($[Ca^{2+}]i$) and cell cycle distribution

BGC-823 cells ($1 \times 10^6$) were seeded in 6-well plate and incubated with RPS for 12 h. A membrane-permeable lipophilic cationic fluorescent carbocyanine dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, was used to probed dissipation of mitochondrial transmembrane potential. Briefly, treated cells were collected and stained with JC-1 for 20 min at 37 °C in the dark. After centrifuged, the cell pellets were washed twice and suspended in pre-warmed assay buffer before measured by flow cytometry. Aggregates and monomers of JC-1 were detected in the FL2 channel (red fluorescence) and FL1 channel (green fluorescence) respectively. To measure the generation of ROS, cells were probed with 10 mM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 20 min. Intracellular calcium ion was

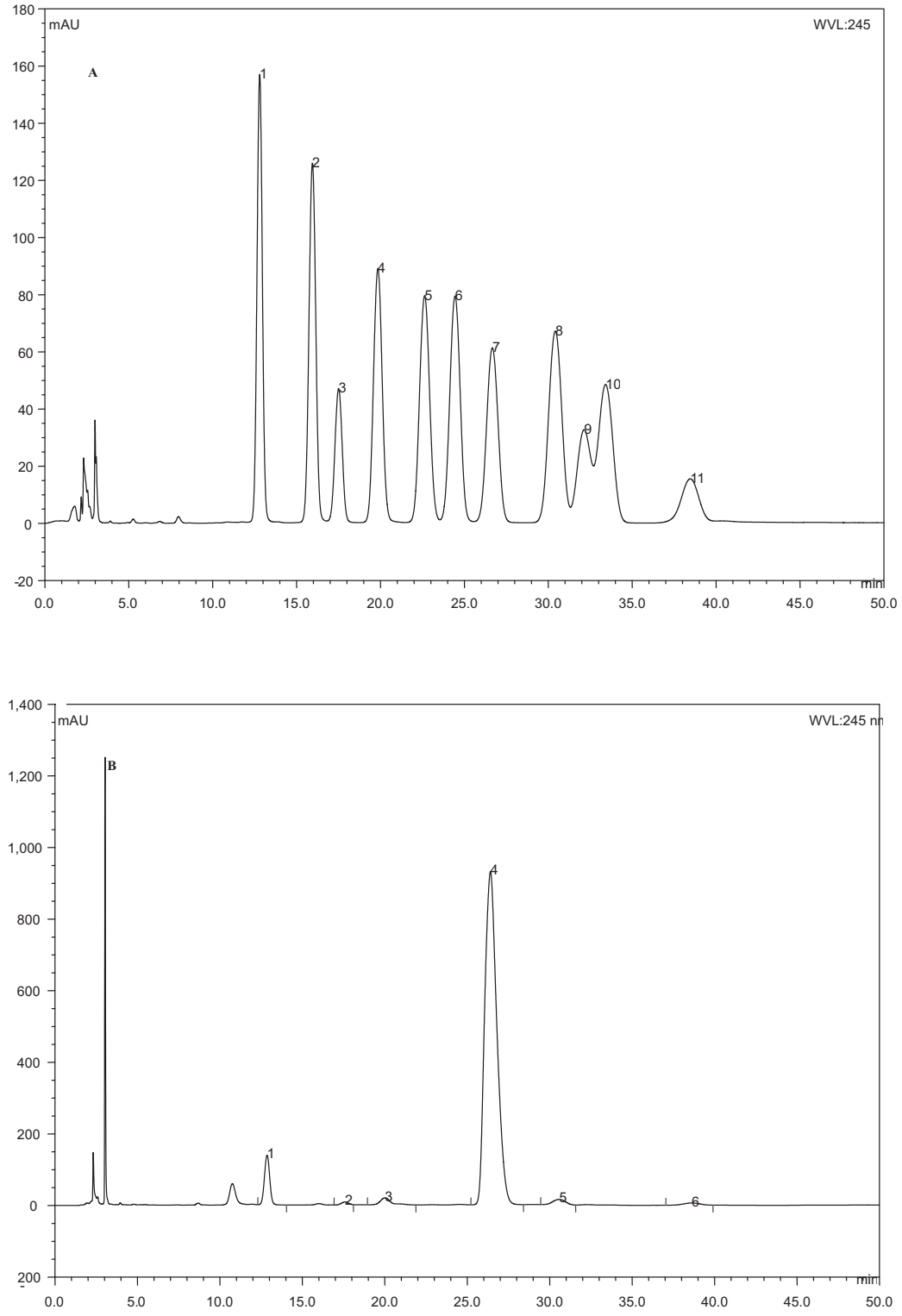


**Fig. 1.** HPLC analyses of PMP derivatives of (A) monosaccharide standard samples (1 – Man, 2 – GlcN, 3 – Rha, 4 – GlcA, 5 – GalA, 6 – GalN, 7 – Glc, 8 – Gal, 9 – Xyl, 10 – Ara and 11 – Fuc) and (B) hydrolysate of RPS.

detected using 1 μM Fluo-3 AM. To investigate cell cycle distribution, cells were fixed with ice-cold 70% ethanol overnight and loaded with staining solution containing propidium iodide (50 μg/ml) and DNase-free RNase (25 μg/ml) for 20 min at 37 °C in the dark. Stained cells were analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA) or fluorescence microscope (Olympus).

### 2.10. Caspase activity assay

The enzymatic activities of caspase-9 and caspase-3 were measured using colorimetric assay kit according to the manufacturer's protocol. Briefly, cells ($1 \times 10^6$) were seeded in 6-well plate and incubated for 12 h with different concentrations of RPS. Treated cells were collected and incubated in 100 μl lysis buffer for 15 min

in ice. After centrifuged, the supernatants were obtained and stored at −70 °C. The lysates were incubated with 20 μM Ac-DEVD-*p*NA (caspase-3 substrate) and Ac-LEHD-*p*NA (caspase-9 substrate) at 37 °C for 2–4 h. Absorbance of the chromophore *p*NA dissociated from the caspase substrates was detected using Microplate Reader at 405 nm. The enzymatic activities of caspases were shown as fold-increase compared with untreated cells.

### 2.11. Statistical analysis

Analysis was performed using one-way analysis of variance (ANOVA) and Student *t* test to determine the significance between groups. $p$-Value $\leq 0.05$ was considered statistically significant. All the results were expressed as mean values ± SD.

## 3. Results and discussion

### 3.1. The monosaccharide analysis of RPS

High performance liquid chromatography (HPLC) was performed to analyze the monosaccharide composition of RPS. As shown in Fig. 1, six peaks were identified as mannose, rhamnose, glucuronic acid, glucose, galactose and fucose in a molar ratio of 5.1:1.0:1.6:92.2:1.3:2.3.

### 3.2. RPS inhibited the proliferation and colony formation of BGC-823 cells

Polysaccharides have been applied in oncotherapy and adjuvant therapy as immunomodulaters for a long time (Zhang, Cui, Cheung, & Wang, 2007). Recent investigations have illustrated the potential mechanisms. These substances govern biologic function and regulation of cytokine networks to mediate host immune responses (Tzianabos, 2000). Besides the immunomodulation function, direct tumor inhibition activity of fungus polysaccharides has provided attractive stimuli on anticancer drug development.

In order to evaluate the effects of RPS on cell proliferation of human gastric cancer, BGC-823 cells were exposed to escalating concentrations of RPS for 12 h, 24 h and 48 h, followed by MTT assay. As shown in Fig. 2A, RPS significantly decreased the cell viability of BGC-823 cells at different time in a does-dependent manner. Furthermore, we conducted clonogenic assay to investigate the anti-tumor activity of RPS. After treatment with RPS, a dose-dependent inhibition in clonogenicity was observed, with approximate 70% inhibition at concentration of 0.6 mg/ml (Fig. 2B). These results indicated that RPS possessed a potent inhibitory effect on the growth of BGC-823 cells.

### 3.3. Morphological characterization

Morphological changes of BGC-823 cells after treatment with RPS were identified using phase contrast microscope. As shown in Fig. 3A, the control cells showed polygonal shapes, while RPS treated cells represented morphological changes including loss of adhesion, cell shrinkage and plasma membrane blebbing. These differences implied that the cells were undergoing apoptosis (Kerr, Wyllie, & Currie, 1972).

To examine whether RPS could induce apoptotic cell death, the BGC-823 cells were stained with Hoechst 33258 and AO/EB, respectively. Results of Hoechst 33258 staining showed that BGC-823 cells underwent typical morphological characteristics of apoptosis such as condensation of chromatin, nuclear pyknosis, formation of apoptotic bodies and nuclear fragmentation after treatment with RPS for 12 h, while cells of control group had normal nuclear morphology (Fig. 3B). AO/EB staining allows distinguishing live, apoptotic and necrotic cells. Live cells possess normal green nuclei. Early apoptotic cells show a bright green nucleus with condensed chromatin and late apoptotic cells display orange fragmented chromatin. Necrotic cells show bright orange intact nucleus. After staining with AO/EB, treated cells showed condensation of the cytoplasm, nuclear fragmentation and increase of cell membrane permeability compared with control group (Fig. 3C). Therefore, apoptosis would probably be involved in the anti-proliferation effect of RPS on BGC-823 cells.

### 3.4. RPS induced apoptosis in BGC-823 cells

For years, apoptosis is recognized as the principal mechanism by which chemotherapeutic agents and radiotherapy kill cancer cells. Apoptosis can be triggered by various forms of physiologic stimuli or stresses, such as cytokines, free radicals and DNA damage (Brunner et al., 2003; Harman, 1992; Roos & Kaina, 2006). Apoptotic cells represent membrane alterations, like transfer of phosphatidylserine from the inner side of the plasma membrane to the outer layers (Bratton et al., 1997). Annexin V is a recombination protein that specifically binds to phosphatidylserine residues with high affinity. It can be combined with specific membrane-impermeant nucleic acid dyes to discriminate apoptosis from necrosis (Koopman et al., 1994).

To validate the results of morphological analysis, we conducted flow cytometry based on Annexin V-FITC/PI staining. The apoptosis

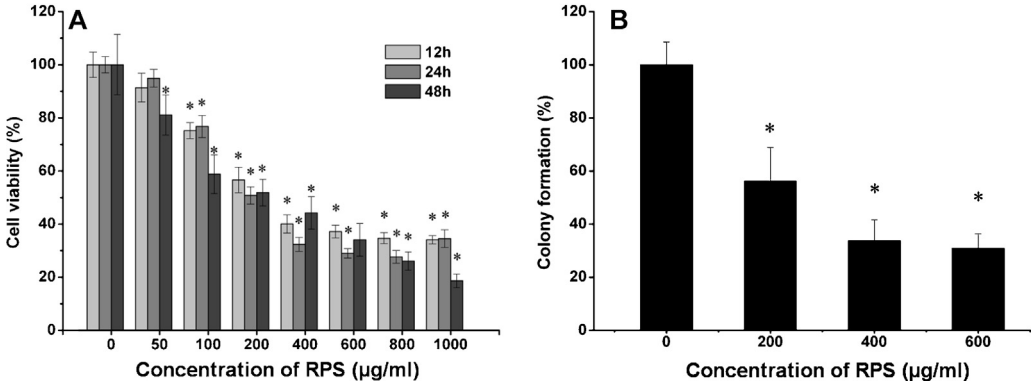


**Fig. 2.** Effects of RPS on cell viability and colony formation in BGC-823 cells. (A) Cells were cultured in 96-well plate and treated with different doses of RPS (0–1000 μg/ml) for 12 h, 24 h and 48 h. The cell viability was analyzed by MTT assay. (B) The clonogenic assay was performed as described in Section 2. Cells were treated with 200, 400, 600 μg/ml RPS for 24 h and incubated with fresh medium for an additional 10 d, and then stained with crystal violet. Results were expressed as mean values ± SD of three independent experiments (*$p \leq 0.05$ versus control group).

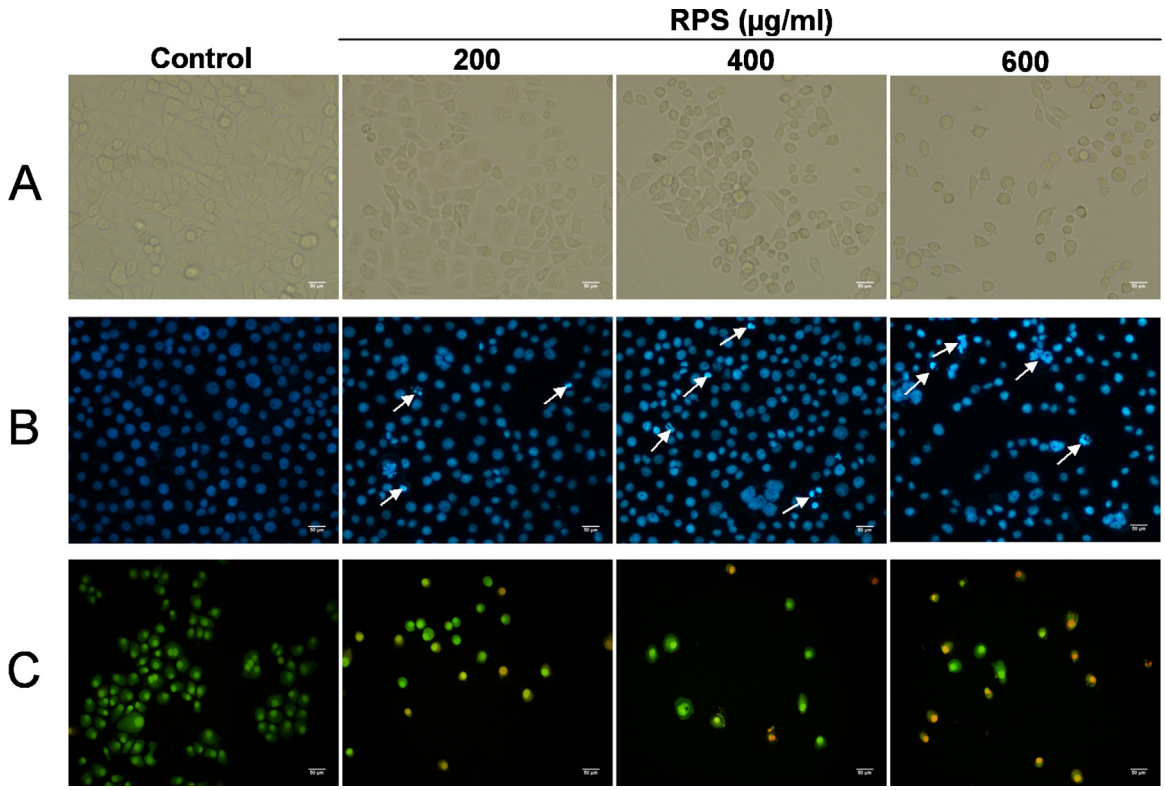


**Fig. 3.** Effects of RPS on morphology of BGC-823 cells. Cells were treated with 0, 200, 400 and 600 μg/ml RPS for 12 h. Treated cells were visualized using (A) bright-field microscope and fluorescence microscope after stained with (B) Hoechst 33258 and (C) AO/EB mixed dye. White arrows indicated the condensation and fragmentation of chromatin (*Bar* = 50 μm).

of BGC-823 cells was effectively induced by 12 h of RPS treatment. As shown in Fig. 4, the percentage of early apoptotic cells (Annexin V-positive/PI-negative) increased from 2.24% in untreated group to 24.93% in treated group. Moreover, gradual increase of non-viable apoptotic cells and necrotic cells was also observed. These results indicated that induction of apoptosis accounted for the growth inhibition of BGC-823 cells.

### 3.5. RPS induced loss of mitochondrial membrane potential (MMP)

There are two classic apoptosis signaling pathways: mitochondrial pathway and death receptor pathway. The mitochondrial pathway can be activated by diverse stress signals, such as toxins, reactive oxygen species and genotoxic stress, resulting in collapse of mitochondrial membrane potential. The loss of the mitochondrial membrane potential is recognized as a key event in the early apoptosis via mitochondrial pathway (Zamzami et al., 1995).

To characterize the mitochondrial pathway by which RPS induced apoptosis, we measured the mitochondrial membrane potential using a non-toxic fluorescent probe JC-1. As shown in Fig. 5A, the population of cells emitted red fluorescence was decreased with a parallel increase of cells emitted green fluorescence after 12 h of RPS treatment. As the dose of RPS increased, we found FL2/FL1 ratio was significantly reduced, which indicated the loss of mitochondrial membrane potential (Fig. 5B). Collapse of mitochondrial membrane potential causes the release of cytochrome *c* and autoactivation of caspase-9, eventually triggering apoptosis (Saelens et al., 2004). Therefore, mitochondrial dysfunction might participate in the apoptosis induced by RPS.

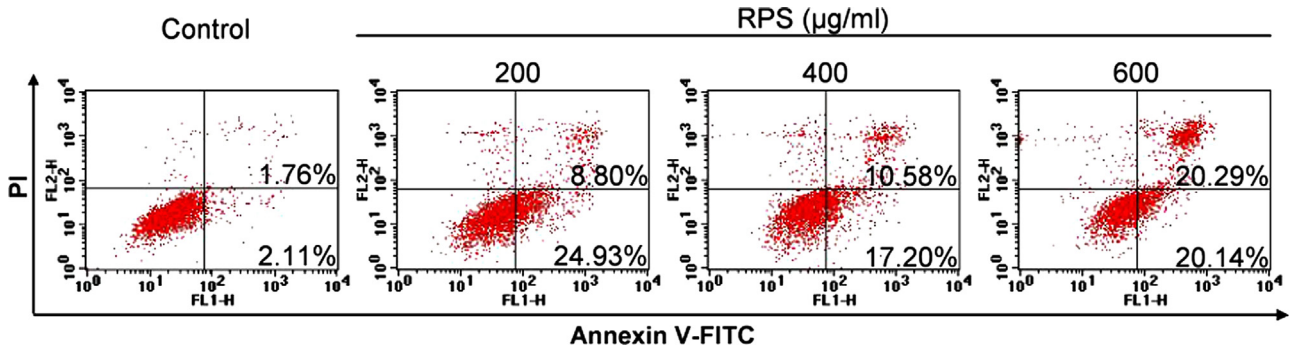


**Fig. 4.** RPS induced apoptosis in BGC-823 cells. Cells were treated with the indicated concentrations of RPS for 12 h. Treated cells were stained with propidium iodide and Annexin V-FITC according to the manufacturer's manual, and detected using flow cytometry. The cells shown in the right lower quadrant (Annexin V+/PI−) were undergoing apoptosis, and cells of right upper quadrant (Annexin V+/PI+) were considered as non-viable apoptotic cells and necrotic cells.

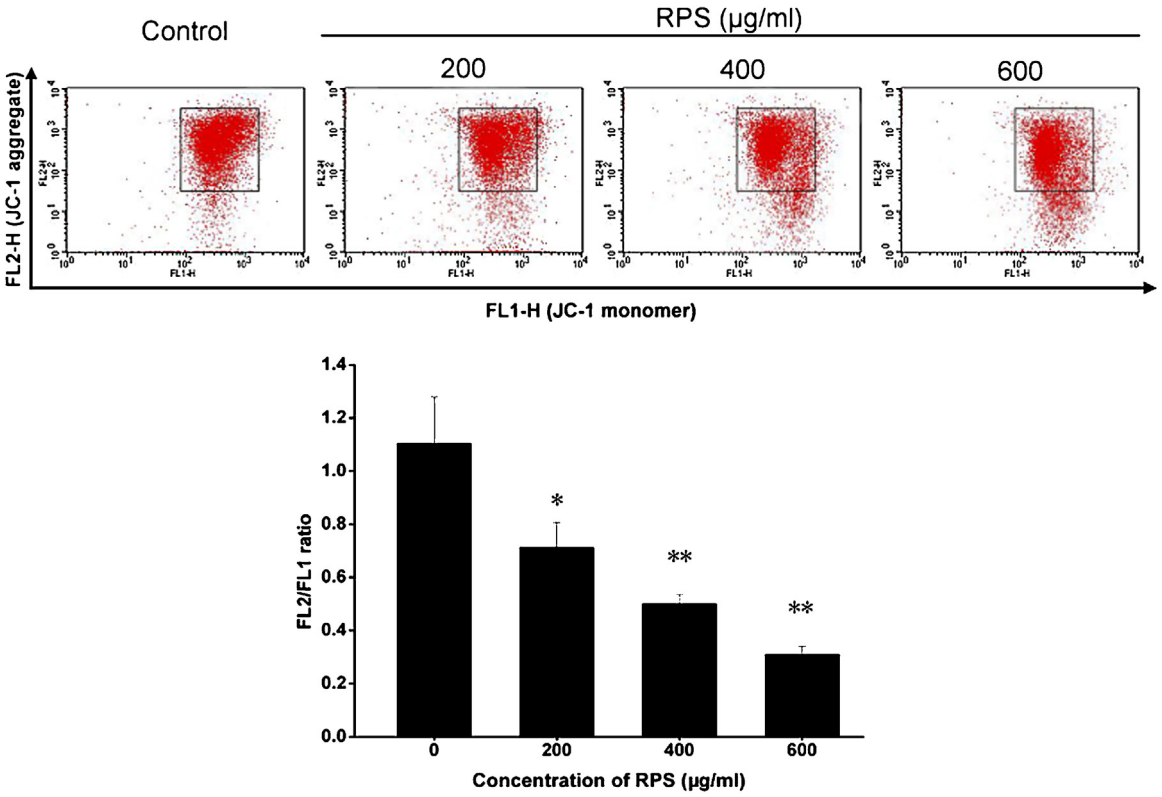


**Fig. 5.** RPS induced loss of mitochondrial membrane potential. Cells were treated with variety concentrations of RPS for 12 h. (A) Treated cells were harvested, stained with JC-1, and assayed by flow cytometry. (B) Histogram illustrated the ratio of FL2/FL1. The data were shown as mean values ± SD of three independent experiments ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ versus control group).

### 3.6. RPS induced activation of caspase-9 and caspase-3 in BGC-823 cells

Caspases are aspartate-specific cysteine proteases and execute selective, limited cleavage of key cellular components (Alnemri et al., 1996; Fuentes-Prior & Salvesen, 2004). The caspase-cascade system plays a pivotal role in regulating diverse biological processes especially programmed cell death (Li & Yuan, 2008). Caspases associated with apoptosis are classified as apoptosis activator (caspase-8, -9, -10) and apoptosis executioner (caspase-3, -6, -7) (Creagh & Martin, 2001). The active form of caspase-9 activates caspase-3, resulting in disintegration of the cytoskeletal and nuclear proteins (Wolf & Green, 1999).

In our previous study, BGC-823 cells treated with RPS displayed chromatin condensation and mitochondrial dysfunction. Caspase-3 is considered as the key protease involved in DNA fragmentation and chromatin condensation (Porter & Janicke, 1999). We measured the enzyme activity of caspase-3 using colorimetric assay in BGC-823 cells. As shown in Fig. 6, the activity of caspase-3 was significantly increased in a concentration-dependent manner after exposed to RPS for 12 h. Caspase-9 is the apical protease of the mitochondrial pathway. To further elucidate whether activation of caspase-3 was involved in mitochondrial apoptotic pathway elicited by RPS, the activity of caspase-9 was also detected. A 1.72 fold increase of caspase-9 activity was observed relative to untreated cells (Fig. 6). Our findings suggested that RPS induced apoptosis through mitochondrial pathway in BGC-823 cells.

### 3.7. RPS induced generation of ROS and elevation of intracellular calcium ion

Mitochondria are recognized as the major source of cellar ROS and modulator for $Ca^{2+}$ signaling (Feissner, Skalska, Gaum, & Sheu, 2009; Turrens, 1997). Intracellular ROS and $Ca^{2+}$ are frequently produced during program cell death and act as second messages (Ferri & Kroemer, 2001). Reactive oxygen species are the byproducts of respiratory chain, including superoxide, hydroxyl radical, hydrogen peroxide and singlet oxygen. ROS have destructive actions on various cellular components including nucleic acids, proteins, and lipid membranes. Accumulation of intracellular ROS may elicit dysfunction of mitochondria. Many studies have shown that treatment by low does oxidants induced apoptosis which can be prevented by

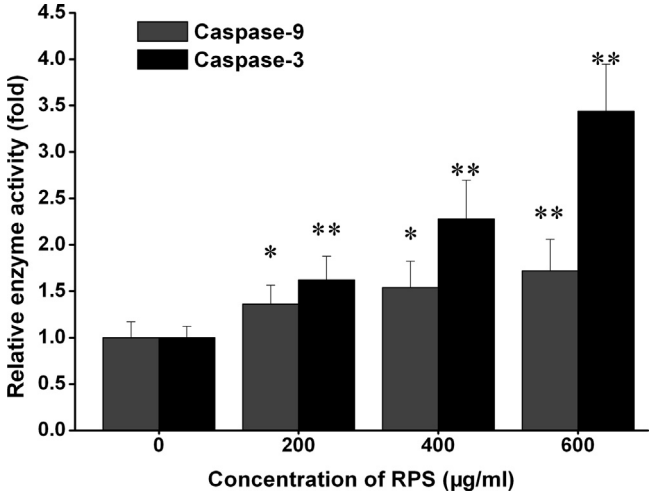


**Fig. 6.** RPS induced activation of caspase-9 and caspase-3 in BGC-823 cells. BGC-823 cells were treated with increasing concentrations of RPS for 12 h. The total cytosolic fraction of the cells was prepared. The activities of caspase-9 and caspase-3 were assayed by caspase colorimetric assay kit and shown as fold-increase compared with control group ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ versus control group).

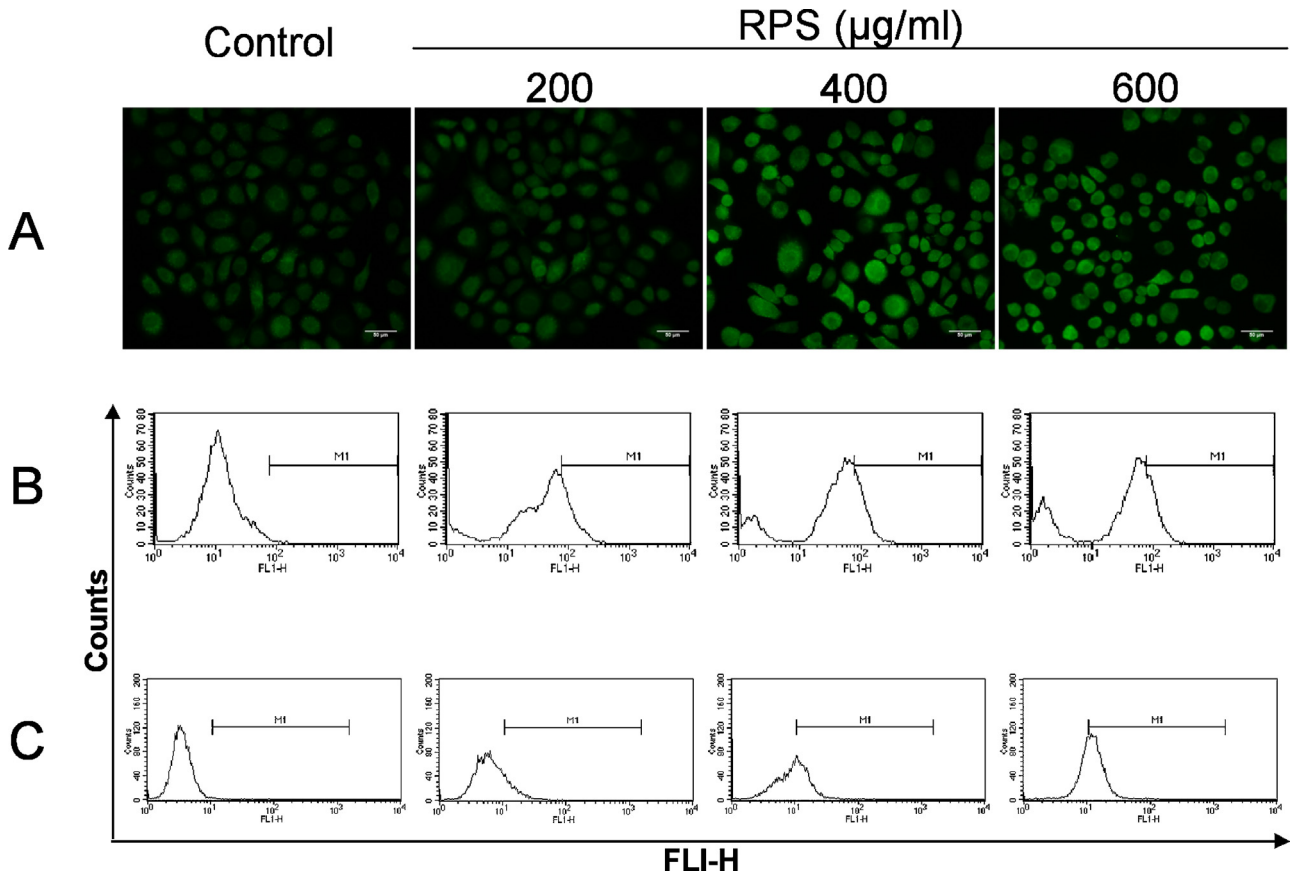


**Fig. 7.** RPS induced generation of ROS and elevation of intracellular calcium in BGC-823 cells. Cells were incubated with RPS for 12 h and stained with DCFH-DA, followed by analyzed with (A) fluorescence microscope and (B) flow cytometry. (C) The treated cells were probed with Fluo-3 AM for 30 min and analyzed using flow cytometry (*Bar* = 50 μm).

antioxidants (Kasahara et al., 1997; Pierce, Parchment, & Lewellyn, 1991). On the other hand, intracellular $Ca^{2+}$ can be sequestered into mitochondria and promote oxidative phosphorylation. However $Ca^{2+}$ overload triggers mitochondrial depolarization and leads to the release of apoptosis promoting factors (Kroemer, Galluzzi, & Brenner, 2007).

To determine whether ROS production conferred apoptotic response to RPS, we conducted fluorochrome-based fluoroscopy and flow cytometry to examine the intracellular ROS level. The results revealed that fluorescence intensity increased in a does-dependent manner compared with the control cells after treated with RPS for 12 h (Fig. 7A). The generation of ROS was remarkably stimulated by RPS treatment (Fig. 7B). In addition, Fluo-3 AM was used to investigate the flux of intracellular calcium ion. As shown in Fig. 7C, treatment with RPS increased the proportion of fluorescence positive cells from 0.45% in control group to 11.05%, 24.12% and 39.07% in treated groups respectively, which indicated the elevation of $[Ca^{2+}]i$. Calpains are cytoplasmic cysteine protease

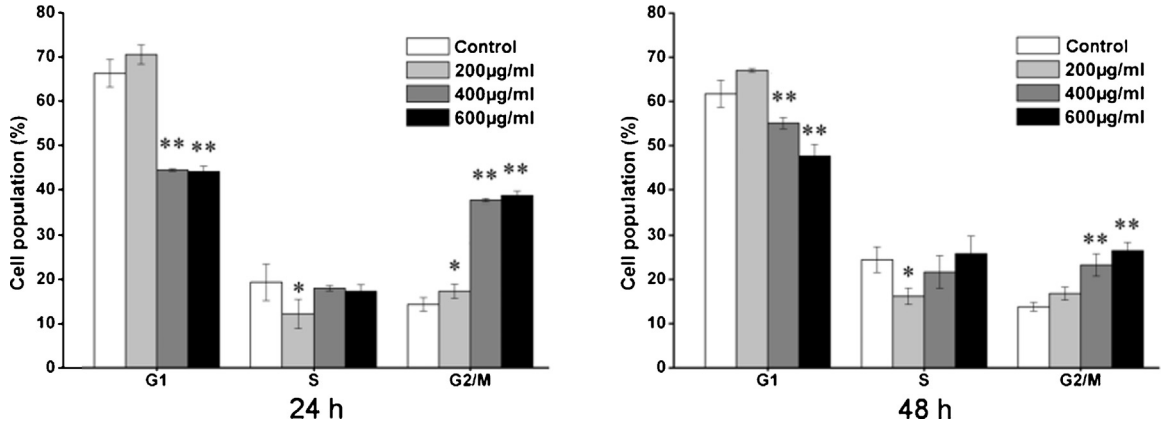


**Fig. 8.** RPS induced G2/M phase cell cycle arrest in BGC-823 cells. Cells were treated with different concentrations of RPS (0, 200, 400, 600 μg/ml) for 24 h and 48 h. After treatment, cells were fixed with ice-cold 70% ethanol, and then stained with propidium iodide (50 μg/ml) and DNase-free RNase (25 μg/ml). Stained cells were analyzed using flow cytometry. Results were shown as mean values ± SD of three independent experiments ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ versus control group).

which can be activated by elevation of intracellular calcium. The active forms of the calpains can serve as upstream triggers of mitochondrial membrane permeabilization and participate in caspase cascade (Varghese, Radhika, & Sarin, 2001; Waterhouse et al., 1998). These results showed that generation of ROS and elevation of $[Ca^{2+}]i$ were associated with mitochondria-mediated apoptosis triggered by RPS.

### 3.8. RPS induced G2/M phase cell cycle arrest in BGC-823 cells

Uncontrolled cell proliferation is the typical character of cancer (Hanahan & Weinberg, 2011). Therefore, cell-cycle regulation is considered as an important target for cancer therapy. Cell cycle checkpoints are the key regulatory pathways monitoring phase transition of the cell cycle (Hartwell & Weinert, 1989). Response to cell stresses such as toxicant or DNA break, they could stop the aberrant cell division of cancer cells (Bjelland & Seeberg, 2003).

To gain further insights into the mechanisms involved in antiproliferative activity of RPS, we investigated the distribution of cell cycle phase using flow cytometry. The results indicated that RPS treatment induced the G2/M phase cells accumulation with a parallel decrease of G1-phase cells after 24 h and 48 h treatment at concentration of 400 μg/ml and 600 μg/ml in BGC-823 cells (Fig. 8). RPS predominantly induced G2/M arrest in BGC-823 cells and probably activated potential blockade of cell cycle progression at G2 checkpoint and/or spindle checkpoint.

## 4. Conclusions

In this study, we found that polysaccharides extracted from fermentation mycelia of *R. nigricans* suppressed the proliferation and colony formation of BGC-823 cells by triggering apoptosis and G2/M phase cell cycle arrest. RPS induced mitochondria-mediated apoptosis via loss of mitochondrial membrane potential and activation of caspase-9 and caspase-3. In addition, generation of intracellular reactive oxygen species and elevation of intracellular calcium ion were also involved in the apoptosis caused by RPS. Our findings will benefit the future studies and drug development of RPS in the treatment of gastric cancer.

## Acknowledgements

This study was financially supported by the Major State Basic Research Development Program of China (973 Program) (No. 2012CB822102) and the High Technology Research and Development Program of China (863 Program) (No. 2012AA021501).

## References

Adamsen, L., Quist, M., Midtgaard, J., Andersen, C., Moller, T., Knutsen, L., et al. (2006). The effect of a multidimensional exercise intervention on physical capacity, well-being and quality of life in cancer patients undergoing chemotherapy. *Supportive Care in Cancer*, *14*(2), 116–127.

Alarcón, J., Águila, S., Cornejo, F., & Alderete, J. (2007). Biotransformation of 5α-hydroxy-14-eudesm-11-en-3-one by *Rhizopus nigricans*, *Cunninghamella elegans* and *Mucor plumbeus*. *Journal of Molecular Catalysis B: Enzymatic*, *48*(1–2), 23–27.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., et al. (1996). Human ICE/CED-3 protease nomenclature. *Cell*, *87*(2), 171.

Bjelland, S., & Seeberg, E. (2003). Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis*, *531*, 37–80.

Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., & Henson, P. M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *Journal of Biological Chemistry*, *272*(42), 26159–26165.

Brunner, T., Wasem, C., Torgler, R., Cima, I., Jakob, S., & Corazza, N. (2003). Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. *Seminars in Immunology*, *15*(3), 167–176.

Creagh, E. M., & Martin, S. J. (2001). Caspases: Cellular demolition experts. *Biochemical Society Transactions*, *29*(6), 696–702.

Dai, J., Wu, Y., Chen, S., Zhu, S., Yin, H., Wang, M., et al. (2010). Sugar compositional determination of polysaccharides from *Dunaliella salina* by modified RP-HPLC method of precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone. *Carbohydrate Polymers*, *82*(3), 629–635.

Feissner, R. F., Skalska, J., Gaum, W. E., & Sheu, S. S. (2009). Crosstalk signaling between mitochondrial $Ca^{2+}$ and ROS. *Frontiers in Bioscience*, *14*, 1197–1218.

Ferri, K. F., & Kroemer, G. (2001). Organelle-specific initiation of cell death pathways. *Nature Cell Biology*, *3*(11), E255–E263.

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

Fuentes-Prior, P., & Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochemical Journal*, *384*(2), 201–232.

Guillermo, S.-H., Carlos, A., Marcela, K., Jaime, A. R., & Cristina, T. (2007). Biotransformations of imbricatolic acid by *Aspergillus niger* and *Rhizopus nigricans* cultures. *Molecules*, *12*(5), 1092–1100.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674.

Harman, D. (1992). Role of free radicals in aging and disease. *Annals of the New York Academy of Sciences*, *673*, 126–141.

Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: Controls that ensure the order of cell cycle events. *Science*, *246*(4930), 629–634.

Huang, Q., Jin, Y., Zhang, L., Cheung, P. C. K., & Kennedy, J. F. (2007). Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter. *Carbohydrate Polymers*, *70*(3), 324–333.

Huang, T., Lin, J., Cao, J., Zhang, P., Bai, Y., Chen, G., et al. (2012). An exopolysaccharide from *Trichoderma pseudokoningii* and its apoptotic activity on human leukemia K562 cells. *Carbohydrate Polymers*, *89*(2), 701–708.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*(2), 69–90.

Kasahara, Y., Iwai, K., Yachie, A., Ohta, K., Konno, A., Seki, H., et al. (1997). Involvement of reactive oxygen intermediates in spontaneous and CD95 (Fas/APO-1)-mediated apoptosis of neutrophils. *Blood*, *89*(5), 1748–1753.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4), 239–257.

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., & van Oers, M. H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*, *84*(5), 1415–1420.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological Reviews*, *87*(1), 99–163.

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.

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, J., & Yuan, J. (2008). Caspases in apoptosis and beyond. *Oncogene*, *27*(48), 6194–6206.

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

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Nicholson, D. W. (2000). From bench to clinic with apoptosis-based therapeutic agents. *Nature*, *407*(6805), 810–816.

Park, H. S., Kim, G. Y., Nam, T. J., Deuk Kim, N., & Hyun Choi, Y. (2011). Antiproliferative activity of fucoidan was associated with the induction of apoptosis and autophagy in AGS human gastric cancer cells. *Journal of Food Science*, *76*(3), T77–T83.

Pierce, G. B., Parchment, R. E., & Lewellyn, A. L. (1991). Hydrogen peroxide as a mediator of programmed cell death in the blastocyst. *Differentiation*, *46*(3), 181–186.

Porter, A. G., & Janicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation*, *6*(2), 99–104.

Roos, W. P., & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends in Molecular Medicine*, *12*(9), 440–450.

Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene*, *23*(16), 2861–2874.

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

Taraphdar, A. K., Roy, M., & Bhattacharya, R. K. (2001). Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Current Science*, *80*(11), 1387–1396.

Turrens, J. F. (1997). Superoxide production by the mitochondrial respiratory chain. *Bioscience Reports*, *17*(1), 3–8.

Tzianabos, A. O. (2000). Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biologic function. *Clinical Microbiology Reviews*, *13*(4), 523–533.

Varghese, J., Radhika, G., & Sarin, A. (2001). The role of calpain in caspase activation during etoposide induced apoptosis in T cells. *European Journal of Immunology*, *31*(7), 2035–2041.

Wagner, A. D., Grothe, W., Haerting, J., Kleber, G., Grothey, A., & Fleig, W. E. (2006). Chemotherapy in advanced gastric cancer: A systematic review and meta-analysis based on aggregate data. *Journal of Clinical Oncology*, *24*(18), 2903–2909.

Wasser, S. P., & Weis, A. L. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushrooms: Current perspectives. *International Journal of Medicinal Mushrooms*, *1*, 47–50.

Waterhouse, N. J., Finucane, D. M., Green, D. R., Elce, J. S., Kumar, S., Alnemri, E. S., et al. (1998). Calpain activation is upstream of caspases in radiation-induced apoptosis. *Cell Death and Differentiation*, *5*(12), 1051–1061.

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: Apoptotic cell death by caspase family proteinases. *Journal of Biological Chemistry*, *274*(29), 20049–20052.

Yu, Z., Yin, L., Qian, Y., & Yan, L. (2009). Effect of *Lentinus edodes* polysaccharide on oxidative stress, immunity activity and oral ulceration of rats stimulated by phenol. *Carbohydrate Polymers*, *75*(1), 115–118.

Zaidman, B. Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*, *67*(4), 453–468.

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. *Journal of Experimental Medicine*, *181*(5), 1661–1672.

Zeng, J., Dai, P., Ren, L., Song, B., Chen, X., Wang, X., et al. (2012). Apoptosis-induced anti-tumor effect of *Curcuma kwangsiensis* polysaccharides against human nasopharyngeal carcinoma cells. *Carbohydrate Polymers*, *89*(4), 1067–1072.

Zhang, M., Cui, S., Cheung, P., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*, 4–19.