Effect of *Sargassum fusiforme* polysaccharide on apoptosis and its possible mechanism in human erythroleukemia cells

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Introduction

Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries [¹]. Recent statistics show that malignant tumors are still one of the main causes of death among residents in China [²]. According to the China Statistical Yearbook 2017 released by the National Bureau of Statistics of China, in 2016, the mortality rate of malignant tumors among urban residents was 160.07/100 000 and that among rural residents was approximately 155.83/100 000. Cancer is also one of the most common diseases in developed countries. In 2017, the National Institutes of Health estimated 1.6 million new cases in the United States, while in 2018, an increase of 1.74 million cases was estimated and 0.61 million died due to cancer-related cases [³,⁴].

Leukemia belongs to a group of heterogeneous neoplastic white blood cell disorders that affect the blood, bone marrow, and lymphoid system. Leukemia includes four main types, acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, and chronic myeloid leukemia [⁵,⁶]. According to the World Health Organization, leukemia occurred among 0.352 million people globally and caused 0.265 million deaths in 2012 [⁷]. The current conventional treatment for leukemia involves chemotherapy, radiation therapy, targeted therapy, bone marrow transplant, and supportive therapy. Despite the major therapeutic advances in the developed world over the past decades, the variation in long-term outcomes among patients with leukemia remains significant. Among young patients with acute myeloid or lymphoid leukemia, the five-year survival rate has ranged from 30%–50%, while the treatment of elderly patients remains a challenge [⁸,⁹]. Chemotherapy is one of the common therapeutic modalities with less satisfactory results due to its severe adverse effects [¹⁰]. These adverse effects are invariably associated with disabling side effects, including anemia, immunosuppression, and alopecia. Some show only mild side effects, such as fever, headaches, and rashes. However, others are associated with severe side effects, such as high blood
pressure, bleeding, and kidney damage caused by bevacizumab. Thus, a novel antitumor substance with little toxicity to patients is required.

Previous studies indicated that natural polysaccharides possess antitumor and immunomodulating properties with fewer side effects [1]. These natural polysaccharides were also found to have the potential for pharmaceutical application in the prevention of cancer. Moreover, extracted from Inonotus taiwanensis [13], Laminaria japonica [13], Laurencia obtusa [14], Fucus vesiculosus [15], and so forth, polysaccharides have been reported to inhibit proliferation or induce apoptosis in lymphoid or myeloid cell lines. Thus, these polysaccharides may provide a potential alternative treatment for leukemia. Sargassum fusiforme (S. fusiforme) has been cultivated in China and Japan for its application as edible food and therapeutic over thousand years [16-18]. It contains various bioactive components with health benefits, including polysaccharides, such as alginate and sulfated polysaccharide (fucoidan) [19]. Recently, polysaccharides from natural sources have been found to be effective and non-toxic substances with a wide variety of biological activities and have attracted more attention for biochemical and medical applications [20]. The sulfated polysaccharide from S. fusiforme is a semi-synthetic chemical compound and contains sulfate groups of natural polysaccharide derivatives. Also, it has shown multiple medical functions, such as antihyperlipidemic agent [21], antioxidant [22], immunity enhancer [23, 24], antiviral [25], hypoglycemic agent [26, 27], and anti-tumor agent [28, 29]. Erythroleukemia is a type of leukemia. However, the apoptosis of human erythroleukemia leukemia (HEL) cells induced by S. fusiforme polysaccharide (SFPS) has not been reported, and the mechanism by which SFPS induces apoptosis has not been clarified. Moreover, the cytotoxicity of purified SFPS has rarely been reported. In this study, a HEL cell line was used as the research object, and the molecular mechanism by which SFPS induces proliferation and apoptosis in leukemia cells was preliminarily investigated, providing the experimental basis of the treatment for leukemia using SFPS.

Materials and Methods

Materials

S. fusiforme was collected in Dongtou, Zhejiang Province, China. AB-8 and D311 macroporous resins were obtained from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). DEAE-52 cellulose, Sephadex G-500 columns, and fetal bovine serum (FBS) were from GE Healthcare Life Sciences (Logan, UT, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Cell Counting Kit-8 (CCK-8), dimethyl sulfoxide (DMSO), protein extraction kits, bicinchoninic acid (BCA) protein assay kits, and tris-buffered saline with tween 20 (TBST) were from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Penicillin G, streptomycin, Roswell Park Memorial Institute (RPMI)-164 and Dulbecco’s Modified Eagle’s medium (DMEM) were from Gibco, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fluorescein isothiocyanate (FITC) annexin V apoptosis detection kits (cat. No. 556547) and propidium iodide (PI)/ribonuclease (RNase) staining buffer (cat. No. 550825) were purchased from BD Biosciences (San Diego, CA, USA). PrimeScript reagent kits with genomic DNA (gDNA) eraser (cat. No. RR047A) and SYBR Premix Ex Taq II (cat. No. RR820A) were from Takara Biomedical Technology Co., Ltd. (Beijing, China). GAPDH rabbit monocalonal antibody (mAb; cat. No. 5174), Caspase-3 antibody (cat. No. 9662), Bcl-2 (D55G8) rabbit mAb (cat. No. 4223), Bcl-xL (54H6) rabbit mAb (cat. No. 2764), Bax antibody (cat. No. 2772), Bad antibody (cat. No. 9292), p53 antibody (cat. No. 9282), and horseradish peroxide (HRP)-conjugated goat anti-rabbit immunoglobulin G (goat anti-rabbit IgG-HRP; cat. No.7074) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). All other reagents were of analytical grade.

Extraction of polysaccharides from S. fusiforme

S. fusiforme was cleaned and dried before ultrasonic extraction of polysaccharides as previously described [30, 31]. A 250 g portion of algal powder was pretreated with water (1 : 35, W/V) at 87 °C, and extracted for 13 min at a power of 900 W. The filtrate was collected, centrifuged at 20 000 g, and passed through successive columns of AB-8 and D311 macroporous resin to remove pigments and small lipophilic molecules, such as polyphenols and proteins. The water extracts were combined, filtered, concentrated, and precipitated with 100% ethanol (5 : 2, V/V) at 4 °C overnight. The resulting supernatant was collected, and absolute ethanol was added for a final concentration of 1 : 4 (V/V) for precipitation for approximately 12 h at 4 °C. The sediment was collected by centrifugation, and the protein was removed as previously described [32]. Finally, the deproteinated supernatant was lyophilized to yield the crude polysaccharides. The crude extracts were purified on DEAE-52 cellulose and Sephadex G-500 columns to yield three S. fusiforme polysaccharides (SFPS I, II, and III), which were lyophilized for further study. The average molecular weights of the SFPS extracts were determined by high-performance gel permeation chromatography to be 180 kDa. SFPS I, II, and III were confirmed by Gas chromatography that mainly composed of L-xylose, D-fucose, D-galactose, and D-mannose. The sulfate ester content of SFPS I, II, and III were 4.87%, 26.47%, and 34.25%, respectively.

Cell lines and culture

Human embryonic lung (MRC-5) cells and HEL cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. MRC-5 cells were cultured in DMEM containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg·mL⁻¹) at 37 °C in a humidified atmosphere with 5% CO₂. HEL cells were maintained in RPMI-1640 medium supplemented with antibiotics (100 IU·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin) and 10% FBS at 37 °C under humidified air containing 5% CO₂. MRC-5 and HEL cells were inoculated into a 96-well culture plate at a density of 5 × 10³ cells per well. When the cultured cells were
80% confluent, the medium was replaced with DMEM or RPMI-1640 containing various concentrations of SFPS I, SFPS II, or SFPS III and cultured for another 24 h. The fresh medium was passed through 0.45 μm filters before being added to cell cultures.

**Cell viability assay**

Logarithmic phase cells were divided into an experimental group and a control group. The negative control group was no drug. The HEL cells in logarithmic growth phase were inoculated into 96-well plates at a density of 5 × 10^4 cells per mL. After 24 h of culture, the S. fusiforme polysaccharides (SFPS I, SFPS II, and SFPS III) were added at a concentration gradient of 10–100 μg mL⁻¹, cisplatin was used as a positive control and the blank control group was only added an equal volume of 10% FBS RPMI-1640 medium. After 24 h of treatment, 10 μL of CCK-8 solution was added to each well and placed in the incubator for 4 h at 37 °C. The absorbance of each well was read at 450 nm using a microplate reader (SpectraMax 190, MD, USA).

**MTT cytotoxicity assay in MRC-5 cells**

The effect of SFPS on MRC-5 cell toxicity was determined using the MTT tetrazolium dye assay kits. In brief, cells were plated in 96-well plates at a density of 5 × 10^3 cells per well in 100 μL of complete culture medium. After complete adherence, cells were treated with SFPS I, SFPS II, and SFPS III (10–100 μg mL⁻¹) for 24 h. Following treatment, MTT solution (final concentration of 500 μg mL⁻¹) was added to each well and incubated for 4 h at 37 °C. The absorbance of each well was read at 490 nm with a microplate reader (SpectraMax 190, MD, USA).

**Apoptosis analysis using Hoechst 33258 staining**

Hoechst 33258 staining was used to investigate the nuclear morphologic changes of apoptotic cells. HEL cells were seeded in 12-well plates at a density of 1 × 10⁴ cells per well in 1 mL of complete culture medium and incubated for 24 h. Then, the polysaccharide solutions (SFPS I, SFPS II, and SFPS III) were added at concentrations of 30, 60, and 90 μg mL⁻¹ for another 24 h. The cells were collected by centrifugation at 500 g for 10 min, and 500 μL of the fixative was added to each well and incubated for 37 °C. The absorbance of each well was read at 450 nm using a microplate reader (SpectraMax 190, MD, USA).

**Flow cytometry analysis of apoptosis**

The induction of apoptosis by the polysaccharides (SFPS I, II, and III) was verified by flow cytometry (BD FACSMVerse, BD, USA) using a commercially available annexin V-FITC/PI apoptosis detection kit. HEL and MRC-5 cells (1 × 10⁴ cells per well) were seeded into 12-well plates and cultured for 24 h before being treated with 10–100 μg mL⁻¹ SFPS I, II, or III in complete DMEM containing 10% FBS for 24 h. Cells were washed twice with cold PBS and resuspended in 1x binding buffer at 1 × 10⁶ cells per mL, and 100 μL aliquots containing 1 × 10⁵ cells each were transferred to 5 mL culture tubes. After being added 5 μL FITC annexin V and 5 μL PI, the cells were gently vortexed and incubated for 15 min at room temperature (25 °C) in the dark. Assays were performed in triplicates.

**Cell cycle analysis**

The cell cycle analysis experiment was performed to quantify the DNA content of cells by flow cytometry. PI was used as a nuclear DNA binding agent. Based on the fluorescence intensity, the different cell cycle phases of the cells were identified. HEL and MRC-5 cells were harvested after 24 h drug treatment and washed twice with PBS, centrifuged at 500 g for 10 min, and fixed overnight in 70% ethanol at −20 °C. Cells were stained with 0.5 mL PI/RNase staining buffer at room temperature (25 °C) for 15 min and assayed by flow cytometry.

**Western blot analysis**

Total protein was extracted from HEL and MRC-5 cells with a protein extraction kit, and the protein concentration was determined using a BCA assay kit. After protein quantification, 20 μg aliquots of cytoplasmic or nuclear extracts were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk powder for 2 h at room temperature (25 °C) and then incubated overnight at 4 °C with anti-Bcl-xL (dilution 1 : 1500), Caspase-3 (dilution 1 : 2000), Bad (dilution 1 : 1500), Bax (dilution 1 : 1500), Bcl-2 (dilution 1 : 1500), p53 (dilution 1 : 1500) primary antibodies, and the anti-GAPDH (dilution 1 : 2000) control. The membranes were subsequently washed three times with TBST for 10 min and then incubated with HRP-conjugated secondary antibodies (dilution 1 : 1000) for 1 h at room temperature (25 °C). Antibody binding was visualized by electrochemiluminescence (BeyoECL Star; Beyotime, China) and photographed with a ChemiDoc imaging system (BIO-RAD, USA).

**Quantitative real-time reverse transcription-polymerase chain reaction (qPCR)**

qPCR was performed as previously described. Total RNA was extracted from the cells with TRIzol reagent. Reverse transcription was performed using a PrimeScript RT reagent kit. qPCR was performed using a CFX96 Real-Time PCR Detection System. The 25 μL PCR reaction included 2 μL of complementary DNA (cDNA), 12.5 μL of 2X SYBR Premix Ex Taq II, 1 μL of PCR forward and PCR reverse primer, and 8.5 μL distilled water (dH₂O). The reactions were incubated in 96-well optical plates at 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, and 60 °C for 30 sec.
qPCR reactions were performed in triplicates. The specificity of the amplification was determined using melting curve analysis. Data were collected and expressed as a function of the threshold cycle (Ct). The samples for the qPCR assay were evaluated with a single predominant peak as quality control. Relative messenger RNA (mRNA) expression was determined by the comparative Ct (2^ΔΔCt) method and normalized against β-actin. The primers used in the study are shown in Table 1. All kits were used following the manufacturer’s instructions.

**Results**

**Effect of SFPS on HEL cell viability**

Tumor cell proliferation is one of the most important mechanisms for tumor incidence and development. HEL cell proliferation was evaluated using the CCK-8 assay. The normal MRC-5 cells were treated with SFPS (I, II, and III) to investigate the cytotoxicity, and MTT assay was performed to measure the growth of the cells. As shown in Fig. 1, the SFPS samples reduced the viability of HEL cells after 24-hour treatments in a dose-dependent manner. Among the SFPS samples, SFPS III had the highest sulfate ester content and the strongest effect on the viability of HEL cells, which declined from 92.80% ± 3.46% to 27.93% ± 4.23% (Fig. 1C). Contrastingly, Fig. 1A shows that HEL cells treated with SFPS I displayed the poorest viability (65.88% ± 4.67%) and lowest sulfate ester content. For the rest of the samples, SFPS II-treated HEL cells showed relatively poor viability (42.33% ± 5.10%) and lower sulfate ester content (Fig. 1B). As shown in Figs. 1A, 1B, and 1C, the highest concentration of SFPS (I, II, and III) induced much less toxic effect on normal MRC-5 cells, indicating a favorable selectivity towards cancer cells. However, interestingly the lowest concentration of SFPS I significantly increased cell proliferation (123.42% ± 6.22%). The concentration of SFPS III which reduced 50% of HEL cell viability (IC50) was the lowest (66.38 ± 5.17 µg·mL⁻¹), whereas the IC50 of SFPS I was 138.81% ± 7.33% (Fig. 1D), indicating that the higher sulfate ester content could provide the enhanced antitumor effect. We used cisplatin as a positive drug control, and the cisplatin reduced the viability of HEL cells after 24-hour treatment in a dose-dependent manner while it also induced much toxic effect on normal MRC-5 cells. As a result, the fact that SFPS III was able to reduce the viability of HEL cancer cells selectively, but not normal MRC-5 cells, strongly nominated SFPS III as a chemotherapeutic candidate.

**Effect of SFPS on the apoptotic morphological changes of HEL and MRC-5 cells**

Hoechst 33258 is a cell-permeable benzimidazole dye that stains DNA by binding to the minor groove of adenine and thymine-rich sequences. It emits blue fluorescence when bound to double-stranded DNA, which is useful as a nuclei marker for cell cycle studies and to distinguish nuclear morphology in apoptotic cells. Thus, we used Hoechst 33258 to stain the nucleus of HEL and MRC-5 cells to analyze the inhibitory effects of SFPS I, II, and III on cell apoptosis. As shown in Fig. 2, the nucleus of a normal cell shows diffuse homogeneous blue fluorescence, while apoptotic cells present with strong blue fluorescence. The control treatment did not appreciably induce apoptotic cells; whereas, significant apoptotic morphological features were observed in the nucleus of cells treated with SFPS I, II, and III. Typical morphological changes were observed, as displayed in the images, with nuclear fragmentation, chromosomal condensation, and cell shrinkage. Moreover, as the concentration of SFPS III increased, not only did the number of cells in the field of view decreased, but the number of cells with strong blue fluorescence increased. From Fig. 2, we found that MRC-5 cells treated with different concentrations of SFPS I, II, and III for 24 h did not exhibit the typical features of apoptosis upon Hoechst staining, such as chromatin condensation and morphology changes, as well as cell shrinkage and membrane blebbing.

**Effect of SFPS on the apoptosis of HEL cells**

In order to quantify the apoptosis in HEL cells, an annexin V-FITC/PI staining experiment was performed to examine
the occurrence of phosphatidylserine externalization on the cell surface. Such staining with annexin V is typically used in conjunction with a vital dye, such as PI, to identify the early stages of apoptotic cells (annexin V+, PI−) and the later apoptosis stages (annexin V+, PI+). Flow cytometry analysis showed that SFPS (I, II, and III) could induce HEL cell apoptosis in vitro, but did not influence MRC-5 cell apoptosis.

Fig. 3 shows that, at a low concentration of SFPS I, the proportion of early apoptotic HEL cells increased, but not significantly. Moreover, the results indicate that SFPS II markedly induced apoptosis, as the population of apoptotic cells was 63.50% higher than the untreated samples. Fig. 3 also showed that the proportion of apoptotic cells significantly increased after treatment with SFPS III. When the concentration of
SFPS III was 0, 30, 60, and 90 μg·mL⁻¹, the percentage of apoptotic HEL cells was 6.97%, 9.85%, 50.76%, and 42.01%, respectively. Furthermore, cells treated with 90 μg·mL⁻¹ SFPS III presented apparent later apoptosis (33.50%) compared to cells in early apoptosis (8.51%). These results demonstrate that SFPS I, II and III could induce HEL cell apoptosis in a dose-dependent manner.

**Effect of SFPS on cell cycle distribution of HEL cells**

To further investigate the mechanism underlying the SFPS inhibitory activity on HEL cancer cells, we investigated its effects on cell cycle progression. Hence, we monitored the cell cycle phase distribution of HEL and MRC-5 cells after treatment with SFPS I, II, and III for 24 h. Cell cycle profiles were analyzed by measuring the DNA content using flow cytometry. Fig. 4 shows the cell cycle arrest of HEL cells at different SFPS treatment concentrations. As shown in Fig. 4, treating cells with 60 and 90 μg·mL⁻¹ of SFPS I caused substantial inhibition of cell cycle progression. The cell population in S phase decreased from 49.21% to 32.89%. Moreover, SFPS II induced cell cycle arrest in the G₀/G₁ phase, with values of 39.47%, 57.08%, and 65.87% after 30, 60, and 90 μg·mL⁻¹ of treatment, respectively. Cells treated with SFPS III showed a decrease in the cell population at S phase compared to untreated cells, while the number of cells at G₀/G₁ phase increased by 67.21% compared to untreated cells (38.71%). These results suggest that SFPS could disturb cell cycle progression in HEL cells, leading to the accumulation of cells in the G₀/G₁ phase. SFPS did not influence MRC-5 cell cycle progression.

**Induction of apoptotic and cell cycle gene expression in HEL cells by SFPS**

Critical exposure of different concentrations of SFPS (I, II, and III) to HEL and MRC-5 cells molded different expression profiles for apoptotic (Caspase-3, Bax, Bcl-xL, Bcl-2, and Bad) and cell cycle gene (p53) mRNA levels. After treatment with different concentrations of SFPS I on HEL and MRC-5 cells for 24 h, Caspase-3, Bax, Bcl-xL, Bcl-2, Bad, and p53 genes showed little difference in expression compared to the control treatment (Figs. 5A and 5B). As shown in Fig. 5C, Bcl-2 in HEL cells treated with SFPS II did not have differential expression compared to the control treatment. Regarding Bcl-xL, p53, Bax, Bad, and Caspase-3 genes, we observed a significant decrease in gene expression of p53 and Bcl-xL and a significant increase in gene expression of Bad

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**Fig. 3 Sargassum fusiforme polysaccharide (SFPS) I, II, and III-induced apoptosis in human erythroleukemia (HEL) cells.** Apoptosis was assessed by flow cytometry after HEL cell treatment with 0, 30, 60, and 90 μg·mL⁻¹ of SFPS I, II, and III for 24 h. Q1 indicates dead cells (FITC annexin V-negative and PI-positive); Q2 indicates cells in end-stage apoptosis (FITC annexin V- and PI-positive); Q3 indicates cells undergoing apoptosis (FITC annexin V-positive and PI-negative); Q4 indicates viable cells not undergoing apoptosis (FITC annexin V- and PI-negative).
and Caspase-3. In MRC-5 cells treated with SFPS II (Fig. 5D), Bcl-2 and Bcl-xL showed no significant changes in expression, and p53, Bax, and Caspase-3 gene expression showed little change, while pro-apoptotic gene, Bad, decreased significantly. It was observed that in HEL cells treated with SFPS III (Fig. 5E), Bcl-xL and p53 expression showed a significant reduction, while Bax, Bad, and Caspase-3 gene expression was significantly increased. Fig. 5F illustrates that MRC-5 cells were treated with SFPS III, Bcl-xL, Bax, and Bad showed little difference in expression compared to the control treatment, and no significant difference was seen in Bcl-2, p53, and Caspase-3 expression.

**SFPS regulates the expression of cancer apoptosis-associated proteins**

To further delineate the mechanism by which SFPS (I, II, and III) induced apoptosis in HEL and MRC-5 cells, we examined the expression of apoptosis-associated proteins, such as Bcl-2, Bax, Bad, Caspase-3, Bcl-xL, and p53 by Western blot assay. Incubation of HEL cells in the presence of SFPS I for 24 h increased the expression of Bax and Caspase-3 but did not significantly change the expression of other proteins (Fig. 6A). As shown in Fig. 6A, the results revealed that when HEL cells were exposed to different concentrations of SFPS II, the protein expression levels of Bcl-2, Bcl-xL, and p53 decreased; whereas, the expression levels of Bax, Bad, and Caspase-3 increased. However, the expression level of p53 increased, and the expression of Bad decreased; whereas, the expression of other proteins did not change significantly in MRC-5 cells (Fig. 6B). As shown in Fig. 6A, after treatment with SFPS III, the expression of pro-apoptotic proteins, Bax and Bad, was increased, while the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, appeared to be markedly decreased in a dose-dependent manner in HEL cells. These differences were statistically significant compared to the control group. Moreover, a significant increase of Caspase-3 was detected in HEL cells following SFPS III treatment, while the expression of most proteins was not affected in MRC-5 cells as shown in Fig. 6B. The statistical data of western blot should be added in Supplementary Fig. D. These data indicate that SFPS-induced apoptosis in HEL cells is partly mediated through the mitochondrial pathway.

**Discussion**

Natural products have long been widely used as a significant source of therapeutically effective drugs, and their importance in the prevention and treatment of tumors is becoming increasingly evident [49]. Current chemotherapeutic drugs available for the therapeutic management of cancer are associated with significant adverse effects, such as alopecia, anemia, immunodeficiency, fatigue, peripheral neuropathy, fertility issues, and neurological problems, just to name a few of them. Polysaccharides have emerged as potential chemical entities exhibiting good anticancer activity across a variety of cancer cell lines. Moreover, as polysaccharides possess selective activity against tumor cells with minimal toxic side effects, they can be developed as alternatives to existing cancer...
Chemotherapeutic agents. Polysaccharides isolated from plants, fungi, microorganisms, and marine sources have been reported to act on malignant cells mainly via induction of apoptosis [49]. Previous studies have reported the potential of polysaccharides from S. fusiforme to induce apoptosis in leukemia, gastric, bladder, and breast cancer cells [25]. However, the underlying cellular and molecular mechanisms for the antitumor properties of purified polysaccharides from S. fusiforme have not been elucidated. In the present study, we demonstrated that different purified polysaccharides (SFPS I, II, and III) were cytotoxic towards HEL cells via apoptosis induction, while being non-toxic for non-carcinogenic MRC-5 cells.

Moreover, there was a considerable difference in the sensitivity of HEL cells to the different purified components from SFPS, of which SFPS III possessed the strongest antitumor activity. However, the concentrations of the samples have been normalized, suggesting that the different effects on cell viability were associated with the polysaccharide purity and components, rather than the dose. SFPS III contained the highest amount of sulfate content. A recent study has also reported that a polysaccharide fraction from Sargassum thunbergii contained plenty of sulfate content and exhibited good antitumor activity [50]. Based on our previous studies [32], sulfate content mainly exists as a bioactive component of SFPS. Thus, we speculated that the sulfate content might be involved in the apoptosis-associated cytotoxicity of SFPS against HEL cells.

Cell apoptosis, a programmed cell death process, is widely contended to be one of the major parameters that incur growth loss to cancer cells. To further determine if this growth inhibition by purified polysaccharide was associated
with the apoptosis induction, we used Hoechst 33258 staining to examine morphological changes in HEL and MRC-5 cells, and then quantified the percentage of apoptotic cells using flow cytometry assay with annexin V-FITC/PI dual-labeling. After 24-hour treatment in the presence of SFPS I, II, and III, distinct apoptotic morphological abnormalities, including cell shrinkage, chromatin condensation, and formation of apoptotic bodies, were observed with Hoechst 33258 staining by a fluorescence microscope, compared to the control. Programmed cell death or apoptosis is characterized by certain morphological cell changes, such as loss of plasma membrane integrity, in addition to internucleosomal DNA cleavage. One of the earliest apparent changes in cells undergoing apoptosis is the translocation of the cell membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This change in the cell membrane is now recognized as an early, essential feature of apoptosis [39]. This translocation exposes phosphatidylserine to the external cellular environment, and this feature can be measured by exposing cells to fluorochrome-conjugated phospholipid-binding proteins, such as phycoerythrin (PE)-labelled annexin V (PE-annexin V). Annexin V-FITC is used to detect early-stage apoptosis. Viable cells with intact membranes exclude PI; whereas, the membranes of dead and damaged cells undergoing apoptosis are permeable to PI. Thus, PI can permeate the cell membrane to stain cells that are in the middle and late stages of apoptosis. Therefore, when annexin V-FITC is used in combination with PI, cells in different stages of apoptosis can be distinguished. Flow cytometry analysis showed that SFPS II and III (30, 60, and 90 μg·mL\(^{-1}\)) induced apoptosis in HEL cells, and the number of apoptotic cells increased as the dose increased. These results demonstrated the occurrence of apoptosis in SFPS-treated HEL cells and were in accordance with the application of other polysaccharides on cancer cells. For example, it has been reported that the apoptotic effect of polysaccharides in cancer cells was related to the generation of reactive oxygen species, which in turn could lead to DNA damage and result in cell death [12]. The plant extracts caused the inability of the cells to replicate damaged DNA, leading to incomplete DNA replication; due to this replication, the cells were arrested at the \(G_0/G_1\) or \(G_2/M\) checkpoint and prevented entry into mitosis. Additionally, Bao et al. [51] demonstrated that RAW 264.7 cells were arrested at the \(G_2/M\) phase by polysaccharide,
which was associated with induction of p53 and controlled by a decrease in the expression of downstream cell cycle regulatory proteins. Moreover, Xu et al. [35], Siddiqui et al. [40], and Abdel-Megeed et al. [44] also mentioned that this type of polysaccharide causes cell cycle arrest at the G2/M phase. As chemopreventive agents, polysaccharides are active at different stages of cancer development, interfering with the overall process through various mechanisms, such as up/downregulation of apoptotic genes and proteins. This study has been performed to examine the mechanism of apoptosis induction by SFPS II and III in HEL cells. We found that, following activation of Caspase-3, Bel-2 and Bel-xL expression was downregulated, and Bax and Bad expression was upregulated, leading to apoptosis. These results preliminarily proved that the Caspase pathway might be involved in SFPS II and III-induced cell apoptosis. However, the intrinsic and extrinsic pathways cannot be determined based on the data presented, and the anti-tumor effects of the different sulfate content in the SFPS III treatment also require further investigation. In summary, an agent like SFPS II or SFPS III could efficiently inhibit proliferation and induce apoptosis of cancer cells and would be as a hopeful alternative medicine to suppress cancer progression and reduce mortality.

References


[29] Chen F, Ran LW, Mi J, et al. Isolation, characterization and antitumor effect on du145 cells of a main polysaccharide in pol-