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Research Article

Effects of *Lycium barbarum* Polysaccharides on the Apoptosis of Human Hepatoma SMMC-7721 Cells

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Abstract: *Lycium barbarum* polysaccharide is a natural functional component which has a variety of biological activities. The composition and apoptosis inducing activities on human hepatoma SMMC-7721 cells of *Lycium barbarum* Polysaccharide fraction (LBP1) was investigated. The results showed that LBP1 consisted of neutral sugars, uronic acid and protein. LBP1 was composed of three kinds of monosaccharides including mannose, galactose and glucose. The molecule weight of LBP1 was 367 KDa. SMMC-7721 cells were cultured in the presence of LBP1 at various concentrations (50-400 mg/L) for 2 days, 4 days and 6 days and the percentage of cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. We found that anti-proliferative effect of LBP1 was associated with apoptosis on SMMC-7721 cells by morphological changes and cell cycle arresting at G0/G1phase. Furthermore, LBP1 could induce cell apoptosis which was closely accompanied with the enhancement of Reactive Oxygen Species (ROS) level, dissipation of Mitochondria Membrane Potential (MMP), increase expression of Bax mRNA and decrease expression of Bcl-2 mRNA. These results suggested that SMMC-7721 cell apoptosis induced by LBP1 mainly was mediated by mitochondrial pathways. However, further research on the molecular mechanisms of LBP1 effecting on the cells' mitochondria is necessary.

Keywords: Apoptosis, Bcl-2/Bax, composition, hepatoma SMMC-7721 cells, *Lycium barbarum* Polysaccharide (LBP), MMP, ROS

INTRODUCTION

Hepatocellular carcinoma rates are continuously increasing in Asia due to persistent high incidences of hepatic diseases and hepatocellular carcinoma even has become one of the most dangerous malignancies in China. Thus, a number of effective prevention measures and novel therapeutic targets need to be sought for the successful treatment of hepatocellular carcinoma (Hu *et al*., 2009).

Fruit from *Lycium barbarum* L. is a kind of traditional Chinese herbal medicine, which is popular as a functional dietary supplement in Western countries now. A broad series of components including polysaccharides, flavonoids and carotenoids from *Lycium barbarm* L. fruit have been shown to have immunomodulation, hypoglycemia, anti-hypertension, anti-aging, lipotropic, antioxidant, anti-apoptotic, anticancer, anti-fatigue and so on (Luo *et al*., 2004; Li *et al*., 2007; Chen *et al*., 2008). However, the polysaccharide constituents still remained uncertain because of its complex monosaccharide composition with a wide range Molecular Weight (MW) (Zou *et al*., 2010).

Lycium barbarm Polysaccharide (LBP) isolated from aqueous extracts of *Lycium barbarm* L. has been identified as one of the major active component that is responsible for the biological activities of the plant. Polysaccharides isolated from *Lycium barbarum* fruits played a role in anti-aging function in fruit flies and mice (Wang *et al*., 2002). LBP treatment inhibited growth of MGC-803 and SGC-7901 cells, with cell-cycle arrest at the G0/G1 and S phase, respectively (Miao *et al*., 2010). Mao *et al*. (2011) found that CDK2 in colon cancer cells contributes to the anticancer activity of LBP. Another in vivo research has shown that LBP has antitumor and immunomodulatory activities in S180-bearing mice (Gan *et al*., 2004). Recent experimental evidence suggested that the increase of intracellular calcium in apoptotic system may participate in the antiproliferative activity of LBP in QGY7703 cells (Zhang *et al*., 2005). It was also reported that spherical molecular shape of LBP was benefit to LBP's apoptosis inducing activity (Zhang *et al*., 2013).

Apoptosis is a selective process of physiological cell deletion that regulates the balance between cellular replication and death, which is characterized by a series

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of morphological and biochemical changes including membrane blebbing, cell shrinkage, loss of mitochondrial membrane potential ($\Delta\Psi$), chromatin condensation and DNA fragmentation (Wu *et al*., 2014). Two major signaling pathways leading to cell death by apoptosis have been identified and are the extrinsic pathway (or the death receptor pathway) and the intrinsic pathway (or mitochondrial pathway) (Sun *et al*., 2011). Recently, Apoptosis has become a focus of interest in oncology and may also shed light on cancer therapy (Marx, 1993). Searching for lower toxicity from nature and anti-cancer drugs with higher bioactivities has been very intensive in recent years. So far, there are few information available about the anticancer effects of LBP on human hepatocellular carcinoma cells and its underlying mechanism of action is still largely unknown.

In this context, we therefore purified one water-soluble polysaccharide from *Lycium barbarm* L. (LBP1) and investigated the effects of LBP1 on proliferation and apoptosis in SMMC-7721 cells. The mechanism involved in these processes was also studied.

MATERIALS AND METHODS

Materials: Fruits of *Lycium barbarum* L. were purchased from Shihezi city, Xinjang province of China. Sephadex G-50 and DEAE-52 were purchased from Pharmacia Biotech. Trifluoroacetic Acid (TFA) was purchased from E. Merck. The human hepatic cancer cell line SMMC-7721 was obtained from Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Culture medium RPMI-1640 was from Hyclone Co. (Logan, UT, USA). Fetal Bovine Serum (FBS) was purchased from Biyhjm Biosciences Inc. The chemicals 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide were purchased from Sigma Chemical. (St. Louis, MO, USA). Rhodamine123 and DCFH-DA were obtained from Sigma Chemical Co. Real-time PCR Master Mix (SYBR Green) was purchased from Toyobo Co., Ltd. All other chemical reagents used in this experiment were of analytical grade and purchased locally.

Extraction and purification of LBP1: The dried fruits of *Lycium barbarum* L. were smashed to powders. *Lycium barbarum* L. powder (800 g) were mixed with 600 mL of 70% ethanol (v/v) for 0.5 h at 70°C to remove most of the polyphenols, pigments and monosaccharide and the treatment was repeated fourth. After removing the supernatant, the residues were dried and then extracted with 600 mL of deionized water at 100°C for 90 min four times. The filtrate was concentrated in a 50°C water bath under a vacuum to 200 mL. The concentrated solution was added with 320 mL of 95% ethanol for precipitation at 4°C for 12 h and then centrifuged 3400 r/min for 15 min. The supernatant was precipitated by adding 600 mL of 95% ethanol and centrifuged (3400 r/min, 15 min). The obtained supernatant was precipitated once again. The crude polysaccharide was deproteined according to the Sevage method (Staub, 1965). Briefly solution of the crude polysaccharide was treated with sevage reagent (1-butanol:chloroform = 1:5) fully oscillated and centrifuged. The process was repeated until the supernatant was no free protein. The crude polysaccharide was obtained by freezing dry of the supernatant. The crude polysaccharides (150 mg) were re-dissolved in distilled water and loaded onto DEAE–cellulose column (2.5×50 cm). The sample was eluted with distilled water and then with a gradient of NaCl (0-0.2 M) at a flow rate of 0.5 mL/min. The main fractions were collected, dialyzed, lyophilized and further purified with gelfiltration chromatography on a column of Sephadex G-50, eluted with distilled water at a flow rate of 1mL/min and 4.0 mL eluant per tube. Each tube was assayed by phenol-sulfuric acid method. Meanwhile, an aliquot of the fractions was tested for total carbohydrate by Phenol-H_2SO_4 method. Only one peak was found in the elution pattern. The main fraction containing polysaccharides was pooled, collected and lyophilized to obtain the purified polysaccharide (named LBP1).

Uronic acid, protein and total sugar content: Uronic acid contents were determined by measuring the absorbance at 523 nm using the m-hydroxybiphenyl colourimetric procedure and with d-glucuronic acid as the standard (Filisetti-Cozzi and Carpita, 1991). The proteins in the polysaccharides were quantified according to the Bradford method using BSA as the standard (Bradford, 1976). The content of total sugar was determined by the method of phenol-sulfuric acid (Dubois *et al*., 1956).

Homogeneity and molecular weight determination: The homogeneity and molecular weight of LBP1 were evaluated by gel permeation chromatography (GPC) on an OHpak SB-803 HQ column (8×300 mm) with standard dextrans (50,000, 80,000, 150,000, 410,000, 670,000 and blue dextran) and glucose. The mobile phase was ultrapure water and the flow rate was 0.8 mL/min at 25°C, with 1.0 mPa. 20 μL aliquot was injected for each run. The elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of LBP1 was plotted in the same graph and the molecular weight was measured. All samples were filtrated through a 0.22 μm pore diameter membrane (Millipore, USA) prior to analysis.

Cell culture: Human hepatoma cells (SMMC-7721) were obtained from Shanghai Cell Biology Institute, China. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The culture medium consisted of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin.

Cell viability assay: Viability of cells was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay (Mosmann, 1983). Briefty, SMMC-7721 cells (5×10^5/well) was seeded into each well of a 96-well culture plate. After incubation for 24 h, various concentrations of LBP1 (50-400 mg/L) were added into each well and 12 replicate wells were used at each point in the experiments. After 2, 4 or 6 days, MTT solution (5 mg/mL in PBS) stored at 4°C in a dark bottle was added to each well and plates were incubated for 4 h at 37°C and the formazan product was solubilized with dimethylsulfoxide (DMSO). The absorbance was detected by the microplate reader (Multiskan MK3, Thermo, USA) at 590 nm and measured values were expressed as mean ± SD.

Morphology examination: SMMC-7721 cells were treated by LBP1 at the indicated concentrations (100 mg/L) for 4 days and 6 days, respectively. Then, the nuclei were labeled by Acridine Orange (AO) for 15 min at room temperature. After washing by PBS, they were subjected to laser scattering confocal microscope (C1 plus, Nikon, Japan).

Cell-cycle analysis: To investigate the effect of LBP1 on the cell cycle distribution and the apoptotic rate, SMMC-7721 cells (1×10^6 cells/well) were seeded onto 6-well plate and treated with LBP1 (100 mg/L) for 4 days and 6 days, respectively. The treated cells were harvested, washed twice with ice-cold PBS, fixed overnight at 4°C with 70% EtOH and stained with 500 μL PI solution consisted of 50 μg/mL PI and 100 μg/mL RNase A at 37°C in the dark for 30 min. The stained cells were analyzed using FCM (BD FACSCalibur, USA). 1×10^6 cells were collected for analysis by ModFit LT 3.0 software.

Measurement of mitochondrial membrane potentials (ΔΨm): To observe the changes in ΔΨm, flow cytometry was performed with mitochondrial prober Rhodamine123. SMMC-7721(1×10^5 cells/mL) cells were cultured in 100 mg/L LBP1 for 4 days and 6 days, respectively and harvested with trypsin/EDTA. After washing by cold PBS twice, they were incubated with Rhodamine-123 at a final concentration of 5 μg/mL at 37°C for 30 min in the dark, rinsed by cold PBS twice and analyzed by a flow cytometer (BD FACSCalibur, USA). The probes were excited with a laser at 488 nm and the fluorescence emission was measured through a 520 nm bandpass filter. Loss in Rh123 staining indicates an association to the disruption of mitochondrial membrane integrity.

Detection of ROS: Intracellular ROS production was measured by use of a reactive oxygen species assay kit. Briefly, SMMC-7721 cells were seeded at a density of 5×10^5 in culture plate and allowed to attach and proliferate for 24 h. These cells were exposed to 100 mg/L LBP1 for 4 days and 6 days, respectively. After exposure, cells were harvested by trypsinization and washed with PBS solution and finally resuspended in 1 ml 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) solution (a final concentration of 10 μM). The cell suspensions were incubated at 37°C for 30 min and analyzed for fluorescence intensity by flow cytometry (BD FACSCalibur, USA).

Quantitative real-time reverse transcription-PCR analysis: The mRNA expression levels for Bcl-2 and Bax were determined by quantitative real-time reverse transcription-PCR (Ma *et al*., 2013; Hou *et al*., 2008). After SMMC-7721 cells were treated with 100 mg/L LBP1 for 4 days, total cellular RNA was isolated from Trizol reagent according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA was synthesized using random primer and Prime Script reverse transcriptase. For quantitative analysis of mRNA of Bcl-2 and Bax, the primers used in the reaction and β-actin gene served as endogenous reference were as follow:β-actin, 5' GAC GTG GAC ATC CGC AAA G3' and 5' CGG ACT CGT CAT ACT CCT GCT3'; Bax, 5' GCT GGA CAT TGG ACT TCC TC3' and 5' AAA GAT GGT CAC GGT CTG C3', Bcl-2, 5' GGA GGA TTG TGG CCT TCT TTG3' and 5' TGTGCAGGTGCCGGTTCAG3'. Quantitative PCR for target gene was carried out using SYBR green qPCR kit on a fluorescent temperature cycler. cDNA templates (2 μL) were amplified in a final volume of 20 μL containing the SYBR Green PCR Master mix and primer. Melt-curve analysis was used to confirm amplicon specificity. The length of the amplified product was confirmed using 2% agarose gel electrophoresis. Relative quantification was performed using the $2^{-\Delta\Delta t}$ method (Livak and Schmittgen, 2001). Relative expression ratios were expressed as fold changes of mRNA abundance in the treatment group compared with the model control group.

Statistical analysis: All the data was expressed as the mean±Standard Deviation (S.D.). The p-value of <0.05 was adopted as statistically significant.

RESULTS AND DISCUSSION

Chemical analysis: The chemical composition of LBP1 was given in Table 1. In general, LBP1 possessed high neutral sugars (82.20%), relatively low protein (0.77%). The uronic acid content of LBP1 was 7.50%. According to the monosaccharide composition analysis, LBP1 was composed of three kinds of monosaccharide including mannose, galactose and glucose with the

Table 1: Analysis of components and molecular weight of LBP1

Sample	LBP1
Neutral sugar (%)	82.20±2.10
Protein (%)	0.770±0.11
Uronic acid (%)	7.500±1.10
M_w (kDa)	367
Sugar component (mol%)	
Man	1.00
Gal	1.79
Glu	3.08

Table 2: Inhibition activities of LBP1 on SMMC-7721 cells

LBP1	Time (d)	Concentration (mg/L)	n	Inhibition ratio (%)
	2	400	10	17.01%±4.60%^{A}
		200	10	8.52%±3.61%^{B}
		100	10	13.28%±4.84%^{B}
		50	10	5.90%±2.30%^{B}
	4	400	10	20.59%±6.30%^{a}
		200	10	13.88%±7.17%^{a}
		100	10	24.80%±5.38%^{a}
		50	10	10.30%±3.26%^{a}
	6	400	10	15.80%±5.14%^{B}
		200	10	19.42%±4.03%^{B}
		100	10	21.63%±6.76%^{A}
		50	10	6.16%±2.40%^{B}

Different letter showed significant differences. A, B: $p<0.1$. a, b: $p<0.05$

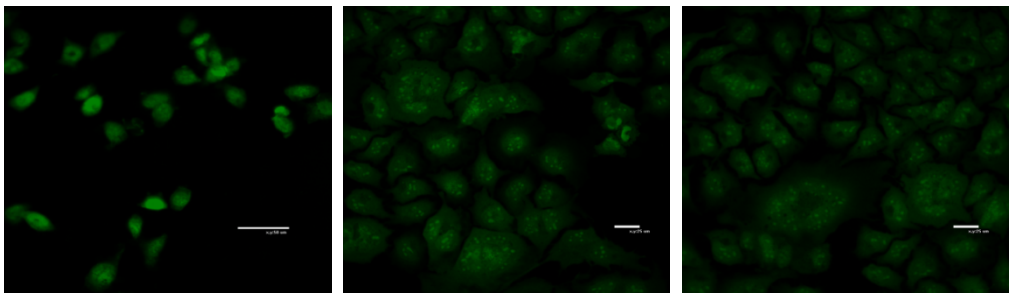
molar ratios of 1.00, 1.79 and 3.08. The above results were different with previous reports which concluded that LBP consisted of nine kinds of monosaccharides (Wu *et al*., 2012). Polysaccharide samples are a mixture of many kinds of polysaccharide fractions with different compositions. Extraction and purification methods could make the polysaccharide moleculars that constitute the polysaccharide samples different, so could change the monosaccharide composition.

The molecular weight of the polysaccharide was determined by high performance liquid chromatography. The equation of the standard curve was Log Mw = 10.38+0.785 t (Mw: the peak molecular weight, t: retention time). The molecular weight of LBP1was estimated to be 367 KDa.

Effect of LBP1 on SMMC-7721 cells viability: The MTT assay measures the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipheny l tetrazolium bromide to a colored formazan product. After incubated with LBP1 at the concentrations from 50 to 400 mg/L for 2 days, 4 days and 6 days, the inhibition effects of SMMC-7721 cells were observed and compared with the control group. The inhibition ratios of LBP1 in different concentrations against the human hepatoma SMMC-7721 cells were summarised in Table 2. The LBP1 exhibited some inhibition effect at the concentrations from 50 to 400 mg/L. There was no dose-dependency relationship between the inhibition ratios and concentrations. It was deserved to note that inhibition activities of LBP1 on SMMC-7721 cells reach to maximum (24.8%, 100 mg/L), when SMMC-7721 cells were cultured for 4 days. These results indicated that LBP1 effectively inhibited the proliferation of SMMC-7721. Zhang and Huang (2006) reported that MAP, a novel polysaccharide from the loach, inhibited SMMC-7721 cell growth in a time-dependent and concentration-dependent manner. However, it was slightly different from our results, which may be due to the different structural characteristics and action modes of polysaccharide. Finally, we choose 100 mg/L for the following experiments.

Morphology changes of SMMC-7721 cells treated with LBP1: The morphological changes of apoptotic cell nucleus were analyzed by AO staining. At the concentration of 100 mg/L LBP1 for 4 days and 6 days, SMMC-7721 cells exhibited the characteristic morphological changes of apoptosis including membrane blebbing, chromatin condensation and the formation of apoptotic bodies, which was suggestive of apoptosis (Fig. 1).

Cell cycle phase analysis by flow cytometry: To prove whether LBP1-induced apoptosis was related to arrest cell cycle progression, FCM was used to quantify the induction of apoptosis and the cell cycle distribution affected by LBP1. The changes of the cell cycle distribution were shown in Table 3. When incubation with 100 mg/L LBP1 for 4 days, a significant increase of the cell population in the G0/G1 phase was observed



(a) (b) (c)

Fig. 1: Morphological observation of SMMC-7721 cells treated by LBP1 by laser scanning confocal microscope (400×); (a): Control group; (b): 100mg/L LBP1 for 4 days; (c): 100mg/L LBP1 for 6 days

Table 3: Effect of LBP1 on cell cycle of SMMC-7721 cells

	G0/G1 (%)	S (%)	G2/M (%)
Control group	42.89±0.81	36.09±1.44	21.02±0.22
LBP1 (4d)	59.79±1.78**	28.00±1.78*	12.21±0.02*
LBP1 (6d)	51.42±0.59*	34.35±1.21	14.22±0.95*

*: $p<0.05$; **: $p<0.01$

from 42.89 to 59.79%, G2/M phases was observed from 21.02 to 12.21%. When incubated with 100 mg/L LBP1 for 6 days, cells appeared to be accumulated at G0/G1 phase (42.89% for control; 51.42% for 100 mg/L LBP1), with a concomitant decrease in the percentage of cells in the S and G2/M phases compared with control group (Table 3).

The results suggested that the inhibitory effect of LBP1 against SMMC-7721 cells proliferation correlated with G0/G1 phase cell cycle arrest.

Effect of LBP1 on mitochondrial membrane potential (ΔΨm) and ROS change: Recent investigations have shown that an alteration in mitochondrial ΔΨm was implicated in the induction of apoptosis and the disruption of the mitochondrial membrane potential was a critical step occurring in cells undergoing apoptosis. Mitochondria are the major source to produce ROS. If the mitochondria are damaged, excessive ROS may be released from mitochondria, which may cause mitochondria further damage and result in the loss of the membrane potential, thus inducing the execution of apoptosis (Ricci *et al*., 2003; Simizu *et al*., 1998).

To identify if cell apoptosis induced by LBP1 was involved mitochondrial pathways, the effect of LBP1 on mitochondrial membrane potential was measured.

In our research, we investigated the change of ΔΨm of SMMC-7721 treated by LBP1 using rhodamine-123 retention. Depolarization of ΔΨm drives the down-regulation of the accumulation of rhodamine-123 in mitochondria. Thus, the amount of rhodamine-123 uptake of mitochondria in cells is proportional to mitochondrial membrane potential.

Compared with the corresponding control, LBP1 caused obvious decrease of mitochondrial membrane potential in SMMC-7721 cells. When the cells were cultured with LBP1 at the concentration of 100 mg/L for 4 days, the percentages of cells in the M2 gate (high mitochondrial membrane potential) increased from 29.76 to 40.17%, which were significant lower compared to the control group (45.69%) (Fig. 2). The level of ΔΨm in cells treated with LBP1 had a little decrease for 6 days compared with control group, which was decreased by 5.52% (Fig. 2).

It could be concluded that compared with the corresponding control, LBP1 caused an obvious decrease of mitochondrial membrane potential in SMMC-7721 cells, meanwhile, ΔΨm reached to minimum after 4 days.

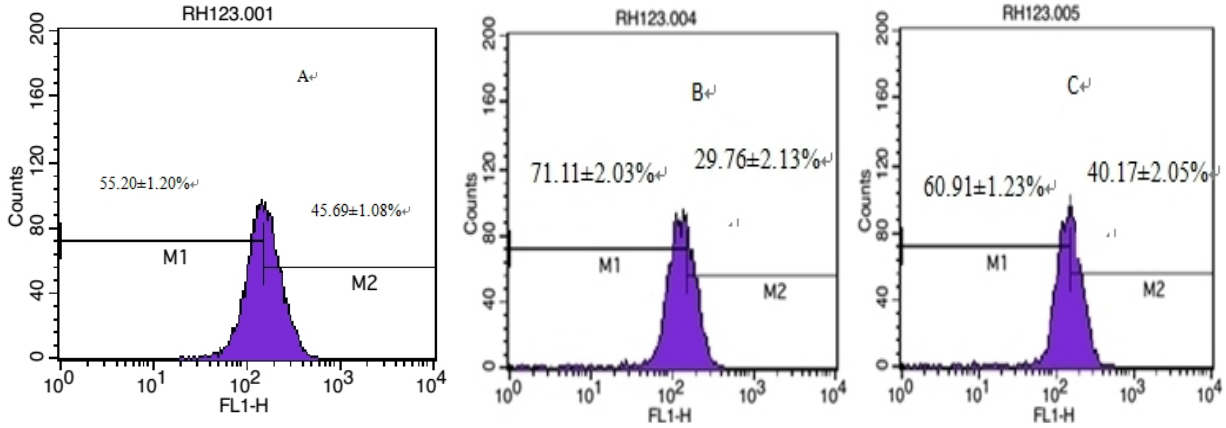
The central role of mitochondria in apoptosis was already established. Mitochondria mediate extrinsic apoptotic pathways and thus played a central role in integrating and propagating death signals inside the cell (Ly *et al*., 2003; Loeffler and Kroemer, 2000). Rhodamine-123 uptake into mitochondria is driven by mitochondrial membrane potential (ΔΨm) that allows the determination of cell population with active integrated mitochondrial functions. ΔΨm loss can induce depolarization of mitochondrial membranes and the opening of permeability transition pores in mitochondria and several apoptogenic factors are released from mitochondria to cytosol by apoptosis-inducing stimuli, which is considered as an initial and irreversible step toward apoptosis (Loo *et al*., 2002).

Flow cytometry was used to investigate the loss of mitochondrial membrane potential which had been shown to play an essential role in mediating apoptosis (Desagher and Martinou, 2000; Kroemer *et al*., 1998). Our research showed that the decrease in ΔΨm was with concentration (100 mg/L) of LBP1, which indicated that the mitochondrial apoptotic death-signal pathway played a critical role in LBP1-induced apoptosis in SMMC-7721 cells.

ΔΨm loss is an early event in apoptosis induction and this reduction in ΔΨm is often accompanied by the production of Reactive Oxygen Species (ROS) (Kroemer *et al*., 1998). To further address the possibility that LBP1-induced apoptosis could be related to contributions from the mitochondrial pathway, we detected the production of ROS induced by LBP1. Induction of ROS was detected by flow cytometry using 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Thus, the intensity of fluorescence in cells treated with DCFH-DA is proportional to the amount of ROS (Thannickal and Fanburg, 2000). As shown in Fig. 3, treated with LBP1 for 4 days, the level of ROS had a rapid and significant increase from 40.52% (control group) to 66.19%. These data demonstrated that LBP1 significantly increased ROS production in SMMC-7721 cells.

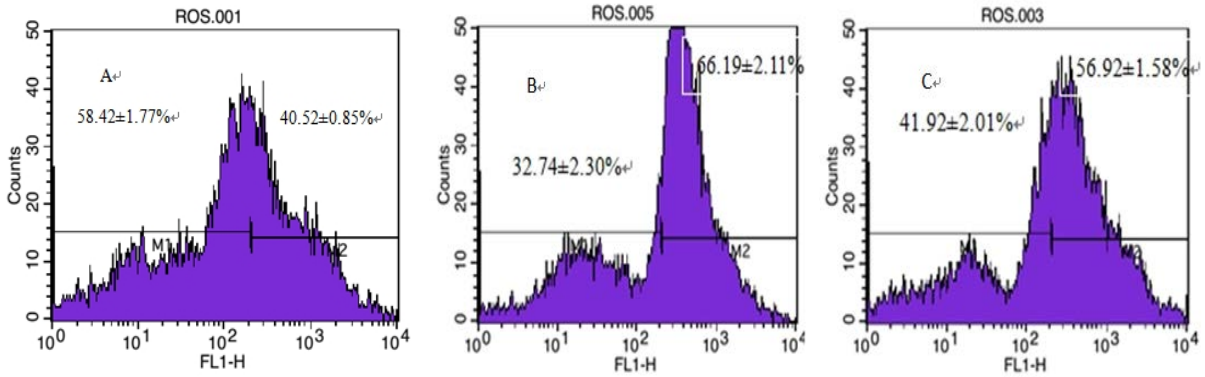
The level of ROS in cells treated with LBP1 had a little decrease from 4 to 6 days, which were decreased by 9.27%, which was consistent with the alteration of ΔΨm in cells. It was observed that the ROS production was high after the 4-day treatment with LBP1, which was coincident with the high apoptosis percentage in our research. We found that LBP1 increased the level of ROS in cancer cells, which was coincident with the data published recently (Ortiz-Sánchez *et al*., 2009).

ROS production plays an important role in apoptosis and several groups have shown that molecules that stimulate the formation of ROS can lead to apoptosis (Heussler *et al*., 1999; Kelso *et al*., 2001). A consequent loss of mitochondrial membrane potential (ΔΨm) and production of Reactive Oxygen Species (ROS) were reported as typical phenomena in the process of apoptosis related to mitochondria (Vaux and Korsmeyer, 1999).



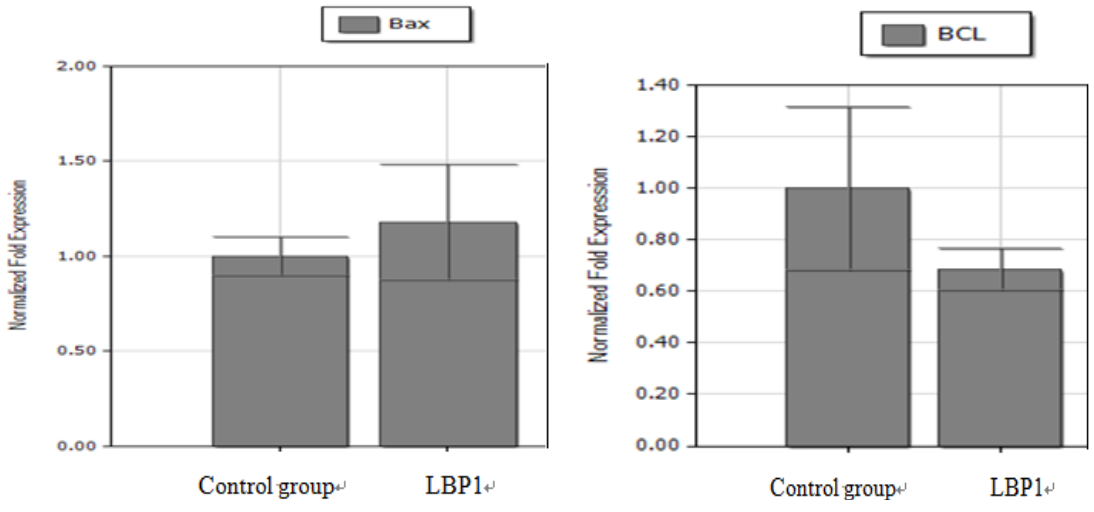
(a) (b) (c)

Fig. 2: Mitochondrial membrane potential changes of cells treated by LBP1; (a): Control group; s (b): 100 mg/L LBP1 for 4 days; (c): 100 mg/L LBP1 for 6 days



(a) (b) (c)

Fig. 3: ROS changes of cells treated by LBP1; (a): Control group; (b): 100 mg/L LBP1 for 4 days; (c): 1000020mg/L LBP1 for 6 days



(a) (b)

Fig. 4: Relative mRNA levels of Bax and Bcl-2 in LBP1-treated SMMC-7721 cells

Mitochondrion plays an important role in the regulation of cell death in mammals (Lam and del Pozo, 2000) and also is a main source of ROS when it is damaged. A variety of apoptotic stimuli can alter mitochondria permeability, inducing the release of proapoptotic proteins normally localized in the mitochondrial inter membrane space, which result in an increased production of ROS, observed upon induction of apoptosis (Martinou and Green, 2001; Tsujimoto, 2000; Zamzami and Kroemer, 2001, Cai and Jones, 1998; Cai *et al*., 2000). The effect of LBP1 on SMMC-7721 cells demonstrated that LBP1 boosted generation of ROS, which implied that LBP1 is an apoptotic stimulus.

Reduction and collapse of Mitochondrial Membrane Potential (MMP) that results in Mitochondrial Permeability Transition (MPT) is among the very first intracellular events preceding the execution phase of apoptosis via the mitochondria-mediated death pathway (Budihardjo *et al*., 1999; Takahashi and Loo, 2004). We detected the influence of LBP1 on MMP and ROS. The reduction of the MMP was accompanied with the increase of ROS which suggested that apoptosis induced by LBP1 was related to mitochondria.

Effects of LBP1 on Bax and Bcl-2 mRNA expressions in SMMC-7721cells: In order to explore the possible mechanisms of LBP1-induced apoptosis, the mRNA expression levels of apoptosis-related genes such as Bax and Bcl-2 in SMMC-7721 cells were examined using real-time fluorescence quantitative PCR. As shown in Fig. 4, after treatment for 4 days with 100 mg/L of LBP1, relative mRNA expression levels of Bax significantly increased by 0.22-fold, compared with that of the control. In contrast, relative mRNA expression levels of Bcl-2 gene markedly decreased by 0.32-fold for the treatment with 100 mg/L of LBP1. The Bcl-2 family of proteins plays a critical role in the mitochondrial pathway of apoptosis (Reed, 2001; Wang and Reed, 1998). The antiapoptotic Bcl-2 family proteins like Bcl-2 and Bcl-xL inhibit the release of certain pro-apoptotic factors from mitochondria. In contrast, pro-apoptotic Bcl-2 family molecules like Bax and Bak induce the release of mitochondria apoptogenic molecules into the cytosol (Yang *et al*., 2009). Genetic studies showed that the pro-apoptotic proteins Bax and Bak are absolutely required for apoptosis induction by diverse intrinsic death stimuli (Wei *et al*., 2001). It has been shown that many cells undergo a reduction of mitochondrial membrane potential before they exhibit signs of nuclear apoptosis (Hockenbery *et al*., 2002).

All the results suggested that LBP1 might induce apoptosis of SMMC-7721 cells through up-regulating of Bax mRNA levels and down-regulating of Bcl-2 mRNA levels and then possibly affect levels of corresponding proteins.

Furthermore, based on the good performance of LBP1 on MTT assays and the results in SMMC-7721 cell cycle analysis and quantitative real-time reverse transcription-PCR analysis, the most likely mechanisms of action are the arrest of the cell cycle and the induction of apoptosis, with the latter involving excessive production of ROS and the decrement of MMP, which followed by up-regulating of Bax mRNA levels and down-regulating of Bcl-2 mRNA levels. Although the exact mechanisms by which these agents bring about arrest in the cell cycle and induce apoptosis require further investigation, this study may offer possible molecular bases for the further development of LBP1 as a clinical alternative treatment for hepatocellular carcinoma patients. Furthermore, the exact correlation between the chemical characteristics and bioactivities of polysaccharides needs further investigation.

CONCLUSION

In the present study, one water-soluble polysaccharide, LBP1, was successfully isolated and purified from the fruiting bodies of *Lycium barbarum.* L, with an average molecular weight of 367 KDa and LBP1 contained a high neutral sugar of 82.20% with 7.50% uronic acid and 0.77% protein. Monosaccharide component analysis indicated that LBP1 was composed of mannose, galactose and glucose with the molar ratios of 1.00, 1.79 and 3.08. MTT assay showed that LBP1 treatment had an obvious inhibitory effect on the proliferation of SMMC-7721 cells. Furthermore, LBP1-induced apoptosis is associated with the excess ROS, the loss of mitochondrial membrane potential and the regulation of apoptosis-associated gene expressions such as Bcl-2 and Bax.

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