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Research Article

Induction of Apoptosis in Human Hepatoma SMMC-7721 Cells by Polysaccharides from *Lycium barbarum* L.

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Abstract: *Lycium barbarum* L, a traditional Chinese herb rich in functional components such as polysaccharide, has been widely consumed in Asian countries. The purpose of this study was to evaluate the inhibitory effect of *Lycium barbarum* Polysaccharide (LBP2) on human hepatoma SMMC-7721 cells and investigate the possible mechanism of apoptosis. An acidic polysaccharide LBP2 isolated from the fruit of *Lycium barbarum* L. was purified by ion-exchanged column. Chemical analysis indicated LBP2 was composed of three kinds of monosaccharides including mannose, galactose and glucose with the molar ratios of 1.32, 1.79 and 7.38 with average molecular weight of 358 kDa, and consisted of neutral sugars, uronic acid and protein. SMMC-7721 cell viability was assessed by MTT assay. The effect on morphology was observed with a inverted microscopy. The cell cycle was analyzed by flow cytometry. GJIC was measured using laser scanning confocal microscopy. LBP2 significantly inhibited the growth of SMMC-7721 cells and induced cell apoptosis. Cell cycle analysis revealed that LBP2 caused cell cycle arrest at the G0/G1 phase. Moreover, induction of apoptosis was associated with an increase in GJIC. The study demonstrated that LBP2 has a potential for inducing apoptosis and promoting GJIC.

Keywords: Apoptosis, cell cycle, composition, CJIC, hepatoma SMMC-7721 cells, *Lycium barbarum*, polysaccharide

INTRODUCTION

Hepatocellular carcinoma is one of the most dangerous malignancies in China. Detection of hepatocellular carcinomas can be difficult and they are often at an advanced stage when detected (Han *et al*., 2009). The majority of the patients diagnosed with HCC have low recovery rates, and conventional and modified therapies now available are rarely effective, such as surgical resection and chemotherapy (Kern *et al*., 2002). Therefore, it is essential to seek out techniques or remedies which could reduce side effects for hepatocellular carcinoma. Numerous studies carried out suggested that bioactive natural compounds found in medicinal plants could in theory serve as alternatives to chemically designed anticancer agents (Ramirez-Mares *et al*., 2004).

The fruit of *Lycium barbarum* L. in the family Solanaceae is a well-known herb in the East, and is widely used as a popular dietary supplement in Western countries now (Li *et al*., 2007a). Several lines of evidence suggested that the polysaccharide (LBP) is the important bioactive component in this herb (Lin *et al*., 2008). For instance, Gan *et al*. (2004) reported that the crude LBP could exhibit antitumor activity in vivo. LBP extracted from *Lycium barbarum* L have been shown to inhibit the growth of human bladder carcinoma cell line BIU87 and induce BIU87 apoptosis, and has the ability to protect against damage from oxidative stress in vitro and in vivo (Mang *et al*., 2011). Apoptosis has recently become a focus of interest in oncology and may also shed light on cancer therapy (Marx, 1993). Apoptosis is a cascade process of programmed cell death regulated by endogenous genes, enzymes and intracellular signaling (Dowling *et al*., 1997). Furthermore, conditions that either inhibit or enhance apoptotic rates have been associated with many disease states (Thompson, 1995). Gap Junctional Intercellular Communication (GJIC) has been hypothesized to regulate growth control and apoptosis (Trosko and Chang, 2001). So far, there are few information available about the anticancer effects of LBP and GJIC on human hepatocellular carcinoma cells. Searching for anti-cancer drugs with higher bioactivities and lower toxicity from nature has been very intensive in recent years (Zhang and Huang, 2005).

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In this study, a water-soluble polysaccharide was isolated with a DEAE Cellulose-52 and Sephadex G-50 named LBP2. The present paper is concerned with the chemical characterisation and evaluation of the anticancer activity of LBP2. Furthermore, the apoptosis mechanism of LBP2 on SMMC-7721 cells was investigated in the present study.

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 and Lucifer yellow were purchased from Sigma Chemical. (St. Louis, MO, USA).

Extraction and purification of LBP2: 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). 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) 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 acidic fraction of polysaccharide eluting with 0.05 mol/L of NaCl 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 LBP2).

General analysis: The neutral polysaccharide was determined by the phenol–sulfuric acid method (Dubois *et al*., 1956) and uronic acids were determined by the sulfuric acid carbazole assay (Dische, 1947). Protein content was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

Molecular weight and monosaccharide composition analysis: The molecular weight of LBP2 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. 10 μL aliquot was injected for each run. The elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of LBP2 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.

LBP2 was treated with 2 mol/L Trifluoroacetic Acid (TFA) at 120°C for 6 h, after that, TFA was eliminated by vacuum rotary evaporation to dryness. The hydrolyzed polysaccharide sample with Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), Glucose (Glu), Sorbose (Sor) and Ribose (Rib) as monosaccharide standard, were dissolved in distilled water (2 mL), reduced by sodium borohydride ($NaBH_4$, 30 mg) for 1.5 h , treated with glacial acetic acid (AcOH) to decompose excessive NaBH4, and dried by rotary evaporation under reduced pressure at 60°C, then added with 2 mL 0.1% (v/v) HC1-methanol, and washed with a small amount of distilled water by oscillation (repeating 4 times). The residue was dried at 105°C and added with 0.5 mL pyridine and 0.5 mL acetic anhydride, then heated to complete the reaction at 100°C for 1 h. The mixture was subjected to GC analysis.

GC conditions: OV1701 silica capillary column (Tokyo, Japan): 30 m × 0.32 mm × 0.5 μm; initial column temperature: 150°C, increased to 240°C at the rate of 10°C/min; inlet temperature: 250°C; helium flow rate: 1 mL/min.

Cell culture: Human hepatoma cell line (SMMC 7721 cells) was purchased from Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China), maintained in RPMI 1640 culture medium plus 10% FBS, 100 units/ml penicillin and 100 μg/mL streptomycin in a 37°C incubator supplied with 5% CO_2. After confluency, the cells were trypsinized with 0.25% trypsin (AMRESCO, dissolved in PBS, pH 7.4), counted and placed down at a needed density for treatment.

Cell proliferation assay: Cell proliferation was determined by MTT assay. Cells in their exponential growth phase were seeded into flat-bottomed 96-well plates at a density of 5×10^4 cells per well and incubated for 24 h at 37°C in CO_2 incubator. LBP2 ranging from 50 to 400 mg/L was added to the wells and the plates were incubated for 2 days, 4 days and 6 days. After removing the supernatants, 30 μL of MTT was added to each well and the plates were incubated at 37°C for 4 h. Again the supernatant was carefully removed and 150 μL of Dimethyl Sulphoxide (DMSO) was added into each well to dissolve the MTT formazan at the bottom of the wells. After 10 min, the absorbance was read at 490 nm using Enzyme-Linked Immunosorbentassay (ELISA) plate reader (MultiskanMK3, Thermo, USA). The inhibition ratio of the treated cells was calculated based on the following formula: (1-the A_{490} value for treated cells/the A_{490} value of untreated cells)×100%. All experiments were carried out in triplicate.

Cell morphological assay: SMMC-7721 cells in exponential growth were plated at a density of 5×10^4/well in a 6-well chamber slide and allowed to attach and proliferate for 4 h. The cells were exposed to 100 mg/L LBP2 for 4 days at 37°C. After three washes with PBS, the cells were observed under an inverted microscope (TS-10, Nikon, Japan).

Cell cycle analyzed by flow cytometry: Based on the results in MTT assays, LBP2 exhibited better inhibitory effect on SMMC-7721 cell. Therefore, the working mechanisms of LBP2 on SMMC-7721 cell were further studied. The effect of LBP2 on SMMC-7721 cell cycle phase distribution was assessed using flow cytometry. SMMC-7721 (8×10^5 cells/mL) cells were cultured in 100 mg/L LBP2 for 4 days, then collected and washed with PBS, fixed with 70% alcohol overnight, then stained with PI (50 μg/mL) in the presence of 100mg/L RNAase A for 30 min before analysis with flow cytometry (FACSCalibur, Becton Dickinson, USA). The data were analyzed with Modift and CellQuest software.

GJIC assay: GJIC was assayed by the scrape-loading and dye transfer (SL/DT) method of El-Fouly *et al*. (1987). Briefly, cells treated with 100 mg/L LBP2 for 4 days were washed with PBS, scraped, incubated at 37°C for 2-3 min with Lucifer yellow (a fluorescent dye that penetrates gap junction), washed with PBS, and fixed in 2 mL of 4% formalin. The trajectory of LY was visualized under a laser scattering confocal microscope (C1 plus, Nikon, Japan), measured using a Fluoview analytical program. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Chemical analysis: The chemical composition of LBP2 was given in Table 1. In general, LBP2 possessed high neutral sugars (74.50%), 14.30% of uronic acid, and relatively low protein. According to the monosaccharide composition analysis, LBP2 was composed of three kinds of monosaccharides including mannose, galactose and glucose with the molar ratios of 1.32, 1.79 and 7.38. The above results were different with previous reports which concluded that LBP consisted of nine kinds of monosaccharides (Wu *et al*., 2012). In a similar study, Li *et al*. (2007b) reported the presence of rhamnose, arabinose, xylose, fucose, glucose and galactose in polysaccharide of *L. barbarum* L. with the molar ratio at 1:2.14:1.07:2.29:3.59:10.06, implying both glucose and galactose were the major

Table 1: Analysis of components and molecular weight of LBP2

Sample	LBP2
Neutral sugar (%)	74.50±0.90
Protein (%)	1.60±0.120
Uronic acid (%)	14.30±1.20
M_w (kDa)	358
Sugar component (mol %)	
Man	1.00
Gal	1.32
Glu	7.38

Table 2: Inhibition rate of SMMC-7721cell cultured with LBP2

LBP2	Time (d)	Concentration (mg/L)	n	Inhibition ratio (%)
	2	400	10	14.89%±5.80%^{a}
		200	10	10.59%±3.40%^{a}
		100	10	9.44%±5.10%^{a}
		50	10	10.08%±6.90%^{a}
	4	400	10	24.12%±6.14%^{A}
		200	10	19.59%±2.03%^{B}
		100	10	27.26%±4.76%^{A}
		50	10	17.44%±7.40%^{B}
	6	400	10	22.44%±7.40%^{A}
		200	10	15.81%±4.65%^{B}
		100	10	16.78%±4.10%^{B}
		50	10	19.56%±6.57%^{B}

AB: $p<0.01$; ab: $p<0.05$

Table 3: Effect of LBP2 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
LBP2 (4 d)	56.24±1.22**	34.02±0.94	7.89±0.10**

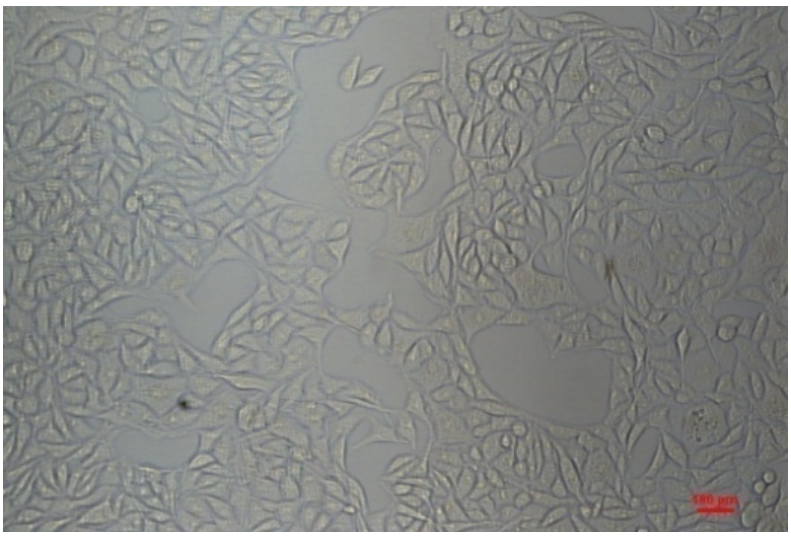
*: $p<0.05$; **: $p<0.01$

sugars. The difference in molar ratio of monosaccharides in our *L. barbarum* L. sample should be caused by variation in variety of *L. barbarum* sample, the analytical condition employed, extraction method and purification method.

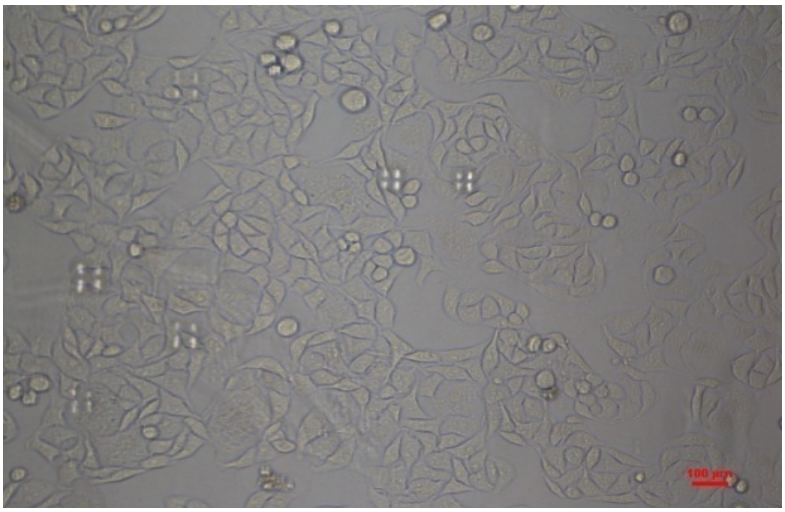
The molecular weight of the polysaccharide was determined by high performance liquid chromatography. The equation of the standard curve was Log Mw = 10.38 + 0.785 t (Mw: the peak molecular weight, t: retention time). The molecular weight of LBP2 was estimated to be 358 KDa.

LBP2 inhibits the cell proliferation: To investigate the anti-tumor activity of LBP2, SMMC-7721 cells were treated either with various concentrations of LBP2 (50, 100, 200 and 400 mg/L) for different periods (2, 4 and 6 days). Using MTT assay, the growth of SMMC-7721 cells was inhibited by LBP2 treatment. As shown in Table 2, four different concentrations of LBP2 inhibited proliferation of SMMC-7721 cells. A dramatic decrease in proliferation was observed until 4 days after LBP2 treatment (50, 100, 200 and 400 mg/L), especially at 4 days for 100 mg/L LBP2 where the percentage of inhibition reached maximum (27.26%, $p< 0.01$). Finally, we choose 100 mg/L for the following experiments.

Morphology changes of SMMC-7721cells treated with LBP_2: The results of morphological observation using an inverted microscope were shown in Fig. 1.

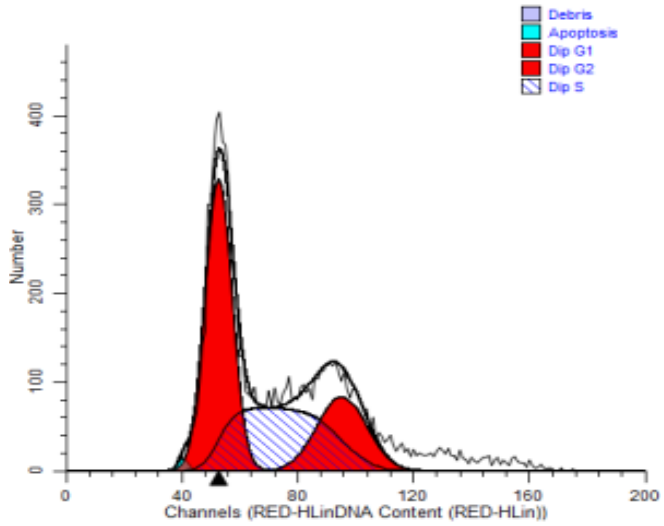


(a)

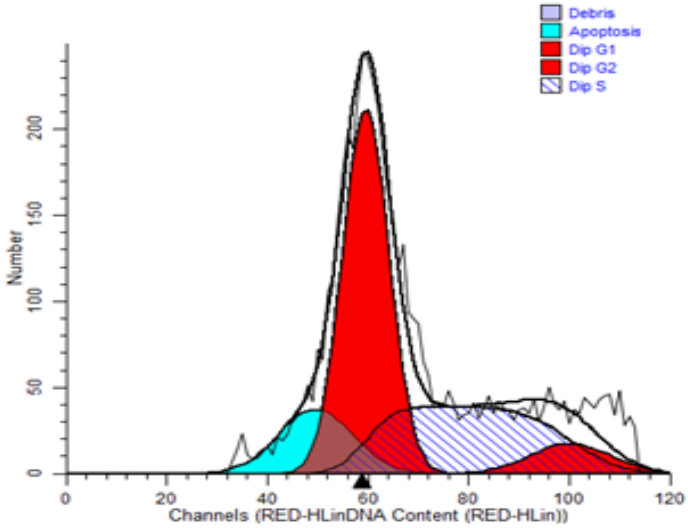


(b)

Fig. 1: Morphological observation of negative control group SMMC-7721 cells under inverted microscope（100×); (a): The Control group for 4 days; (b): 100mg/L LBP2 for 4 days



(a)



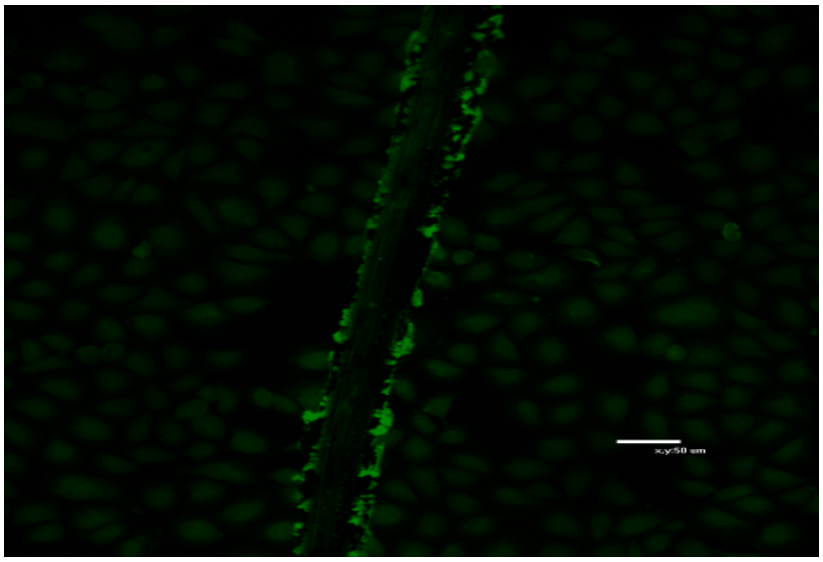
(b)

Fig. 2: Effect of LBP2 on cell cycle of SMMC-7721 determined by flow cyometer; (a): Control group; (b): 100mg/L LBP2 for 4 days

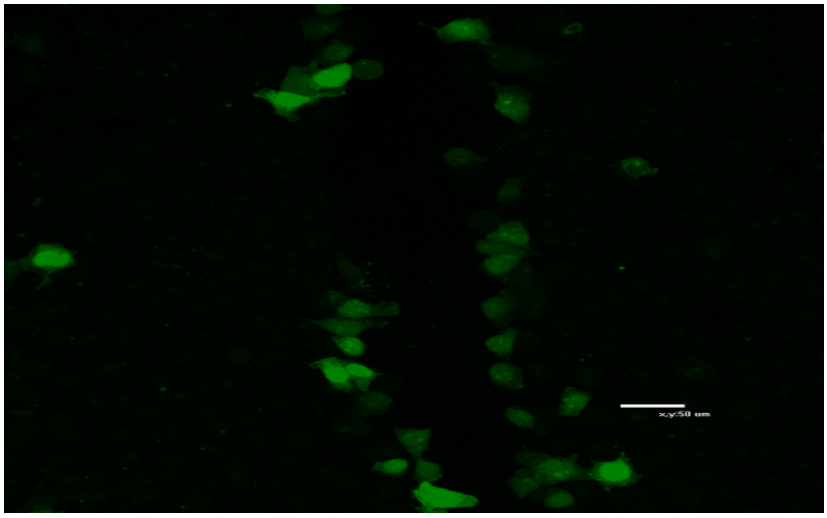
Treated with 100 mg/L LBP2, the number of cells having unaltered morphology gradually decreased. Additionally, the cell morphology began to change as round-shaped, shrank and detached from culture plate (Fig. 1a). The control group cells displayed shuttle, normal shape, regular and round-shaped nuclei (Fig. 1b).

Apoptosis had the typical characteristics of morphological alterations such as membrane bleeding, cell shrinkage, chromatin condensation, and nuclear fragmentation with formation of apoptosis bodies (Elmore, 2007). In the present study, several typical morphologies of cell apoptosis were observed in the LBP2 treated SMMC-7721 cells. The similar phenomena were also found in the honokiol treated human SMMC-7721 cancer cell (Han *et al*., 2009).

Flow cytometry analysis of cell cycle: To examine the effect of LBP2 on the cell cycle progression, cell cycle analysis was performed in SMMC-7721 human cancer cells using flow cytometry. Compared with control group, cells treated with 100 mg/L of LBP2 moderately accumulated in the G0/G1 phase of the cell cycle from 42.89 to 56.24% ($p<0.01$), with a concomitant decrease in the percentage of cells in the S and G2/M phases compared with control group (Table 3). The sub-G1 peak, an indicator of apoptotic cell death, also appeared in all of tested cells, which meant that LBP2 induced apoptosis in these cells (Fig. 2). Hence,



(a)



(b)

Fig. 3: Transfer of fluorescence of SMMC-7721 cells treated with LBP2; (a): Control group; (b): 100mg/L LBP2 for 4 days

Table 4: Effect of LBP2 on gap junctional communication of SMMC-7721 cancer cells

			GJIC level			
Group	Time (day)	Cell number	+	++	+++	Average level
Control	4	30±1.00	0	0	0	0
LBP2	4	31±2.08	24±1.53	5±0.58	2±0.58	1.29

+: LY concentrated around the scratch; ++: LY transferred into 1-2 cell layers; +++: LY transferred into 2-3 cell layers; +, ++ and +++ were quantified to 1, 2 and 3, and took the average

LBP2 exerted growth-inhibitory effects on SMMC-7721 cells via G0/G1 phase arrest.

Determination of GJIC: As shown in Fig. 3a, the control group of fluorescent material (LY) was concentrated around the scratch without intrusion into the cells, which illustrated that GJIC function of SMMC-7721 cells was weak. In contrast, incubation with 100 mg/L LBP2, lucifer yellow was rapidly disseminated throughout the GJIC-active SMMC-7721 cell population (Fig. 3b), with a significant increase of GJIC level, and the average level improved from 0 to 1.23 (Table 4).

Lucifer yellow is a hydrophilic fluorescent molecular dye. Once inside a cell, it can only be transmitted to adjacent cells through intact gap junction

intercellular channels-it cannot diffuse through cell membranes into adjacent cells (El-Fouly *et al*., 1987). Lucifer yellow is the ‘‘gold-standard’’ fluorescent molecule by which functional GJIC in a cell population is determined (Frank *et al*., 2005). Gap junctions have been linked to the apoptotic process (Wilson *et al*., 2000) as well as to the tumor promotion process (Trosko and Goodman, 1994). The transfer of small molecular weight molecules and ions are mediated through gap junctions, which are composed of two juxtaposed connexons consisting of a hexamer of proteins, connexins (Loewenstein, 1979; Bruzzone *et al*., 1996). Several anti-tumor promoting chemicals such as dexamethasone and retinoids (Grassilli *et al*., 1992; Alles and Sulik, 1989), which are known to facilitate apoptosis, can enhance GJIC. Our results showed that firstly, SMMC-7721 cells had lost functional GJIC activity; secondly, LBP2 could promote GJIC of SMMC-7721.

In the present study, 100 mg/L of LBP2 promoted GJIC of SMMC-7721 cells suggested a possible mechanistic link between apoptosis and GJIC. GJIC may facilitate signal transduction needed for the apoptotic process in cells contacting each other in the tissues.

CONCLUSION

In the current study, we isolated a water-soluble polysaccharide from *Lycium barbarum* L, which is a polysaccharide consisting of mannose, galactose and glucose with the molar ratios of 1.32, 1.79 and 7.38 with average molecular weight of 358 kDa. The neutral sugar content of LBP2 was 74.5%, with 14.30% of uronic acid and 1.6% of protein. MTT assay showed that LBP2 treatment had an obvious inhibitory effect on the proliferation of SMMC-7721 cells. In the analysis of cell cycle distribution, SMMC-7721 cells were treated with LBP2 was retarded at G0/G1 phase, with a considerable decrease in the percentage of cells at S and G2/M phase. Our results clearly demonstrate that the antitumor mechanism of LBP2 might be mediated by promotion of GJIC in SMMC-7721 cells, thus resulting in the apoptosis.

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