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Extraction, purification of *Lycium barbarum* polysaccharides and bioactivity of purified fraction

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ABSTRACT

Polysaccharides was extracted from dried *Lycium barbarum* fruits. The ion-exchange chromatography was used to isolate and purify the polysaccharides to afford a complex water-soluble polysaccharide, named LBPF5 with a molecular mass of 5.3×10^4 Da. LBPF5 consisted of arabose, mannose, xylose, glucose and rhamnose. Preliminary tests in vitro showed strong free radicals scavenging activities. The oxidative hemolysis of erythrocytes induced by AAPH was suppressed by the LBPF5 in a concentration- and time-dependent manner. In addition, LBPF5 and anthocyan could inhibit the growth of human bladder carcinoma cell line BIU87 and induce BIU87 apoptosis. Furthermore, anthocyan displayed stronger antitumor activities. From these results, it was demonstrated that LBPF5 has the ability to protect against damage from oxidative stress in vitro and in vivo.

1. Introduction

Lycium barbarum L., a traditional Chinese herb possessing vital biological activities, such as prevention of cancer and age-related macular degeneration (AMD), is widely used in Asian countries. A broad series of components including polysaccharose and glycine betaine from *L. barbarum* fruit have been identified as an important class of bioactive natural products, possessing many important properties of pharmacological relevance. Many functional components in *L. barbarum*, including flavonoids, carotenoids and polysaccharides, have been reported to be closely associated with the health-enhancing effect (Sheng et al., 2007; Yang, Zhao, Yang, & Ruan, 2008).

The main active components of wolfberry have been identified as *Lycium barbarum* polysaccharides (LBP) and zeaxanthin, and other small molecules such as betaine, cerebroside, beta-sitosterol, p-coumaric acid, and various vitamins (Chang & So, 2008). LBP extracted from *L. barbarum* have been shown to protect neurons against beta-amyloid peptide toxicity in neuronal cell cultures (Ho et al., 2007; Yu et al., 2005, 2007), to protect retinal ganglion cells in an experimental model of glaucoma (Chan et al., 2007), and to attenuate stress kinases and pro-apoptotic signaling pathways (Chang & So, 2008). A milk-based formulation of wolfberry, containing both the water-soluble LBP and the fat-soluble zeaxanthin, has been demonstrated in a human clinical study to give a three-fold enhanced bioavailability of zeaxanthin compared with a traditional preparation of wolfberry (Benzie, Chung, Wang, Richelle, & Bucheli, 2006).

Based on growing interest in free-radical biology and lack of effective therapies for most chronic diseases, the usefulness of natural antioxidants from plant materials have been evaluated for therapeutic efficiency against diseases related to oxidative stress. Plants are rich source of bioactive components, the most important of these are flavonoids and polyphenolic compounds. They exhibit high antioxidant properties that terminate free-radical mediated reactions by donating hydrogen atom or an electron to the radicals (Havsteen, 2002; Snoog & Barlow, 2004). This aspect reinforces the idea that the dietary inclusion of natural antioxidants present in plant foods is an important health-protecting and disease-preventing factor in humans.

Bladder cancer is one of the most commonly diagnosed malignancies in Western societies and its incidence and prevalence are still rising. Eighty percent of these tumors are superficial transitional cell carcinomas (TCC) but exhibit a high recurrence rate (50–80%). Intense research has been conducted to better understand the progression of these superficial tumors to invasive disease. The early superficial disease stage in 75% of patients is diagnosed due to the finding of gross or microscopic blood in the

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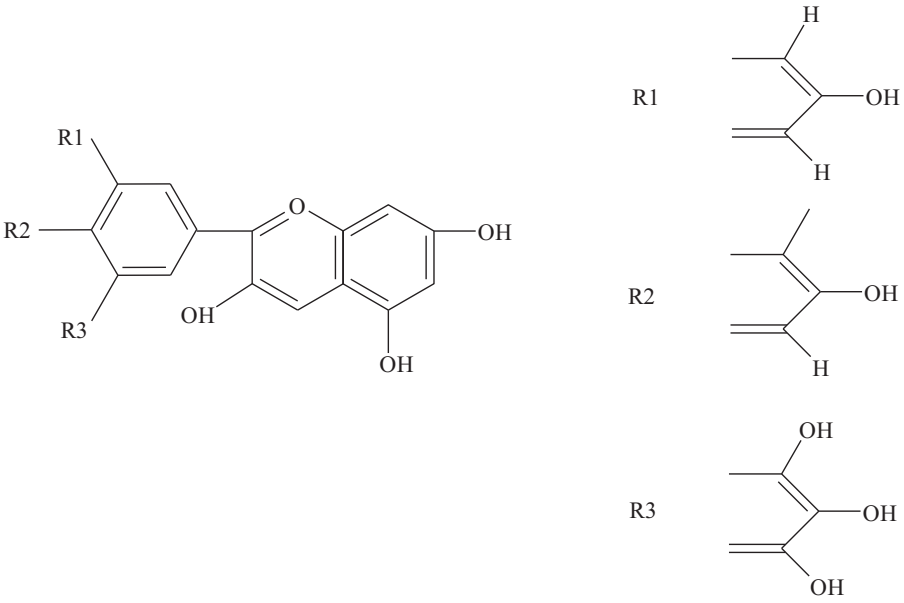

Fig. 1. Chemical structure of anthocyanin.

urine (Alexandroff, Jackson, O'Donnell, & James, 1999). When first diagnosed, 75–85% of the patients have papillary urothelial tumors (Prout, 1977) and more than 70% of patients with these superficial tumors have one or more recurrences after initial therapy (Culter, Heney, & Friedell, 1982). These types of tumors are considered to have low invasive potential with a progression rate to invasive cancer of 15–20% (Heney, Ahamed, & Flanaga, 1983).

Many bioflavonoids including anthocyanin (Fig. 1) extracted from the petals of higher plants are effective in the growth suppression of human cultured malignant cells. Anthocyanins extracted from red glutinous rice and grape rinds most likely have conjugated sugar other than glucose, such as lutinose and rhamnose.

In this study, a water-soluble polysaccharide was isolated with a DEAE Cellulose-52. We named it LBPF5. The present paper is concerned with the isolation, chemical characterisation and evaluation of the antioxidant activity of LBPF5. In addition, LBPF5 and anthocyanins from grape rinds were examined for their antitumor effects in vitro and in vivo. Furthermore, the anti-cell growth activity was analyzed by inspection of histograms obtained by flow-cytometry.

2. Materials and methods

2.1. Plant material, isolation and purification of polysaccharide

L. barbarum fruits were collected in Yinchuan city, China between March and April 2009. The plant material was dried at ambiant temperature and stored in a dry place prior to use.

Dried *L. barbarum* fruits was extracted 3× with 95% EtOH at 75 °C for 5 h under reflux to remove lipid, and the supernatant was removed. The residue was then extracted 4× with 10 vol of distilled water at room temperature for 3 h. After centrifugation (5000 rpm for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (1:5, v/v) at 4 °C for 12 h. The precipitate was washed with absolute ethanol, acetone and ether, respectively. The washed precipitate was the crude polysaccharide, named as CLPB. Ultraviolet spectrum of CLBP showed that absorption peak at 280 nm was not detected, suggesting absence of protein in CLBP (Fig. 2).

The CLBP (100 mg) were dissolved in 0.1 M NaCl (10 mg/ml) and 2 ml of solution were applied to a column of DEAE Cellulose-52 (2.6 × 30 cm), followed by stepwise elution with 0.1, 0.3, and 0.5 M sodium chloride solutions at a flow rate of 60 ml/h. Eluate (5 ml/tube) was collected automatically and the carbohydrates were determined by the phenol–sulfuric acid method, using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Finally, six fractions of polysaccharides, LBP-F1 (2.14%), LBP-F2 (0.98%), LBP-F3 (3.31%), LBP-F4 (2.62%), LBP-F5 (79.53%) and LBP-F6 (7.58%), were obtained, dialyzed with water, and lyophilized for further study. Because LBP-F5 was a major component of LBP, LBP-F5 was further analyzed by GC.

2.2. Monosaccharide composition and molecular weight determination

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. LBPF5 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described and analyzed by GC as previously mentioned. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol (Gerwig, Kamerling, & Vliegenthart, 1979).

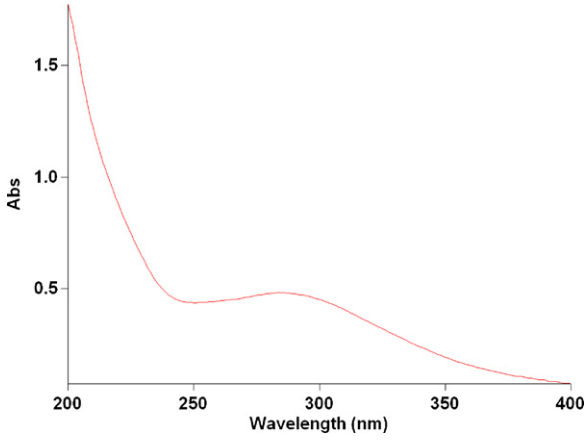

Fig. 2. UV spectrum for CLBP.

Table 1
Chemical composition of LBP fractions.

Sugar	LBP-F1	LBP-F2	LBP-F3	LBP-F4	LBP-F5	LBP-F6
Arabose	–	+	–	+	+	–
Mannose	–	–	+	–	+	–
Xylose	+	+	+	–	+	+
Glucose	+	+	+	+	+	+
Rhamnose	+	–	–	–	+	+

–, not detection; +, detection.

The average molecular weight of LBP-F5 was determined by high-performance size-exclusion chromatography (HPSEC), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PWXL column (7.8 mm ID × 30.0 cm) and a SHIMADZU RID-10A detector. The data were processed by GPC processing software (Millennium32 version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 ml/min at 40 °C. A sample (3 mg) was dissolved in the mobile phase (0.5 ml) and centrifuged (10,000 rpm; 3 min), and 20 μl of supernatant was injected. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular masses (T-130 80, 50, 25, 10). According to the retention time, its molecular weight was estimated to be 5.3×10^4 Da. LBP-F5 was composed of arabose, mannose, xylose, glucose and rhamnose (Table 1).

2.3. Superoxide radical scavenging assay

Superoxide radical (O_2^-) scavenging capacity of LBP-F5 was examined by a pyrogallol autoxidation system with minor modifications (Xiang & Ning, 2008). The reaction mixture contained 70 μl 10 mM pyrogallol, 4.5 ml 50 mM Tris–HCl (pH 8.2) and 0.5 ml various concentrations of LBP-F5 samples. The absorbance at 325 nm was recorded immediately at 30 s and then recorded once every minute. Anthocyan was used as control. The scavenging rate was obtained according to the formula: O_2^- scavenging rate (%) = $[1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of the control (without extract), A_1 was the absorbance in the presence of the extract, A_2 was the absorbance of without pyrogallol.

2.4. Scavenging of hydrogen peroxide

The ability of LBP-F5 to scavenge hydrogen peroxide was determined according to the method of Ruch, Cheng, and Klaunig (1989). A solution of hydrogen peroxide (2 mmol/L) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity 81 $mol/L^{-1}/cm$. LBP-F5 samples (0.4–1.6 mg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of both LBP-F5 extract and standard compounds was calculated.

2.5. $DPPH^-$ radical scavenging

This experimental procedure was adopted from Brand-Williams, Cuvelier, and Berset (1995). The LBP-F5 sample at different concentrations (0.4–1.6 mg/ml) were mixed with the same volume of 0.2 mM methanolic solution of $DPPH^-$ from Sigma. The disappearance of $DPPH^-$ was read spectrophotometrically at 517 nm immediately after mixing and after 1, 5 and 30 min of incubation at room temperature. Anthocyan was used as control. Free radical scavenging capacity was calculated by the following equation:

$$\%\,\text{scavenging} = \frac{100 - \text{Abs sample}}{\text{Abs DPPH}} \times 100$$

Abs sample = Abs measured – Abs control (i.e., absorbance of the sample tested without $DPPH^-$).

2.6. MTT test

Human bladder carcinoma cell line BIU87 provided from the typical culture preservation committee of the Chinese Academy of Science (Shanghai, China) was grown in RPMI-1640 medium (Gibco/Invitrogen Corp., Carlsbad, CA, USA) containing 10% FCS, 100 units/mL penicillin (Sigma, St. Louis, MO, USA) and 50 μg/mL streptomycin (Sigma) and was incubated at 37 °C in 5% CO_2. Cells in 96-wells (2×10^4 cells/well) were treated with the various concentrations of LBP-F5 and anthocyan for 24 h. All the extracts were dissolved in DMSO, and then diluted with RPMI-1640 medium to obtain the required concentration before use, and cells grown in medium containing an equivalent final volume of DMSO (<0.1%, v/v) served as a vehicle control. At the end of treatment, 20 μL of fresh serum-free medium containing MTT (5 mg/mL) reagent was added to each well. Cells were incubated for 4 h at 37 °C, and the medium was then carefully aspirated from the wells. Cells were lysed by addition of 200 μL DMSO, and the optical density (OD) at 570 nm was determined using a Bio-Tek PowerWave 340 microplate scanning spectrophotometer. Data are reported as the average of at least three replicates.

2.7. Flow cytometery analysis

For flow cytometry analysis, BIU87 cells were cultured into 6-well plates at a density of 1×10^6 cells in the presence and absence of the cytotoxic agents for 48 h. All floated and adherent cells were harvested and centrifuged at 200 × *g* for 10 min. Cell pellet was washed with 1× phosphate buffer saline solution and centrifuged at 200 × *g* for 10 min. The cell pellet was then resuspended in 100 μL of Annexin V/FLUOS labeling solution (predilute 20 μL Annexin V/FLUOS labeling reagent in 1 mL incubation buffer and add 20 μL propidium iodide solution), and incubated at 15–25 °C for 10–15 min. It was then employed to analyze the cell population analyzed by flow cytometer (Bio-Rad, USA). Using varying concentration of LBP-F5 and anthocyan giving 50% cytotoxicity (LC_{50}) was selected to evaluate of apoptosis. FL1 and FL2 channels were used for detection of Annexin V/FLUOS labeling solution, respectively. The samples were read in a FACS flow cytometer (USA) using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter >600 nm for propidium iodide detection. Analyses were performed by the software supplied in the instrument.

3. Result

3.1. In vitro antioxidant activities of LBP-F5 sample

Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. Several in vitro methods are available for generation of superoxide radicals (Vani, Rajani, Sarkar, & Shishoo, 1997). Fig. 3 shows the % inhibition of superoxide radical generation of LBP-F5 (0.4–1.6 mg/ml) and comparison with anthocyan. The LBP-F5 have superoxide radical scavenging activity and exhibited lower superoxide radical scavenging activity than anthocyan. Free radical scavenging activ-

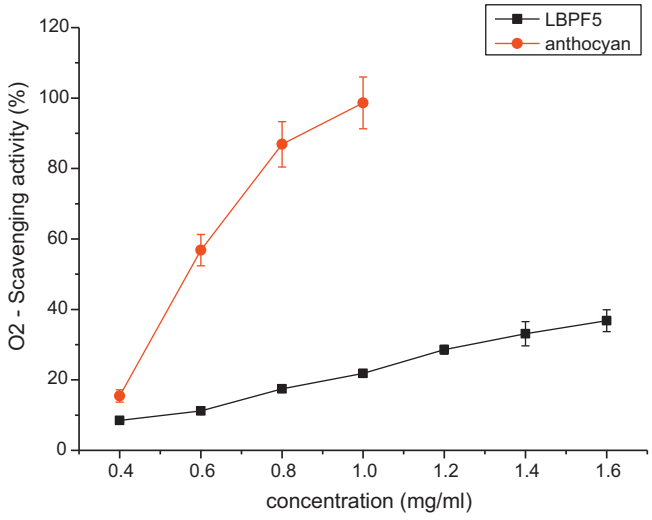

Fig. 3. Superoxide radical scavenging activity of LBP-F5 sample.

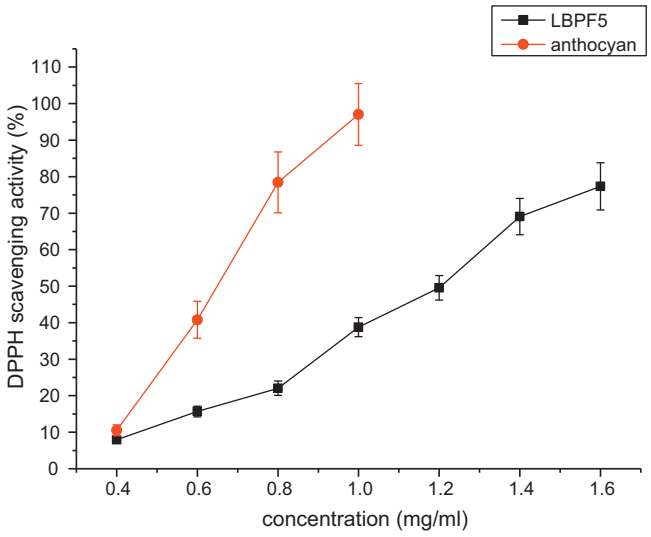

Fig. 5. DPPH radical scavenging activity of LBP-F5 sample.

ity also increased with increasing concentration. The scavenging effect of LBP-F5 on the superoxide radical was 35.6% at the dose of 1.6 mg/ml.

The hydroxyl radical is one of representative reactive oxygen species generated in the body. Fig. 4 depicts the scavenging capacity of LBP-F5. When the concentration is from 0.4 to 20 mg/ml, the percentage scavenging of LBP-F5 ranged from 6.6% to 57.8%. Hydroxyl radical scavenging activity of LBP-F5 was lower than that of anthocyan.

The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals is initiated by the lipid autoxidation (Ingold, Bowry, Stocker, & Walling, 1993). DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dinis, Cunha, & Almeida, 1997). The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm ($P < 0.05$), which is induced by antioxidants. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of LBP-F5 and anthocyan on DPPH radical was compared. On the DPPH radical, LBP-F5 had significant scavenging effects with increasing concentration in the range of 0.4–1.6 mg/ml when compared with that of anthocyan, the scavenging effect of LBP-F5 was lower. The different concentrations of LBP-F5 (0.4–1.6 mg/ml) showed antioxidant activities in a dose dependent manner. In the dose 1.6 mg/ml, LBP-F5 showed 78.73% inhibition on the DPPH radical scavenging assay (Fig. 5).

The antioxidant activity of pure compounds, foods and dietary supplements has been extensively studied, with many new antioxidant and antioxidant-activity assays developed in recent years. Therefore, there is a need for cell models (erythrocytes) to access antioxidant bioactivity. Fig. 6 shows human erythrocyte hemolysis induced by AAPH with the addition of LBP-F5. When erythrocytes were incubated in air at 37 °C as a 5% suspension in phosphate buffered saline (PBS), they were stable with little hemolysis observed for 6 h (6.45 ± 0.31%). When the water-soluble radical generator, AAPH (25 mM), was added to the aqueous suspension of erythrocytes, the hemolysis induction was time dependent. The hemolysis lagged, indicating that endogenous antioxidants in the erythrocytes can trap radicals to protect them against free radical-induced hemolysis, as described previously (Zou, Agar, &

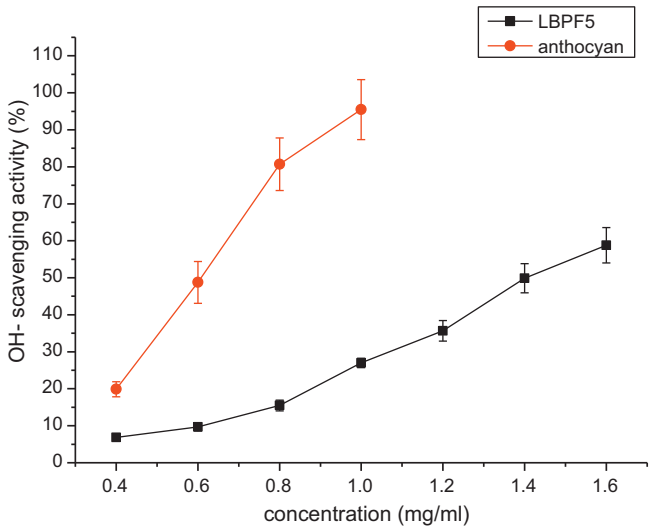

Fig. 4. Hydroxyl radical scavenging activity of LBP-F5 sample.

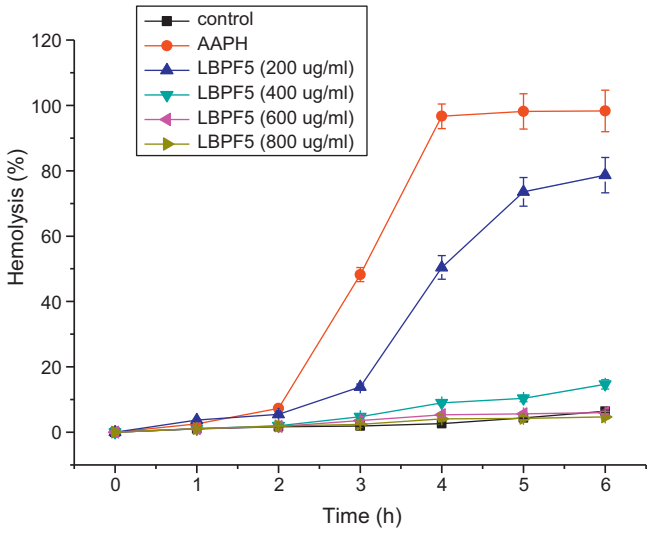

Fig. 6. Effects of LBP-F5 on AAPH-induced hemolysis in erythrocytes. Erythrocyte suspension at 5% hematocrit was incubated with PBS (control) or preincubated with LBPF5 at the indicated concentrations for 30 min. The product was then incubated with 25 mM AAPH for 6 h at 37 °C. Values are expressed as the mean ± SD of three experiments.

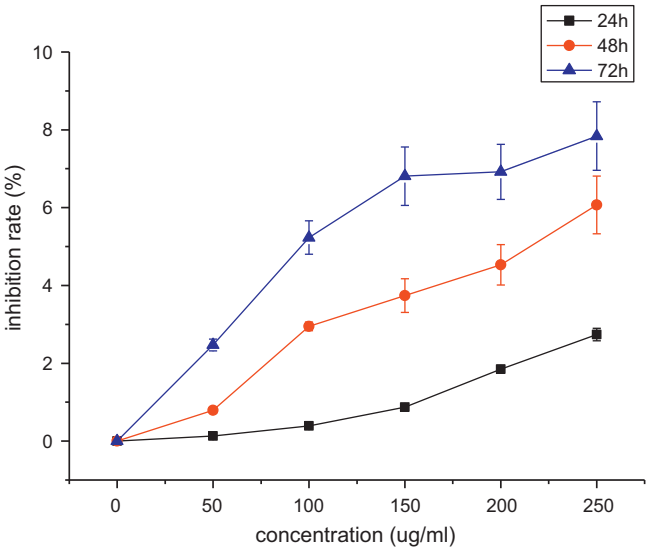

Fig. 7. Inhibition of LBP-F5 with varying concentration against BIU87.

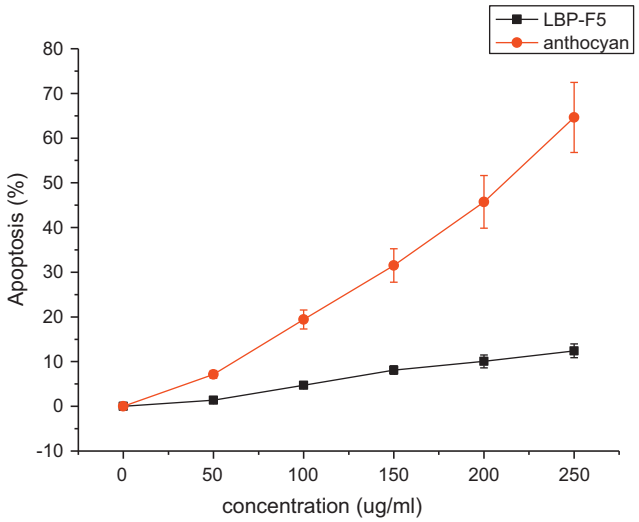

Fig. 9. LBP-F5 and anthocyan inducing BIU87 apoptosis.

Jones, 2001). Fig. 6 shows that LBP-F5 (200–800 μg/mL) decreased AAPH-induced hemolysis. LBP-F5 at 200 μg/mL only slowed down the haemolysis. When the cells were incubated with LBP-F5 (800 μg/mL) alone, hemolysis was maintained at a background level similar to that of the AAPH-untreated samples (data not shown).

3.2. *In vitro antitumor activities of LBP-F5 and anthocyan samples*

Cells in 96-wells (2×10^4 cells/well) were treated with different concentrations of LBP-F5 for 24 h, and anthocyan was used as a positive control (Figs. 7 and 8). The results indicated that LBP-F5 possessed a weak cell inhibition effect within the range of 50–250 μg/ml. Anthocyan from grape rinds displayed strong antitumor activities ($IC_{50} = 246 \pm 31$ μg/mL). As shown in Fig. 6, the inhibition effect was dose dependent.

The flow cytometry experiment was performed. The BIU87 cell line was treated with the LBP-F5 and anthocyan. As shown in Fig. 9, it is shown that 12% and 64% of the cells undergo apoptosis in the presence of the LBP-F5 and anthocyan. Flow cytometry findings reveal that apoptosis is the main mechanism by which the extract brings about cell death.

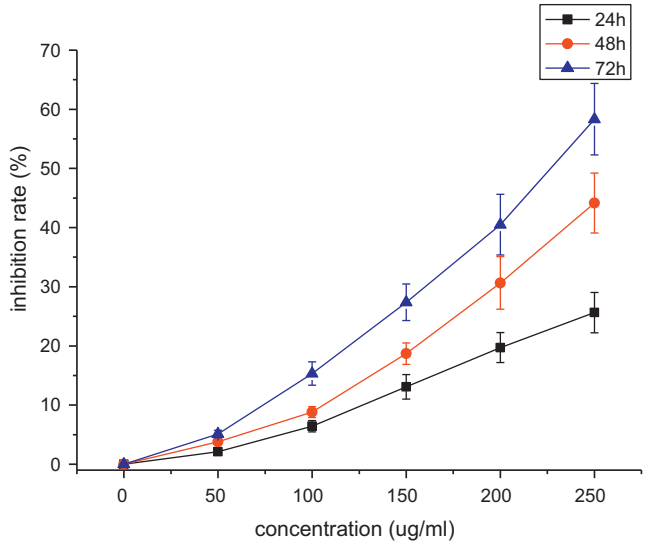

Fig. 8. Inhibition of anthocyan with varying concentration against BIU87 growth.

4. Discussion

L. barbarum, a well-known traditional Chinese medicinal herb, possesses diverse biological activities and pharmacological functions including reducing blood glucose and serum lipids. It has long been used to treat diabetes mellitus and related hyperlipidemia (Gao, Xu, & Li, 2000).

In the present study, polysaccharides isolated from *L. barbarum* fruit and six polysaccharide fractions, named as LBP-F1, LBP-F2, LBP-F3, LBP-F4, LBP-F5 and LBP-F6, were separated. The yield of LBP-F5 was high, so the LBP-F5 was used in the subsequent studies. Result showed that LBP-F5 was composed of arabose, mannose, xylose, glucose and rhamnose. Then, in vitro antioxidant activity of LBP-F5 sample was examined. We found that LBP-F5 could significantly scavenge OH^-, DPPH radicals and decreased AAPH-induced hemolysis. Our results indicate that LBP-F5 possesses strong antioxidant activity.

The relation between the occurrence, growth and decline of tumor and immune states is the essential problem of tumor immunology. The discovery and identification of new antitumor drugs, which can potentiate the immune function has become an important goal of research in immunopharmacology and oncotherapy. This study demonstrates the favourable antitumor effect of LBP-F5 and anthocyan from grape rinds. LBP-F5 could inhibit ($P < 0.05$) the growth of human bladder carcinoma cell line BIU87 after treatment for 24 h and the inhibitory effect was dose dependent. In addition, anthocyan could significantly inhibit the growth of human bladder carcinoma cell line BIU87 after treatment for 24 h and the inhibitory effect was also dose dependent. The flow cytometry analysis showed that LBP-F5 and anthocyan could induce BIU87 apoptosis ($P < 0.05$). Moreover, antitumor effect of anthocyan was by far stronger than that of LBP-F5.

In conclusion, the LBP-F5 and anthocyan shows strong antioxidant activities in vitro. In addition, LBPF5 and anthocyan still dose-dependently inhibit the growth of human bladder carcinoma cell line BIU87.

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