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Inhibition Effect of *Dictyophora* Polysaccharides on Human Hepatocellular Carcinoma Cell Line HCC-LM3

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Data Interpretation D
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Background: It has been reported that polysaccharides have potential novel anti-cancer properties. Previously, we confirmed that *Dictyophora* polysaccharides could significantly inhibit liver transplantation tumors in mice. However, the mechanism of *Dictyophora* polysaccharide action on human liver cancer is unclear. Here, we aimed to clarify the mechanism of *Dictyophora* polysaccharide action on human hepatocellular carcinoma cells, namely the effect on cell proliferation, the cell cycle, and apoptosis, and on the apoptosis-related genes and proteins *in vitro*.

Material/Methods: The HCC-LM3 cell line was incubated with 2.5 mg/mL *Dictyophora* polysaccharides for 24, 48, and 72 h. The cell growth inhibition rate was evaluated using Cell Counting Kit-8. Cell cycle and apoptosis were measured with flow cytometry. The expression of apoptosis-related genes and proteins was measured using real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) and Western blotting, respectively.

Results: The *Dictyophora* polysaccharides inhibited HCC-LM3 cell proliferation in a time- and dose-dependent manner and blocked the cell cycle in the G_2/M phase. In addition, Bax and caspase-3 expression were significantly increased after *Dictyophora* polysaccharides treatment.

Conclusions: To the best of our knowledge, this is the first published study on the mechanism of *Dictyophora* polysaccharide inhibition of HCC-LM3 cell proliferation.

MeSH Keywords: **Apoptosis • Carcinoma, Hepatocellular • Cell Cycle Checkpoints • Cell Proliferation • Polysaccharides**

Abbreviations: **Bax** – Bcl-2-associated death promoter; **Bcl-2** – B cell lymphoma 2; **BCA** – Bicinchoninic acid; **CCK-8** – Cell counting kit-8; **DMEM** – Dulbecco's modified Eagle's medium; **DMSO** – Dimethyl sulfoxide; **FBS** – Fetal bovine serum; **FITC** – Fluorescein isothiocyanate; **FDA** – US Food and Drug Administration; **HCC** – Hepatocellular carcinoma; **PVDF** – Polyvinylidene fluoride; **PBS** – Phosphate-buffered saline; **PI** – Propidium iodide; **qRT-PCR** – Quantitative real-time polymerase chain reaction; **RNA** – Ribonucleic acid; **RIPA** – Radioimmunoprecipitation assay

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Background

Liver cancer is one of the leading causes of cancer death in men in developing countries**.** Around 80% of liver cancer cases are hepatocellular carcinoma (HCC), and there has been a recent increase in new liver cancer cases [1,2]. Although surgery is the preferred method for treating liver cancer, delayed diagnosis means that most patients lose the opportunities for surgery; therefore, chemotherapy is the main therapeutic strategy, and effective therapeutic reagents are vital [3,4]. Although research on HCC has reported that numerous compounds have anti-cancer properties, most have been found to be inefficient in clinical trials. Sorafenib, the unique drug as first-line treatment for advanced hepatocellular carcinoma (HCC), has opened a window of hope after searching for effective agents to combat HCC for decades. However, the overall outcomes are far from satisfactory. Increasing evidence of resistance effects have limited its utilization [5,6].

Recently, the US Food and Drug Administration (FDA) approved a considerable number of low-toxicity compounds extracted from herbs for treating malignant cancer [7–9].

Dictyophora is a valuable edible and medicinal fungus that contains a variety of biologically active ingredients such as polysaccharides, amino acids, salts, and vitamins, and has immunomodulation, antioxidant, anti-tumor, hypotensive, and hypolipidemic bioactivities [10–12]. *Dictyophora* polysaccharides are extracted from *Dictyophora indusiata*. *Dictyophora* polysaccharides have antioxidant and anti-tumor effects [13]. In addition, the polysaccharides have a significant inhibitory effect on transplanted tumors in animal models *in vivo* [14]. Previously, we found that *Dictyophora* polysaccharides significantly inhibited liver transplantation tumors in mice and effectively controlled the growth of ascites tumor in mice. However, research on the effect of *Dictyophora* polysaccharides on human liver cancer *in vitro* has rarely been reported.

Therefore, we aimed to evaluate the effects of *Dictyophora* polysaccharides on HCC cell proliferation, cell cycle, and apoptosis, and on the expression of the apoptosis-related genes and proteins, to explore the possible mechanism of *Dictyophora* polysaccharide inhibition of HCC cells.

Material and Methods

All work reported in this study was performed in full compliance with good laboratory practices (GLP).

Chemicals

RPMI-Dulbecco's modified Eagle's medium (DMEM) cell culture medium, SYBR RT reagent kit with genomic DNA (gDNA) Eraser (Perfect Real Time), and Premix Ex Taq II (Perfect Real Time) were purchased from TaKaRa Dalian Biotech. Trypsin was purchased from Amresco. Fetal bovine serum (FBS) was purchased from Hangzhou Evergreen Biotech. Phosphate-buffered saline (PBS) was purchased from Beijing Zhongsha Golden Bridge Biotechnology. Dimethyl sulfoxide (DMSO) was purchased from Sigma. Propidium iodide (PI), a cell cycle assay kit, and an annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis assay kit were purchased from Nanjing Kaiji Biological Technology Development. PCR primers for caspase-3, Bcl-2, and Bax were synthetized by Shanghai Biological Technology. A bicinchoninic acid (BCA) protein assay kit, protein sample buffer, and Western blot gel preparation kit were purchased from Shanghai Beyotime Biotech. The protein molecular weight marker was purchased from Fermentas (Burlington, Canada). Polyvinylidene fluoride (PVDF) membranes and ECL chemiluminescence kit were purchased from Millipore (Billerica, MA, USA). The cell proliferation-toxicity assay kit (Cell Counting Kit-8, CCK-8); radioimmunoprecipitation assay (RIPA) lysis buffer; rabbit anti-human caspase-3, Bcl-2, and Bax antibodies; and horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Wuhan Boster Biological Engineering. The other reagents used in this experiment were purchased from Sigma (St. Louis, MO, USA) or were of analytical grade.

Preparation of *Dictyophora* polysaccharides

Food-grade Dictyophora was purchased for Sifang Hongye Company (Zhijin, Guizhou, China) in March, 2017. The sample was authenticated by Prof. Qingde Long of the School of Pharmacy, Guizhou Medical University, and voucher specimens (No. Di2018030501) were stored in our Research Laboratory, School of Pharmacy, Guizhou Medical University. For preparation of *Dictyophora* polysaccharides, the fruiting bodies of *D. indusiata* (2 kg) were dried in a hot air-drying oven at 45°C and crushed into powder using a tissue triturator. The powder of fruiting body was extracted by high-pressure ultrasonic-assisted extraction (Xian Taikang Biotechnology Co., China) according to the water-material ratio (1: 20), at 70°C for 3 h. The extract was then concentrated at 50°C using a rotary evaporator (R-215, Buchi, Switzerland). After that, the concentrated extract mixed with 4 volumes of anhydrous ethanol (70% v/v of ethanol in final concentration) at 4°C overnight. The precipitate (4500 r/min, 10 min) was deproteinated by the Savage method and washed with anhydrous alcohol. The residual nucleic acid and protein was detected by UV method and it had not obvious absorbed at 260 nm and 280 nm wave length. The precipitate was then re-dissolved in distilled water and dialyzed (8,000–14,000 Da) in running tap water for 48 h.

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Table 1. PCR Primers used and PCR product sizes.

Target mRNA	Forward primer 5'–3'	Reverse primer 3'–5'	Product size
GAPDH	AGAAGGTGGTGAAGCAGGCATC	CGAAGGTGGAAGAGTGGGAGTTG	111 bp
Bax	CAGGATGCGTCCACCAAGAA	GCAAAGTAGAAGAGGGCAACCAC	197 bp
Bcl2	TGTGGAGAGCGTCAACAGGG	AGACAGCCAGGAGAAATCAAACAGA	175 bp
Caspase 3	GTGGGACTGATGAGGAGATGGC	AAGGGACTGGATGAACCACGAC	131 bp

The final liquid solutions were lyophilized in a vacuum freeze dryer (Alpha 2–4 LSC plus, CHRIST, Germany).

Detection conditions of liquid chromatography

The chromatographic column was a Thermo C18 column (4.6×250 mm, 5 μm), mobile phase A was acetonitrile, mobile phase B was 0.02 mol/l ammonium acetate, gradient elution (0–30 min, mobile phase A was 12–30% for 30–40 min, mobile phase A was 30–20%), flow rate was 1 ml/min, detection wavelength was 250 nm, column temperature is 30°C, and injection volume was 10 μL.

Cell culture

The human hepatocellular carcinoma cell line HCC-LM3 was purchased from Pharmaceutical Technology/BioHermes. Cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell proliferation activity

Cell proliferation was examined using CCK-8 according to the manufacturer's instructions. Briefly, the cells were exposed to 0.5 mg/mL, 1 mg/mL, 2 mg/mL, or 4 mg/mL *Dictyophora* polysaccharides for 0, 24, 48, and 72 h. Optical density (OD) values measured by microplate reader at 450 nm were used to quantitate cell counts. The CCK-8 assay results are stable and widely used in cell proliferation and cytotoxicity assays, drug screening, and cancer susceptibility testing [15].

Investigation of cell cycle

PI single staining was used according to the manufacturer's instructions to measure the cell cycle. An exposure concentration of 2.5 mg/mL *Dictyophora* polysaccharides was prepared for 0-, 24-, 48-, and 72-h incubation.

Apoptosis

The Annexin V-FITC/PI assay kit was used according to the manufacturer's instructions to examine apoptosis in HCC cells exposed to 2.5 mg/mL *Dictyophora* polysaccharides for 0, 24, 48, and 72 h. Fluorescence was read at excitation and emission wavelengths of 488 nm and 530 nm, respectively.

Apoptosis-related genes

The apoptosis-related genes were determined using real-time fluorescence quantitative polymerase chain reaction (RT-qPCR). The HCC cells were exposed to 2.5 mg/mL *Dictyophora* polysaccharides for 0, 24, 48, and 72 h. After mRNA extraction and quality testing, the PrimeScript RT reagent kit was used to synthesize complementary DNA (cDNA) by reverse transcription. Table 1 shows the primer sequences used. The RT-qPCR was prepared according to the reagent kit instructions.

Apoptosis-related proteins

The HCC cells were exposed to 2.5 mg/mL *Dictyophora* polysaccharides for 24 h and Western blotting was performed according to the kit instructions. Rabbit anti-human caspase-3 (1: 1000 diluted with dilution buffer), Bcl-2 (1: 1000), and Bax (1: 1000) antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies (1: 5000) were purchased from Wuhan Boster Biological Engineering.

Data analysis and statistical methods

All data in different experimental groups are expressed as mean±SD. Data shown in the study were obtained from 3 independent experiments (n=3). Statistical analyses were performed using an unpaired, two-tailed *t* test. $P<0.05$ was considered statistically significant.

Results

Determination of polysaccharide in *Dictyophora*

According to the results in Figure 1 and Table 2, the standard curve equation is $y=3.7084x+0.2796$, and the correlation coefficient is $R^2=0.9984$. According to the linear regression equation, the sugar content of the polysaccharide was 54.13%. The results showed that the retention times of D-mannose,

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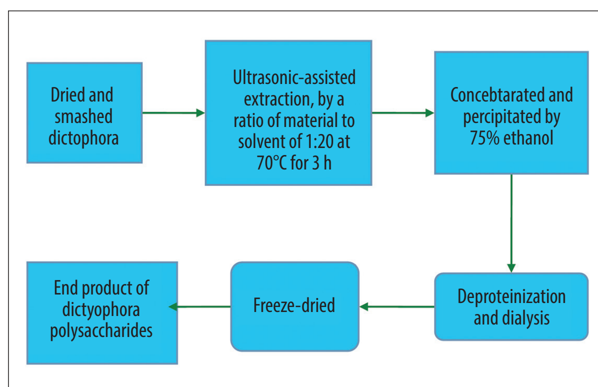

Figure 1. *Dictyophora* polysaccharides extraction process.

Table 2. The standard of gliucose spectrophotomety value.

Group	Glucose standard (ml)	Mean absorbance
1	0.0	0
2	0.2	0.12±0.21
3	0.4	0.15±0.01
4	0.6	0.21±0.12
5	0.8	0.26±0.03
6	1.0	0.31±0.07

Note: 1 and 2 are solvent peaks

Note: 1: D-mannose; 2: ribose; 3: L-rhamnose monohydrate; 4: galacturonic acid; 5: D-glucose; 6: D-galactose; 7: D-xylose; 8: L-fucose

The high performance liquid chromatography (HPLC) figure of reference substance

Figure 2. Determination of polysaccharide in *Dictyophora.*

ribose, L-rhamnose monohydrate, galacturonic acid, D-glucose, D-galactose, d-xylose, and L-fucose were 14.740, 16.290, 16.751, 17.274, 19.506, 20.221, 20.793, and 21.946 min, respectively. In the chromatogram of the mixed control, separation of the peaks was achieved at baseline; and the peak shape was symmetrical and the analysis time was 30 min. This shows that the chromatographic conditions are feasible for the analysis of monosaccharide composition in polysaccharides (Figure 2).

Analysis of the composition and monosaccharide content of polysaccharides from *Dictyophora*

The types of monosaccharides contained in Penny's fungus polysaccharide are D-mannose, ribose, L-rhamnose, galacturonic acid, D-glucose, D-galactose, D-xylose, and L-fucose. The content is 65.89, 5.04, 5.45, 6.46, 955.68, 316.25, 43.10, and 160.50 mg/L (Figure 3, Table 3).

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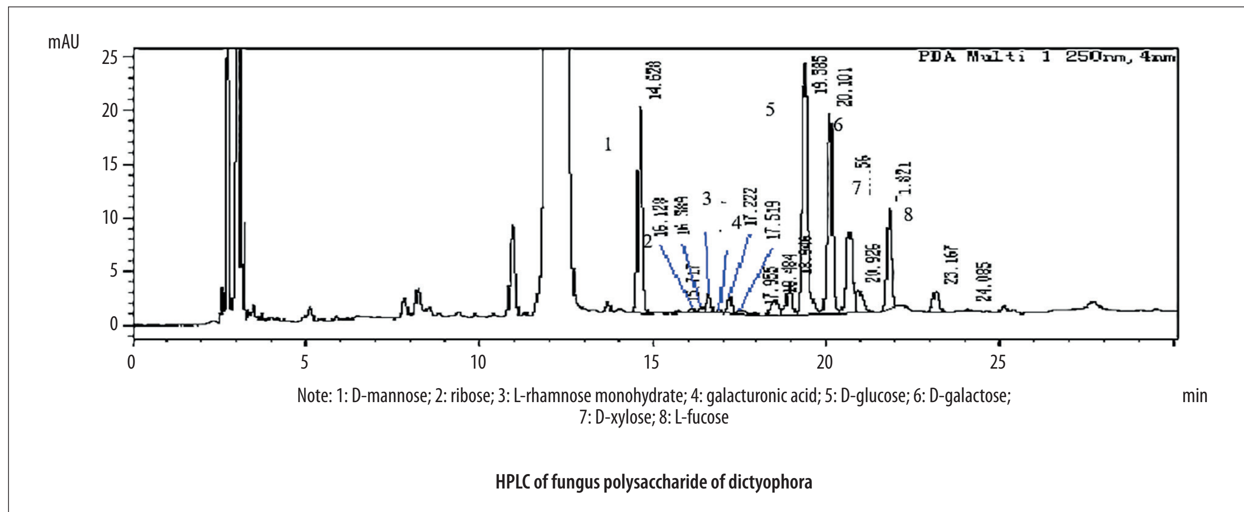

Figure 3. HPLC of each monosaccharide mixed reference.

Table 3. The monosaccharide content determination of polysaccharide fungus of *dictyophora*.

Form	Content (mg/L)	Mole Ratio
D-mannose	65.89	13.07:1.00:1.08:1.28:189.62:62.74:8.55:31.85
Ribose	5.04	
L-rhamnose monohydrate	5.45	
Galacturonic acid	6.46	
D-glucose	955.68	
D-galactose	316.25	
D-xylose	43.10	
L-fucose	160.50	

Determination of cell proliferation

The concentrations of *Dictyophora* polysaccharides used had diverse effects on HCC-LM3 cell viability over the various incubation periods. The OD values in the later periods reflected differing growth compared to the earlier periods. The decrease in OD values paralleled the increase in *Dictyophora* polysaccharide concentration during the incubation period (Figure 4). Each *Dictyophora* polysaccharide concentration caused differing but time- and dose-dependent inhibition of HCC-LM3 cell growth. The cell growth inhibition rate was 3.13% after 24-h treatment with 0.5 mg/mL *Dictyophora* polysaccharides and was up to 62.4% after 72-h treatment with 4 mg/mL *Dictyophora* polysaccharides. Cell proliferation gradually decreased with prolonged incubation and increased polysaccharide concentration (Figure 5). SPSS 16.0 was used to calculate the median inhibitory concentration (IC50) at 24 h (3.713 mg/mL), 48 h (2.409 mg/mL), and 72 h (1.863 mg/mL).

Measurement of cell cycle

Following treatment with 2.5 mg/mL *Dictyophora* polysaccharides, the ratios of cells in G_0/G_1 showed no significant change in each incubation period. The ratios of cells in G_2/M increased after 24-h treatment. The inhibitory effect increased with prolonged exposure. After 72 h, the ratio of control cells in G_2/M increased from 3.58% to 11.55%. The apoptosis peak and other cell cycle arrest phenomena were not observed (Figure 6, Table 4). *Dictyophora* polysaccharides inhibit HCC-LM3 cell growth *in vitro* by causing G_2/M arrest.

Measurement of apoptosis

The rate of HCC-LM3 cell apoptosis increased with incubation time. There was a time-response relationship for 2.5 mg/mL *Dictyophora* polysaccharides. Polysaccharide treatment for 0 and 72 h increased the rate of HCC-LM3 cell apoptosis to 4.57% and 33.49%, respectively (UR+LR); there were more cells in the early apoptosis stage than in late apoptosis (Figure 7, Table 5).

Detection of the expression of apoptosis-related proteins and genes

HCC-LM3 cells were incubated with 2.5 mg/mL *Dictyophora* polysaccharides for 48 h, and then the expression of the apoptosis-related proteins Bax and caspase-3 were analyzed by Western blotting. As shown in Figure 8 and Table 6, the Bcl-2/Bax ratio was decreased ($P<0.05$); the control and treatment group were 1.09±0.07 and 0.8±0.01, respectively. RT-qPCR determined that the expression of the apoptosis-related genes for

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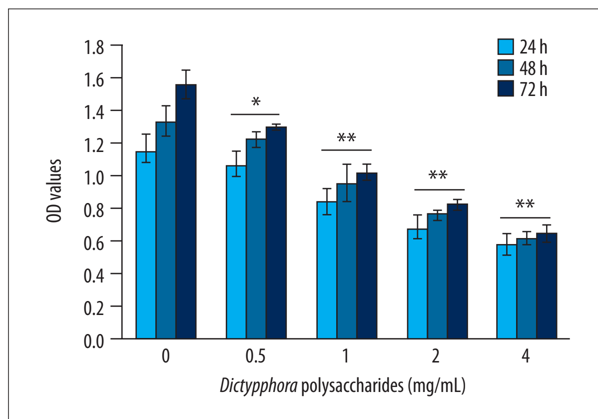

Figure 4. Effect of *Dictyophora* polysaccharides on HCC-LM3 cell growth. * Significant compared with control, * $P<0.05$, ** $P<0.01$, n=3.

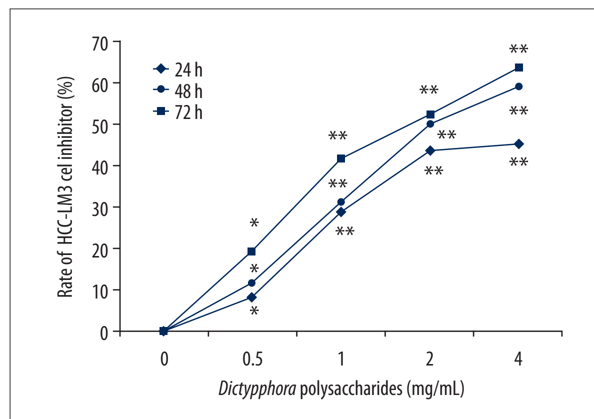

Figure 5. Rate of cell proliferation inhibition according to time. * Significant compared with control, * $P<0.05$, ** $P<0.01$, n=3.

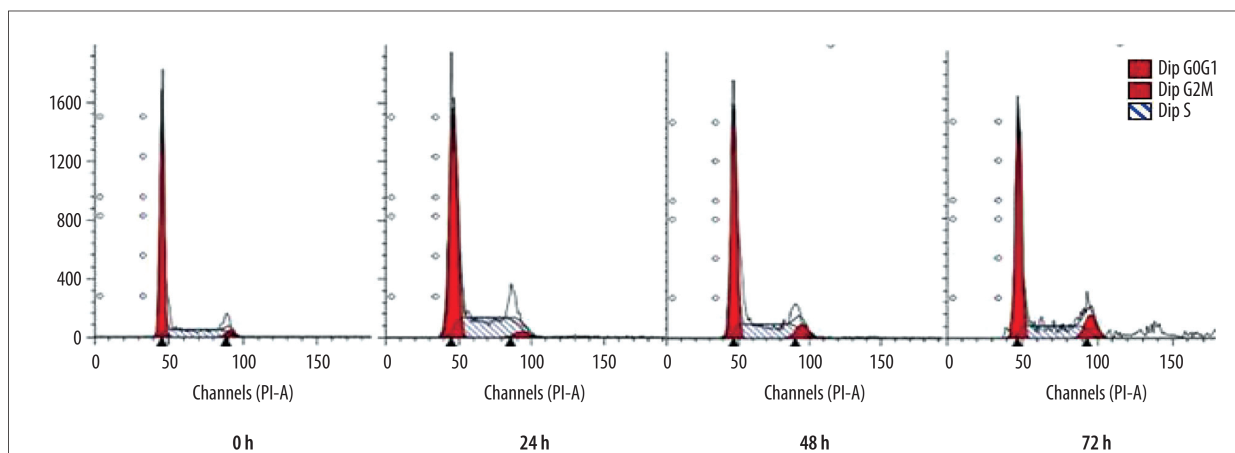

Figure 6. Effect on cell cycle by *Dictyophora* polysaccharides treatment.

Bax and caspase-3 were increased. The Bax and caspase-3 overexpression indicate that *Dictyophora* polysaccharides can induce HCC-LM3 cell apoptosis via a caspase-dependent mitochondrial pathway.

Discussion

In the present study, HCC-LM3 cells were treated with polysaccharides extracted from *Dictyophora* fruiting bodies, which showed a variety of biological activities. Treatment with the *Dictyophora* polysaccharides decreased cell proliferation activity. Treatment with 4 mg/mL *Dictyophora* polysaccharides for 24, 48, and 72 h inhibited the rate of HCC-LM3 cell growth by 45.3%, 59%, and 63.4%, respectively. The cell growth inhibitory effect was more significant with prolonged incubation time, indicating that the effect of the *Dictyophora* polysaccharides was time-dependent. In the 48-h treatment experiment, where the concentration of *Dictyophora* polysaccharides used was increased from 0.5 mg/mL to 4 mg/mL, the growth inhibition rate increased from 12% to 59%, showing an evident dose-response relationship. These findings indicate that *Dictyophora* polysaccharides inhibit HCC-LM3 cell growth in a time- and dose-dependent manner.

Abnormal cell cycle regulation is closely related to tumorigenesis, and the cell cycle as a target for biological treatment has become a new direction for anti-tumor therapy, which has become the focus of life science research in recent years [16–18]. Our study showed that the effects of cell cycle arrest began to appear after 24-h treatment with *Dictyophora* polysaccharides, suggesting that the polysaccharides have an anti-tumor effect. HCC-LM3 cell cycle arrest occurred mainly in the G_2/M phase. Extending the duration of treatment enhanced the blocking effect, which may be related to the activated G_2/M restriction point. Cells can die if the external disruption persists. However, FCM detected no obvious apoptosis peak in HCC-LM3 cells treated with 2.5 mg/mL *Dictyophora* polysaccharides for 24, 48, and 72 h. One reason for this may be the low set of polysaccharide concentrations, but we used a concentration higher

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Table 4. Changes to HCC-LM3 cell cycle phase following incubation with *Dictyophora* polysaccharides (n=3).

Incubation time	Cell cycle		
	G0/G1 (%)	S (%)	G2/M (%)
0 h (control)	63.47±4.00	32.95±4.32	3.58±0.61
24h	62.53±4.10	31.14±4.11	6.33±0.55*
48h	50.63±11.34	41.70±10.75	7.68±0.59*
72h	63.30±6.05	25.15±6.02	11.55±0.82*

* Comparison with control, $P<0.05$.

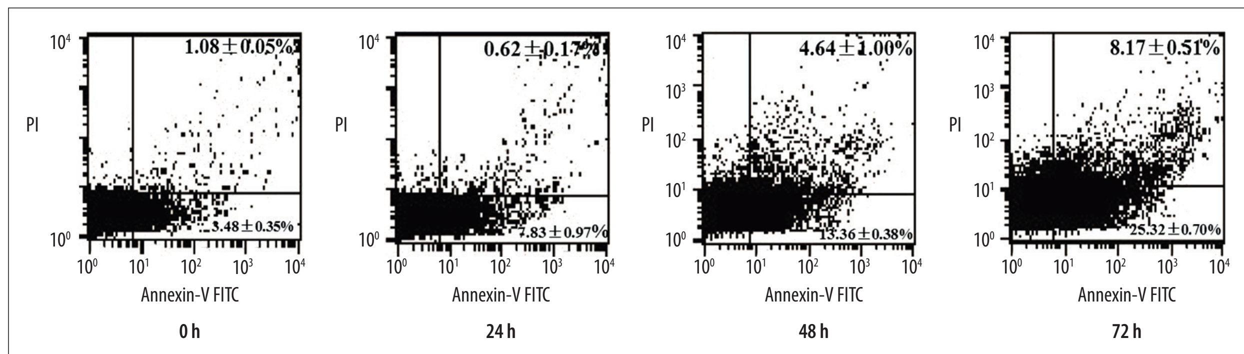

Figure 7. Annexin V–FITC/PI staining of HCC-LM3 cell apoptosis.

Table 5. Results of Dictyophora polysaccharide induction of HCC-LM3 cell apoptosis (n=3).

Incubation time	Annexin V (+)/PI (+) (%)	Annexin V (+)/PI (–) (%)	Total (%)
0 h (control)	1.08±0.05	3.48±0.35	4.57±0.38
24 h	0.62±0.17	7.83±0.97*	8.45±1.13*
48 h	4.64±1.00	13.36±0.38*	18.00±0.97*
72 h	8.17±0.51	25.32±0.70*	33.49±0.73*

* Comparison with the control, * $P<0.05$.

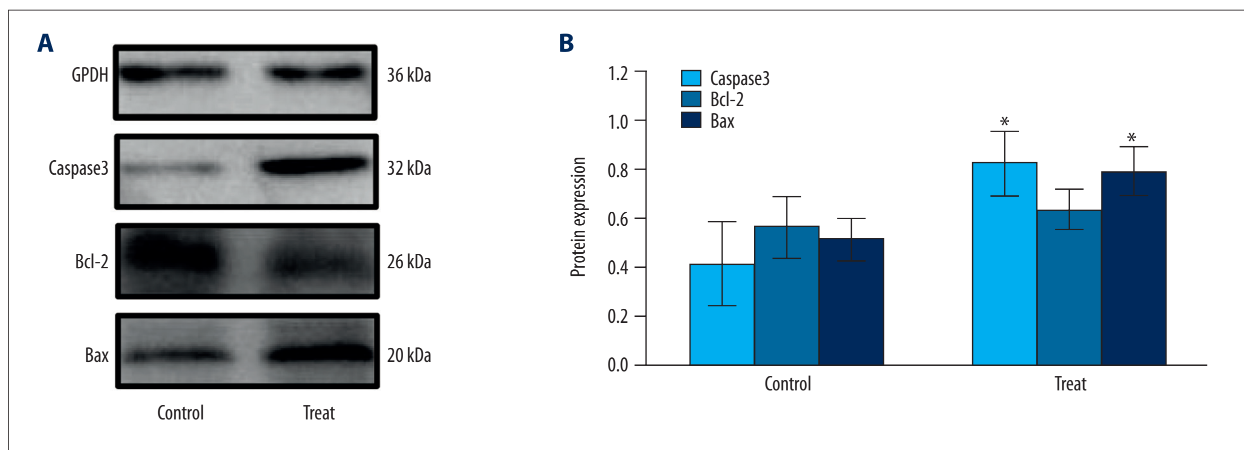

Figure 8. (**A**) Western blotting of apoptosis-related proteins in HCC-LM3 cells treated with *Dictyophora* polysaccharides. (**B**) The expression of apoptosis-related proteins in HCC-LM3 cells treated with *Dictyophora* polysaccharides. * Significant compared with control, * $P<0.05$, n=3.

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Table 6. RT-qPCR results (n=3).

Group	Control		Treatment	
	Mean ΔCt	$2^{-\Delta\Delta Ct}$	Mean ΔCt	$2^{-\Delta\Delta Ct}$
Bax	11.38±1.17	1	8.94±1.21	5.60±1.71*
Bcl2	7.13±0.57	1	6.19±0.84	1.98±0.53
Caspase3	5.64±1.00	1	3.27±1.40	5.30±1.70*

* Significant compared with control, * $P<0.05$. ΔCt – threshold cycle value; $2^{-\Delta\Delta Ct}$ – comparative threshold cycle value.

than the IC50 value at 48 h (2.409 mg/mL). The second reason may be that the DNA damage caused by the *Dictyophora* polysaccharides was too minor to induce HCC-LM3 cell apoptosis, the cause of which we intend to study further.

To further understand the impact of *Dictyophora* polysaccharides on HCC cell apoptosis, we measured apoptosis using annexin V–FITC/PI staining. *Dictyophora* polysaccharides induced apoptosis in the HCC-LM3 cells, and the pro-apoptotic effect was more obvious with longer treatment durations. In this experiment, 48-h treatment of HCC-LM3 cells with *Dictyophora* polysaccharides significantly increased the expression of the caspase-3 gene and protein, suggesting the occurrence of caspase-dependent apoptosis. By acting on the mitochondria, the Bcl-2 family regulates the release of apoptosis-related proteins, of which there are 2 types with opposite effects: the pro-apoptotic proteins (e.g., Bax, Bad, Bak, and Bik) and the anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL, and Bcl-w) [19,20]. Our experimental results showed that the expression of genes and proteins was consistent and that the expression of Bax was significantly increased. Although Bcl-2 expressions in the experimental group *vs*. the control were not significantly different, the Bcl-2/Bax ratio was decreased in the experimental group (<1), indicating that Bax plays a dominant role in promoting apoptosis and in the mitochondria-dependent apoptosis pathway.

Conclusions

This is the first study to evaluate the relationship between DP and apoptosis in HCC-LM3. The mechanism of *Dictyophora* polysaccharides induction of HCC cell apoptosis may be related to the upregulated expression of the genes for Bax and caspase-3 and the decreased Bcl-2/Bax heterodimer formation. The *Dictyophora* polysaccharides regulated the HCC cell cycle and promoted HCC cell apoptosis, suggesting that these polysaccharides have potential value for treating liver cancer. Our results suggest that the induction of HCC cell apoptosis is related to the apoptosis-related genes for Bax, Bcl-2, and caspase-3 and their transcription and translation, but the mechanism involved requires further study.

Conflict of interest

None.

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