The Structure-Activity Relationship and Molecular Mechanism of Anti-tumor Polysaccharide Isolated from *Dendrobium nobile* Lindl.

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Abstract: In this study, structure-activity relationship and molecular mechanism of anti-tumor polysaccharide isolated from the stems of *Dendrobium nobile* Lindl. were investigated. The sulfate and enzymatic analysis were employed to modify polysaccharide DNP-W1A, a fraction of polysaccharide isolated from *D. nobile*. Nine sulfated derivative fragments (S1–S9) and three fragments after enzymatic digestion (PE1, PE2 and PE3) were obtained using chloro-sulfonic acid and enzymatic methods, respectively. The activities experiment demonstrated that S1–S9, PE1, PE2 and PE3 showed significant anti-tumor activities in a dose-dependent manner (P < 0.05) in vitro. Annexin-FITC/PI results indicated that PE2 promoted the apoptosis of HepG2 cells at the highest rate of 19.3% compared with other fragments. Also, PE2 significantly increased the gene expression levels of Bax, Caspase-3 and Caspase-9 and suppressed the gene expression of Bcl-2 (P < 0.01). Meanwhile, HepG2 cells treated with polysaccharide resulted in suppressed P-AKT expression, and PE2 induced the lowest protein level of P-AKT compared with other fragments. The results concluded that polysaccharide DNP-W1A and its derivatives induced HepG2 cells apoptosis by up-regulating the gene expressions of Bax, Bcl-2, Caspase-3 and Caspase-9 and inhibiting the PI3K/AKT signaling pathway.

Keywords: Anti-tumor activities, Molecular mechanism, Polysaccharide from *Dendrobium nobile* Lindl., Structure-activity relationship

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INTRODUCTION

In recent decades, research on the pharmacological activities of polysaccharides obtained from natural products has gradually drawn considerable attention (Tang et al., 2017; Lam et al., 2015). The pharmacological activities of polysaccharides include anti-tumor, anti-oxidative, anti-coagulant, anti-diabetic, anti-obesity, anti-fatigue and anti-viral activities (Xie et al., 2016; Wang et al., 2012; Friedman et al., 2016; Wang et al., 2014). Among the numerous activities, anti-tumor activities have been widely recognized in polysaccharides obtained from various sources. For instance, polysaccharide isolated from the king oyster mushroom and *Saccharomyces cerevisiae* showed strong anti-tumor activities, and it effectively inhibited the growth of tumor cells in vivo (Liu et al., 2015). Also, blueberry polysaccharide showed 73.4% tumor inhibition rate in S180-bearing mice (Sun et al., 2015). Moreover, lentilin injection has been used as an effective anti-tumor drug in patients (Oka et al., 1992). However, polysaccharides from different sources exhibited different biological activities emphasizing the importance of polysaccharide development from new sources (Xing et al., 2013). The anti-tumor activities of natural product polysaccharides were largely related to their molecular weight, branching degree and charge etc. (Meng et al., 2016). Appropriate molecular modification and structural modification, such as sulfate modification (certain hydroxyl groups on the polysaccharide were substituted by sulfate group) and enzymatic modification (reduction of groups) improved the biological activities of the polysaccharide (Wei et al., 2012). Previous studies showed that some sulfated polysaccharide and enzyme-digested polysaccharide fragments significantly increased anti-tumor activities.
compared with unmodified polysaccharide obtained from brown seaweed Sargassum pallidum and polysaccharide from brown alga Turbinaria ornata (Jin et al., 2017; Ye et al., 2008).

Apoptosis was tightly regulated, which involved activation, expression, and regulation of a series of genes (Wang et al., 2014). Many genes and proteins are involved in the process of apoptosis, wherein PI3K/AKT signal transduction pathways played an important role in influencing the biological characteristics of tumor cells such as polysaccharide inhibited downstream targets and expressions of β-catenin (Lin et al., 2017). Phosphatidylinositol 3-kinase (PI3K) belongs to the family of lipid kinases and is divided into two categories: IA subclass and IB subclass (Wang et al., 2015). AKT was an essential serine/threonine protein kinase (Fruman et al., 1998). PI3K and AKT are stimulated by signal and then activate and promote cell proliferation, differentiation and apoptosis (Burgering et al., 2003).

*Dendrobium nobile* is one of the traditional Chinese herbal medicines (one of the ten immortals), which is listed as “top grade” in the “Shen Nong's Herbal Classic” (Luo et al., 2010). In our previous report, polysaccharide DNP-W1A from *D. nobile* obtained by water-alcohol precipitation and purification method showed significant anti-tumor activities against Sarcoma 180 in vivo and HL-60 in vitro (Wang et al., 2010). However, the structure-activity relationship and apoptosis mechanism of polysaccharide from *D. nobile* are still unknown. Besides, whether sulfate modification and enzymatic digestion affected the anti-tumor activities of the polysaccharide DNP-W1A also needs further investigation. In this study, structure-activity relationship and apoptosis mechanism of polysaccharide DNP-W1A and its derivatives were investigated using flow cytometer, and the gene expression assays of Bax, Bcl-2, Caspase-3 and Caspase-9 and protein levels of PI3K/AKT channels in HepG2 cells in vitro.

**MATERIALS AND METHODS**

**Materials and Reagents**

The plant stems of *D. nobile* were collected from the Sichuan province of China. HepG2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). DEAE-cellulose, Sephacryl S-200 and Sephadex G-100 were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). iScript™ cDNA Synthesis kit and iTaq™ Universal SYBR® Green Supermix kit were purchased from BIO-RAD (CA, USA). All the primary antibodies were obtained from the Cell Signaling Technology Inc. (MA, USA). The secondary antibody was obtained from Wuhan Boster Bio-Tech Co. (Wuhan, China). All reagents were of analytical or chromatographic grade.

**Extraction and Purification of Polysaccharide DNP-W1A from *D. nobile***

The dried stems of *D. nobile* were extracted with ethanol to obtain the crude polysaccharide. Further, it was fractionated on anion-exchange chromatography and purified by gel filtration chromatography. The polysaccharide DNP-W1A yield is about 5.3% of the dry weight (Wang et al., 2010). The chloro-sulfonic acid method (certain hydroxyl groups on polysaccharide were the substituted by sulfate group) and enzymatic digestion (reduction of groups) were applied to modify the polysaccharide DNP-W1A.

**Preparation Derivatives of Polysaccharide DNP-W1A**

*Sulfated modification of polysaccharide DNP-W1A*

The sulfated modification of polysaccharide DNP-W1A was carried out using the chloro-sulfonic acid method with some modifications (Liang et al., 2018). The modified fragments were named as S1–S9, respectively. The sulfur content in S1–S9 was determined by the barium chloride-gelatin method with some modifications (Dodgson et al., 1962). The standard curve regression equation for the determination of sulfuric acid content is $y = 0.6508x + 0.0255$, $R^2 = 0.9995$, and dermatan sulfate (DS) calculated according to the following equation:

$$DS = \frac{162 \times S\%}{(32-102 \times S\%)}$$

DS indicated the content of sulfate residues.

Three main factors of sulfate modification of polysaccharide DNP-W1A were selected according to this method with some modifications (Qian et al., 2014). The three factors were the ratios of chlorosulfonic acid to pyridine (1:6, 1:4 and 1:2), reaction temperatures (60°C, 80°C and 100°C) and reaction time (2 h, 3 h and 4 h). The DS was used an index to evaluate the sulfate modification of the polysaccharide DNP-W1A.

**Enzymatic digestion of polysaccharide DNP-W1A**

The cellulose (3500 U/mL), pectinase (20,000 U/mL) and α-amylase (40,000 U/mL) were selected as tool enzymes to digest polysaccharide DNP-W1A. Ten milligrams of polysaccharide DNP-W1A was dissolved in 15 mL citric acid-disodium hydrogen phosphate buffer solution. After incubation for 8 h, the reaction was terminated by boiling for 15 min. The yield of reducing sugar in the reaction system was determined using 3, 5-dinitrosalicylic acid (DNS) assay (Potumarthi et al., 2013). The optimal tool enzyme was chosen as the following experiment.

Three factors of the enzymatic digestion were selected as enzyme concentration (90 U/mL, 120 U/mL and 150 U/mL), reaction temperature (40°C, 50°C and 60°C) and reaction pH (4, 5 and 6). The reaction was carried out under above conditions and eight time points (0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h) in order to obtain the optimal reducing sugar yield in the reaction system. A response surface methodology is used to optimize the conditions of this experiment.

Samples were collected after 1 h, 8 h and 24 h time points at optimal reaction conditions. The polysaccharide fragments after enzymatic digestion obtained through concentrating, centrifugation, freeze-thawing, dialysis, and freeze-drying. Enzymatic fragments were dissolved in distilled water to a final concentration of 100 mg/mL aqueous. The mixture treated with DEAE-cellulose anion exchange column (1.6 cm × 60 cm) which was a combination of Sephacryl S-200 and Sephadex G-100 with distilled water.
as elution solvent. The flow rate of the constant current pump was adjusted to 4 mL/min. Eluent was collected with the automatic dispenser with 1 tube every 3 min. The eluent in each tube was determined by the phenol-sulfuric acid method, named PE1, PE2 and PE3, respectively.

Polysaccharide DNP-W1A and its Derivatives for Particle Size Determination
Around 15 mg of polysaccharide DNP-W1A and its derivatives were dissolved in 1.5 mL distilled water. The particle size was measured using Zetasizer Nano ZS-90 (Malvern Panalytical, Malvern, UK).

MTT Assay
HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose medium (HyClone Laboratories, Utah, USA) containing 10% calf serum and 1% double antibody incubated at 37°C incubator containing 5% CO₂. HepG2 cells in the logarithmic phase digested by trypsin and the cell concentration was adjusted to 1 × 10⁶ cells/mL. Around 90 µL of suspension cells were added to each well of 96-well plates. The cells were incubated for 4 h, and 10 µL of different concentration solutions for polysaccharide DNP-W1A and its derivatives was added to each well with equal volume of phosphate-buffered saline (PBS) as blank control. Then, the cells were incubated for 24 h, 20 µL of 5 mg/mL 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) was added and incubated for 4 h. Then dimethyl sulfoxide (DMSO, 100 µL per well) was added to the wells for 14–16 h. Finally, the absorbance was measured at 570 nm using a micro-plate reader (Multiskan Go 1510, Thermo Fisher Scientific, USA). All experiments were done in triplicate.

Effects of Polysaccharide DNP-W1A and its Derivatives on HepG2 Cells Apoptosis
One mL of suspension cells (1 × 10⁶ cells/mL) were added to the 6-well plates and incubated at 37°C for 4 h. Then 50 µL of different concentration solutions of polysaccharide DNP-W1A and its derivatives were added to each well with equal volume of phosphate buffered saline (PBS) as control and the cells incubated for 24 h. Then the cells were collected and treated with the Apoptosis kit (Shanghai Qihai Feitai Biotechnology Co., Ltd.). The suspended cells were filtered through a 300-mesh cell sieve and then loaded into a flow tube. The samples were pelleted using a Flow Cytometer (BD Accuri™ C6, BD Biosciences, CA, USA).

Effects of Polysaccharide DNP-W1A and its Derivatives on Apoptosis-related Gene Expression in HepG2 Cells
A 1.8 mL cell suspension (1 × 10⁶ cells/mL) was added to 6-well plates. The cells incubated for 4 h. A 100 µL of different concentration solution of polysaccharide DNP-W1A and its derivatives was added to each well and the cells were incubated for 24 h. The upper culture medium was collected in a suitable centrifuge tube and then trypsin was added to each well. After digestion for 2 min, the cells were collected and centrifuged (Thermo Fisher Scientific, Massachusetts, USA) at 1000 × g for 5 min. The cells were washed twice with PBS buffer, and then 300 L of TRIZOL (TaKaRa Bio Inc., Japan) was added to each centrifuge tube to extract the total RNA of the cells. Reverse transcription was performed using a reverse transcription kit (TaKaRa Bio Inc., Japan).

Primer (Sangon Biotech) sequences were as follows: Bax forward, 5′-TGCTTACGGGTTTCATCCAG-3′; reverse, 5′-GGGGCTACCATCCTCTG-3′; BCL-2 forward, 5′-GCTCTGTGAGACTGAGTA-3′; reverse, 5′-GGCCGTACAGTTCCACAAAG-3′, Caspase-9 forward, 5′-AACGGCAAGGACAGAATGT-3′; reverse, 5′-CACGCGAGAATTCACATGG-3′, Caspase-3 forward, 5′-CTGGACGTGTCGATGGAC-3′; reverse, 5′-AACAAGGCTGTGGATGACC-3′. The cDNA was subjected to real time quantitative polymerase chain reaction (RT-PCR) amplification for 30 cycles. The reaction conditions were: 95°C (pre-denaturation), 3 min; 95°C, 15 s and 60°C, 30 s for a PCR reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Relative quantification was processed using the delta-delta Ct method.

Western Blot Analysis
Approximately, 1.8 mL of HepG2 cell suspension (1 × 10⁶) was collected and incubated by the above method. The cells were collected and centrifuged (Thermo Fisher Scientific, Massachusetts, USA) at 1000 × g for 5 min and washed twice with phosphate buffered saline (PBS) buffer. Approximately, 0.3 mL of the frozen radio-immune precipitation assay (RIPA) cells lysate (Sangon Biotech, Shangai, China) containing protein phosphatase inhibitor and protease inhibitor) was added to the cell pellet for 30 min on ice. The mixture was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was transferred to a clean Eppendorf tube and the cell protein concentration in the supernatant was measured by bicinchoninic acid (BCA) protein assay kit. The final protein sample supplemented with loading buffer was boiled at 100°C for 10 min and stored at −80°C.

For Western blot analysis, equal amounts of proteins were subjected to SDS-PAGE and electrophoresis and transferred to a membrane. Specific anti-β-actin, anti-phosphor-AKT, anti-AKT (Cell signaling Technology Co., Ltd) and appropriate secondary antibody (goat anti-rabbit, 1:5000 Ph.D. creatures) were used in the protein blotting analysis. The protein bands were visualized using enhanced electro-chemiluminescence (ECL) assay kit (Thermo Scientific Pierce, Massachusetts, USA).

Statistical Analysis
The values are shown as mean ± standard deviation (SD) of three replicates. All statistical analysis was processed using the SPSS software, and statistical significance was expressed as *P < 0.05 or **P < 0.01.

RESULTS AND DISCUSSION
Preparation Derivatives of Polysaccharide DNP-W1A
Sulfated modification fragments
The optimal conditions of sulfate esterification were determined by L9(3⁴) with the degree of substitution (H⁺ in hydroxyl by sulfate
group) as the index (Table 1). The results showed that the optimal condition was A_B_C with a reaction temperature of 100°C, reaction time of 4 h and the ratio of chloro-sulfonic acid to pyridine at 1:6. Under the optimized condition, DS (%) can reach 8.0%.

**Enzymatic digestion fragments**

The results showed that the highest reducing sugar yield was of pectinase (14.505 μg/mL), followed by cellulose (11.443 μg/mL) and α-amylase (9.941 μg/mL). Therefore, pectinase was selected as the tool enzyme for subsequent experiments.

**Single factor analysis**

The effect of reaction temperature on polysaccharide DNP-W1A degradation capability of pectinase was studied using reducing sugar yield as an index. The optimal reaction temperature was 50°C with the highest reducing sugar yield of 15.211 μg/mL (Fig. 1A). However, the lowest pectinase activity was observed when the temperature reached 70°C. Consistently, the optimal acid pectinase temperature ranged from 30 to 50°C in previous research (Kashyap et al., 2001).

The highest reducing sugar yield (15.105 μg/mL) was at pH 5 (Fig. 1B). However, the reducing sugar yield decreased at pH 3 or pH 7, suggesting that pectinase in the strong acid or neutral environment was partially inactivated. Our results revealed that polysaccharide DNP-W1A was digested by pectinase only in weak acid environment, which is consistent to previous research of pectinase hydrolysis of guar galactomannan (Thornberry et al., 1997).

Enzyme concentration is an important factor affecting polysaccharide hydrolysis efficiency (Wu et al., 2012). Reducing sugar yield in each reaction system increased rapidly at the initial stage, and the rate gradually decreased with reaction time (Fig. 1C).

**TABLE 1** | Orthogonal test and values for yield of sulfur content (%) and DS (%).

<table>
<thead>
<tr>
<th>DNP-W1A</th>
<th>Sulfated conditions</th>
<th>Sulfur content (%)</th>
<th>DS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSA: PD*</td>
<td>Temperature (°C)</td>
<td>Time (h)</td>
</tr>
<tr>
<td>S1</td>
<td>1(1:6)</td>
<td>1(60)</td>
<td>1(2)</td>
</tr>
<tr>
<td>S2</td>
<td>1</td>
<td>2(80)</td>
<td>2(3)</td>
</tr>
<tr>
<td>S3</td>
<td>1</td>
<td>3(100)</td>
<td>3(4)</td>
</tr>
<tr>
<td>S4</td>
<td>2(1:4)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>S5</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>S6</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>S7</td>
<td>3(1:2)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>S8</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>S9</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

K1: 18.9 | K2: 9.7 | K3: 8.3 | k1: 6.3 | k2: 3.2 | k3: 2.8 | Range (R): 3.5 | Primary factors: A > B > C |

Optimal level: A_B_C | A_B_C

*The ratio of CAS to pyridine in sulfating reagent.

**FIGURE 1** | Effect of reaction temperature (A), reaction pH (B) and enzyme concentration (C) on the digestion of polysaccharide DNP-W1A. Data are presented as mean ± SD (standard deviation) (n = 3).
the reducing sugar yield in the reaction system increased slightly. Thus, 120 U/mL of enzyme concentration was used in subsequent experiments. The present result indicated that excessive enzyme did not bring about higher activity, which was consistent to the previous report (Chen et al., 2008).

Response surface analysis
According to single-factor experimental results and previous report, the effects of enzyme concentration, pH and temperature on the enzymatic hydrolysis of polysaccharides were investigated by determining reducing sugar yield (Qian et al., 2014). The optimal conditions were assayed by Box-Behnken Design (BBD) (Zhang et al., 2016).

Based on the multiple regression analysis with the experimental data (Table 2), the final second-order polynomial equation is given as follows:

\[
Y = 16.22 + 0.21 \times A - 0.51 \times B - 0.65 \times C - 0.015 \times AB + 0.029 \\
\times AC - 0.16 \times BC - 0.60 \times A^2 - 0.55 \times B^2 - 1.11 \times C^2
\]

Y indicates the reducing sugar yield, while A, B and C are the coded values of the factors for enzyme activities, reaction time, and reaction pH, respectively.

High F-value (F = 7.89) and a low P-value (P < 0.01) indicated that the statistics of the model was significant and reasonable (Table 3). The coefficients (R²) can be used to check the reliability of the model. R² value was 0.9012, indicating the significant linear relationship between independent variable and dependent variable. R²_A40 (0.7948) was used to check the model adequacy. However, the model can be reproducible depending on the coefficient of variation (C.V.), which should be less than 5.00%. In the study, C.V. was 3.01%, indicating the standard deviation as a percentage of the mean for reducing sugar yield.

The P-value (Table 3) of B, C, A², B² and C² is less than 0.05, indicating that pH and temperature had significant effects on the enzymatic digestion of polysaccharide DNP-W1A. The P-value of A, AB, AC and BC was greater than 0.05, suggesting that enzyme concentration and the interaction of the three factors had no significant effects on enzymatic digestion of polysaccharide DNP-W1A. The absolute value of the regression coefficient of one term was compared, indicating that temperature > pH > enzymatic concentration.

The regression model’s three-dimensional (3D) response surface was used to clarify the effects of independent variables on response interactions (Yoshida et al., 2010).

The yield of reducing sugar increased when the extraction temperature rose from 30°C to 47.4°C (Fig. 2A), and then decreased. At a fixed temperature, when enzyme concentration increased from 90 U/mL to 125.3 U/mL, the yield of reducing sugar increased at first and then decreased. The highest reducing sugar yield was observed under the extraction temperature of 47.4°C and pH 4.6 (Fig. 2B).

The yield of reducing sugar increased with increasing pH and enzyme concentration at 50°C (Fig. 2C). The reason may be as the reaction rate increased with enzyme concentration and pH, the rate of reducing sugar yield also increased. As the reaction progressed, the reaction rate slowed down and the rate of reducing sugar yield decreased when it exceeds the highest point (enzyme concentration was 125.3 U/mL, reaction pH was 4.6). The reason is that the enzyme is inactivated by excessively high pH.

The maximum predicted yield was obtained according to an established mathematical mode as follows: enzyme concentration 125.3 U/mL, reaction temperature 47.4°C, and the reaction pH 4.6. The reducing sugar (yield 16.421 μg/mL) was obtained under the above optimal conditions. Consistent to the prediction, the value obtained at optimal conditions was 16.439 μg/mL (n = 3). The digested fragments PE1, PE2 and PE3 were obtained by the optimal combination in the above experiments.
Structure-activity relationship analysis

MTT assay was used to analyze the activities of HepG2 cells. The principle is that mitochondrial succinate dehydrogenase can reduce exogenous MTT which is precipitated in the cells, while the dead cells are not precipitated. The methyl-chain in live cells can be dissolved in DMSO and the absorbance at 570 nm was measured using a micro-plate reader (Gerlier et al., 1986).

The anti-tumor activities of the polysaccharide DNP-W1A and its derivatives were investigated on HepG2 cells in vitro. The S1–S9 induced HepG2 cells apoptosis in a dose-dependent manner (Table 4). S4 concentration at 1000 μg/mL induced apoptosis of HepG2 cells by 33.70%, which was 3.04 times of the apoptosis effects of polysaccharide DNP-W1A. The anti-tumor activities were higher than other sulfated fragments when DS (%) value was around 2.90% (Table 1), and particle size was 721.026 nm. According to the data available, the particle size of polysaccharide derivative fragments S1–S9 were 1076.589 nm, 657.541 nm, 731.587 nm, 721.026 nm, 798.201 nm, 721.304 nm, 786.165 nm, 676.858 nm and 506.664 nm, respectively. The absorbance of the three enzyme digested fragments, PE1, PE2 and PE3 induced HepG2 cells apoptosis, significantly increased compared to the polysaccharide DNP-W1A at > 200 μg/mL concentration (Table 5). Additionally, the S4 apoptosis rate was 6.36% at 200 μg/mL concentration and the PE2 inhibition rate (8.69%) was greater than S4 at the same concentration. The reason being PE2 particle size (1290.181 nm) was greater than S4, and PE2 structure to more loosen than S4. Polysaccharides DNP-W1A, S4, PE, PE2 and PE3 were used for subsequent experiments to study the molecular mechanism.

Molecular Mechanism Assay

Effects of derivative polysaccharide fragments on HepG2 cells apoptosis

To investigate the anti-tumor effects of S4, PE1, PE2, PE3 and polysaccharide DNP-W1A, apoptosis of HepG2 cells after
different fragments was analyzed using flow cytometer after annexin V-FITC/PI staining. The apoptosis percentage of HepG2 cells was 6.4% (the sum of early apoptosis and late apoptosis in the blank control group (Fig. 3A), whereas sulfated polysaccharide fragments induced increased HepG2 cells apoptosis in a dose-dependent manner (Fig. 3). HepG2 cells apoptosis reached 36.8% when treated with polysaccharides at 1000 μg/mL. The HepG2 cells apoptosis in the control group was 6.6% (Fig. 4A), polysaccharides DNP-W1A, S4, PE1, PE2 and PE3 exhibited stronger pro-apoptosis effect compared to the control group (Fig. 4A) at a concentration of 200 μg/mL reached 8.9% (Fig. 4E), 11.4% (Fig. 3C), 17.7% (Fig. 4B), 19.3% (Fig. 4C) and 15.4% (Fig. 4D), respectively. The results showed that derivative polysaccharide fragments increased apoptosis of HepG2 cells (P < 0.05), suggesting that the derivative polysaccharide fragments could contribute to direct anti-tumor activity in vitro. Furthermore, PE2 significantly exhibited higher ratios of apoptosis compared to other derivative polysaccharide fragments.

Effects of derivative polysaccharide fragments on the expression of apoptosis-related gene in HepG2 cells

To further elucidate the mechanism involved in the pro-apoptosis effect of polysaccharide DNP-W1A and derivative polysaccharide fragments on HepG2 cells, the expression of apoptosis related gene in HepG2 cells were analyzed by real-time quantitative polymerase chain reaction (RT-PCR). Caspases and Bcl-2 families play key roles in the occurrence and regulation of apoptosis (Ghia et al., 1998; Thornberry et al., 1998). In this study, expression of Bax, Caspase-3, Bcl-2 and Caspase-9 genes was investigated. Sulfated polysaccharide fragments significantly increased the expression of Bax (Fig. 5A), Caspase-3 (Fig. 5C) and Caspase-9 (Fig. 5D) genes and significantly down-regulated that of Bcl-2 (Fig. 5B) in a dose-dependent manner (P < 0.01). Besides, HepG2 cells were treated with polysaccharide DNP-W1A and enzyme digested fragments led to significant up-regulation of Bax (Fig. 6A), Caspase-3 (Fig. 6C) and Caspase-9 (Fig. 6D) genes expression, as well as significant
down-regulation of Bcl-2 (Fig. 5B) expression. Apoptosis-related gene expression in HepG2 cells were compared in S4-200 and PE2 polysaccharide fragments. PE2 treatment induced the highest expression of Bax, Caspase-3 and Caspase-9 genes and the lowest expression of Bcl-2 compared with control. The result showed that PE2 were the highest activities fragments by induced HepG2 cells apoptosis.

**Effects of derivative polysaccharide fragments on the expression of apoptotic pathway protein in HepG2 cells**

To further elucidate the mechanism involved in the apoptosis promoting effects of polysaccharide DNP-W1A and its derivative fragments on HepG2 cells, Western blot was used to analyze the expression of apoptosis-related protein. AKT is a serine–threonine kinase which is intimately involved in the regulation of cell survival (Kim et al., 2001). The level of P-AKT protein was significantly down-regulated by the polysaccharide DNP-W1A and its enzymatic digested fragments were compared with the control group, and the protein expression level of P-AKT was the lowest for PE2 (Fig. 7). Meanwhile, sulfated DNP-W1A also increased the protein level of P-AKT in a dose-dependent manner (Fig. 7B).

PE2 significantly decreased the protein expression of P-AKT in the PI3K/AKT signaling pathway, thereby promoting HepG2 cell apoptosis. The molecular weight of the polysaccharide changed for sulfate modified method, whereas enzymatic digestion changed the structure of polysaccharides. With the extension of enzymatic digestion time, the branched-chain glucose of the polysaccharide was released and the molecular weight decreased, but the main chain structure of the polysaccharide was not destroyed by the hydrolysis of pectinase (Zha et al., 2013). According to the study, the binding of β-glucans to complement receptor 3 was well known, while a lot of studies had been conducted on the receptor dectin-1, which recognized exogenous β-1, 3 and β-1, 6 linked plant polysaccharides (Brown et al., 2001). However, the degree of branching and molecular weight were not known for the helical structure of the bound β-glucan. In previous experiments, the structure of polysaccharides from *D. nobile* had been reported, DNP-W1A contains six kinds of sugar residues, in which glucosyl groups existed in the form of 1,4–, 1,6–, 1,4,6–linked, galactose
FIGURE 5 | Effects of sulfated polysaccharide fragments on the expression of gene related to HepG2 cells. All data are representative or average of analysis of eight samples from three independent experiments. *P<0.05, **P<0.01.

FIGURE 6 | Effects of polysaccharide DNP-W1A and enzyme digested fragments on the expression of genes related to HepG2 cells. All data are representative or the average of analysis of eight samples from three independent experiments. *P<0.05, **P<0.01.
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FIGURE 6 | (Continued)

existed in an end-linked manner, and arabinose appears in a terminal and 1,3-linked manner. Its branched chain link was similar to β-glucan (Thornberry et al., 1997). In this study, PE2 showed strongest anti-tumor activities of HepG2 cell. The reason was that enzymatic digestion caused the polysaccharide structure to loosen so that the binding site to the recipient cell was exposed on the molecular surface, and combined with the PI3K/AKT signaling pathway regulates the apoptosis of HepG2 cells by regulating the expression of Bax, Bcl-2 Caspase-3 and Caspase-9 proteins.

CONCLUSIONS

PE2 had the highest anti-tumor activities by comparing with other derivative fragments. Treatment of HepG2 cells with polysaccharide DNP-W1A and its derivatives led to significant up-regulation of Bax, Caspase-3 and Caspase-9 genes expression and a significant down-regulation of Bcl-2 gene expression. Meanwhile, HepG2 cells treated by derivative fragments resulted in suppressed p-AKT expression, and PE2 induced the lowest protein level of p-AKT. PE2 promoted the HepG2 cells apoptosis at the highest rate up to 19.3% (polysaccharides concentration was 200 μg/mL) compared with other derivative fragments. In conclusion, the mechanism of HepG2 cells may be that PI3K/AKT signaling pathway was inhibited by derivative fragments. The polysaccharide DNP-W1A was obtained by enzyme digestion and sulfate-modification method. Enzymatic digestion fragments were more effective than sulfate modification fragments in human tumor cells HepG2 apoptosis.

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