**Supplementary material for**

**A novel polysaccharide from *Paeonia lactiflora* exerts anti-tumor activity via immunoregulation**

**Supplementary methods**

**S1 *Materials and reagents***

The roots of *Paeonia lactiflora* were acquired from Anguo Materia Medica Market (Hebei, China) and identified by Professor Yuanqiang Guo, Nankai University (Tianjin, China). DEAE-Sepharose Fast Flow (DEAE-FF) and Sephadex G-75 were purchased from Huiyan Bio (Wuhan, China) and GE Healthcare (Chicago, USA), respectively. Standard monosaccharides were obtained from Desite Biological Technology Co. Ltd. (Chengdu, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was supplied from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM), Fetal bovine serum (FBS, BI, Israel) were purchased from Lab Biotech Co. Ltd. (Shandong, China). Thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Solarbio (Beijing, China).

**S2 *Isolation and purification of PLP90-1B***

The dried roots (4 kg) were extracted three times with distilled water (1: 10, w/v) at 90 ℃ (3 h each time). The concentrated extract was precipitated with anhydrous ethanol to final concentrations of 50%, 70%, and 90% for 24 h at 4 ℃. After centrifugation (5000 rpm, 10 min), the precipitate was re-dissolved in distilled water and the protein was removed by the Sevage reagent (*n*-butanol: dichloromethane, v: v = 1: 4). After that, the deproteinized solution was dialyzed (*Mw* cut-off: 3500 Da), concentrated, and lyophilized to obtain the crude polysaccharide named PL90.

The crude polysaccharide solution (40 mg/mL) was loaded into DEAE-FF column (Ø 3.5 × 40 cm) and eluted with deionized water, 0.05 M NaCl and 0.15 M NaCl, respectively. Each eluent fraction (10 mL/tube) was collected using an automatic collector (Huxi, Shanghai, China) and monitored with phenol-sulfuric acid method and UV-spectrophotometer at 490 nm. Then, the main elution part of 0.05 M NaCl was subjected to dialysis (*Mw* cut-off: 1000 Da), concentration, lyophilization, and further separation using a gel filtration of Sephadex G-75 (Ø 1.5 × 100 cm) at the flow rate of 0.2 mL/min. The eluent was gleaned according to the results of high-performance gel permeation chromatography (HPGPC) and concentrated to acquire PLP90-1B.

**S3 *Molecular weight and homogeneity analysis***

The molecular weight of PLP90-1B was measured by high performance size exclusion chromatography (HPSEC). PLP90-1B (4 mg) were dissolved by 1 mL NaCl (0.1 M) and loaded into the chromatography system (20 *μ*L). The column temperature was set as 25 ℃ and eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. Preliminary calibration of the column was conducted by using Shodex STD P-82 series with different molecular weights. The retention time of chromatographic peak and the logarithm of molecular weight of standards were used for linear regression to obtain the linear regression equation. The molecular weight (*Mw*) of PLP90-1B was calculated by substituting the retention time of PLP90-1B into the regression equation.

**S4 *Monosaccharide composition analysis***

PLP90-1B (4 mg) was hydrolyzed by 2 M trifluoroacetic acid (TFA, 2 mL) in an oil bath at 120 ℃ for 6 h. After the reaction, it was cooled to room temperature and then evaporated to complete dryness by adding methanol repeatedly to remove the excess TFA. The hydrolysates were re-dissolved in 1 mL distilled water. 100 *µ*L of the above polysaccharide hydrolysate was mixed with sodium hydroxide solution (0.3 M, 100 *μ*L) and PMP in methanol (0.5 M, 100 *μ*L), and the reaction was carried out at 70 ℃ for 30 min. Subsequently, the resulting product was neutralized with hydrochloric acid (0.3 M, 105 *μ*L) and extracted three times with equal volume of chloroform. Finally, the water layer was filtered with 0.45 *μ*m membrane and analyzed by an HPLC system with an ultraviolet detector. The chromatographic conditions were as follows: column, Kromasil 100-5-C18 column (5 *μ*m, 4.6 × 250 mm); column temperature, 25 °C; mobile phase, a mixture of phosphate buffered saline (0.1 M, pH 6.9) and acetonitrile (83: 17, v/v); detector wavelength, 250 nm; flow rate, 0.8 mL/min; volume of sample injection, 20 *μ*L. Mannose, rhamnose, galacturonic acid, glucuronic acid, glucose, galactose, xylose, arabinose, and fucose were used as monosaccharide standards and processed in the same way as the sample.

**S5 *Absolute configuration of monosaccharide analysis***

PLP90-1B (5 mg) was hydrolyzed by 2 M TFA (2 mL) in an oil bath at 120 ℃ for 6 h. After the reaction, it was cooled to room temperature and then evaporated to complete dryness by adding methanol repeatedly to remove the excess TFA. Then, L-cysteine methyl ester (2.5 mg) and pyridine (1 mL) were added in turn and reacted for 1 h at 60 ℃. *O*-tolylisothiocyanate (5 *μ*L) was added to continue the derivatization reaction. Meanwhile, the standard monosaccharides (2 mg) were derivatized by the same method. Finally, the derivatized products were filtered through a 0.45 *μ*m filter and detected with an HPLC system equipped with an ultraviolet detector. The chromatographic conditions were as follows: column, Kromasil 100-5-C18 column (5 *μ*m, 4.6 × 250 mm); column temperature, 25 °C; mobile phase, CH3CN-H2O (25:75, v: v) (0.1% acetic acid); flow rate, 0.8 mL/min; detector wavelength, 250 nm. The configuration of monosaccharide standards was as below: D-arabinose, L-arabinose, D-glucose, L-glucose.

**S6 *Methylation and GC-MS analysis***

Specifically, PLP90-1B (4 mg) was dissolved in 5 mL of anhydrous DMSO in a single-necked flask and sonicated until the sample was completely dissolved. The NaOH-DMSO solution was mixed with the DMSO solution containing the sample, and ultrasonic treatment lasted for 1.5 h. Then, the methyl iodide (CH3I) was added into the mixture under dark conditions three times. The whole methylation process should be controlled at a low temperature. After the reaction was terminated by distilled water, 2 mL of chloroform was used to extract the product, and the chloroform phase was analyzed by IR spectroscopy. The absorption peak without hydroxyl at 3300−3600 cm–1 indicated PLP90-1B was completely methylated.

The methylated PLP90-1B was hydrolyzed with TFA (2 M, 2 mL) for 6 h at 120 ℃, and reduced with NaBD4 (20 mg) for 1 h at 40 ℃. The reaction was then finished with the addition of 100 *μ*L of glacial acetic acid and concentrated to neutral with methanol under reduced pressure. Afterwards, the sample was completely dried and reacted with 2 mL pyridine and 2 mL acetic anhydride in oil bath at 95 ℃ for 2 h. Partially methylated alditol acetates (PMAAs) were dissolved with 1 mL chloroform and extracted by an equal volume of deionized water repeatedly. The chloroform layer was injected into an Agilent 7890A-5975C GC-MS (Agilent, Washington, USA) for determination.

***S7 Anti-tumor activity evaluation using zebrafish xenograft model***

Prior to microinjection, HepG2 cells were labeled with CM-DiI at the final concentration of 2 *µ*M, and suspended in FBS-free medium at a density of 1×107/mL. Then, the labeled HepG2 cells (5 nL) were microinjected into the yolk sac of the anesthetized embryos. After 4 h incubation, zebrafish embryos were randomly divided into five groups (15/group) and placed in a 12-well plate, which were then treated with different concentrations of PLP90-1B for 48 h at 28.5 °C. Lentinan, a polysaccharide used for cancer, was set as a positive control. At 5 days post fertilization (dpf), the proliferation and migration of HepG2 cells in the zebrafish embryos were observed by laser confocal microscopy (Leica, Germany) and quantified using Image J software (NIH, Bethesda, MD, USA).

***S8 In vitro anti-proliferation assay***

In brief, HepG2 cells were seeded into 96-well plate with 100 *μ*L medium at a final density of 5 × 103 cells per well. After 24 h incubation, the cells were treated with different concentrations of PLP90-1B for 48 h. Then, 20 *μ*L MTT solution (5 mg/mL) was added to the cells and incubated continuously for 4 h at 37 °C. After incubation, the medium was removed and replaced with 150 *μ*L dimethyl sulfoxide (DMSO). After the formazan crystals were dissolved, the absorbance was read at 492 nm via a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The inhibitory rates were calculated as the percentage of the control group (100%).

**S9 *Cell viability determination using MTT assay***

RAW264.7 cells were harvested and seeded in 96-well plates (5 × 104 cells/well) and cultured for 24 h at 37 °C. Then, the cells were treated with PLP90-1B (50, 100, and 200 *μ*g/mL) for another 24 h. Subsequently, 20 *μ*L MTT solution (5 mg/mL, Solarbio, Beijing, China) were added in each well. After incubated for 4 hours at 37 ℃, the medium was completely removed, DMSO (150 *μ*L) was added to solubilize the formazan crystals and the absorbance was measured at 492 nm using microplate reader (Thermo Fisher Scientific Inc. America). The experiments were performed three times, the viability was calculated and shown as a percentage relative to the control group.

**S10 *Determination of phagocytic activity by neutral red assay***

RAW264.7 cells were seeded in 96-well plates (5 × 104 cells/well). After incubation for 24 h, the cells were treated with various concentrations of PLP90-1B (50, 100, and 200 *μ*g/mL) and incubated for another 24 h. LPS (1 *μ*g/mL) solution was used as a control. Then, the supernatant was replaced with the neutral red solution (100 *μ*L, 0.075%), and incubated for 1 h. RAW 264.7 cells were lysed with lysis buffer for 1 h at room temperature, the absorbance value was read at 540 nm under the automated microplate spectrophotometer (Thermo Fisher Scientific Inc., America).

**S11 *Measurement of nitric oxide (NO) in RAW264.7 cells***

RAW264.7 cells were seeded in 96-well plate and treated with different concentrations of PLP90-1B (50, 100*,* and 200 *μ*g/mL) for 24 h at 37 °C. LPS (1 *μ*g/mL) solution was used as a control. Then, 50 *μ*L of supernatant was collected in a 96-well plate, and reacted with 50 *μ*L of Griess reagent. The absorbance at 550 nm was recorded with a microplate reader (Thermo Fisher Scientific Inc., America), and a standard curve of sodium nitrite (NaNO2) was generated to calculate the NO concentration.

**S12 *Determination of cytokines in RAW264.7 cells***

RAW264.7 cells were seeded in a 24-well plate and incubated at 37 ℃ for 24 h. Then, the cells were continuously treated with different concentrations of PLP90-1B (50, 100, and 200 *µ*g/mL) and LPS (1 *μ*g/mL) for 24 h. The cell supernatant was collected to determine the cytokine levels by ELISA kits according to the manufacturer's instructions. The optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific Inc., USA).

**S13 *Evaluation of reactive oxygen species (ROS) and NO in the zebrafish model***

Healthy embryos at 7-8 hours post-fertilization (hpf) were selected and placed into 12-well culture plate (15 embryos per well). After treatment with various concentrations of PLP90-1B (50, 100, and 200 *µ*g/mL) for 24 h, the embryo culture medium was changed with fresh medium every 24 h until 3 days post fertilization (dpf). Then, the embryos in 12-well were treated with DCFH-DA solution (20 *μ*g/mL) for 1 h (for the measurement of ROS level) or DAF-FMDA (5 *μ*M) for 2 h (for the measurement of NO level), both in dark at 28 ℃. The photographs of the stained embryos were obtained by the confocal laser microscope (Leica TCS SP8, Leica, Germany). ImageJ software (NIH, Bethesda, Maryland, USA) was used to quantify the fluorescence intensity of a single zebrafish embryo.

**Supplementary figures**

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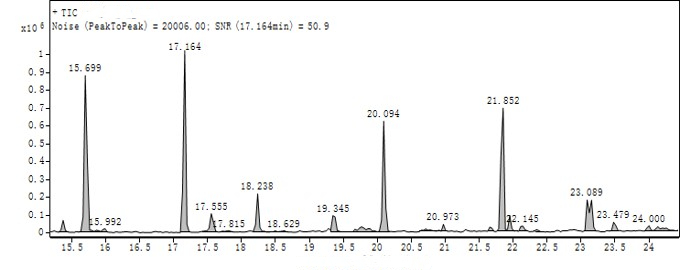
**Fig. S1** The elution curves of PL90 and PL90-1. (A) The elution curve of PL90 on DEAE-FF column (10 mL/tube). (B) The elution curve of PL90-1 on Sephadex G-75 column (5 mL/tube).

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**Fig. S2** The absolute configuration analysis of PLP90-1B.



**Fig. S3** FT−IR spectrum of PLP90-1B (A) and the completely methylated spectrum of PLP90-1B (B).



**Fig. S4** Total ion chromatogram of the methylated products of PLP90-1B.











**Fig. S5** The mass spectra of PMAAs of PLP90-1B.

A. 1,4-di-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-methyl-L-arabinitol.

B. 1,4,5-tri-*O*-acetyl-1-deuterio-2,3-di-*O*-methyl-L-arabinitol.

C. 1,3,4,5-tetra-*O*-acetyl-1-deuterio-2-*O*-methyl-L-arabinitol.

D. 1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl-D-glucitol.

E. 1,2,3,4,5-penta-*O*-acetyl-1-deuterio-L-arabinitol.



**Fig. S6** Congo red experiment of PLP90-1B.

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**Fig. S7** Effects of PLP90-1B on cell viability in HepG2 cells.